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PRINCIPAL INVESTIGATOR:
Massimo Trucco, M.D.

CONTRACTING ORGANIZATION:
Children's Hospital of Pittsburgh
Pittsburgh, PA 15213

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14. ABSTRACT Type 1 diabetes is considered an autoimmune disease characterized by the presence of inflammatory cells in the islets of Langerhans. These cells are T lymphocytes, considered responsible for the destruction of the insulin producing beta-cells present in the islets. When the majority of the beta cells are dead, the disease presents, frequently with an abrupt and clinically serious onset. Hyperglycemia can be induced by chemical destruction of the insulin producing beta cells in monkeys. Following diabetes induction, histological examination of the pancreas shows islet cells with virtually null or sporadic immuno-reactivity for insulin. In our pilot studies, monkeys (<i>macaca fascicularis</i>) were rendered diabetic prior to receiving a xenogeneic porcine islet transplantation. A recovery of endogenous C-peptide production was observed in monkey recipients of steadily functioning pig islet grafts, concurrently to improved metabolic control. Histological analysis of the pancreatic tissue of these monkeys showed: increased proliferative (Ki67+) activity in the pancreas; small aggregates of insulin positive cells detached from the pre-existing "damaged" islets; as well as high numbers of CK19+ cells that also showed strong insulin positive immunoreactivity. Such hallmarks were not seen in diabetic animals kept under insulin daily administrations, nor in recipients that experienced early graft loss. It remains to be demonstrated whether islet cell transplantation, in combination with a regimen of a non-diabetogenic immunosuppression, has a role in triggering endogenous insulin production.				
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INTRODUCTION:

Lesions of the endocrine pancreas, as they occur in Type 1 Diabetes (T1D), were historically considered to be permanent and irreversible since patients, once endogenous insulin production is blunt, require hormone therapy for life. More recently it was proposed that islet beta cells actually retain the ability to heal from an injury, and to proliferate and multiply from precursors into mature beta cells during adult life, in a way similar to that during embryonic development. It was also proposed that possible reparative events affecting the beta cell mass normally occur but in diabetes are opposed by the destructive mechanisms that cause diabetes in first place such as the autoimmune attack (1,2).

In mouse models, evidence was presented that adult animals retain the ability to expand the beta cell mass as a response to various triggers. However, it remains largely unclear through which molecular and cellular mechanism(s) reestablishment of the beta cell mass takes place. Furthermore it was shown in the diabetic prone NOD-mouse that, even after establishment of the clinical diabetic status, it is still possible to recover endogenous pancreatic insulin production. This can be achieved by successfully combining strategies aimed at blocking the autoimmune attack with the use of non-diabetogenic immunosuppressive drugs. Normally an islet transplantation was used in parallel to supply beta cell function for the relatively long periods required to recover beta cell function (3-6).

A spontaneous recovery of the pancreatic beta cell function is also normally reported in patients diagnosed with autoimmune T1D, the so-called “honeymoon” period. Although the honeymoon may vary in duration quite dramatically from one patient to the next, there are also anecdotal cases of complete and permanent recovery. Following diagnosis of T1D and initiation of exogenous insulin treatment these individuals experienced a return of endogenous insulin production, characterized by increased C-peptide secretion, and reduction in the titre of circulating islet auto-antibodies, confirming the return of the immunologic tolerance and a consequent recovery from islet cell destruction (7). Although it is unquestionably proven that some beta cells are still present and able to produce insulin years after the clinical onset of diabetes (8,9), a steady recovery from the diabetic status is quite unique and is worth further investigations. At any rate, the potential for the pancreatic organ to recover substantial endocrine function is quite fascinating and should be exploited for possible clinical applications.

To this aim, the observation that only a limited islet mass is actively engaged in supplying insulin to maintain normoglycemia at a give time point is remarkable. This information seems to indicate that the critical mass required to synthesize and release insulin sufficient for the body's normal needs is far less than that produced by the entire beta cell pool of a healthy pancreas. This observation implicates that even a relatively limited quantity of insulin producing tissue should exert clinically evident effects in patients.

Non-human primates are relevant animal models for pre-clinical studies in general and, in particular, for xenotransplantation experiments. Monkeys and humans present strong similarities although significant metabolic differences do exist. It is therefore of great value to investigate the potentiality of non-human primate pancreatic tissue to resume endogenous insulin production following its destruction, for a better understanding of the human behaviour as well.

In monkeys, a permanent diabetic status can be induced by total pancreatectomy, a major surgical procedure usually not devoid of technical difficulties or, alternatively, by chemical destruction of the insulin producing cells by means of streptozotocin, a potent toxic agent that specifically target the pancreatic beta cells (10).

Our experimental protocol for pig islet xenotransplantation into monkeys involved induction of diabetes in the recipients by streptozotocin, intra-portal transplantation of porcine islets after recipient immunologic pre-conditioning, completed by an adequate non-diabetogenic immunosuppressive therapy. While the majority of recipients exhibited a transitory islet graft function and were sacrificed early after transplantation, in some recipients' islet graft function (indirectly assessed by measurable levels of porcine C-peptide) lasted for months (11). At a certain point in time, concurrently to a gradual reduction of the C-peptide (porcine) graft output, we observed a stepwise increased in endogenous C-peptide (monkey) levels. Metabolic clinical improvement and peculiar histological features of the pancreatic tissue were also found associated to this change. To note, no

spontaneous recovery of endogenous function was observed in transplanted monkeys with transient function of the graft or diabetic monkeys maintained under exogenous insulin for up to 1 year.

Our data provide evidence that the pancreatic tissue is able to re-establish endogenous insulin production after chemically induced beta cell specific injury. Additional investigation is required to understand whether conditions such as improved glucose metabolic control and an appropriate immunosuppressive regimen, in association with functional islet transplantation could play a role in beta cell rescue or regeneration, and if so, through which mechanism(s) (12).

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BODY:

In our first quarterly scientific progress report (06/01/06 – 08/31/06) for the first year of our award, we presented a lot of progress made in the mouse towards the aims of an independently funded grant (i.e., JDRF 7-2005-1154, “Gene and Cell Based Approaches for Abrogation of Autoimmunity in Diabetes”, Project #3, “MHC Tailored for Diabetes Cell Therapy”) that let us to believe in an easy transfer of pertinent information into the monkey’s model.

Furthermore, results obtained in the monkey in the pursue of a different project (i.e., JDRF 4-2004-786, “Alpha 1,3 GT DKO Pigs as Donors for Xenogeneic Islet Transplants”) can be used to properly address some of the aims here originally proposed.

More precisely, in the pursuit of the original

Task 1. To isolate – using appropriate antibodies and cell sorting – bone marrow (BM) cell precursors from a diabetic (i.e., streptozotocin [STZ] treated) cynomolgus monkey. The isolated precursors will then be transfected *ex vivo* with an *Mhc* class II beta chain gene conferring resistance to the disease, and re-infused in the BM depleted animal to determine the safety of this maneuver.

a) This protocol will be implemented immediately after diabetes onset, in association with allogeneic islet transplantation to guarantee the diabetic animal’s euglycemia until the regenerative process brings about sufficient beta cells to make the islet graft obsolete.

b) In parallel experiments, insulin-based therapy will precede transplantation of transfected BM cells and the protocol will be implemented after a protracted insulin therapy. Exogenous insulin administration will continue after protocol’s implementation.

We were able to produce retroviruses containing the selected beta chains of the MHC, able to transfect isolated bone marrow-derived progenitor cells.

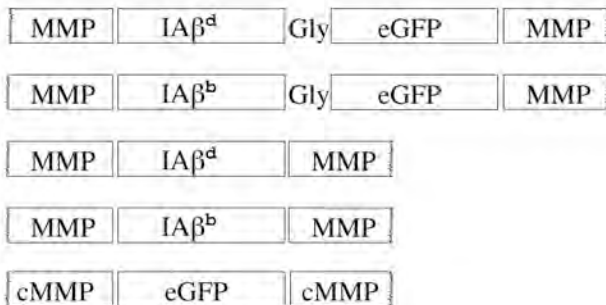


Figure 1. The MMP retroviral vector, derived from MFG, was used to generate IAb expressing retroviral plasmids. The IAb sequences from the b and d haplotypes were fused at their carboxyl termini with eGFP via an intervening glycine codon. Vectors excluding the eGFP were also prepared. The cMMP-eGFP plasmid (Harvard Gene Therapy Initiative) is used as a parallel control for the production of retrovirus.

We prepared three constructs useful for the mouse project: The first is a retrovirus carrying only the GFP gene. This vector is useful to set the best conditions for successfully transfecting BM derived hematopoietic precursor cells. The second is carrying the GFP encoding gene plus the I-A β chain gene of the haplotype H2^d. The NOD mouse is carrying an I-A molecule constituted by an α^d chain and a β^{Ag7}. The third one is carrying instead the GFP encoding gene plus the I-A β chain gene of the haplotype H2^b. For additional experiments, retroviruses were prepared carrying the I-A β chain gene but omitting the GFP segment so that they can be used in the presence of other fluorescently labeled cells (Figure 1).

The ability of these vectors to transfect BM derived hematopoietic precursor cells was also successfully tested. To confirm the feasibility of preventing diabetes progression in diabetic NOD animals by transfected bone marrow precursor cell reconstruction, we combined AutoMACs with Flow Sorter approaches to isolate

Sca1+, c-Kit+ and Lin- bone marrow cells for BM transplant into myeloablated recipients. The BM (total number 126×10^6 cells) was from diabetic females. The cells were re-suspended in 500mL of AutoMACs running buffer (PBS with 0.5% BSA) to follow the lineage depletion kit protocol. A total of 3×10^6 lineage-negative (**Lin-**) cells were recovered. They were then labeled with anti-Sca1 and anti-c-Kit antibodies and subjected to flow sorting. The sorted cells were collected. Their purity was about 70%.

Precursor cells were efficiently infected when an MOI of 100 was used (**Figure 2**). This is the concentration of the virus we then used for all the subsequent experiments.

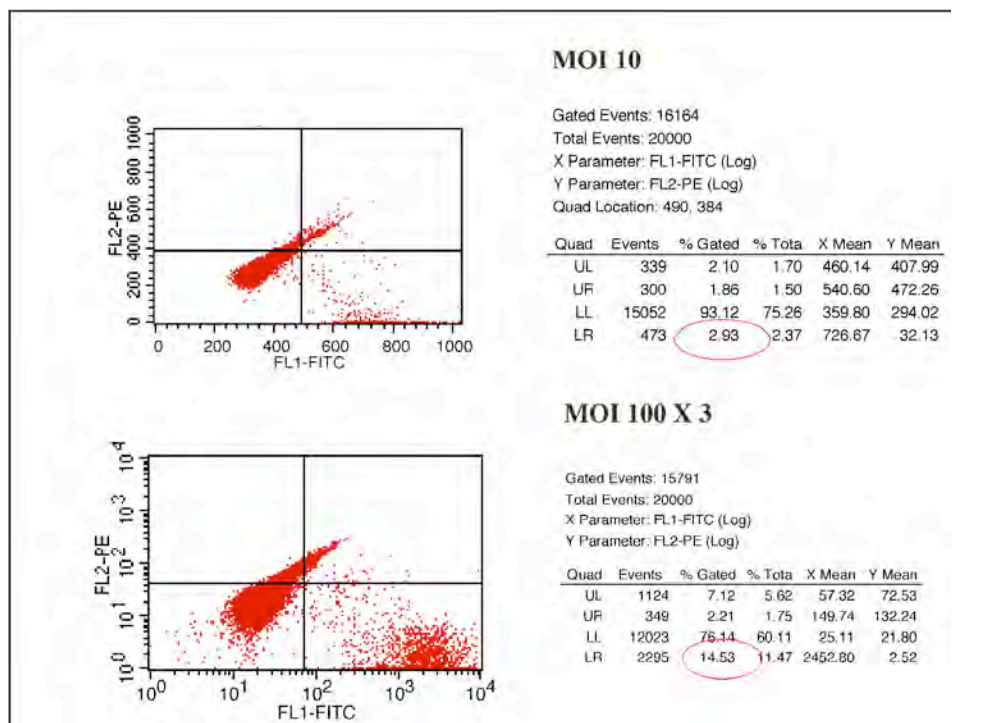


Figure 2

Once infected, the cells were expanded in vitro before re-injecting them into the diabetic recipient depleted of its BM. A total of 500,000 successfully transfected precursors were then injected per mouse with obtaining an hematopoietic chimerism of 83% at two weeks and of 60% at four weeks from transplant (**Figure 3**). We will use the same strategy to transfect cell progenitors in the monkey.

Task 2. To ascertain the efficacy of engraftment and repopulation capabilities of the engineered hematopoietic precursor cells, following non-radiation based pre-conditioning.

a) We will systematically substitute to irradiation an antibody-based, immuno-reductive conditioning protocol, testing different quantities and well-defined injection schedules.

In independent experiments in which a xenotransplant of pig islets was performed into the macaca, a protocol without irradiation (**Figure 4**) was successfully used to preserve the graft. At day -4 and -3, ATG was administered with *Solumedrol*, cortisone necessary to limit the consequences of the cytokines' storm due to the massive death of the majority of white cells of the animal (1). This protocol will be first used in BM reconstitution experiments to substitute irradiation with an antibody based preconditioning.

Analysis of Peripheral Blood of Diabetic NOD 2567 after Treatment

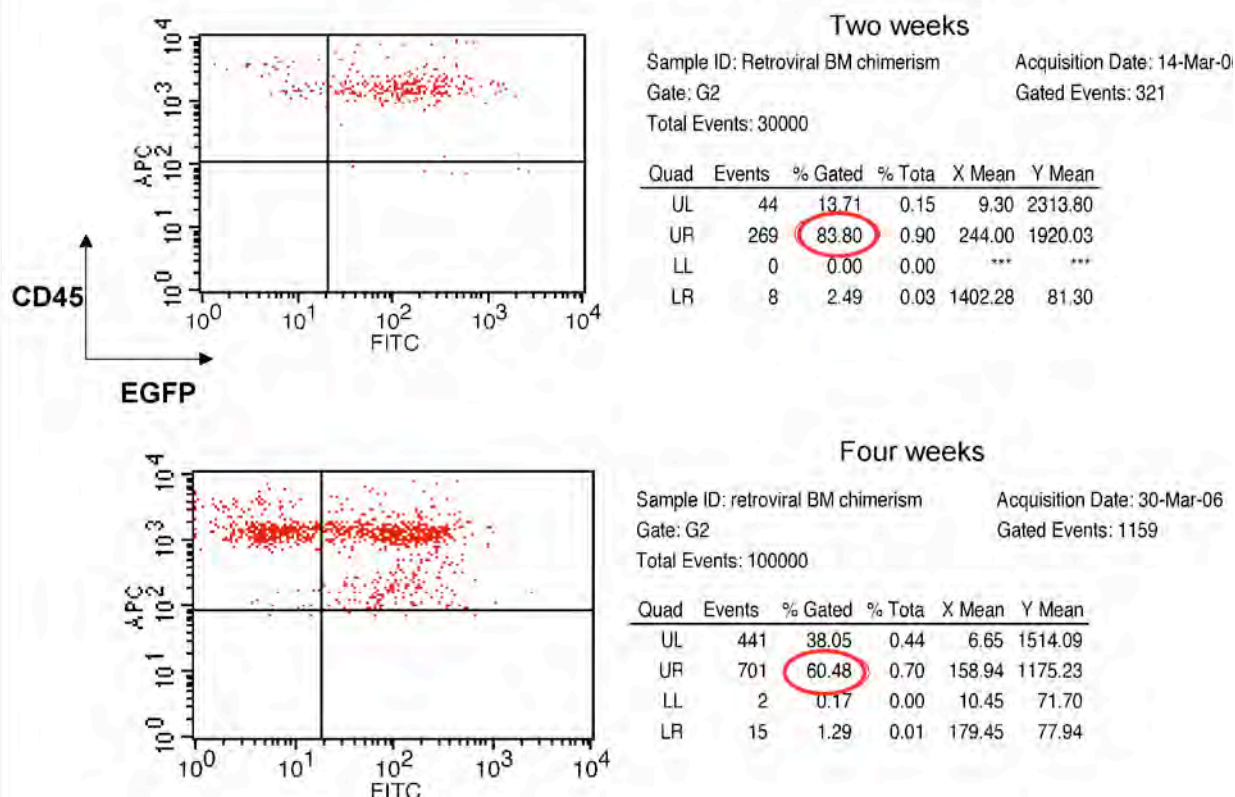


Figure 3

PROTOCOL GROUP II: (M48 to M52)

- M48-52: Islets intraportally (40 000/kg)
- subrenal capsular (16 000/kg) and in omental pouches (40 000/kg)

IBMIR:

- Cobra venom factor
- Prostacyclin
- (Aspirin), 105 U/kg Heparin with islets

Immunosuppression:

- **ATG**
- Anti-CD 154 mAb
- Mycophenolate mofetil

Figure 4

Task 3. To utilize phage integrases to guide the stable and irreversible insertion of DNA at specific locations within the genome to satisfy the need for an everlasting synthesis of the beta chain conferring resistance, even in the offspring of the successfully transfected BM precursor cells.

- a) Determine if any of the three DirectIt™ recombinases integrate plasmid DNA at pseudo *att* sites in monkey chromosomes.
- b) Determine if the best recombinase identified in Task 1 integrates plasmid DNA into mesenchymal stem cells (MSC) of cynomolgus monkey.
- c) Identify the pseudo *att* sites in MSC of cynomolgus monkey.
- d) Optimize the transfection and gene targeting procedure for integrating plasmid in to CD34⁺ cells of cynomolgus monkey.

For this aim, BM from the vertebral bones of four sacrificed monkeys have been provided to Reogene to isolate hematopoietic precursor cells and then determine in how many different sites of the cell genome the retrovirus will be found inserted and whether these insertions are in not dangerous locations of the non-human primate genome.

Task 4. To infuse *in situ* appropriate factors (e.g., PAX4, EGF, LIF, HGF, GLP, and IGF) able to speed up the physiologic regenerative process.

- a) A topical route via the pancreatic duct by retrograde delivery, similar to a commonly used clinic technique ERCP (endoscopic retrograde cholangio-pancreatography) will be used.

Towards this aim the method has been tuned up in the mouse (2). We are planning to reproduce this successful experiment in the monkeys where, considering the dimensions of the bigger animal, we don't expect serious difficulties.

Task 5. To test with a proteomics approach whether the successfully infused target tissue secrete, during regeneration, the product(s) of the transfected gene(s) and additional, spontaneously-generated, adjuvant factors.

- a) Isolated endocrine and exocrine tissues obtained at the autopsy, will be used any time they become available.
- b) The pancreatic fluid will be collected from the duct of the treated monkeys, by using the ERCP technique and its content analyze in detail in terms of relative quantities of each of the proteins present.

These experiments were not initiated yet, due to the lack of already treated monkeys.

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[In our second quarterly scientific progress report \(09/01/06 – 11/30/06\) for the first year of our award, we presented our regeneration results obtained in transplanted monkeys.](#)

In the year 2000, the clinical possibility of transplanting islets into the livers of diabetic patients was documented; rejection was avoided, thanks to an immunosuppressive regimen that reduced the use of tacrolimus and sirolimus, removed the use of steroids, and instead used daclizumab, an antibody against the interleukin-2 receptor molecule (1). However, the Edmonton protocol soon showed its limits. The first limiting factor was the immunosuppressive protocol, which was associated with side effects and allowed this type of transplantation in certain adult recipients only (2). The second limiting factor was the need for more than one islet donor for each recipient.

To respond to the latter limiting aspect of the Edmonton protocol, some groups looked at a theoretically

unlimited source of transplantable islets. An unlimited source of islets can be found in animals able to produce insulin very similar to human insulin and in quantities that may satisfy the insulin requirements of an individual of an average body weight. Based on these two parameters, the pig seemed to be the animal of choice. There is only one amino acid difference between human and pig insulin, and the pig is large enough to supply large amounts of donor islets. Pig insulin was successfully used to treat diabetic children for years before recombinant human insulin became available. Also, evidence that pig islets can be used for human transplantation was provided by studies conducted, in particular, in the 1990s in Sweden (3). This possibility was not further explored when it became clear that the alpha1,3 galactose (alpha1,3Gal) epitopes present on pig tissues were the targets of antibodies, normally found in human serum, that are able to quickly reject xenotransplants. This rapid, deleterious reaction is known as “hyperacute rejection” (HAR). HAR is the major cause of tissue destruction within a few hours after xenotransplantation. The best way to obviate HAR was to work toward the generation of pigs genetically deprived of the activity of the enzyme alpha1,3 galactosyltransferase (alpha1,3GT) and, consequently, free of alpha1,3Gal epitopes at their cell surface (4). In the spring of 2003 (5), our effort of many years (6,7) to generate alpha1,3GT double knockout (DKO) pigs was successfully completed. DKO pigs are better suited as donors for xenotransplantation than their wild-type counterparts because, once their tissues are transplanted into humans or Old World monkeys, they are not targets for a HAR. Adult islet cells from wild-type animals express only low levels of alpha1,3Gal epitopes (8). However, other cells contaminating each preparation used for transplantation do express alpha1,3Gal epitopes at high levels. Experiments in chemically diabetic (i.e., STZ-treated) monkeys indicated that pig islets can substitute for endogenous islets, producing enough insulin (monitored by pig C-peptide) to control the recipient animal glycemia (9,10).

More pertinent to our proposed aims, however, has been the observation that, using a non-calcineurin inhibitor-based immunosuppressive protocol, the monkeys’ own pancreatic endocrine tissue is able to regenerate within a period of time similar to that determined for the diabetic mouse (11,12).

Preliminary studies show that all the insulin-positive and Glut-2-positive cells disappear in the pancreata of monkeys treated with STZ, but insulin-positive and Glut-2-positive cells reappear after 3-4 months of treatment. After STZ treatment, the endocrine pancreas of the monkey was no longer able to produce sufficient quantities of insulin to satisfy the need of the animal, which consequently became diabetic. Monkey C-peptide levels remained <0.5 ng/ml for the entire duration of all experiments in which conventional immunosuppressive cocktails were used, and the arginine stimulation test was always blunted when performed during follow-up. Regenerative properties may have been overpowered by the effects of the diabetogenic calcineurin inhibitors administered to the monkey. Also, regeneration did not spontaneously take place, at least at a detectable pace, because STZ-treated nontransplanted monkeys continued to need insulin injections after the induction of diabetes. In contrast, in the absence of diabetogenic immunosuppressive agents, using instead an anti-CD154 monoclonal antibody to block the recipient’s immune rejection (13), the monkeys transplanted with DKO pig islets not only produced pig C-peptide but eventually (more than 3 months after STZ treatment) recovered the ability to produce monkey C-peptide. New insulin-producing cells are appearing with time in the monkey’s endogenous pancreas, eventually forming islet-like conglomerates of cells.

If regeneration can occur not only in rodents but also in the monkeys, we can also expect the endocrine tissue to regenerate in humans once autoimmunity has properly and successfully been abrogated. To determine whether monkey C-peptide evaluation is a reliable marker for regeneration we compared different species, diabetic versus non-diabetic monkeys and tested pig versus monkey C-peptide in transplanted diabetic monkeys.

1. Comparison among species

Fasting C-peptide, blood glucose, insulin and glucagon values in monkeys, pigs and humans are presented in **Figure 1**. Blood glucose in fasting non-diabetic monkeys ranges from 39 to 74 mg/dL and appear to be significantly lower than the corresponding values in pigs (72-94 mg/dL) ($p < 0.001$). Mean serum C-peptide and insulin values in monkeys are instead 10 times higher than in pigs (C-peptide 4.23 ± 0.29 ng/mL in monkeys versus 0.49 ± 0.12 ng/mL in pigs $p < 0.001$; insulin 0.61 ± 0.07 ng/mL in monkeys versus 0.05 ± 0.01 ng/mL in pigs $p = 0.01$). Fasting glucagon values were higher in Cynomolgus monkeys compared to pigs and humans (**Figure 1**).

Notably, human blood glucose values appear to be higher than monkey values, even after correcting them for the different type of sample tested (i.e. whole blood in monkeys and plasma in humans) (14-16).

Consistently, human C-peptide is lower than monkey C-peptide and it resembles more closely pig C-peptide ranges. Instead, insulin values in humans are as high as those in Cynomolgus monkeys. The ratio insulin/C-peptide is approximately 2.5 in humans while it is approximately 10 in monkeys and pigs. The difference of blood glucose, C-peptide, insulin and glucagon values between monkeys and pigs are even more evident after i.v. glucose stimulus (Figure 2).

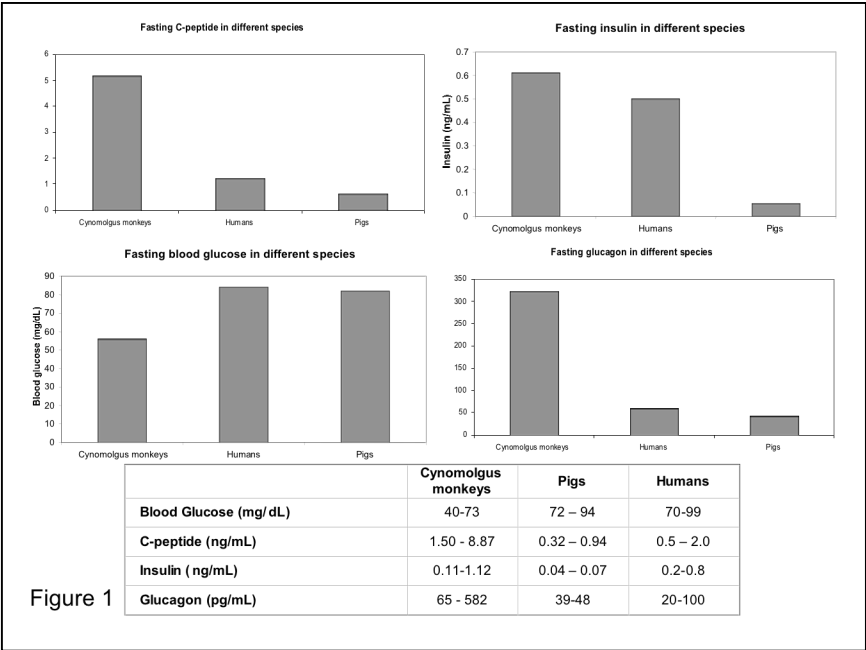


Figure 1

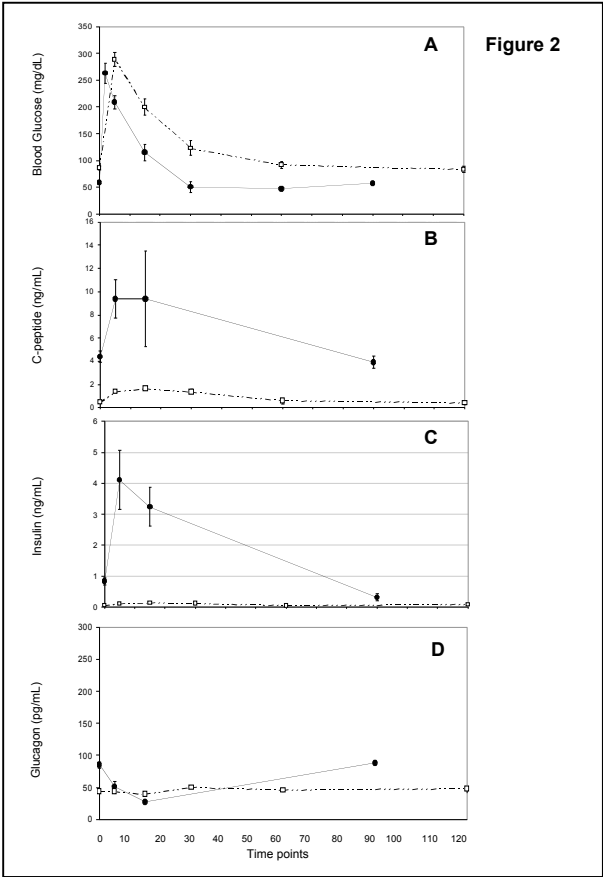


Figure 2

Figure 2: Metabolic parameters during IVGTT in non-diabetic monkeys and non-diabetic pigs. Mean $\pm$ SE of blood glucose (A), C-peptide (B), insulin (C) and glucagon (D) during IVGTT in non-diabetic monkeys (solid lines) and non-diabetic pigs (dashed lines). SE in pigs are too small to be seen in the graph. Monkey blood glucose values decrease faster than pig values. C-peptide and insulin response to IVGTT is higher and more pronounced in monkeys compared to pigs.

However, the C-peptide (Figure 2B) increase ratio at 5 and 15 minutes is higher in pigs, being 2.8 and 3.3 times the fasting value compared to respectively 2.1 and 2.1 in monkeys. The corresponding ratios in humans are 3.2 and 2.1 (17).

The mean increase ratio for insulin at 5 and 15 minutes was instead higher in monkeys, being respectively 5.0 and 4.0 times the basal values, while in pigs was 2.0 and 2.3 (Figure 2C). The corresponding ratios in humans are 1.8 and 2.0 (17).

Insulin secretion is able to rapidly control blood glucose values in both monkeys and pigs, as shown in Figure 2A. However, blood glucose drops slower in pigs than monkeys as indicated by lower K_G values (K_G in monkeys 3.27-8.22 [mean 6.17], K_G in pigs 2.75-6.70 [mean 3.64]). Glucagon drops at 15 minutes with the lowest point at 30 minutes (Figure 2D), and this drop is more pronounced in monkeys than in pigs.

Data from the literature show that human response to i.v. glucose challenge lays always between the pig and the monkey response for both C-peptide and insulin (17).

2. Diabetic versus non-diabetic monkeys

In Figure 3, the same tests are shown for normal versus diabetic monkeys.

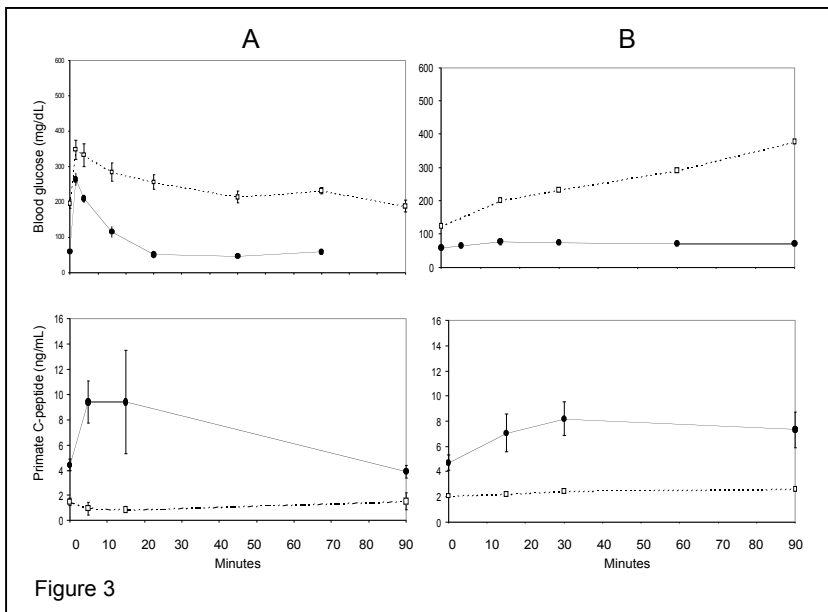


Figure 3: Metabolic tests in non-diabetic and diabetic monkeys

Mean $\pm$ SE of blood glucose levels (top panels) and primate C-peptide levels (bottom panels) during IVGTT (A), OGTT (B), AST (C) and MMT (D) in normal (solid line) and diabetic monkeys (dotted line). SE were not calculated when less than 3 measurements were available (3C). C-peptide response to physiological stimuli is completely abolished in diabetic monkeys and this is accompanied by rise of blood glucose levels.

During IVGTT (Figure 3A), the rise in glucose was higher in diabetic monkeys compared to non-diabetic monkeys with a peak of 347 ± 27 mg/dL 2 minutes after glucose infusion. The glucose slowly decreased never reaching the normal values, as expected. K_G in diabetic animals was lower compared to normal animals, ranging from 0.6 to 1.5. C-peptide response was absent with a drop 5 and 15 minutes after the stimulus.

During OGTT (Figure 3B) for healthy monkeys, the rise in blood glucose was minimal (mean 77 ± 8 mg/dL at 15 minutes). Insulin and C-peptide rose at 15 minutes and reached a maximum at 30 minutes (insulin 3.32 ± 0.65 ng/mL and C-peptide 8.20 ± 1.37 ng/mL respectively). The curve shape of the glucagon response is the mirror-image of the C-peptide and insulin response with the lowest point at 30 minutes. C-peptide and insulin responses in humans are comparable to those detected in Cynomolgus monkeys (18) in terms of fold of variation.

OGTT in diabetic monkeys (Figure 3B) showed a progressive increase of blood glucose reaching the highest value 90 minutes after oral administration of glucose. No response of C-peptide was measured.

During AST for healthy monkeys (Figure 3C), the blood glucose remained stable while C-peptide, insulin, and glucagon values rose at 2 minutes and then returned to pre-stimulus values at 5 minutes. AI_{Arg} ranges from 0.3 to 1.9 ng/mL and ACR_{Arg} ranges from 0.6 to 2.7 ng/mL. Human AI_{Arg} (2.38 - 3.08 ng/mL) is higher than Cynomolgus monkeys AI_{Arg} , while ACR_{Arg} (1.00 - 1.40 ng/mL) is similar to that in Cynomolgus monkeys (17,19). In diabetic monkeys, no changes of blood glucose were seen during AST. Blood glucose values, in fact, remained stable around 250 mg/dL. The response of C-peptide to arginine was absent (and the ACR_{Arg} ranged between -1.12 and 0.06 ng/mL) with a drop after stimulus.

During the MMT (Figure 3D) in healthy monkeys, the blood glucose was stable and the C-peptide and insulin rose slowly with a peak at 120 minutes after the meal (10.2 ± 2.9 ng/mL). Similarly, insulin significantly rose 60 minutes (2.63 ± 0.75 ng/mL) and it reached a plateau?peak 120 minutes after the meal (3.55 ± 1.47 ng/mL). In humans, similar insulin and C-peptide values are obtained, however if we consider in term of fold increase the response is stronger because it starts from lower basal values (17,18,20). Data from the literature show that the response to MMT in pigs is at least 2 folds higher than in monkeys even if the absolute values are lower (21) showing that oral stimuli are more potent than glucose in pigs than in monkeys. In diabetic monkeys, the blood glucose reached its highest value (441 mg/dL) 120 minutes after the meal. No response of C-peptide was seen.

In summary, low levels of fasting endogenous C-peptides are measured in diabetic monkeys corresponding to 12 to 33% of the C-peptide levels pre-STZ. This C-peptide is obviously not able to keep the monkey blood sugar within the normal range as shown by the high blood glucose values reached when insulin therapy was stopped and by the needs of insulin to prevent DKA and maintain blood glucose below 200 mg/dL. Any residual endogenous C-peptide did not respond to physiological stimuli, as shown by the results of the dynamic tests and by the absence of correlation between endogenous C-peptide level and blood glucose level at the time of sampling. No correlation was found between endogenous C-peptide levels and mean daily requirement of exogenous insulin per kg, while attempting to maintain blood glucose below 200 mg/dL (data not shown).

3. Transplanted monkeys

Following porcine islet transplant, 5 monkeys improved their metabolic control (Table 1). This was defined as: exogenous insulin therapy ≤ 0.50 IU/Kg/day and average blood glucose < 160 mg/dL, lower than the levels before transplant in spite of the reduction of insulin dose. In two monkeys, insulin therapy was completely stopped for 34 and 16 days.

The mean porcine C-peptide in these five monkeys was greater than 0.6 ng/dL (Case 8 mean porcine C-peptide 0.64 ng/mL, Case 2 mean porcine C-peptide 0.80 ng/mL, Case 7 mean porcine C-peptide 2.82 ng/mL, Case 9 mean porcine C-peptide 0.97 ng/mL, Case 10 mean porcine C-peptide 0.86 ng/mL).

Figure 4A shows the porcine C-peptide levels in monkeys with improved metabolic control compared to those that did not experience any improvement. Notably, Case 1 was borderline for the criteria of improved metabolic control (blood glucose 197 mg/dL, insulin 1.38 IU/kg/day). This animal's mean porcine C-peptide level was 0.56 ng/dL.

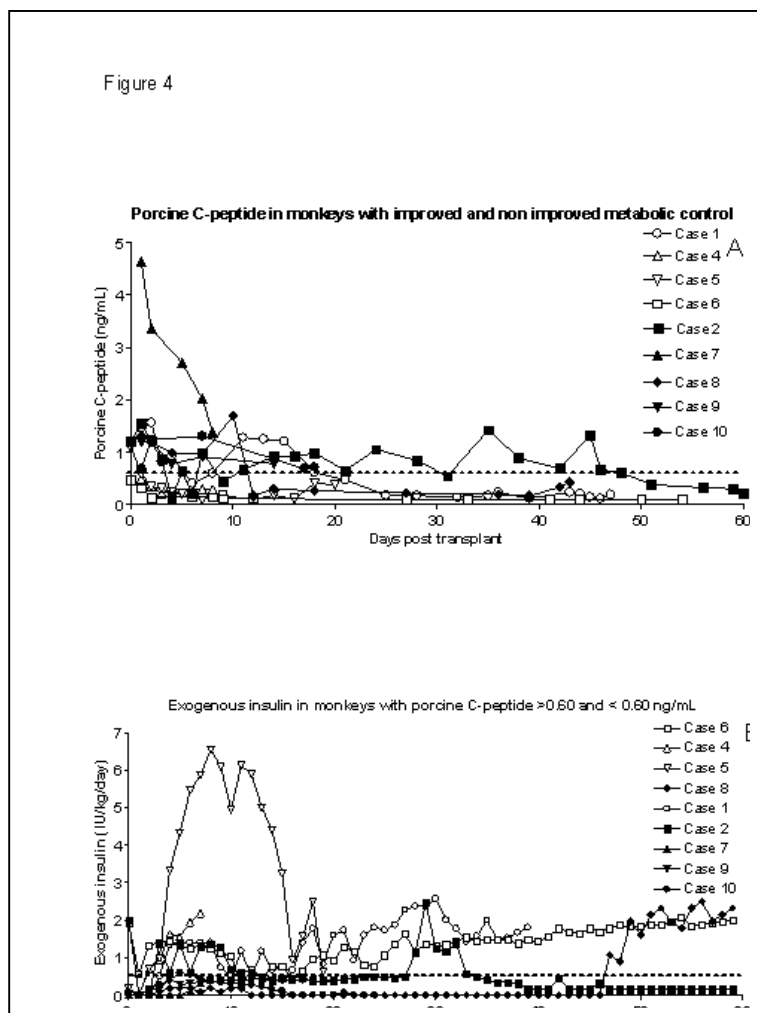


Figure 4: Porcine C-peptide level and Insulin requirement in transplanted monkeys.

A: Porcine C-peptide levels in transplanted monkeys with improved metabolic control (filled symbols) are higher than those detected in monkeys without improvement in metabolic control (empty symbols). C-peptide in monkeys with improved metabolic control is mostly above 0.60 ng/mL.

B: Insulin requirement in transplanted monkeys with mean porcine C-peptide above 0.6 ng/mL (filled symbols) is lower than that in monkeys with mean porcine C-peptide below 0.6 ng/mL (empty symbols). Insulin requirement in monkeys with mean porcine C-peptide above 0.6 ng/mL is mostly below 0.5 IU/kg/day.

Table 1: Mean metabolic values before STZ, during diabetic state and after porcine islet transplant.

Monkey	Before STZ		AFTER STZ – TIABETIC MONKEYS				After islet transplant					
	Mean BG mg/dL	Primate C- pept ng/mL	Mean BG mg/dL	% above 200 mg/dL	Mean insulin IU/kg/day	Primate C-peptide ng/mL	Mean BG mg/dL	% above 200 mg/dL	Mean insulin IU/kg/day	Primate C- peptide ng/mL	Porcine C- peptide ng/mL	Follow up after Tx
Case 1	62	3.26	217	55	2.67	0.50 (15%)	197	46	1.38 (52%)	1.13	0.56	39
Case 3	57	4.74	236	77	2.17	1.33 (28%)						
Case 4	39	3.02	207	47	1.03	1.00 (33%)	170	27	1.28 (124%)	0.74	0.34	8
Case 5	53	2.65	184	51	0.58	0.68 (26%)	176	35	3.05 (521%)	<0.10	0.22	19
Case 6	60	-----	235	73	0.75	2.58	284	76	2.20 (293%)	1.43	0.11	120
Case 2	83	4.24	192	48	2.25	0.50 (12%)	159	21	0.50 (22%)	0.71	0.80	60
Case 7	70	0.51	241	59	0.46	0.99	122	7	0.06 (13%)	0.98	2.82	8
Case 8	71	3.49	164	32	0.96	0.73 (21%)	95	0	0.04 (4%)*	1.21	0.64	43
Case 9	52	6.78	147	23	0.80	1.79 (26%)	154	7	0.35 (44%)	2.06	0.97	21
Case 10	54	6.61	232	63	1.04	1.52 (23%)	123	7	0.15 (14%)*	1.92	0.86	30

Primate C-peptide after STZ is expressed as raw values and, in parenthesis, as percentage of the basal non-diabetic value. Mean insulin dosage after transplant is expressed as a raw value and, in parenthesis, as percentage of the dosage before transplant.

Monkey CT5B was not transplanted because of intolerance to immunosuppressive drugs.

* Insulin therapy was stopped 2 weeks after porcine islet transplant in Case 8 and Case 10.

A complementary view of the same phenomenon is shown in **Figure 4B**, where the insulin requirement after porcine islet transplantation in the same monkeys is presented. The insulin requirement of the monkeys, who had a mean porcine C-peptide >0.60 ng/dL, was well below the insulin requirement in monkeys that did not show any improvement of metabolic control. Exception to this rule is Case 8. Even if its porcine C-peptide values dropped after 14 days post transplant to values below 0.60 ng/mL, it did not need any exogenous insulin and its blood glucose was well below 150 mg/dL (mean 95±3 mg/dL) until day 48 when the animal experienced sudden metabolic decompensation.

Insulin requirement was reduced to less than half of the requirement before transplant in the monkeys that experienced an improvement in metabolic control (Case 2 22%, Case 7 6%, Case 8 4%, Case 9 44%, Case 10 14%), while it remained stable or even increased in those that did not (1.2 to 5.2 folds of increase), possibly due to the stress induced by the immunosuppressive therapy and the surgical procedure. Case 1, with a partial improvement (mean porcine C-peptide 0.56 ng/dL, mean blood glucose 197 mg/dL and mean insulin requirement 1.38 IU/kg/ day), had a reduction of insulin need to 52% of the pre-transplant therapy.

The monkeys with improved metabolic control, not only had lower insulin requirement, but also had a more stable glucose values as expressed by the reduction of hyperglycemic peaks. The prevalence of blood glucose above 200 mg/dL ranged between 23% and 63% before transplant while dropped to 0% to 21% after transplant.

Any residual endogenous C-peptide did not seem to have any effect on the metabolic control of the transplanted monkeys. Achievement of better metabolic control was in fact independent from the levels of residual endogenous C-peptide. The 5 monkeys that had an improvement of metabolic control had mean endogenous C-peptide of 1.92, 0.71, 2.06, 0.98 and 1.21 ng/mL, while those that did not improve had mean endogenous C-peptides of 1.43, 1.13, 0.74 and <0.1 ng/mL.

Three of the transplanted monkeys that experienced improved metabolic control, had a sum of C-peptide >2.5 ng/mL and all of them had sum of C-peptides within the range for non-diabetic monkeys. Even if all of the transplanted monkeys had sum of C-peptides within the range for non-diabetic monkeys, the primate C-peptide negative response to the metabolic challenges suggests that it doesn't contribute to the improvement. The concept that residual endogenous C-peptide does not take part in the metabolic control of the monkeys is also evident from the analysis of the IVGTTs performed after transplant in 4 of the monkeys with evidence of graft function. Even if none of these IVGTTs was completely normal (K_G ranges from 1.09 to 3.04, below the normal values even if higher than the diabetic response), a clear porcine C-peptide response was seen. Meanwhile the response of endogenous C-peptide was inexistent, with a drop after the stimulus as seen in diabetic monkeys.

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In our second quarterly scientific progress report, we presented evidence that if regeneration can occur not only in rodents but also in the monkeys, we can also expect the endocrine tissue to regenerate in humans once autoimmunity has properly and successfully been abrogated. While we were focused to determine whether monkey C-peptide evaluation is a reliable marker for regeneration comparing different species, diabetic versus non-diabetic monkeys and testing pig versus monkey C-peptide in transplanted diabetic monkeys, we discovered some properties of the C-peptide before disregarded yet possibly physiologically and therapeutically important.

In our third quarterly scientific progress report (12/01/06 – 02/28/06) for the first year of our award, we then reported interesting properties of the C-peptide.

Effect of glucose on microangiopathy and atherosclerosis

Both type 1 (T1D) and type 2 diabetes (T2D) are well-established risk factors for vascular diseases, including microangiopathy and atherosclerosis. Therefore, it is not surprising that diabetes is associated with multiple vascular complications, which account for the majority of deaths and disability in patients affected by diabetes (1,2). Hyperglycemia is considered an important etiologic factor in the development of these vascular complications (3,4). Several studies have focused on the direct effects of C-peptide on the vasculature, often with contradicting results.

The vascular lesions characteristic of human and experimental diabetes suggest a primary involvement of the endothelium and underneath smooth muscle cells. High glucose exerts a variety of adverse effects *in vitro* on several human endothelial cell lines. For example, short-term incubation with high glucose induces delayed replication and excess cell death in cultured vascular endothelial cells (5-7). High glucose also modulates expression of endothelial cell adhesion molecules and promotes binding of circulating monocytes to the endothelium, one of the first steps in atherosclerosis plaque formation (8-9). Circulating monocytes from T1D patients also show increased expression of adhesion molecules, which enhances their adhesive capacity to endothelial cells. Other effects of glucose to the endothelium include stimulation of reactive oxygen species (ROS) production (10) and secretion of inflammatory cytokines (11), both of which are toxic to endothelial cells.

Vascular smooth muscle cells are also affected by high glucose. Rat and porcine vascular smooth muscle cell lines proliferate in response to high glucose *in vitro* (12-16). One report also demonstrates hyperproliferation of a human aortic smooth muscle cell line under high glucose conditions (17). Proliferation of smooth muscle cells is a key process in atherosclerosis (18-20). Thus, an emerging strategy for the prevention or the treatment of vascular complications in diabetes is directed towards inhibition of smooth muscle cell proliferation.

The discovery of insulin has greatly improved the metabolic control of diabetes; however, vascular complications are still common among diabetic patients despite intensive insulin treatment and well-controlled glucose levels (21). One feature of both T1D and late stage T2D patients is a decreased plasma level of C-peptide (22). This peptide is cleaved from proinsulin and released from the pancreas into the circulation in amounts equimolar with insulin. C-peptide was initially believed to have no biological effects apart from its role in insulin biosynthesis. However, recent findings of the physiologic effects of C-peptide on a variety of cell types and beneficial effects of C-peptide replacement in patients with T1D have prompted renewed interest (23-25). Studies have indicated that in patients with T1D who lack endogenous C-peptide, the administration of C-peptide improves renal function (26-28), stimulates skeletal muscle microcirculation (29-31), and improves neuronal dysfunction (28,32,33). C-peptide could therefore represent a pivotal factor in preserving vascular integrity or in reversing vascular damage induced as a result of diabetes. In line with this idea is the evidence that T1D patients receiving pancreas and islet transplantations demonstrate benefits in reversal of diabetic complications as compared to patients who have not undergone transplantation and control their hyperglycemia with injections of insulin only (34).

While there seems to be concordance on the vasodilatory properties of C-peptide (31,35), it is still controversial whether C-peptide exerts pro-atherogenic effects on the vasculature (36,37) or vice versa. Other observations rather point to a protective role of C-peptide on vascular dysfunction in diabetes. For example, Kobayashi *et al.* (16) demonstrated that 3 days exposure to C-peptide inhibited high glucose-induced hyperproliferation of rat aortic smooth muscle cells. In addition, another group showed that C-peptide inhibits leukocyte-endothelium interaction *in vivo* in rat microvascular endothelium (38). To our knowledge, no studies are available investigating possible effects of C-peptide on high glucose-induced dysfunction of human vascular endothelial and smooth muscle cells. Therefore, in this study, we examined the effect of short-term exposure of C-peptide on glucose-induced apoptosis of human aortic endothelial cells (HAEC) and glucose-induced proliferation of human umbilical artery smooth muscle cells (UASMC) *in vitro*.

Effects of high glucose on apoptosis of HAEC

We found that exposure of HAEC to high glucose (25mmol/l) for 48 hours resulted in a significant increase in cellular apoptosis ($p=0.002$ versus 5.6mmol/l-treated cells). HAEC treated with 25mmol/l mannitol (as osmotic control) for 48 hours failed to induce HAEC apoptosis (Figure 1A).

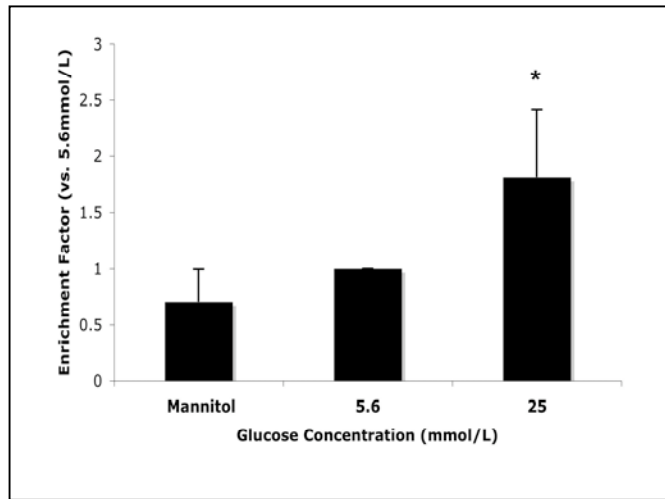


Figure 1A. Induction of apoptosis of human aortic endothelial cell (HAEC) after exposure to high glucose. HAEC were treated with normal glucose (5.6mmol/l), high glucose (25mmol/l) glucose, and high mannitol (25mmol/l) (osmotic control) at 37°C, 5% CO₂ for 48 hours. Apoptosis was assessed by using an ELISA kit (Roche), which evaluates cytoplasmic histone-associated DNA fragments. A specific enrichment of mono- and oligonucleosomes released in the cytoplasm (enrichment factor) is calculated based on the ratio between the absorbance value of the experimental sample and the absorbance of the negative control (cells incubated with 5.6mmol/l glucose). High glucose induced a significant apoptosis of HAEC compared to control cells in 5.6mmol/l glucose ($p=0.002$). Data are expressed as means $\pm$ SD of 4 independent experiments. Statistical analysis was compared with 5.6mmol/l glucose (* $p=0.002$).

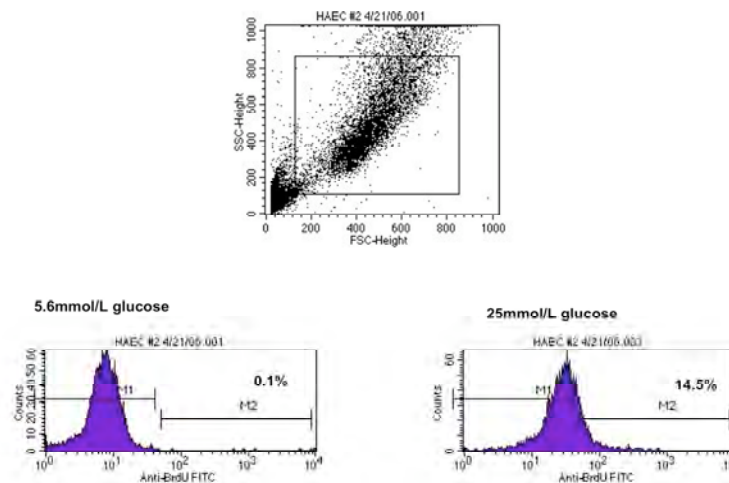


Figure 1B. Induction of apoptosis of human aortic endothelial cell (HAEC) after exposure to high glucose. HAEC were treated with normal glucose (5.6mmol/l), high glucose (25mmol/l) glucose, and high mannitol (25mmol/l) (osmotic control) at 37°C, 5% CO₂ for 48 hours. Here it is shown a representative example (of 2 independent experiments) of percent of apoptotic HAEC cultured for 48 hours in low (5.6mmol/l) and high (25mmol/l) glucose as detected by Br-dUTP incorporation and analyzed by flow cytometry. M2 indicates apoptotic population of HAEC.

Apoptosis of HAEC under high glucose was also confirmed by Br-dUTP incorporation analyzed by flow cytometry. Br-dUTP can be used as a marker of DNA strand breaks, which is a typical feature of apoptotic cells. We found that while the percent of apoptotic HAEC exposed to normal glucose (5.6mmol/l) was 0.1%, exposure to 25mmol/l of glucose increased percent of apoptotic HAEC to 14.5% (Figure 1B).

Effect of C-peptide on apoptosis of HAEC

As shown in **Figure 2**, administration of C-peptide for 48 hours significantly suppressed the high glucose-induced HAEC apoptosis. C-peptide significantly reduced apoptosis of HAEC at a dose of 1nmol/l ($p=0.002$), 0.5nmol/l ($p=0.004$), 0.3nmol/l ($p=0.016$), and 0.03nmol/l ($p=0.013$) compared to the high glucose control.

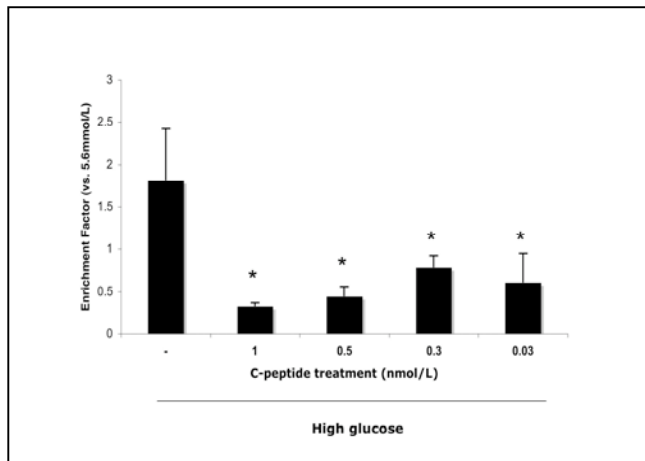


Figure 2. C-peptide reduces high glucose-triggered apoptosis of HAEC. HAEC were treated with high glucose (25mmol/l) glucose with or without C-peptide (1-0.03nmol/l) at 37°C, 5% CO₂ for 48 hours. Apoptosis was assessed by using an ELISA kit (Roche), which evaluates cytoplasmic histone-associated DNA fragments (see materials and methods). A specific enrichment of mono- and oligonucleosomes released in the cytoplasm (enrichment factor) is calculated based on the ratio between the absorbance value of the experimental sample and the absorbance of the negative control (cells incubated with 5.6mmol/l glucose). C-peptide significantly decreased HAEC apoptosis compared to cells in 25mmol/l glucose. Data are expressed as means±SD of 4 independent experiments. *represents significant values compared with 25mmol/l glucose.

Effect of glucose on proliferation activities of UASMC

In **Figure 3A**, we examined the influence of high glucose on UASMC proliferation (DNA synthesis) by measuring the nuclear incorporation of BrdU. We found that exposure of UASMC for 24 hours to (high) 25mmol/l glucose increased BrdU incorporation by 100% compared with control cells exposed to (low) 5.6mmol/l glucose ($p<0.000$). In accord with these results, when we counted the number of UASMC exposed to 25mmol/l glucose, we found an increase in cell number (42625 ± 9672) relative to control cells in 5.6mmol/l glucose (28000 ± 8337) ($p=0.001$) (**Figure 3B**).

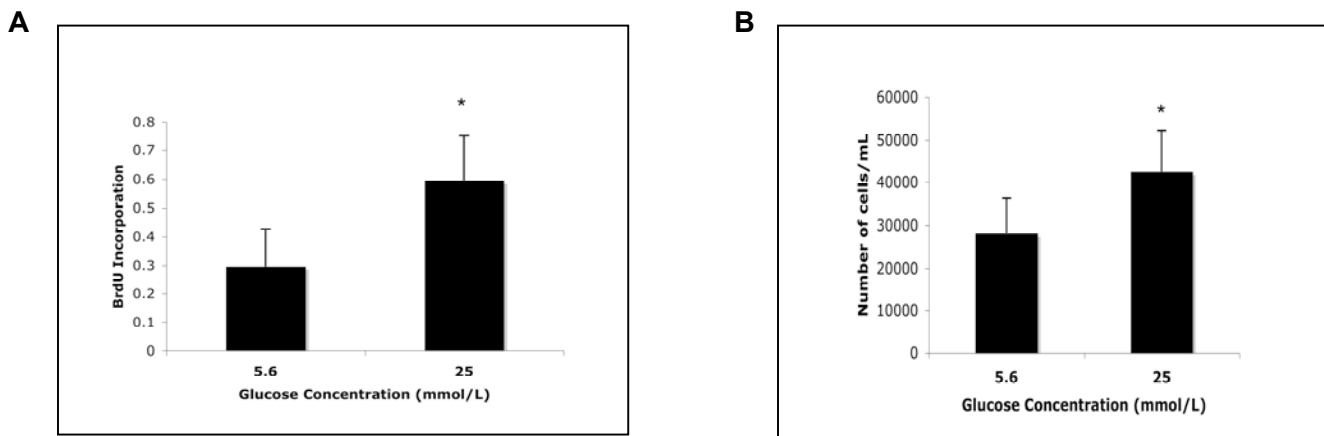


Figure 3. Umbilical artery smooth muscle cells (UASMC) proliferate in response to high glucose. In **A**, it is shown that high glucose (25mmol/l) stimulates BrdU uptake in UASMCs after 24 hours incubation ($p<0.000$ versus control in 5.6mmol/l glucose). Values are means±SD of 10 different experiments. Each experiment was run in triplicate. A similar result was obtained when counting UASMC exposed to 25mmol/l for 24 hours (**B**). The increased number of UASMC exposed to 25mmol/l was statistically significant compared to 5.6mmol/l glucose ($p=0.001$). Values are means±SD of 10 different experiments. * represents significant difference compared to 5.6mmol/l glucose.

Effect of C-peptide on proliferation of UASMC

As shown in **Figure 4**, administration of C-peptide for 24 hours significantly suppressed the high glucose-induced increase in BrdU incorporation in UASMC at a dose of 0.5 (decrease of 20%) ($p=0.022$), 0.3

(decrease of 26%) ($p=0.006$), and 0.03nmol/l (decrease of 29%) ($p=0.008$) compared to cells kept in high glucose only.

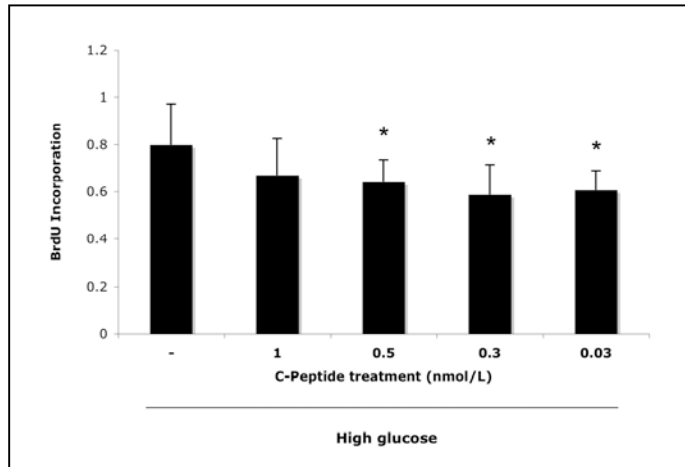


Figure 4. C-peptide reduces BrdU uptake in UASMC exposed to high glucose. UASMC were incubated with high glucose (25mmol/l) either in the presence or in the absence of different doses of C-peptide (1, 0.5, 0.3, and 0.03nmol/l) for 24 hours. Values are means $\pm$ SD of 10 different experiments. Each experiment was run in triplicate. * represents significant difference compared to 25mmol/l glucose.

Similar results were obtained when UASMC were counted using an hemocytometer. Again, short-term exposure to C-peptide at a concentration of 0.5nmol/l ($p=0.049$), 0.3nmol/l ($p=0.000$), and 0.03nmol/l ($p=0.000$), significantly decreased number of UASMC compared to cells exposed to high glucose only in the absence of C-peptide (Figure 5). In contrast, C-peptide had no statistically significant effects on the proliferation activities of UASMC under normal glucose (5.6mmol/l) conditions (not shown).

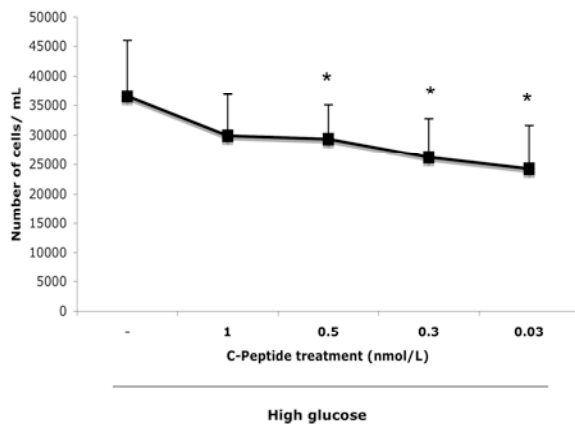


Figure 5. C-peptide reduces the number of UASMC exposed to high glucose. The number of UASMC cultured in high glucose (25mmol/l) with or without different doses of C-peptide (1, 0.5, 0.3, and 0.03nmol/l) for 24 hours. At the end of incubation, UASMC were harvested and counted using an hemocytometer. C-peptide administration significantly reduced the number of UASMC compared to 25mmol/l glucose only. Values are means $\pm$ SD of 10 different experiments. * represents significant difference compared to 25mmol/l glucose only.

As an additional indication of cellular proliferation, we performed immunofluorescence staining to determine presence of Ki67⁺ cells exposed to normal (5.6mmol/l) and high glucose (25mmol/l) in the absence or in the presence of C-peptide (0.5nmol/l) for 24 hours (Figure 6A-C). By determining the Ki67-labeling index of UASMC under the different experimental conditions, we found presence of a significant reduction when cells were exposed to 25mmol/l glucose (26.8% $\pm$ 2.91) in the presence of 0.5nmol/l C-peptide (19.8% $\pm$ 2.88) ($p=0.02$) (Figure 7).

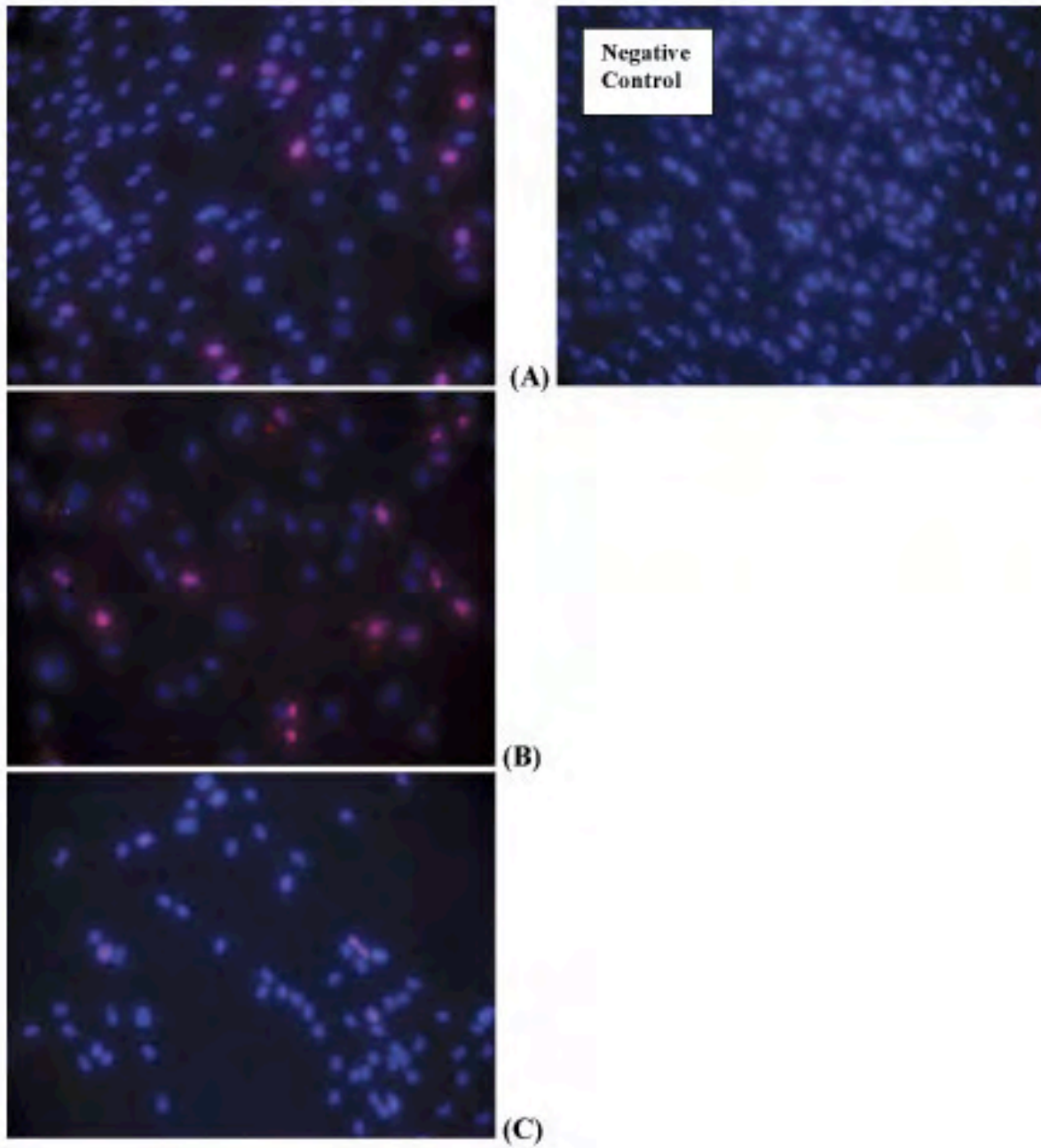


Figure 6. Ki67-immunostaining of UASMC exposed to high glucose and C-peptide. Images of Ki-67-immunostaining (in red) in UASMC. Ki-67 is a marker of proliferating cells being expressed only in S, G₂ and M phase of the cell cycle, but is absent in G₀. Hoechst stain was used to stain the nuclei (in blue). (A) UASMC cultured for 24 hours in 5.6 mmol/l glucose, (B) UASMC cultured for 24 hours in 25 mmol/l glucose, and (C) UASMC cultured for 24 hours in 25 mmol/l glucose + 0.5 nmol/l C-peptide. UASMC were incubated for 24 hours for all conditions. C-peptide addition to the high glucose medium reduced the number of Ki67-positive cells compared to high glucose alone.

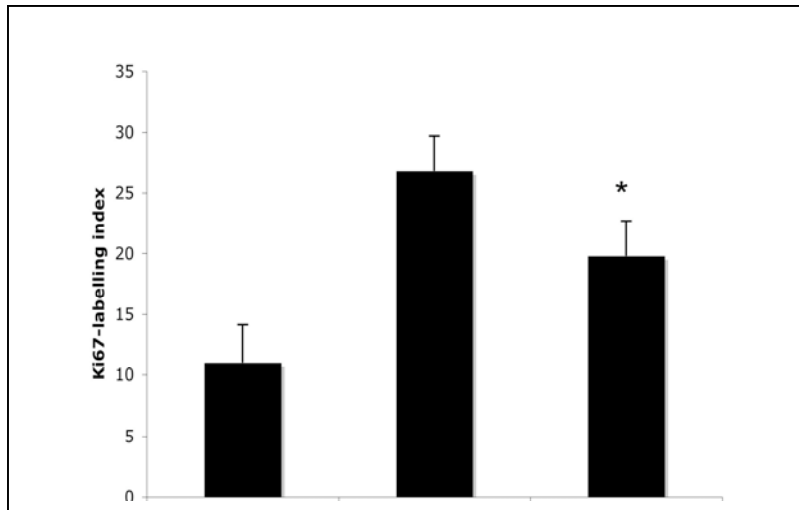


Figure 7. Ki67-labeling index of UASMC exposed to high glucose in the presence of C-peptide. Quantitation of Ki67-positive cells in UASMC exposed to basal (5.6mmol/l), high (25mmol/l), and high glucose in the presence of 0.5nmol/l of C-peptide for 24 hours. Addition of C-peptide at 0.5nmol/l significantly reduced the number of proliferating cells ($p=0.02$). *represents significant difference compared to 25mmol/l glucose. Four different experiments were performed.

Unique to this work was our finding that short-term exposure to C-peptide protects from high glucose-triggered apoptosis of HAEC. This was demonstrated by a significant decrease in apoptosis of endothelial cells cultured for 48 hours in a high glucose ambience together with a range of C-peptide concentrations *in vitro*. We found that C-peptide was able to significantly prevent glucose-induced apoptosis of HAEC within a range of concentrations of 1-0.03nmol/l. This result supports other findings showing an anti-apoptotic effect of C-peptide in several cellular systems. For example, using the neuroblastoma SH-SY5Y cell line, Li *et al.* demonstrated that C-peptide decreased high glucose-induced apoptosis (25). The same group also reported a protective effect on hippocampal neuronal apoptosis in the spontaneously T1D BB/Wor rats (25). C-peptide has also been shown to protect kidney proximal tubular cells from Tumor necrosis factor(TNF)-alpha-induced apoptosis (39). Another evidence of the anti-apoptotic effect of C-peptide is on isolated human islets, as reported by Bugliani *et al.* (40). All together, these findings support the hypothesis that C-peptide has a cytoprotective effect on several cellular models, endothelium included.

The mechanisms underlying the effects of C-peptide on the human vasculature are still largely unknown. There is evidence that C-peptide binds to specific, yet unidentified cell surface receptors and stimulates intracellular signaling processes, such as Na-K-ATPase or mitogen activated protein kinase (MAPK) (41,42). This latter signaling pathway has been shown to mediate suppression of the proliferation of a rat smooth muscle cell line under chronic C-peptide exposure (16). It is not clear whether this pathway is also affected by short-term C-peptide exposure in human smooth muscle cells. Riegler (43) has shown that C-peptide binds to specific G-protein-coupled-receptors on human saphenous vein endothelial cell membrane. However, these studies have been recently disputed by more recent findings suggesting that C-peptide may bind to insulin receptor itself at a different ligand site (44). This is consistent with C-peptide signaling by ways of the insulin signaling pathways. Here, our results are in line with those of Kobayashi *et al.* (16) demonstrating that C-peptide has an opposite effect to that of insulin on the growth of smooth muscle cells. While insulin stimulates the growth of smooth muscle cells, C-peptide inhibits it. Further studies are required to address these questions.

Diabetic patients have an increased risk of developing atherosclerosis compared to the non-diabetic population. In diabetic patients with atherosclerotic coronary artery disease, vascular lesions are particularly marked by exuberant vascular smooth muscle cell proliferation. Proliferating smooth muscle cells migrate from the media into early atherosclerotic lesions where they secrete pro-inflammatory mediators, up-regulate cell adhesion molecules, and promote synthesis of matrix molecules required for the retention of lipopoliproteins (19). There is evidence suggesting that smooth muscle cells may also be important for the stability of the atherosclerotic plaque through a formation of a firm fibrous cap (45).

Thus far, we have been able to demonstrate that short-term exposure to C-peptide inhibited hyperproliferation of human UASMC induced by high glucose *in vitro* while reducing glucose-promoted

apoptosis of endothelial cells. Evidence of the inhibitory effect of C-peptide on the proliferation of UASMC included a decrease in BrdU uptake, a reduced number of cells, and a decreased expression of the nuclear antigen Ki67, a marker of cellular proliferation, in cultures of UASMC exposed to C-peptide. Importantly, the effect of C-peptide on UASMC was independent of that of insulin, since insulin was not included in the culture medium.

We found that C-peptide produced significant effects on proliferation of human UASMC at a concentration of 0.5, 0.3, and 0.03nmol/l. These concentrations are within the range of the low physiological C-peptide concentrations (0.9nmol/l) reported by different groups (41,46,47). Although this evidence in human UASMC is reported here for the first time, a suppressive effect of C-peptide on proliferation of smooth muscle cells has been described in a different model. Using a rat aortic smooth muscle cell line, Kobayashi *et al.* (16) found that C-peptide reduced the growth of muscle cells induced by high glucose. Our and Kobayashi results support the view that C-peptide may exert important direct physiological actions on vascular smooth muscle cells and is not just the products of beta-cell function.

In this context, our findings of a suppressive effects of C-peptide on glucose-induced proliferation of smooth muscle cells strengthen the idea that administration of C-peptide to diabetic patients, who lack endogenous C-peptide, and who are at risk of developing vascular disease could be beneficial as C-peptide targets an important pathological change in the atherosclerotic vessel. This protective effect of C-peptide on the vasculature is also important in view of our findings of an increase adhesive capacity of circulating monocytes of T1D patients to vascular endothelial cells. C-peptide could reduce the risk of diabetes vascular complications by both acting on the vasculature and reducing the inflammatory cellular infiltrate that constitute the early developing atherosclerotic plaque. A beneficial effect of C-peptide replacement therapy has already been demonstrated by clinical studies on T1D patients where C-peptide has been shown to ameliorate chronic myocardial, renal, and neuronal complications (10,26-28).

In conclusion, the present study shows that C-peptide has major physiological effects on human vascular endothelial and smooth muscle cells. While C-peptide inhibits apoptosis of endothelial cells, it suppresses hyperproliferation of smooth muscle cells under high glucose conditions. These are two major components of vascular dysfunction during atherosclerosis in diabetes. Therefore, these findings support the idea that C-peptide has a biological function on human vasculature especially in conditions of diabetes insult of the vasculature.

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In the fourth and final quarterly scientific progress report (3/01/07 – 05/30/07) of Year 01 we now report on direct evidence of regeneration in the monkey tissue.

Metabolic evolution of islet recipients.

The metabolic evolution and the C-peptide levels (graft and endogenous) of the monkey recipients of porcine islet grafts that exhibited a partial function after transplantation are shown in **Figure 1**. To note, raising levels of endogenous C-peptide were measured after transplantation but at the time the graft function begun to fail, such endogenous production appeared to take over and it was likely responsible for the maintenance of a stable metabolic control. In monkey recipients of islet graft that failed immediately after implant, or in monkeys sacrificed shortly (within 10 days after transplantation) therefore without sustained graft function, no stable increase in endogenous production was observed. In one monkey where the graft had a partial function, a trend to raising endogenous C-peptide production was noted but after only two weeks graft failed, hyperglycemia returned and also endogenous C-peptide dropped.

Histological analysis of the pancreatic tissue following re-establishment of the endogenous production of C-peptide.

Proliferative activity in the pancreas. Ki67 immunostaining

Pancreatic tissue was immuno-stained with anti-Ki67 antibody, a nuclear marker of active proliferation. Normal, healthy control pancreatic tissue contained occasional Ki67 positive cells, scattered throughout the entire organ. The incidence of positive cells was quantitatively similar in streptozotocin-diabetic, non-transplanted, as well as in transplanted monkeys in which the islet graft had stopped functioning. On the contrary, substantial increased numbers of positive cells were observed in the monkeys that had had a functioning graft and showed return of endogenous C-peptide production. Further analysis demonstrated that Ki67 positive cells could be co-stained with the ductal marker cytokeratin-19 (CK19) or with markers of fibroblast and endothelial phenotypes, but never with insulin positive cells, suggesting that proliferation was not occurring in phenotypically mature beta cells, either pre-existing or newly generated beta cells (Figure 2).

Figure 1

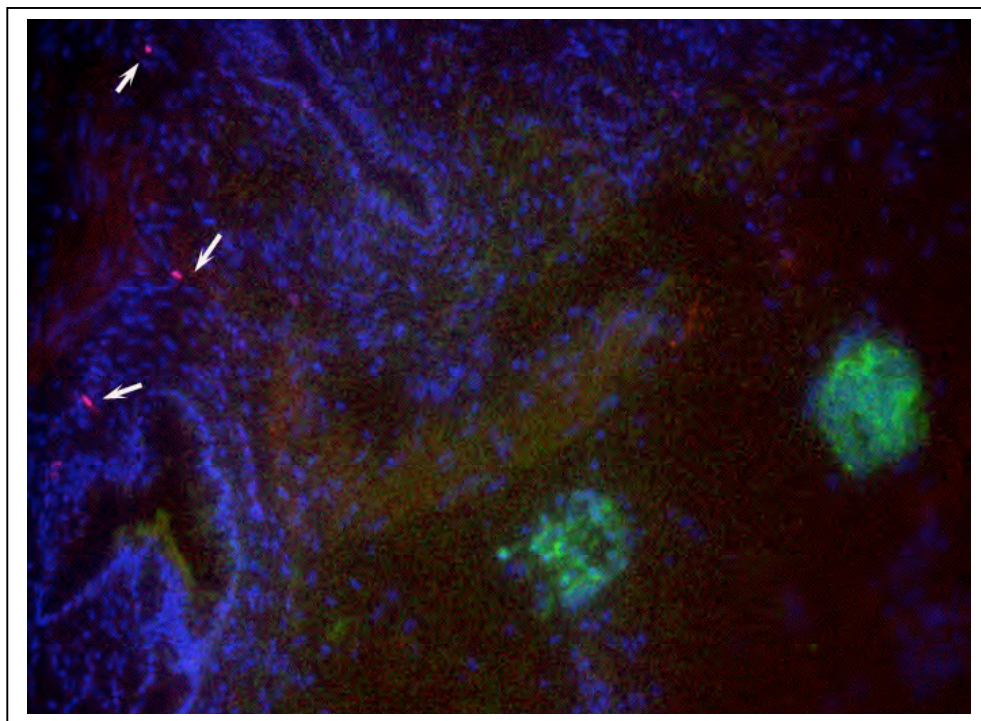
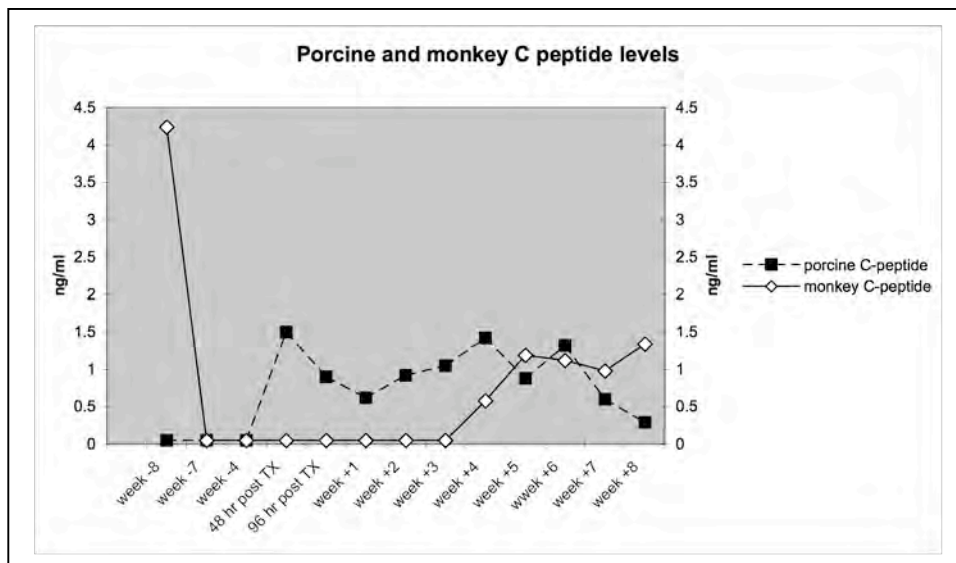


Figure 2 Ki67 in red, Glucagon in green.

Insulin/Proinsulin immunostaining

The pancreas of these monkeys that re-establishment endogenous C-peptide production, was analysed for the presence of insulin producing cells. Insulin positive cells were found throughout the entire pancreatic section, mostly as single cells, not organized in large, islet-like clusters, except for rare small oligo-cellular aggregates. No clear information was obtained whether these cells existed before streptozotocin treatment or, on the contrary, appeared after injury could be accrued. From a speculative point of view, whilst fully formed, streptozotocin-treated islets are typically positive only for glucagon, small clusters were constituted mostly of insulin positive cells. To confirm that immunoreactivity with insulin antibody, and rule out the effect of phenomena of insulin absorption, we also stained the sections with a pro-insulin antibody that confirmed co-localization with previously insulin positive cells (**Figure 3**).

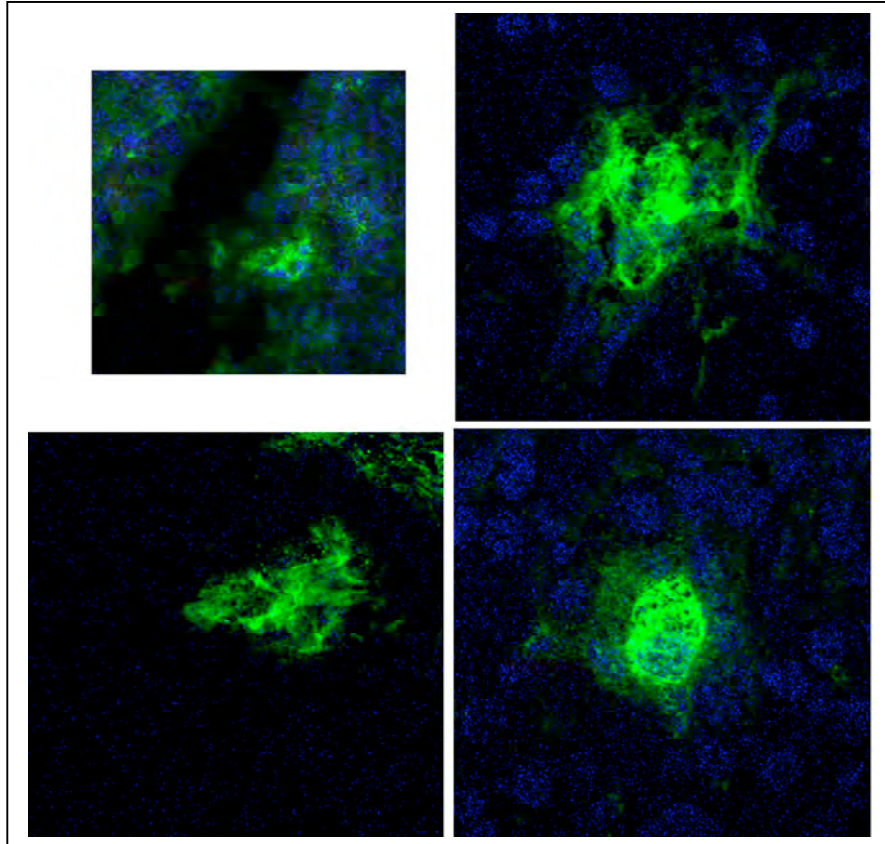


Figure 3

The distribution of insulin positive tissue in the pancreatic organ was also analysed. **Figure 4** show details of insulin/proinsulin positive cell aggregates in areas where glucagone positive cells are not present. The presence of such cell aggregates, positive exclusively for insulin was not reported on control diabetic monkeys or healthy, non diabetic pancreas control tissue. Since streptozotocin spares alpha cells, it is likely that insulin positive cells not associated to alpha cells are not part of a pre-STZ existing islet of Langerhans.

Glut-2 immunostaining

Glucagone and Glut-2 co-localize in the normal control monkey (see figure)

CK19 immunostaining

Anti-CK19 antibody staining was used to identify the ductal/epithelial cells in the pancreatic tissue. The pancreas of those monkeys that showed recovered endogenous production of C-peptide following transplantation, also showed higher numbers of CK19 positive cells in the pancreas compared to control, healthy and diabetic not transplanted monkeys. Interestingly, the monkeys with long-term function of the graft, associated to increased endogenous insulin production, showed a subpopulation of cells staining positive for CK19 as well as insulin. (**Figure 5**). No co-localization between alpha and beta phenotype (glucagon and insulin) was found.

Figure 4

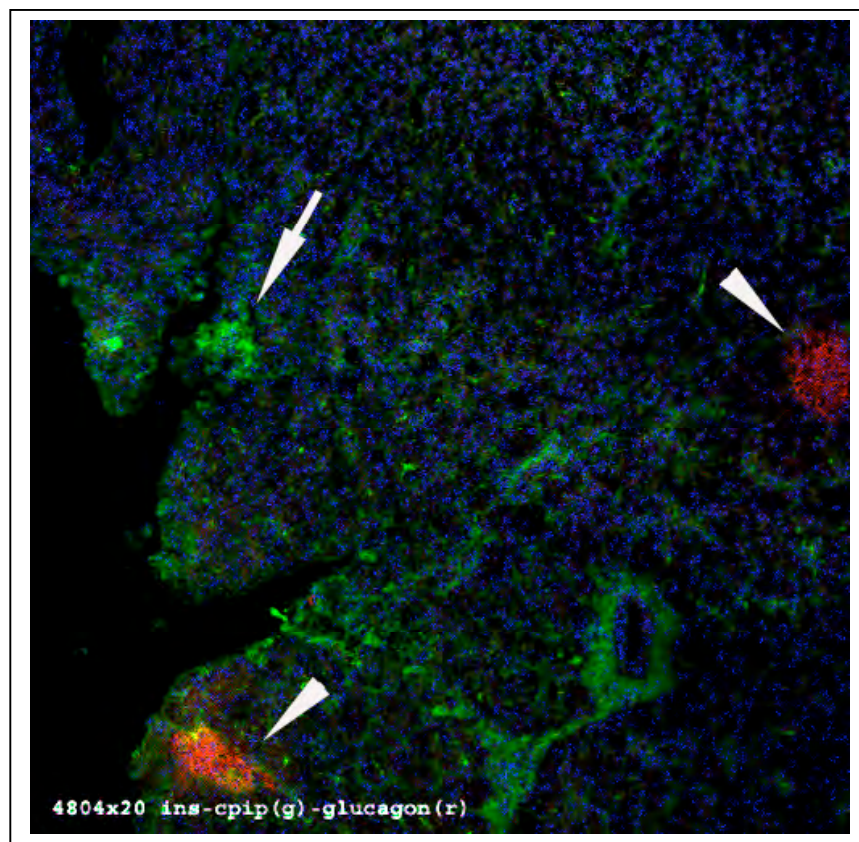
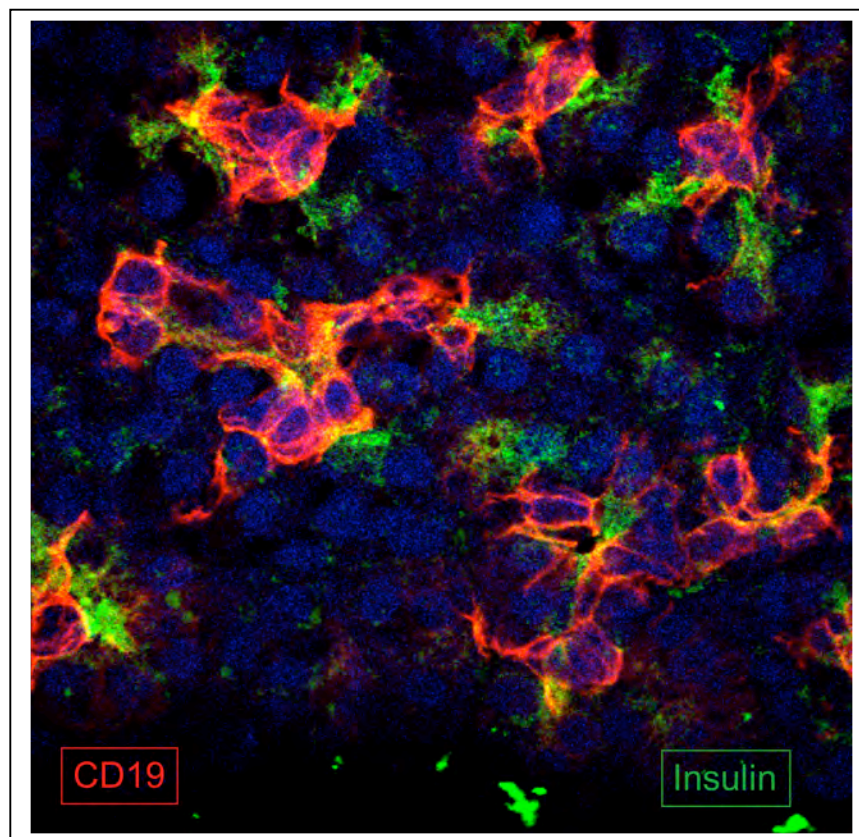


Figure 5



KEY RESEARCH ACCOMPLISHMENTS

- Streptozotocin successfully induces hyperglycemia following administration in rodents as well as in non-human primates.
- Diabetes induction is characterized by the establishment of a hyperglycemic status that, in non-human primates, requires exogenous insulin treatment to prevent fatal cheto-acidosis. Following streptozotocin administration, a drastic reduction of endogenous circulating C-peptide is observed, and histologic analysis of the pancreas shows virtual absence of insulin positive cells. Even in those monkeys where low but detectable C-peptide levels were measured after streptozotocin, histological findings confirmed the destruction of insulin positive cells, and in vivo functional studies suggested that any residual insulin producing mass is not responsive. We consequently observed that hyperglycemia and diabetes management were not influenced by the residual C-peptide amounts.
- No report of a spontaneous remission of the hyperglycaemic status in diabetic monkeys even months after streptozotocin administration was found. It appears therefore that in non-human primates, similarly to rodents, exogenous insulin itself does not trigger a healing process of the pancreatic beta cell mass, even in the absence of an autoimmune contest, thus after chemically-induced diabetes.
- On the putative source of cells that contribute to reform functional insulin producing tissue in the recipient, quite interesting speculation have been put forwards, however, in our experiments as well in those of other groups no direct evidence that an external, donor origin cell source is really needed, supporting the concept that the pancreatic organ can potentially heal itself. It remains scope for investigations the metabolic effect of the islet graft, that in addition to providing insulin, essential to maintain the animal alive, may further provide additional factors able to affect/trigger the recovery of endocrine function. In the absence of the autoimmune mechanisms (no evidence of autoimmunity is accrued in streptozotocin diabetic monkeys), our experiments suggest that diabetic monkeys that receive xenogeneic islet support with sustained detectable biological activity for sometime, they may recover a self-driven endocrine function. Such function is evidenced not only by stepwise increasing levels of endogenous C-peptide, but also by amelioration of the metabolic control, not simply explained by the effect of the graft. In this contest, the xenotransplantation model offers the advantage to allow discernment between graft and endogenous insulin production. In several monkey recipients, following transplantation we observed an increased in endogenous C-peptide levels, however only two recipients maintained graft function long enough and endogenous C-peptide substantial recovery occurred relatively late over time, consistent with the kinetics of recovery described in rodent models.
- This recovery of the endogenous insulin production was associated with quite peculiar histological features of the pancreatic tissue. We found a generalized augmented proliferative activity in the pancreatic tissue, spread throughout the organ and associated to fibroblast positive, ductal and endothelial phenotype, but not specifically to insulin positive cells. We also observed that in monkeys that exhibited endogenous production of C-peptide, pancreatic ducts were hypertrophic. Interestingly, substantial numbers of ductal cells (CK19+) showed also co-staining with proinsulin. More close analysis of the pancreas in the monkey that presented double CK19-proinsulin phenotype, indicated the presence of small size insulin positive cell aggregates. Such clusters appeared not even partially damaged, and differed morphologically from those that clearly showed regressed signs of injury, commonly associated with undamaged alpha cells (glucagon positive cells). Moreover proinsulin positive aggregates were often found not in correspondence of glucagon positive cells suggesting that they were not part of pre-existing islet of Langerhans.
- Since such effects were not found in monkeys that did not receive a transplant, and monkeys recipient of non functional graft, but partially present in monkeys with longer survival of the graft, we hypothesize that the transplantation may play a role. Whether a functional graft is exerting an effect on recovery, it remains unclear but in this line there are reports in human clinical islet allografts that indicate an amelioration of the metabolic conditions, often accompanied by increased endogenous C-peptide production, in patients that received a transplant, even if the graft was rejected or insufficient to establish normoglycemia.

CONCLUSION

Overall our data suggest that the monkey pancreas retains the ability to recover even after a chemical damage that specifically destroys the islet beta cells inducing a irreversible status of hyperglycemia. Such ability seems to be associated with activation of proliferative events in the pancreatic organ, and hypertrophy of the ductal cells. Double phenotype, epithelial and endocrine on the same pancreatic cells further indicate that a direct relation there exist between these two cell types. Recovery of the insulin producing function, in association with the histological findings may provide indirect supportive evidence that epithelial/ductal cells play a role in the generation of insulin producing beta cells. The metabolic enhancement achieved by even partially functioning islet transplantations compared to mere exogenous insulin administration may contribute to promote return of endogenous production of C-peptide and it is desirable to further investigate the multiple biological events that accompany engraftment. Further studies will clarify whether the reparative events involve ex-novo generation of endocrine tissue from pancreatic precursor cells, or, alternatively it involves the recovery of previously damaged cells. In both cases, considering that monkeys are the closest animal species to humans, useful information can be accrued on the dynamic properties of the endocrine pancreas in men.

APPENDICES

Wang Z, Zhu T, Rehman KK, Bertera S, Zhang J, Chen C, Papworth G, Watkins S, **Trucco M**, Robbins PD, Li J, Xiao X: Widespread and stable pancreatic gene transfer by AAV vectors via different routes. **Diabetes** 55:875, 2006.

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BODY:

Our first quarterly scientific progress report (06/01/07 – 08/31/07) for the second year of this project, detailed the following steps forward in reaching the aims of our study.

Lesions of the endocrine pancreas, as they occur in Type 1 Diabetes (T1D), were historically considered to be permanent and irreversible since patients, once endogenous insulin production is blunt, require hormone therapy for life. More recently it was proposed that islet beta cells actually retain the ability to heal from an injury, and to proliferate and multiply from precursors into mature beta cells during adult life, in a way similar to that during embryonic development. It was also proposed that possible reparative events affecting the beta cell mass normally occur but in diabetes are opposed by the destructive mechanisms that cause diabetes in first place such as the autoimmune attack (1,2).

In mouse models, evidence was presented that adult animals retain the ability to expand the beta cell mass as a response to various triggers. However, it remains largely unclear through which molecular and cellular mechanism(s) reestablishment of the beta cell mass takes place. Furthermore it was shown in the diabetic prone NOD-mouse that, even after establishment of the clinical diabetic status, it is still possible to recover endogenous pancreatic insulin production. This can be achieved by successfully combining strategies aimed at blocking the autoimmune attack with the use of non-diabetogenic immunosuppressive drugs. Normally an islet transplantation was used in parallel to supply beta cell function for the relatively long periods required to recover beta cell function (3-6).

A spontaneous recovery of the pancreatic beta cell function is also normally reported in patients diagnosed with autoimmune T1D, the so-called “honeymoon” period. Although the honeymoon may vary in duration quite dramatically from one patient to the next, there are also anecdotal cases of complete and permanent recovery. Following diagnosis of T1D and initiation of exogenous insulin treatment these individuals experienced a return of endogenous insulin production, characterized by increased C-peptide secretion, and reduction in the titre of circulating islet auto-antibodies, confirming the return of the immunologic tolerance and a consequent recovery from islet cell destruction (7). Although it is unquestionably proven that some beta cells are still present and able to produce insulin years after the clinical onset of diabetes (8,9), a steady recovery from the diabetic status is quite unique and is worth further investigations. At any rate, the potential for the pancreatic organ to recover substantial endocrine function is quite fascinating and should be exploited for possible clinical applications.

To this aim, the observation that only a limited islet mass is actively engaged in supplying insulin to maintain normoglycemia at a give time point is remarkable. This information seems to indicate that the critical mass required to synthesize and release insulin sufficient for the body’s normal needs is far less than that produced by the entire beta cell pool of a healthy pancreas. This observation implicates that even a relatively limited quantity of insulin producing tissue should exert clinically evident effects in patients.

Non-human primates are relevant animal models for pre-clinical studies in general and, in particular, for xenotransplantation experiments. Monkeys and humans present strong similarities although significant metabolic differences do exist. It is therefore of great value to investigate the potentiality of non-human primate pancreatic tissue to resume endogenous insulin production following its destruction, for a better understanding of the human behaviour as well.

In monkeys, a permanent diabetic status can be induced by total pancreatectomy, a major surgical procedure usually not devoid of technical difficulties or, alternatively, by chemical destruction of the insulin producing cells by means of streptozotocin, a potent toxic agent that specifically target the pancreatic beta cells (10).

Our experimental protocol for pig islet xenotransplantation into monkeys involved induction of diabetes in the recipients by streptozotocin, intra-portal transplantation of porcine islets after recipient immunologic pre-conditioning, completed by an adequate non-diabetogenic immunosuppressive therapy. While the majority of recipients exhibited a transitory islet graft function and were sacrificed early after transplantation, in some recipients’ islet graft function (indirectly assessed by measurable levels of porcine C-peptide) lasted for months

(11). At a certain point in time, concurrently to a gradual reduction of the C-peptide (porcine) graft output, we observed a stepwise increased in endogenous C-peptide (monkey) levels. Metabolic clinical improvement and peculiar histological features of the pancreatic tissue were also found associated to this change. To note, no spontaneous recovery of endogenous function was observed in transplanted monkeys with transient function of the graft or diabetic monkeys maintained under exogenous insulin for up to 1 year.

Our data provide evidence that the pancreatic tissue is able to re-establish endogenous insulin production after chemically induced beta cell specific injury. Additional investigation is required to understand whether conditions such as improved glucose metabolic control and an appropriate immunosuppressive regimen, in association with functional islet transplantation could play a role in beta cell rescue or regeneration, and if so, through which mechanism(s) (12).

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In the aim of completing the studies presented in the first year quarterly reports, we characterized the inflammatory response in diabetes by analyzing expression of a panel of activation markers on the surface of peripheral blood monocytes in recently-diagnosed Type 1 diabetes (T1D) patients. Compelling evidence implicates inflammation in the pathogenesis of T1D and associated vascular complications. Obesity is also characterized by a low-grade systemic inflammation. The potential role of glycemic control and of body mass index (BMI) on monocyte phenotype was then investigated by using flow cytometry to analyze the expression of CD11b, CD49d, CD54, CD62L, and CD64 antigens on monocytes in a cohort of 51 T1D patients (≤ 2 months from diagnosis).

We found that circulating monocytes from T1D patients tested at the clinical onset of the disease (i.e., within 1 week from diagnosis) had higher CD11b expression compared to patients analyzed after 2 months from diagnosis ($p=0.02$). The highest CD11b levels were detected in patients with $HbA_{1c} > 8\%$ ($p=0.04$ vs. patients with $HbA_{1c} < 8\%$). In T1D children analyzed after 2 months from diagnosis, we found that those overweight (BMI $\geq 85^{\text{th}}$ percentile) had higher levels of monocyte activation than those not overweight (BMI $\leq 85^{\text{th}}$ percentile) ($p=0.03$). CD11b and HbA_{1c} were significantly correlated (correlation coefficient 0.329, $p=0.02$).

From these studies we can conclude that circulating immune cells from T1D patients display many aspects of a proinflammatory state, as indicated by primed or activated monocytes. Obesity is an important factor in monocyte activation during diabetes.

Introduction

Compelling evidence demonstrates that components of the innate immune system, including natural killer cells (NK) and monocytes are involved in the autoimmune response characteristic of T1D both in humans and in the non-obese diabetic (NOD) mouse [1-4]. The primary role of monocytes in T1D has been demonstrated by showing that these cells are the first to accumulate in the pancreatic islets of prediabetic BB rats [5]. Subsequent T and B lymphocyte infiltration is dependent upon prior monocyte invasion of the islets [5]. These data suggest a role for monocytes in the early stages of T1D pathogenesis [6].

Monocytes are pivotal cells in inflammatory responses as they serve as the principal reservoir of pro-inflammatory cytokines and are the first cells to be engaged in nonspecific immune responses, such as those triggered by environmental factors. Recent studies reported evidence of increased monocytic activity, biomarkers of inflammation and oxidative stress in adult T1D patients well after the onset of diabetes [7,8]. In these patients, monocytes also released higher levels of pro-inflammatory cytokines as compared to non-diabetic subjects, suggesting presence of an inflammatory response in T1D. Interestingly, elevated circulating levels of the pro-inflammatory cytokine interleukin(IL)-8 were found in children with recent-onset T1D (<1year) who were also overweight [9]. Other evidence of monocyte involvement in T1D includes studies showing aberrant constitutive and lipopolisaccharide (LPS)-stimulated expression of monocyte cyclooxygenase (COX)-2 expression in monocytes of T1D patients, a defect which may predispose to a chronic inflammatory response in T1D [4,10]. The direct consequences of monocyte activation in T1D are unknown, but theoretically could involve release of pro-inflammatory cytokines, endothelial activation with increased monocyte adherence to the vascular endothelium, such as that of the pancreatic islets or kidney and retina capillaries.

During cellular activation, monocytes undergo phenotypic modifications with changes in expression of adhesion molecules on the cellular surface. These changes allow adhesion to the endothelial cells and movement of the monocytes through the endothelial layer toward inflammatory sites. The integrin Mac-1 (CD11b) is one of the most studied leukocyte adhesion molecules mediating tight binding to endothelial cells and migration through the vascular wall. While it is known that hyperglycemia, as seen in T1D, upregulates the expression of endothelial cell adhesion molecules [11-13], changes in adhesion molecule on the circulating monocytes have not been as well studied as those on the vascular endothelium. One report demonstrates increased expression of monocyte CD11b in adult T1D patients [14]. This increased CD11b expression was associated with higher monocyte adhesion to human aortic endothelial cells (HAEC) *in vitro* [14]. In contrast, other reports show either no increase [7,15] or increased binding of monocytes to endothelial cells compared to healthy subjects [16].

The mechanism involved in the recruitment of monocytes in the peri islet vasculature during diabetes is not fully understood. This is of particular importance in human subjects because it is not possible to directly follow the different stages of the islet inflammatory process. Changes that affect both the vasculature and the circulating monocytes in the early stages of T1D may play a crucial role in promoting leukocyte adherence to the endothelium and ongoing infiltration of the islets. Another factor that could affect monocyte activation in T1D patients is changes in body weight, as obesity has been associated with the presence of leukocyte abnormalities and inflammation [9,17,18]. In this study, we therefore investigated the potential role of glycemic control and body mass index (BMI) on monocyte expression of a panel of adhesion molecules in recently-diagnosed T1D children (≤ 2 months from diagnosis).

Results

Monocytes from new-onset T1D patients have an activated phenotype

Analysis of the mean fluorescence intensity (MFI) of the monocyte marker CD11b in children with T1D, showed a higher expression in those who were tested at clinical onset of diabetes (n=38; within 1 week from diagnosis) (878 ± 83 SEM; range 210-2403, median 698) compared to those tested at 2 months from diagnosis (n=13) (595 ± 132 SEM; range 180-1663, median 442), ($p=0.02$) (Figure 1 and Figure 2).

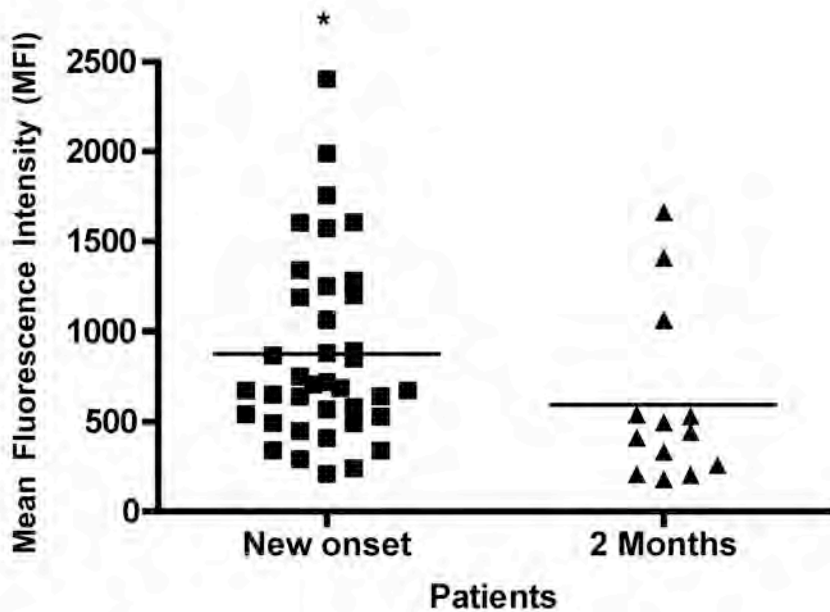


Figure 1. Expression of CD11b on monocytes of T1D patients. Column-scatter-plot showing changes in mean fluorescence intensity (MFI) of CD11b expression on circulating monocytes in recently-diagnosed T1D patients. Analysis was performed by flow cytometry. Monocyte from T1D patients at the onset of the disease show (n=38) shows higher expression of the adhesion molecule CD11b then T1D patients assayed at 2 months of diabetes (n=13) ($p=0.02$). Horizontal bars show mean CD11b values. *represents significant difference of surface expression for CD11b between the two groups.

Because diabetes ketoacidosis (DKA) has been described as an inflammatory condition characterized by elevated levels of C-reactive protein, within our cohort of newly-onset T1D patients, we found that monocyte expression of CD11b in the children with DKA tended to be higher (1007 ± 188 SEM) compared to those without DKA (797 ± 82 SEM), however this was not statistically significant ($p=0.67$). When we compared those without DKA at onset (n=21) with those without DKA at 2 months (n=13), monocyte expression of CD11b was statistically significantly different between the two groups (797 ± 82 versus 595 ± 132 respectively, $p=0.03$).

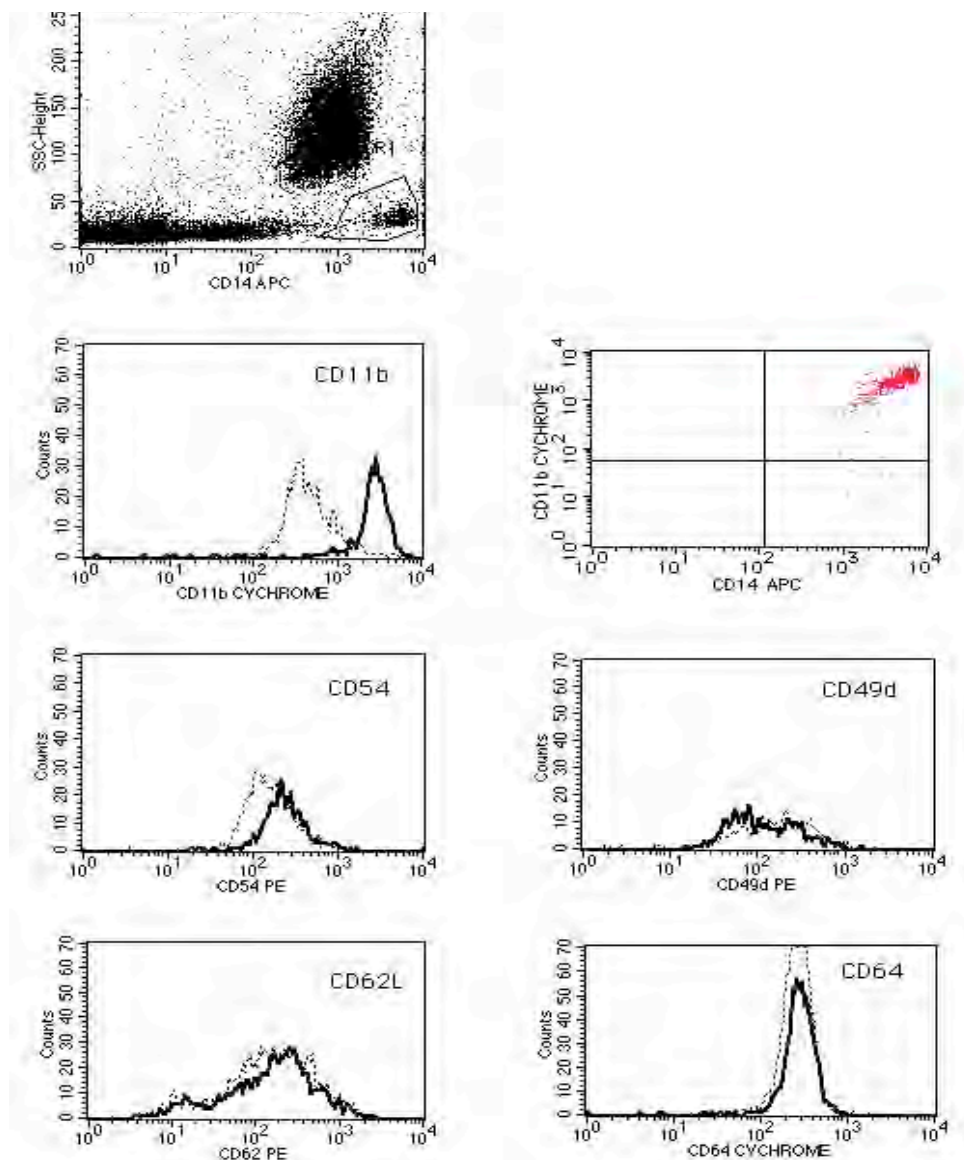


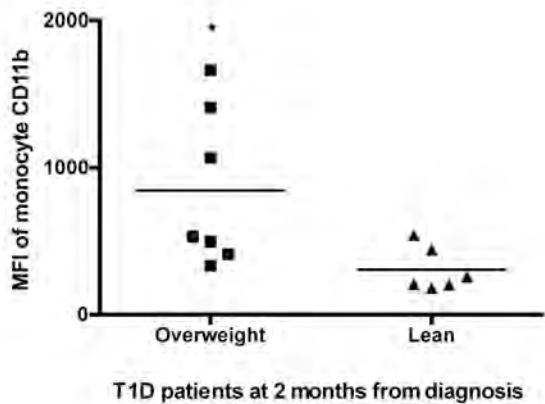
Figure 2. Representative flow cytometry analysis of CD11b expression in a T1D patient. In (A) representative dot-plot graph of CD11b expression on circulating monocytes (CD14⁺) of T1D patients at the onset of the disease. For each sample tested, dot-plot graphs of SSC versus CD14⁺ cells were drawn, and a tight gate was created, encompassing the “bright” elements for this specificity. Within the CD14 gate, CD14⁺ elements expressing the different adhesion molecules were analyzed by drawing separate dot-plots. Percentage and MFI of positive cells was then defined by setting lower-limits for adhesion molecule positivity from each antibody combination using negative isotype controls. In A it is also shown a representative histogram of CD11b expression in T1D patient at the onset of the disease and after 2 months from diagnosis. Dotted line= T1D patient after 2 months from diagnosis; solid line= T1D patient after 2 months from diagnosis. Expression of CD11b was higher in the T1D patient at the onset of diabetes than after 2 months. In (B) representative histograms of adhesion molecule expression in T1D patient at the onset of the disease and after 2 months from diagnosis. The x axis is fluorescence intensity of the stated adhesion molecule. Dotted line= T1D patient after 2 months from diagnosis; solid line= T1D patient after 2 months from diagnosis. There was no significant differences in expression of CD54, CD49d, CD62L, and CD64 on monocytes between the two studied groups.

As expected, glycemic control measured by HbA_{1c} was significantly higher in the onset group compared to the group tested at 2 months (11.3%±2 versus 7.4%±0.9, $p=0.0001$). To test whether patients with higher HbA_{1c} had also higher monocyte activation, we compared expression of monocyte CD11b in T1D patients according to HbA_{1c} levels. We found that T1D patients with higher HbA_{1c} levels (HbA_{1c}>8%) ($n=35$) had significantly higher expression of CD11b (896±75 SEM) as compared to those with lower HbA_{1c} levels (HbA_{1c} ≤8%) (563±103 SEM) ($n=12$) ($p=0.04$). In addition, CD11b and HbA_{1c} were significantly correlated (correlation coefficient 0.329, $p=0.02$).

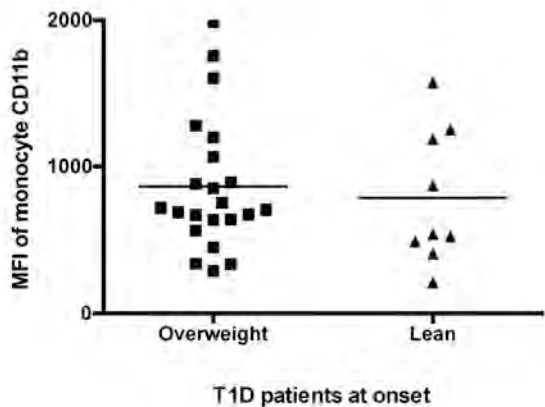
Analysis of the expression of the surface antigens CD49d, CD54, CD62L and CD64 on circulating monocytes in the different subgroups of patients did not show any significant differences. A representative example of the expression of the stated markers in T1D patients at the onset of the disease and after 2 months from diagnosis is shown in **Figure 2**.

Overweight children with T1D have high monocyte expression of CD11b

There is evidence that obesity is associated with a systemic inflammatory process involving both the leukocytes and the body fat [17,18]. We therefore analyzed changes in monocyte CD11b expression in T1D according to BMI. We found that among T1D children who were tested after 2 months from diagnosis (n=13), the expression of CD11b was significantly higher overweight children (BMI $\geq$ 85th percentile) (n=7) (844 $\pm$ 201 SEM; median 531, range 332-1663) as compared to children who were not overweight (BMI $\leq$ 85th percentile) (n=6) (305 $\pm$ 61 SEM; median 233, range 180-540) (p=0.03) (**Figure 3A**).



3A



3B

Figure 3. Monocyte CD11b expression among T1D patients is higher in those who are overweight. In 3A, column-scatter-plot showing changes in mean fluorescence intensity (MFI) for the molecule CD11b on monocytes according to Body mass index (BMI) in thirteen patients tested at 2 months from diagnosis (Lean n=6; Overweight n=7). In 3B, same analysis performed in thirty-one T1D patients at the onset of the disease (Lean n=22; Overweight n=9). Horizontal bars show mean CD11b values. *represents significant difference of cell surface expression for CD11b between T1D patients who are overweight (BMI $\geq$ 85th percentile) and lean (BMI $\leq$ 85th percentile).

These two groups were not different in terms of their glycemic control as measured by HbA_{1c} (7.1% $\pm$ 0.3 in the overweight group versus 7.6% $\pm$ 4.5 in the non-overweight group, p=0.33) and none of them were in DKA at the time of the evaluation.

Same analysis performed in patients at the onset of the disease (n=31) did not show significant differences in monocyte CD11b between overweight (BMI $\geq$ 85th percentile) (n=9) (864 $\pm$ 97 SEM; median 713, range 291-1989), and lean (BMI $\leq$ 85th percentile) (784 $\pm$ 153 SEM; median 543, range 210-1574) (n=22) patients p=0.66 (Figure 3B). These two groups were not different in terms of their control as measured by HbA_{1c} (10.4% $\pm$ 0.5 in the overweight group versus 11.6% $\pm$ 0.4 in the non-overweight group, p=0.33) or the presence of DKA (50 versus 31%, p=0.65). CD11b and BMI percentile were not statistically significantly correlated (correlation coefficient 0.027; p=0.86).

Conclusions

Little is known about monocyte phenotype and function in children with recently-diagnosed T1D. In this study, we analyzed the expression of a panel of adhesion molecules on circulating monocytes in T1D children within 2 months from clinical diagnosis and evaluated the potential role of glycemic control and BMI on cell phenotype. We found that among the markers analyzed, only the expression of CD11b was significantly higher in the cohort of new-onset diabetic patients (i.e., within 1 week from diagnosis) as compared to subjects tested after 2 months from diagnosis. CD11b is a polypeptide α -chain linked to the β_2 -subunit of CD18 that constitute the CD11/CD18 β_2 -integrin family. Resting monocytes constitutively express integrins, which are important signal transducers for virtually all monocyte functions by mediating cell adhesion, chemotaxis, migration, phagocytosis, and oxidant production. After monocyte activation, new copies of CD11b/CD18 are rapidly translocated to the cell surface from the intracellular granules [21]. Our finding of increased CD11b expression on monocytes at the onset of T1D suggests presence of immune activation at such an early stage of the disease. This result also supports an earlier finding of increased monocyte CD11b expression in T1D patients [14].

Within the cohort of new-onset T1D patients, children who presented with DKA had the highest levels of monocytes CD11b detected. Although these data did not reach statistical significance most likely due to small numbers, it supports a previous report showing presence of an inflammatory response in T1D children with DKA [22]. However, other factors, besides DKA, appear to be involved in triggering monocyte activation at the onset of diabetes. In fact, significantly higher monocyte CD11b expression was detected in new-onset T1D children without DKA versus those without DKA at 2 months. These results seem to suggest that the onset of diabetes is *per se* an inflammatory condition. Further studies on a larger number of patients at the onset of T1D are required to better define the inflammatory state present in DKA.

We found that diabetic children tested after 2 months from diagnosis who were overweight (BMI $\geq$ 85th percentile) displayed higher CD11b values when compared to lean diabetic children. This finding supports the association between obesity and inflammation [17,18]. Recently, it has been shown that obesity is characterized by abnormalities in peripheral leukocyte counts [17], increased circulating levels of the pro-inflammatory cytokine interleukin(IL)-8 [9], and by an accumulation of immune cells, especially macrophages, in the adipose tissue [18]. The fact that we could not detect the same changes of monocyte CD11b between overweight and lean diabetic children within the cohort of patients tested at the onset of the disease is probably due to the confounding effect of the inflammatory response associated with the onset of the disease.

The factor(s) triggering up-regulation of CD11b on the monocytes in diabetes and its biological significance are not known. One hypothesis would be that the increased expression of CD11b on monocytes in diabetes is simply a marker of an inflammatory response, which becomes more pronounced in overweight children and in children who present with DKA. Alternatively, CD11b up-regulation could reflect an increased monocyte activation in response to poor glycemic control and insulin-deficiency likely present at the onset of diabetes, which is supported by the fact that HbA_{1c} levels were significantly higher at the onset than at 2 months and by the fact that there was a significant correlation between the two variables. In addition, diabetic children with higher HbA_{1c} (HbA_{1c}>8%) also had higher CD11b. A third explanation envisions that intercurrent infections may have precipitated the diagnosis (due to increased insulin requirements) and be responsible for immune activation. At this point, we cannot decide to which extent the inflammatory state is a pathogenic mechanism contributing to the development of T1D or is the response to a metabolic derangement. In the first case, the inflammatory state should precede overt diabetes and in this context the study of pre-diabetic subjects (i.e., autoantibody-positive individuals) should be characterized in respect to the inflammatory state.

Our findings of increased CD11b on the monocytes of T1D patients at the clinical onset of diabetes would strengthen the idea that these cells have the potential to adhere to the endothelium and exit the circulation at such an early stage of the disease. In fact, current investigations in our laboratory show that monocytes isolated from peripheral blood of recently-diagnosed T1D patients (who were free from any vascular complications) have increased adherence to Human Umbelical Vein Endothelial Cells (HUVEC) *in vitro* (unpublished preliminary findings) (Figure 4).

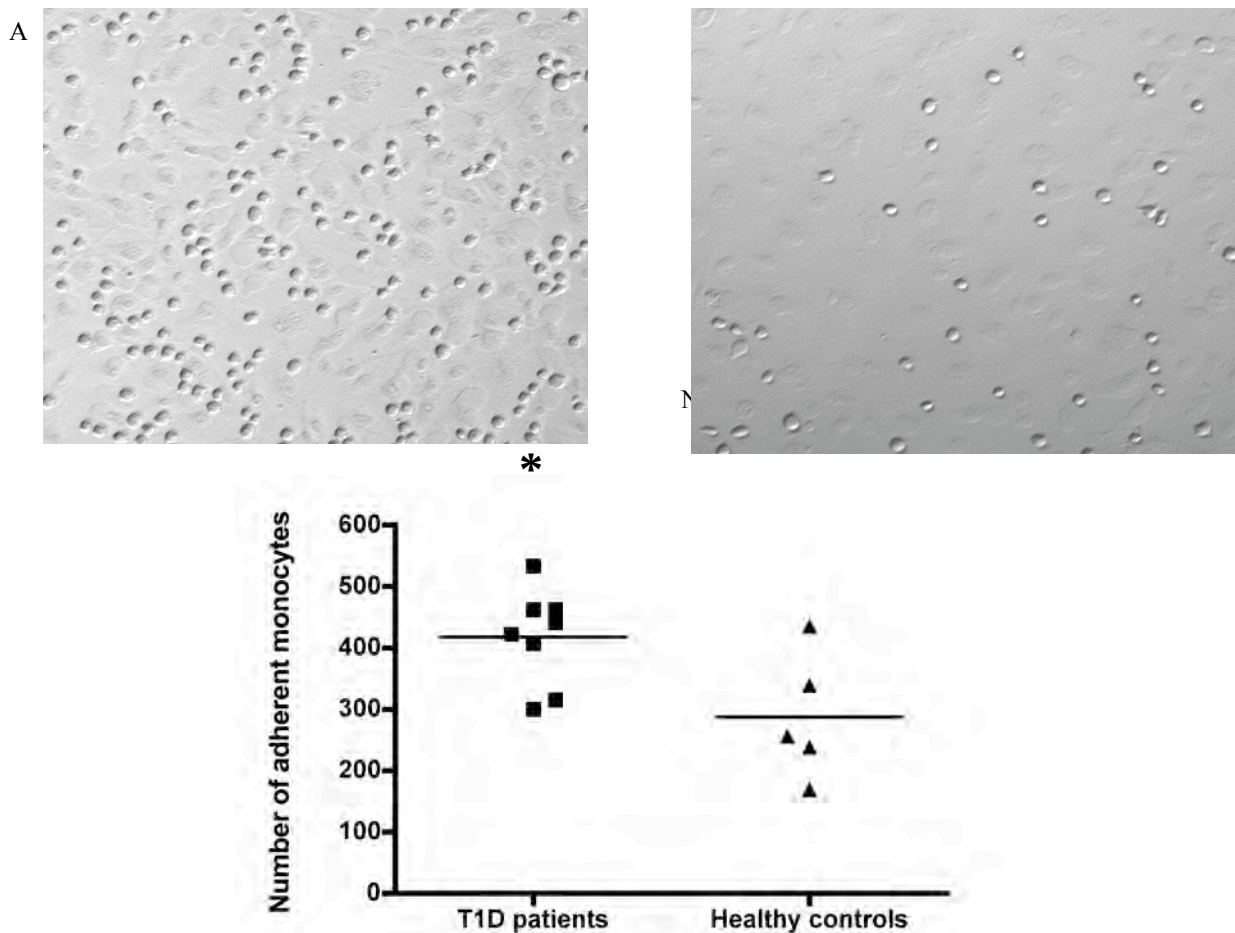


Figure 4. Monocytes from T1D patients have increased adherence to HUVEC. Freshly isolated monocytes from new onset T1D patients (n=8) and from healthy controls (n=5) were added to confluent HUVEC and counted, as described in Materials and Methods. In (A) 40x photographs of monocytes adherent to HUVEC. In (B) Columns-scatter-dot-plot showing number of monocytes adherent to HUVEC in 5 random 40x fields per well. Horizontal bars show mean number of monocytes. The number of monocytes of T1D patients adherent to the wells was significantly higher than in normal controls ($p = 0.04$).

These findings, although preliminary, would support the hypothesis that monocytes from recent-onset T1D patients have increased adhesive capacity and the potential to exit the circulation and infiltrate the islets of Langerhans. Protracted inflammation with increased expression of the same adhesion molecules could be the basis for late diabetes-associated atherosclerotic vascular complications.

In conclusion, the clinical onset of T1D is associated with changes in activation-related markers on the circulating monocytes as part of a generalized inflammatory response. We have identified the leukocyte integrin CD11b/CD18 as a crucial molecule up-regulated on circulating monocytes, especially in overweight children. Enhanced monocyte CD11b would support the notion that monocytes of recently-diagnosed T1D patients have the potential to adhere to the endothelium, most likely in the vasculature of the pancreas, and accumulate in the islets.

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In our second quarterly scientific progress report (09/01/07 – 11/30/07, we presented our regeneration results obtained in transplanted monkeys.

Type 1 diabetes (T1D) is an autoimmune disease the clinical onset of which most frequently presents in children and adolescents who are genetically predisposed. In light of accumulating evidence that: a) the endocrine pancreas has regenerative properties (1-7); b) hematopoietic chimerism can abrogate destruction of β cells in autoimmune diabetes (8,9), so that physiologically-sufficient endogenous insulin production can be restored in non-obese diabetic (NOD) mice, even after the disease clinically presented (10-12) – this strain of mouse spontaneously develops T1D with etiopathogenetic characteristics very similar to the disease in humans; and c) these regeneration properties of the endocrine pancreas have also been seen, even if only sporadically, in humans (13,14), we propose here to test reliable and more clinically translatable alternatives able to achieve these same goals in non-human primates.

T1D is prevented by transfecting a “diabetes-resistant” MHC class II β chain gene allele into the hematopoietic stem cells of genetically susceptible (i.e., carrying a “diabetes-susceptible” allele) mice (15). The expression of the newly formed diabetes-resistant molecule in the re-infused hematopoietic cells, is sufficient to prevent T1D onset in the NOD mouse, even in the presence of the native, diabetogenic molecule. This approach to obtain autoimmunity abrogation facilitates the recovery of autologous insulin production also in the already-diabetic individual. Safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

Our working hypothesis is currently tested by using bone marrow (BM) enriched hematopoietic precursor cells (instead of a non-fractionated BM cell population used by Tian et al.), transfected with an MHC class II β chain gene that confers resistance to the disease, to abrogate autoimmunity. Also, already diabetic -- rather than pre-diabetic -- mice are treated by the re-infusion of transfected BM enriched precursors. The enriched precursors are able to generate the right derivative cells and in sufficient numbers to efficiently repopulate the thymus. By negatively selecting possibly autoreactive T cell clones, and making peripheral tolerance, mediated by T regulatory cells, more efficient, autoimmunity is abrogated. In the absence of autoimmunity and of diabetogenic immunosuppressive protocols, by adopting auxiliary means to correct hyperglycemia, the regenerative property of the autologous endocrine pancreas repopulates the gland with enough insulin-producing cells to restore euglycemia. Also, to avoid the use of radiation to eliminate the activated T cell clones present in the diabetic patient, an antibody-based preconditioning is used instead. Finally, we are determining how long after its onset disease reversal remains possible.

However, the evidence generated in rodents must be confirmed in non-human primates to be allowed to quickly transfer this protocol to humans. Even in a non-human primate that does not spontaneously develop autoimmunity nor T1D, safety and regenerative issues can be properly addressed.

To this goal we propose:

Task 1. To isolate – using appropriate antibodies and cell sorting – bone marrow (BM) cell precursors from a diabetic (i.e., streptozotocin [STZ] treated) cynomolgus monkey. **The isolated precursors will then be transfected ex vivo with an Mhc class II b chain gene conferring resistance to the disease, and re-infused in the BM depleted animal to determine the safety of this maneuver.**

a) This protocol will be implemented immediately after diabetes onset, in association with allogeneic islet transplantation to guarantee the diabetic animal's euglycemia until the regenerative process brings about sufficient β cells to make the islet graft obsolete.

b) In parallel experiments, insulin-based therapy will precede transplantation of transfected BM cells and the protocol will be implemented after a protracted insulin therapy. Exogenous insulin administration will continue after protocol's implementation.

Task 2. To ascertain the efficacy of engraftment and repopulation capabilities of the engineered hematopoietic precursor cells, following non-radiation based pre-conditioning.

a) We will systematically substitute to irradiation an antibody-based, immuno-reductive conditioning protocol, testing different quantities and well-defined injection schedules.

Task 3. To utilize phage integrases to guide the stable and irreversible insertion of DNA at specific locations within the genome to satisfy the need for an everlasting synthesis of the b chain conferring resistance, even in the offspring of the successfully transfected BM precursor cells.

a) Determine if any of the three DirectIt™ recombinases integrate plasmid DNA at pseudo *att* sites in monkey chromosomes.

b) Determine if the best recombinase identified in Task 1 integrates plasmid DNA into mesenchymal stem cells (MSC) of cynomolgus monkey.

c) Identify the pseudo *att* sites in MSC of cynomolgus monkey.

d) Optimize the transfection and gene targeting procedure for integrating plasmid in to CD34⁺ cells of cynomolgus monkey.

Task 4. To infuse *in situ* appropriate factors (e.g., PAX4, EGF, LIF, HGF, GLP, and IGF) able to speed up the physiologic regenerative process.

a) A topical route via the pancreatic duct by retrograde delivery, similar to a commonly used clinic technique ERCP (endoscopic retrograde cholangio-pancreatography) will be used.

Task 5. To test with a proteomics approach whether the successfully infused target tissue secrete, during regeneration, the product(s) of the transfected gene(s) and additional, spontaneously-generated, adjuvant factors.

a) Isolated endocrine and exocrine tissues obtained at the autopsy, will be used any time they become available.

b) The pancreatic fluid will be collected from the duct of the treated monkeys, by using the ERCP technique and its content analyze in detail in terms of relative quantities of each of the proteins present.

In summary our final goal is to obtain autoimmunity abrogation in a diabetic patient *via* an autotransplant of precursor cells transfected with HLA class II b chain genes conferring resistance to the disease and, while correcting his/her hyperglycemia using conventional insulin administrations, leave “nature” to heal the rest. We also propose to speed up the natural process of healing by endoscopic retrograde intraductal delivery of factors known to promote b cell regeneration. Should this approach work satisfactorily, our young patients will be cured for good, without any need for long drug therapies associated with troublesome consequences.

Towards Task #1 we focused on the isolation of “**BM cell precursors from a diabetic (i.e., streptozotocin [STZ] treated) cynomolgus monkey**”.

With regards to the STZ treatment we can say that so far we have accumulated evidence that regeneration of the endogenous pancreas, in a chemically (STZ) induced, diabetic monkey -- transplanted with a1,3-galactosyltransferase double knockout (DKO) (26) pig islets, in which these islets can substitute for endogenous islets producing enough insulin (monitored by pig C-peptide) to control the recipient animal glycemia -- starts to take place a few months after the total destruction of the b cells, provided that diabetogenic immunosuppressants are not used. In Figure 1 we show how after STZ treatment the endocrine pancreas of the monkey is not any longer able to produce sufficient quantities of insulin to satisfy the need of the animal that consequently becomes diabetic.

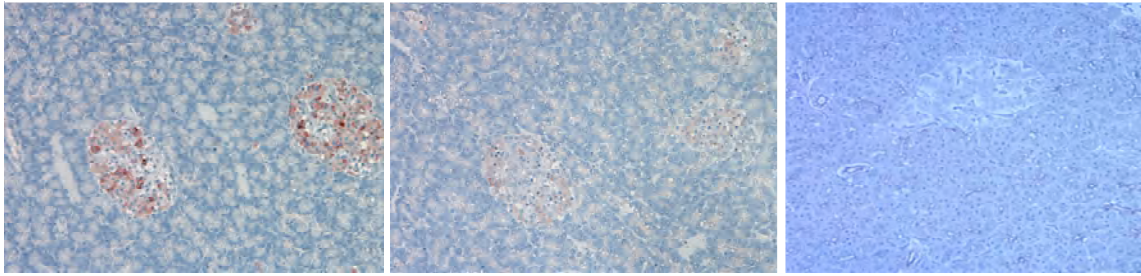


Figure 1. Monkey pancreas 4 hours, 7 hours, and 4 weeks after 150mg/kg STZ infusion. Insulin in red.

Regeneration properties are overpowered by the effects of diabetogenic immunosuppressive cocktails: the monkey C peptide levels remained lower than 0.5ng/ml for the entire duration of all these experiments and Arginine-stimulation-test was always blunted when performed during follow-up (Figure 2).

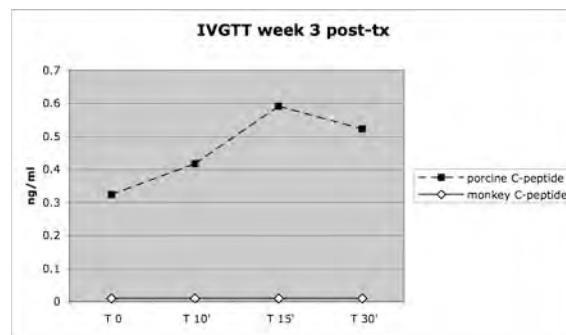


Figure 2. IVGTT carried out on monkey recipient 3 weeks following pig islet xenotransplantation. Porcine (graft) and monkey (endogenous) C peptide levels are shown.

More than three months after STZ treatment -- in the absence of diabetogenic immunosuppressant agents and using instead an anti-CD154 monoclonal antibody to block the recipient's immune system -- the monkeys not only keep producing pig C-peptide but eventually recovered the ability to produce monkey C-peptide. New insulin-producing cells are appearing with time in the monkey endogenous pancreas eventually forming insulin-producing conglomerates of cells (Figure 3).

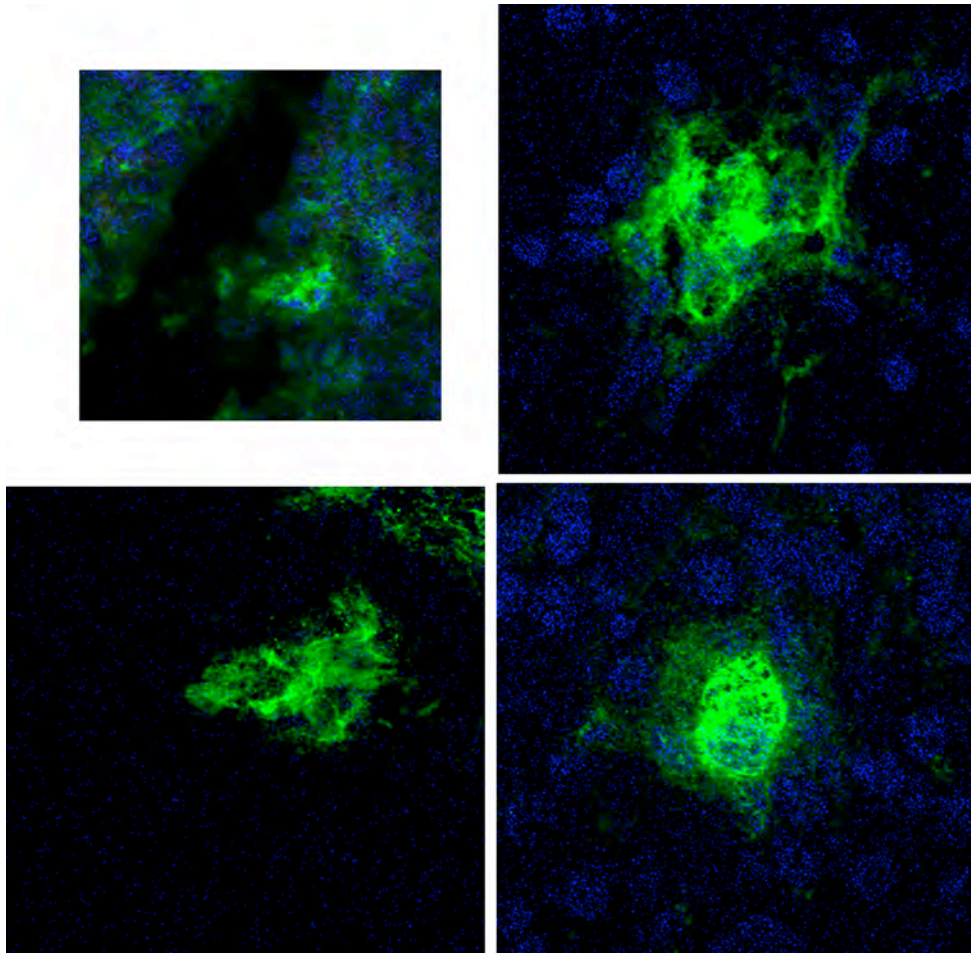


Figure 3. Monkey pancreas four months after transplantation. Insulin in green.

Towards Task #1 we also tested the possibility to isolate BM cell precursors from diabetic (i.e., streptozotocin [STZ] treated) cynomolgus monkeys and our ability to isolate, expand and transfect them.

Isolation: Vertebrae were surgically removed from monkeys (*Cynomolgus Macaque*) euthanized for islet transplantation studies. Connective tissues and muscles were removed with scalpel and the cleaned vertebrae were crushed to release the bone marrow cells. Dead cells and red blood cells were further removed by Ficoll-gradient centrifugation. A total of $5 \pm 2 \times 10^9$ bone marrow cells were harvested from each monkey and subjected to magnetic bead-based progenitor cell isolation. We tested two commercially available CD34+ progenitor cell isolation systems: the DYNAL CD34+ progenitor cell selection system (Invitrogen) and the CD34+ progenitor cell isolation kit from Miltenyi. As shown in Figure 4, we were able to isolate hematopoietic progenitor cells from the monkey bone marrow cells at a recovery rate around $1.1 \pm 0.2\%$ with the DYNAL kit. The isolated progenitor cells were aliquoted and stored in liquid nitrogen for future use.

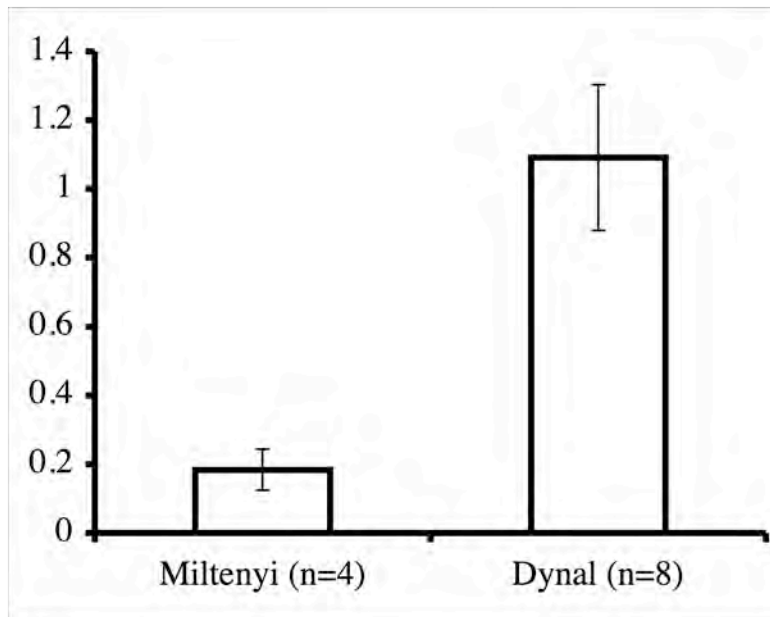


Figure 4. Isolation of Monkey CD34⁺ Progenitor Cells. Bone marrow cells from monkey vertebrae were harvested and stained with anti-CD34 antibody anchored on magnetic beads. We tested two commercially available systems for the isolation procedure, and found that the DYNAL system is superior to the Miltenyi one, with a much higher recovery rate.

Expansion: We were able to recover and grow the liquid nitrogen-stored monkey progenitor cells with growth factor supplements (StemSpan CC110 cytokine cocktail from Stemcell Technologies, including 100 ng/mL rh Flt-3 ligand, 100 ng/mL rh stem cell factor and 100 ng/mL rh thrombopoietin). Under such condition, these progenitor cells can expand more than 20 times with a doubling time about 72 hours (Figure 5).

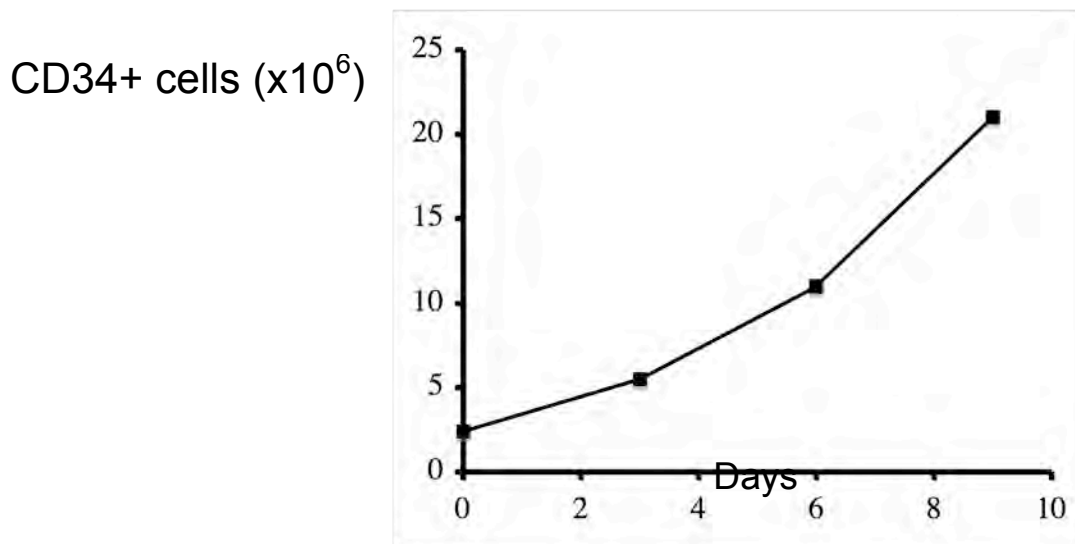


Figure 5. *In vitro* Culture of Monkey CD34⁺ Progenitor Cells. CD34⁺ progenitor cells harvested were cultured *in vitro*, supplemented with growth factors (human recombinant SCF, TPO and Flt-3). The doubling time is about 72 hours.

Transfection: To further investigate the probability of subjecting the cultured CD34+ monkey bone marrow progenitor cells to therapeutic use, we used the human hematopoietic progenitor Nucleofector kit from AMAXA GmbH to introduce the green fluorescent protein (GFP) expressing plasmid pmaxGFP into these cells. It was shown in previous studies that the Nucleofector technology could be successfully used to introduce transgenes, transiently as well as stably, into a variety of primary stem cell populations, such as adipose progenitor cells, neuronal progenitor cells, and human bone marrow progenitor cells. Seventy-two hours after electroporation, bone marrow cells were analyzed by fluorescein-activated cell sorting (FACS) analysis. More than 50% of bone marrow cells were GFP positive, indicating high-efficient transfection of the pmaxGFP plasmid (Figure 6).

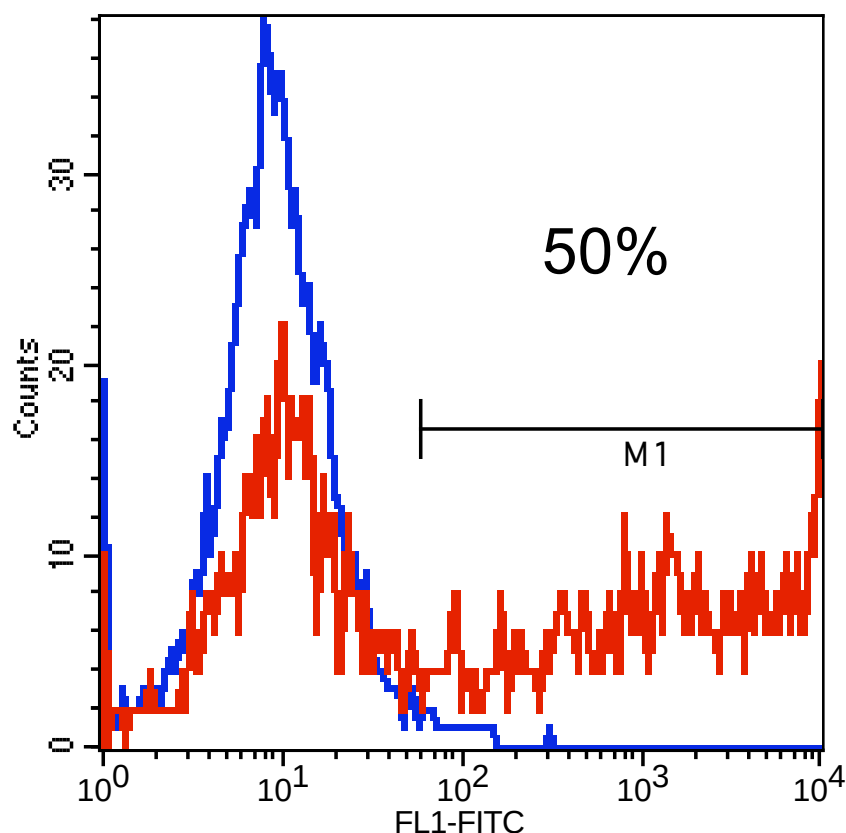


Figure 6. High Efficient Transfection of Monkey CD34+ Cells. *In vitro* cultured CD34+ progenitor cells were transfected with pmaxGFP plasmid, using the Amaxa Nucleoporation system. 72 hours after electroporation, about 50% cells are GFP+.

Taken together, we have overcome all the technical hurdles, and are able to isolate, store, expand and manipulate (introducing transgenes) CD34+ monkey bone marrow progenitor cells *in vitro*.

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In our third quarterly scientific progress report (12/01/07 – 02/29/08) we then reported interesting properties of the C-peptide.

Type 1 diabetes (T1D) is an autoimmune disease the clinical onset of which most frequently presents in children and adolescents who are genetically predisposed. In light of accumulating evidence that: a) the endocrine pancreas has regenerative properties (1-7); b) hematopoietic chimerism can abrogate destruction of β cells in autoimmune diabetes (8,9), so that physiologically-sufficient endogenous insulin production can be restored in non-obese diabetic (NOD) mice, even after the disease clinically presented (10-12) – this strain of mouse spontaneously develops T1D with etiopathogenetic characteristics very similar to the disease in humans; and c) these regeneration properties of the endocrine pancreas have also been seen, even if only sporadically, in humans (13,14), we propose here to test reliable and more clinically translatable alternatives able to achieve these same goals in non-human primates.

T1D is prevented by transfecting a “diabetes-resistant” MHC class II β chain gene allele into the hematopoietic stem cells of genetically susceptible (i.e., carrying a “diabetes-susceptible” allele) mice (15). The expression of the newly formed diabetes-resistant molecule in the re-infused hematopoietic cells, is sufficient to prevent T1D onset in the NOD mouse, even in the presence of the native, diabetogenic molecule. This approach to obtain autoimmunity abrogation facilitates the recovery of autologous insulin production also in the already-diabetic individual. Safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

Our working hypothesis is currently tested by using bone marrow (BM) enriched hematopoietic precursor cells (instead of a non-fractionated BM cell population used by Tian et al.), transfected with an MHC class II β chain gene that confers resistance to the disease, to abrogate autoimmunity. Also, already diabetic -- rather than pre-diabetic -- mice are treated by the re-infusion of transfected BM enriched precursors. The enriched precursors are able to generate the right derivative cells and in sufficient numbers to efficiently

repopulate the thymus. By negatively selecting possibly autoreactive T cell clones, and making peripheral tolerance, mediated by T regulatory cells, more efficient, autoimmunity is abrogated. In the absence of autoimmunity and of diabetogenic immunosuppressive protocols, by adopting auxiliary means to correct hyperglycemia, the regenerative property of the autologous endocrine pancreas repopulates the gland with enough insulin-producing cells to restore euglycemia. Also, to avoid the use of radiation to eliminate the activated T cell clones present in the diabetic patient, an antibody-based preconditioning is used instead. Finally, we are determining how long after its onset disease reversal remains possible.

However, the evidence generated in rodents must be confirmed in non-human primates to be allowed to quickly transfer this protocol to humans. Even in a non-human primate that does not spontaneously develop autoimmunity nor T1D, safety and regenerative issues can be properly addressed.

To this goal we proposed:

Task 1. To isolate – using appropriate antibodies and cell sorting – bone marrow (BM) cell precursors from a diabetic (i.e., streptozotocin [STZ] treated) cynomolgus monkey. The isolated precursors will then be transfected ex vivo with an Mhc class II b chain gene conferring resistance to the disease, and re-infused in the BM depleted animal to determine the safety of this maneuver.

a) This protocol will be implemented immediately after diabetes onset, in association with allogeneic islet transplantation to guarantee the diabetic animal's euglycemia until the regenerative process brings about sufficient b cells to make the islet graft obsolete.

b) In parallel experiments, insulin-based therapy will precede transplantation of transfected BM cells and the protocol will be implemented after a protracted insulin therapy. Exogenous insulin administration will continue after protocol's implementation.

Task 2. To ascertain the efficacy of engraftment and repopulation capabilities of the engineered hematopoietic precursor cells, following non-radiation based pre-conditioning.

a) We will systematically substitute to irradiation an antibody-based, immuno-reductive conditioning protocol, testing different quantities and well-defined injection schedules.

Task 3. To utilize phage integrases to guide the stable and irreversible insertion of DNA at specific locations within the genome to satisfy the need for an everlasting synthesis of the β chain conferring resistance, even in the offspring of the successfully transfected BM precursor cells.

a) Determine if any of the three DirectIt™ recombinases integrate plasmid DNA at pseudo *att* sites in monkey chromosomes.

b) Determine if the best recombinase identified in Task 1 integrates plasmid DNA into mesenchymal stem cells (MSC) of cynomolgus monkey.

c) Identify the pseudo *att* sites in MSC of cynomolgus monkey.

d) Optimize the transfection and gene targeting procedure for integrating plasmid in to CD34⁺ cells of cynomolgus monkey.

Task 4. To infuse *in situ* appropriate factors (e.g., PAX4, EGF, LIF, HGF, GLP, and IGF) able to speed up the physiologic regenerative process.

a) A topical route via the pancreatic duct by retrograde delivery, similar to a commonly used clinic technique ERCP (endoscopic retrograde cholangio-pancreatography) will be used.

Task 5. To test with a proteomics approach whether the successfully infused target tissue secrete, during regeneration, the product(s) of the transfected gene(s) and additional, spontaneously-generated, adjuvant factors.

a) Isolated endocrine and exocrine tissues obtained at the autopsy, will be used any time they become available.

b) The pancreatic fluid will be collected from the duct of the treated monkeys, by using the ERCP technique and its content analyze in detail in terms of relative quantities of each of the proteins present.

In summary our final goal is to obtain autoimmunity abrogation in a diabetic patient *via* an autotransplant of precursor cells transfected with HLA class II β chain genes conferring resistance to the disease and, while correcting his/her hyperglycemia using conventional insulin administrations, leave “nature” to heal the rest. We also propose to speed up the natural process of healing by endoscopic retrograde intraductal delivery of factors known to promote β cell regeneration. Should this approach work satisfactorily, our young patients will be cured for good, without any need for long drug therapies associated with troublesome consequences.

Towards Task #1 we checked whether sources other than BM may be suitable for generating surrogates of pancreatic beta cells.

INTRODUCTION

Type 1 diabetes is an autoimmune disease characterized by the selective loss of the beta cell mass in the pancreas and the subsequent lack of a single secreted and circulating protein, i.e., insulin (1). As an example of a mono-cellular deficiency state diabetes has tremendous appeal as a target for cell replacement therapy. Whole pancreas and isolated islet transplantation are currently the two available alternatives for beta cell replacement, although their feasibility is severely limited by the shortage of donors and the need for lifelong immunosuppressive therapy (2). In 2000, Shapiro et al. provided the undeniable “proof of principle” that the reestablishment of an adequate beta cell mass via transplantation of isolated human islets can restore euglycemia in patients with insulin-dependent T1D (3). Unfortunately, two or more pancreata per recipient were necessary to achieve insulin independence (4). Thus, many efforts are now aimed at finding alternative sources of insulin-producing cells which satisfy the need for a large number of transplantable cells while preserving the physiological function of the beta cell, i.e. the ability to sense blood glucose levels and consequently release the appropriate amount of hormone. Stem cells, which can be isolated from embryonic, fetal, and adult tissues might represent such an unlimited source because of their ability to self-renew by symmetric division while retaining the potential of differentiating under the proper conditions into the desired phenotype (5).

EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst which forms several days after an egg is fertilized (6). ESCs can be defined as totipotent; they are able to generate all of the tissues of the developing organism. Evidence of this property was demonstrated by the injection of mouse ESCs of one strain into the blastocyst of a second strain. Following re-implantation into mice, the ESCs contribute to the formation of all the tissues of the embryo chimera (7). For this reason, human ESCs (hESCs) might represent an excellent source of large numbers of transplantable cells to be used for cell replacement therapies. Human ESCs express telomerase activity and many specific biomarkers such as Oct-4, Nanog, Thy-1, stage specific embryonic antigen-3 and -4, and tumor-rejection antigen-1-60 and -1-81 (8). These markers have been used to characterize hESCs in comparison to other stem cells with less plasticity and more limited differentiation potential. In addition, cultured ESCs can easily be manipulated genetically via ectopic transgene expression or homologous recombination-based approaches, thereby providing powerful model systems for the study of mammalian embryogenesis and disease processes (8). Despite these advantages, the therapeutic use of hESCs has major drawbacks in clinical application, not limited to ethical concerns relative to embryo manipulation (9). Main obstacles are the allogeneic nature of the cells with their consequent chance of immune rejection reactions, and the possibility of teratoma/teratocarcinoma formation after implant in ectopic sites, which is a natural consequence of their broad differentiation potential (8). Therefore, at least to prevent the latter issue, cells derived from ESCs should be depleted of the undifferentiated population before their safe transplantation.

A large number of studies described the derivation of surrogate beta cells from mouse and human ESCs. In 2000, Soria et al. first used the so-called “promoter trapping” approach to enhance the survival of insulin-producing cells taking advantage of spontaneous differentiation of mouse ESCs (10). Spontaneous differentiation was also reported in human ESCs (11). However, the major challenge remained the one of directing *in vitro* the differentiation of ESCs into a specific target cell and at the same time preventing or suppressing all other unnecessary alternatives of the pluripotent repertoire. In 2001 Lumelski et al. claimed that functional islet-like structures could be produced from mouse ESCs using a relatively simple five-step

procedure (12). Influenced by the similarities existing between neural and islet cells -- including their shared expression of the intermediate filament protein Nestin and the transcription factors Isl1, Ngn3, Pax6, Pax4, and beta2NeuroD -- their approach was based on protocols developed to differentiate mouse ESCs towards neurons. A major critique, elegantly demonstrated by Melton and his group in 2003, was that insulin positive staining of supposedly differentiated ESCs was an artifact due to absorption of the hormone from the media.

Co-culturing of human ESCs with developing murine pancreas to facilitate their maturation into beta-like cells was another method tested (16). More recently, the dramatic evolution in the discipline of developmental biology and the deeper understanding of the physiologic ontogeny of the pancreas, has led to more precise protocols for directing differentiation of embryonic stem cells towards insulin-producing cells. Achievement of this goal was reported in 2006 using a five-step protocol designed to mimic pancreatic organogenesis (17). The authors were able to differentiate a consistent fraction of hESCs into islet-like cells capable of synthesizing the hormones insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin. The insulin content per cell and the frequency with which beta-like cells were obtained was quite remarkable, however glucose responsiveness was not assessed nor were *in vivo* studies performed (18).

ADULT STEM CELLS AND PROGENITOR CELLS

Somatic or adult stem cells (ASCs) are present within post-natal tissues and are thought to be more limited in their differentiation ability than totipotent ESCs. They are defined as multipotential and they possess some advantages over ESCs, such as their use for autologous transplantation which virtually bypasses the need for immunosuppression, their nearly nonexistent tumorigenic behavior, and the absence of related ethical concerns (19).

The physiologic role of ASCs is to support cell turnover under homeostatic conditions or exceptional circumstances, i.e., regeneration of damaged tissues after severe injuries. ASCs are generally considered not terminally differentiated but pre-committed to only generate cells of the tissue with which they share the embryological origin. However, it has been reported that some ASCs can give rise to cell types other than their default ones. Accumulating lines of evidence revealed for example that bone marrow (BM) harbors not only hematopoietic stem cells, which are committed to differentiate into blood cells, but also the less differentiated mesenchymal stem cells (MSCs) (20). When transplanted in NOD mice, MSCs were reported to be able to differentiate into glucose-competent pancreatic endocrine cells (21), although confirmatory studies resulted in controversial outcomes. Indeed, it has been suggested that MSCs do not become insulin-producing cells *per se*, rather they take part in islet vascularization eventually promoting beta-cell regeneration (22). It has also been proposed that BM cells promote the functional recovery of residual beta cell mass. This is suggested by the fact that allogeneic transplantation of BM cells from autoimmunity-free strains of mice abolishes the autoimmune process in NOD mice before and even after the clinical onset of the disease, creating a hematological chimera that allows the physiologic recovery of sufficient recipient insulin-producing cells to re-establish euglycemia in the treated diabetic animals (23). Transplantation of human MSCs was also shown to induce repair of pancreatic islets and renal glomeruli in NOD/scid mice with STZ-induced diabetes (24).

Since a "resident" expandable stem cell would be an advantageous starting point for the generation of new beta cells, there have been studies which focus on the characterization of possibly-existing pancreatic progenitor cells. In 2000 Ramiya et al. (25) claimed that long-term cultivation of pancreatic ductal epithelial cells isolated from adult NOD mice contained stem cells able to differentiate into islets of Langerhans. These surrogate islets responded *in vitro* to glucose challenge, and reversed insulin-dependent diabetes after being implanted into diabetic NOD mice. Similar observations were reported using more defined culture conditions in which isolated human pancreatic duct preparations led to formation and propagation of human islet-like structures (26). Whether these results can be explained by the existence of proper pancreatic stem cells (and not surviving endocrine contaminants) has not been rigorously determined, although other studies based on selective human duct labeling confirmed that endocrine progenitors might reside in ducts (27). Besides the ductal progenitors, it has been proposed that pancreatic stem cells may also reside within the islets or that the beta cells themselves can regenerate (28).

Progenitor cells found in the liver can be converted into pancreatic endocrine hormone-producing cells (29). Pancreas and liver share the same embryological origin and it has been reported that trans-differentiation of pancreas into liver occurs both naturally *in vivo* and in animal models after a number of experimental treatments. It has been argued that the reverse inter-conversion of liver into pancreas should also be possible (30). Zalzman et al. (31) were able to immortalize a population of human fetal liver epithelial progenitor cells

that, once transfected with the *pdx1* gene, gave rise to a stable population of insulin-producing cells. Intra-peritoneal transplantation of these cells into NOD/scid mice led to reversal of diabetes for 80 days. Therapeutically the process of trans-differentiation is very interesting and attractive, since it involves de-differentiation towards a common progenitor-like stage with an associated proliferative potential which can eventually be guided towards a predetermined differentiated cell type. Gershengorn and collaborators first described such phenomenon as epithelial-to-mesenchymal transition (EMT) of islet-derived tissue cultures, characterized by their extensive proliferation and eventual re-differentiation towards endocrine cells (32). However, many critiques were advanced from other groups claiming that, at least in mouse pancreatic cultures, islet-derived fibroblast-like cells are not derived via EMT from pancreatic beta cells (33, 34). Later Gershengorn confirmed the existence of differences between human and mouse cultures (35).

FETAL AND UMBILICAL CORD BLOOD STEM CELLS

Fetal (placental, amniotic, trophoblastic) and umbilical cord blood (UCB) stem cells are considered multipotent and share with ASCs the same advantages over ESCs. Fetal and UCB stem cells also possess other characteristics, which make their use a very appealing alternative. UCB is a convenient source of stem cells because of the potentially unlimited donor pool, its easy accessibility, the absence of distress for the donor when harvesting, and the low risk of viral transmission (36). In addition, UCB stem cells seem to have less restrictive transplant requirements in comparison to adult BM cells, possibly due to their low expression of human leukocyte antigens (HLA) (37). Published data about the phenotype of the subpopulation of UCB able to differentiate into non-hematopoietic cells is scarce. Kogler et al. in 2004 first characterized a pluripotent adherent CD45- population of cells, called “unrestricted somatic stem cells” (USSCs), able to differentiate *in vitro* into osteoblasts, chondroblasts, adipocytes, hematopoietic, neural and hepatic cells (38). More recently, other adherent UCB stem cell populations, with both embryonic and hematopoietic characteristics, have been identified (39,40). These cells not only showed several ESC-specific molecular markers, including the transcription factors OCT4 and Nanog and the embryonic antigens SSEA-3 and SSEA-4, but also possessed the ability to differentiate *in vitro* into cells with characteristics of the three embryonic layers. More notably, in our laboratory they were successfully directed to become insulin-producing cells (Figure 1 and 2). *In vivo* experiments confirmed these results: once transplanted into an NOD/scid mouse, these same cells were able to generate human insulin (41). However, it has been argued that the demonstrated restoration of metabolic function could merely be the result of cell fusion with host-derived beta cells rather than a true *de novo* cell generation, i.e., neogenesis.

Stem cells were isolated from amniotic fluid which is known to contain multiple cell types derived from the developing fetus (42). These cells, named AFS, are claimed to be pluripotent, able to give rise to tissue from all of the three germ layers. Recently, stem cells isolated from human placental tissues -- placental-derived multipotent stem cells (PDMSCs) -- were induced to differentiate into insulin positive cells (43). PDMSCs, which express the ESC markers OCT4 and Nanog, were cultured in conditioning media for 4 weeks and gradually formed 3D spheroid bodies. PCR analysis of the bodies revealed the expression of the early pancreatic markers PDX1 and Foxa2, followed by appearance of the mature pancreatic markers insulin, glucagon and somatostatin. When transplanted in STZ-treated mice PDMSCs were able to normalize blood glucose. Insulin secretion was also successfully assessed *in vitro*.

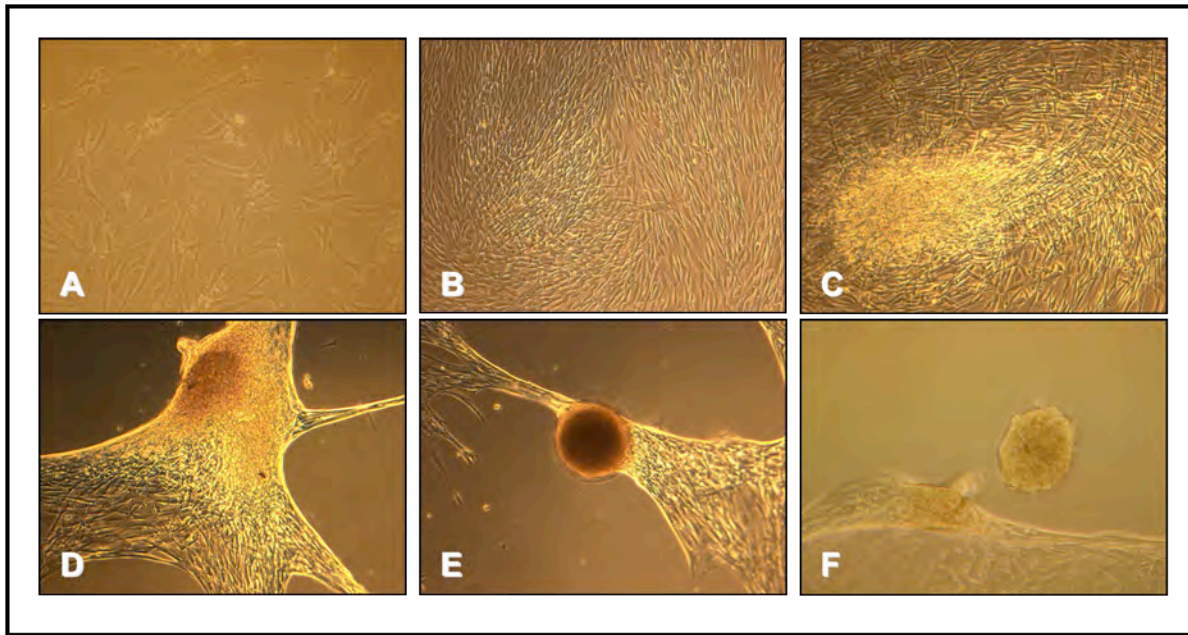


Fig 1. USSCs in various phases of expansion and differentiation. (A) undifferentiated cells at peak of logarithmic growth; (B) cells at confluency; (C) cells gather and (D) start to form islet-like structures; (E) and (F) show differentiated islet-like bodies. (A): cells cultured in basal media; (B)-(F): cells cultured in differentiation media

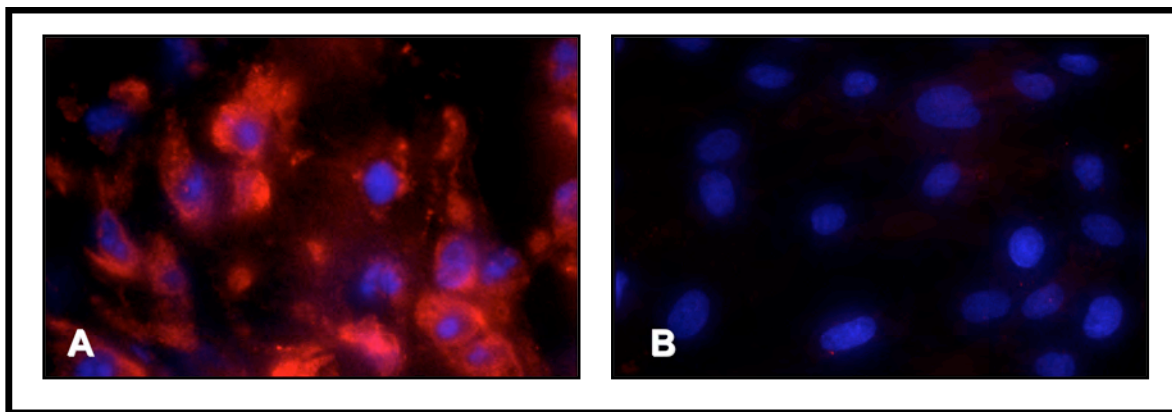


Fig 2. immunofluorescence analysis of USSCs (A) differentiated islet-like bodies stain positive for insulin; (B) undifferentiated USSCs are negative. Red: insulin; Blue: nuclei

CONCLUSIONS

Type 1 diabetes is an excellent candidate for cell replacement therapy. The last few years have seen an increase in the publication of experiments carried out to obtain surrogate insulin-producing cells. Stem cells, which have been isolated from embryonic, fetal, and adult tissues, hold great promise for the treatment and potential cure of diabetes. However, despite the enormous scientific potential of human stem cell research, its clinical application is still limited by many challenges.

First, regardless of their nature, the stem cells must be guided efficiently to differentiate into therapeutically relevant cells, i.e., possessing the ability to sense blood glucose levels and consequently release insulin in physiologically appropriate quantities. The primary challenge for ESCs remains the differentiation *in vitro* into a specific target cell while preventing or suppressing all other alternatives of their pluripotent repertoire. Strategies include attempts to recapitulate or exploit the developmental program that

orchestrates pancreatic islet ontogeny, as well as directing cells along new pathways not experienced by that cell type *in vivo*. Although promising, the majority of these studies have no immediate clinical applicability because of the low production of insulin or the lack of glucose-regulated secretion even in successfully treated cells.

Second, though ESCs possess the widest differentiation potential and should therefore be the best candidates for cell replacement therapy, they are ethically problematic to some, may give rise to teratomas when implanted outside their physiologic niche, and are by definition only allogeneic. On the other hand, ASCs are ethically acceptable to most people, but lack the pluripotency of ESCs. Furthermore, recent sensational publications suggest that it is possible to approach the otherwise unparalleled pluripotency of the ESCs by reprogramming differentiated human somatic cells (e.g., adult human dermal fibroblasts) by transduction of four defined transcription factors, *OCT4* and *SOX2*, plus either *Klf4*, and *c-Myc* (44) or *Nanog* and *Lin28* (45). These procedures should allow the creation of patient-specific stem cells which would be virtually free of rejection concerns. These studies await full confirmation since the lack of published studies using true clonal analysis raises fundamental scientific concern regarding the legitimacy of attributing extensive pluripotency to these tissue-derived somatic stem cells. However, using these induced pluripotent stem cells (iPS), Hanna et al. (46) were able to rescue mice from their humanized sickle cell anemia. Autologous iPS were guided *in vitro* to differentiate into hematopoietic progenitors -- after their genetic defect was corrected through homologous recombination -- and then transplanted into the same affected animal which donated the original fibroblasts. The authors of this study correctly added that “problems associated with using retroviruses and oncogenes for reprogramming need to be resolved before iPS cells can be considered for human therapy”.

Finally, in the case of T1D, any suggested cell therapy will also have to deal with the underlying autoimmune disease, since the autoreactive T-lymphocytes will eventually kill new beta cells generated to replace those lost. Cord blood or placental stem cells might address these concerns because they express histocompatibility antigens in very limited amounts.

In conclusion, although human stem cell research carries with it enormous scientific potential in the treatment and possible cure of T1D, it is still hard to say when this approach will be successfully applied in the clinic. However, the rapid progression of science in this particular challenging field of investigation has been especially reassuring.

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In the fourth and final quarterly scientific progress report (3/01/08 – 05/30/08) of Year 02 we now report on direct evidence of regeneration in the monkey tissue.

Metabolic evolution of islet recipients.

The metabolic evolution and the C-peptide levels (graft and endogenous) of the monkey recipients of porcine islet grafts that exhibited a partial function after transplantation are shown in [Figure 1](#). To note, raising levels of endogenous C-peptide were measured after transplantation but at the time the graft function begun to fail, such endogenous production appeared to take over and it was likely responsible for the maintenance of a stable metabolic control. In monkey recipients of islet graft that failed immediately after implant, or in monkeys sacrificed shortly (within 10 days after transplantation) therefore without sustained graft function, no stable increase in endogenous production was observed. In one monkey where the graft had a partial function, a trend to raising endogenous C-peptide production was noted but after only two weeks graft failed, hypoglycemia returned and also endogenous C-peptide dropped.

Histological analysis of the pancreatic tissue following re-establishment of the endogenous production of C-peptide.

Proliferative activity in the pancreas. Ki67 immunostaining

Pancreatic tissue was immuno-stained with anti-Ki67 antibody, a nuclear marker of active proliferation. Normal, healthy control pancreatic tissue contained occasional Ki67 positive cells, scattered throughout the entire organ. The incidence of positive cells was quantitatively similar in streptozotocin-diabetic, non-transplanted, as well as in transplanted monkeys in which the islet graft had stopped functioning. On the contrary, substantial increased numbers of positive cells were observed in the monkeys that had had a functioning graft and showed return of endogenous C-peptide production. Further analysis demonstrated that Ki67 positive cells could be co-stained with the ductal marker cytokeratin-19 (CK19) or with markers of fibroblast and endothelial phenotypes, but never with insulin positive cells, suggesting that proliferation was not occurring in phenotypically mature beta cells, either pre-existing or newly generated beta cells (Figure 2).

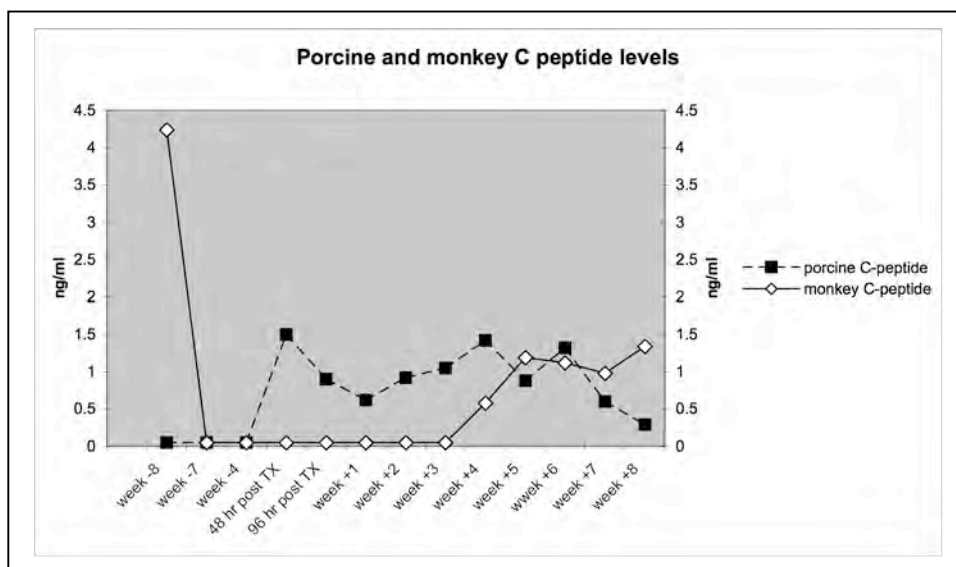


Figure 1

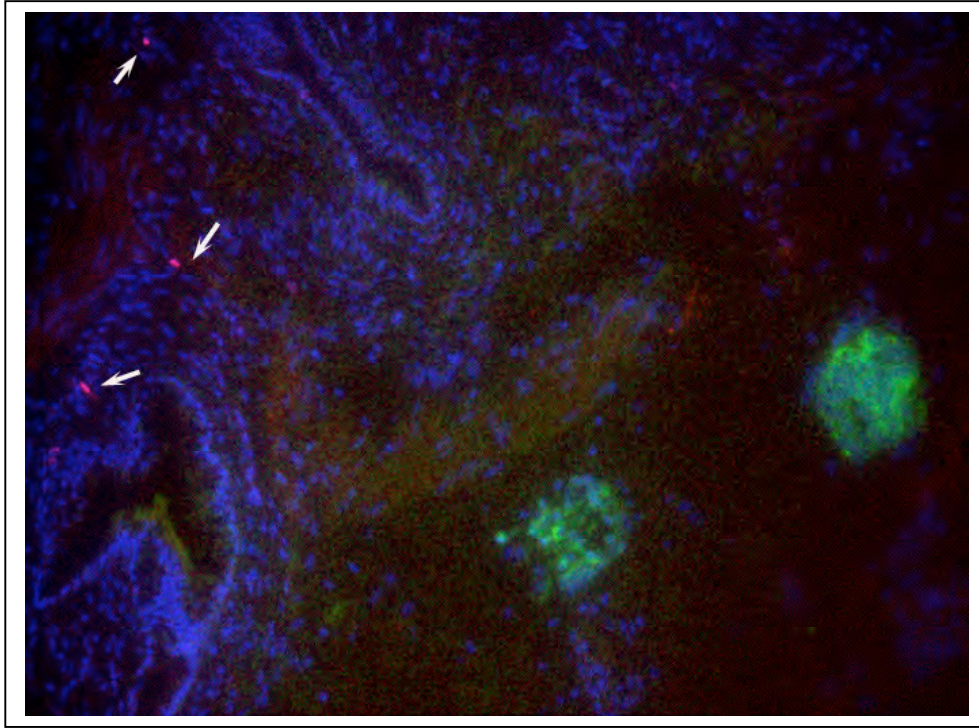


Figure 2 Ki67 in red, Glucagon in green.

Insulin/Proinsulin immunostaining

The pancreas of these monkeys that re-establishment endogenous C-peptide production, was analysed for the presence of insulin producing cells. Insulin positive cells were found throughout the entire pancreatic section, mostly as single cells, not organized in large, islet-like clusters, except for rare small oligo-cellular aggregates. No clear information was obtained whether these cells existed before streptozotocin treatment or, on the contrary, appeared after injury could be accrued. From a speculative point of view, whilst fully formed, streptozotocin-treated islets are typically positive only for glucagon, small clusters were constituted mostly of insulin positive cells. To confirm that immunoreactivity with insulin antibody, and rule out the effect of phenomena of insulin absorption, we also stained the sections with a pro-insulin antibody that confirmed co-localization with previously insulin positive cells ([Figure 3](#)).

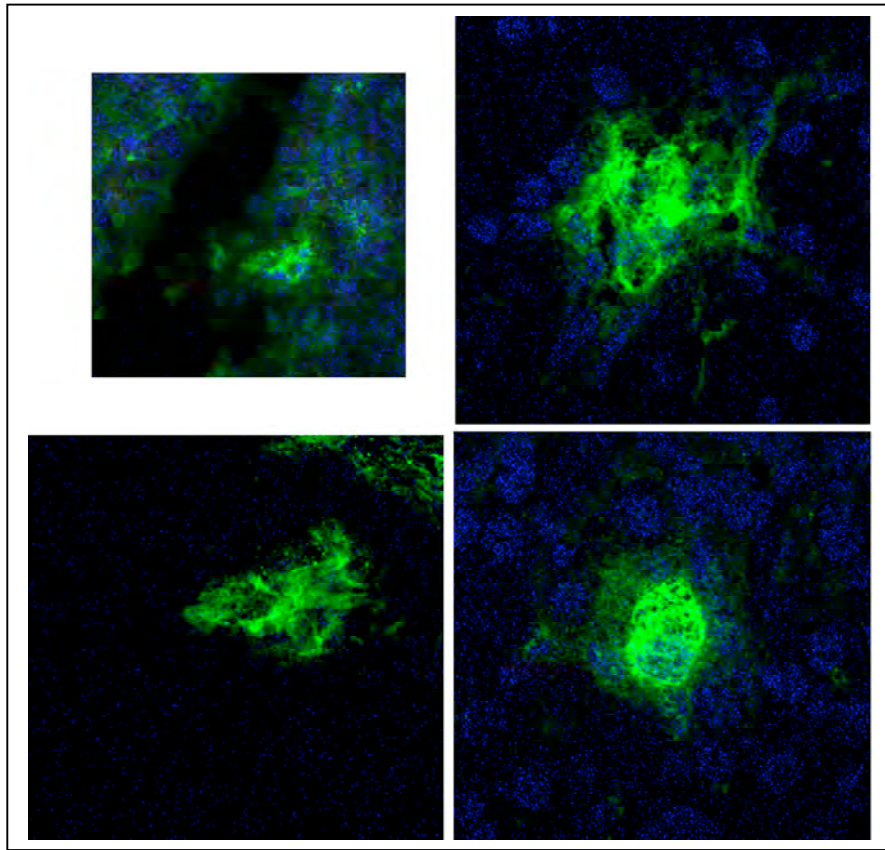


Figure 3

The distribution of insulin positive tissue in the pancreatic organ was also analysed. **Figure 4** show details of insulin/proinsulin positive cell aggregates in areas where glucagone positive cells are not present. The presence of such cell aggregates, positive exclusively for insulin was not reported on control diabetic monkeys or healthy, non diabetic pancreas control tissue. Since streptozotocin spares alpha cells, it is likely that insulin positive cells not associated to alpha cells are not part of a pre-STZ existing islet of Langerhans.

Glut-2 immunostaining

Glucagone and Glut-2 co-localize in the normal control monkey (see figure)

CK19 immunostaining

Anti-CK19 antibody staining was used to identify the ductal/epithelial cells in the pancreatic tissue. The pancreas of those monkeys that showed recovered endogenous production of C-peptide following transplantation, also showed higher numbers of CK19 positive cells in the pancreas compared to control, healthy and diabetic not transplanted monkeys. Interestingly, the monkeys with long-term function of the graft, associated to increased endogenous insulin production, showed a subpopulation of cells staining positive for CK19 as well as insulin. (**Figure 5**). No co-localization between alpha and beta phenotype (glucagon and insulin) was found.

Figure 4

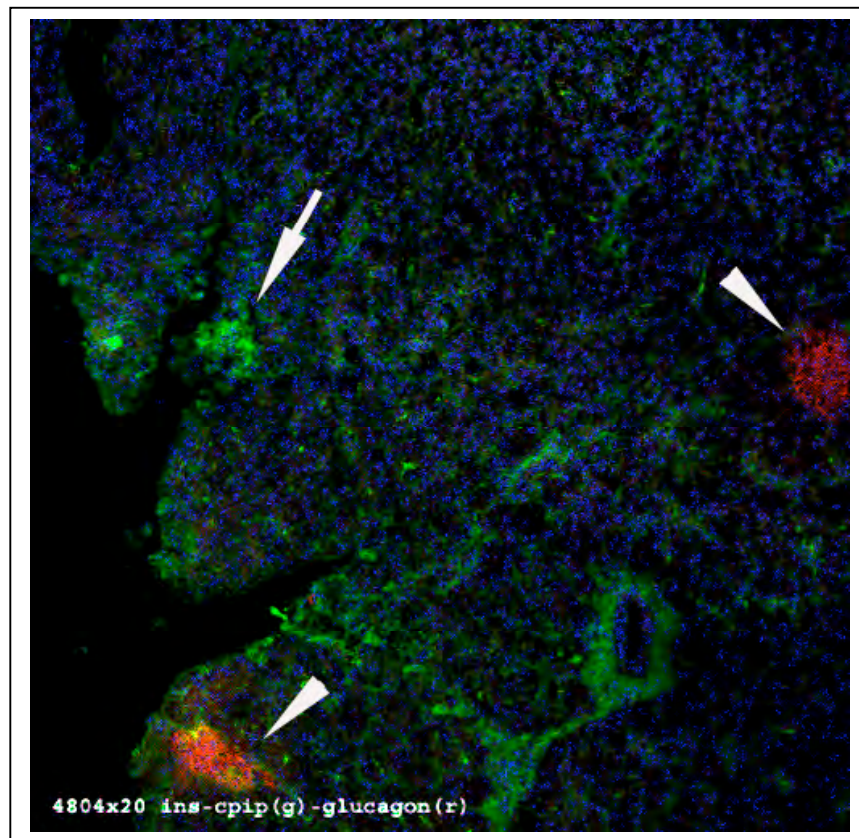
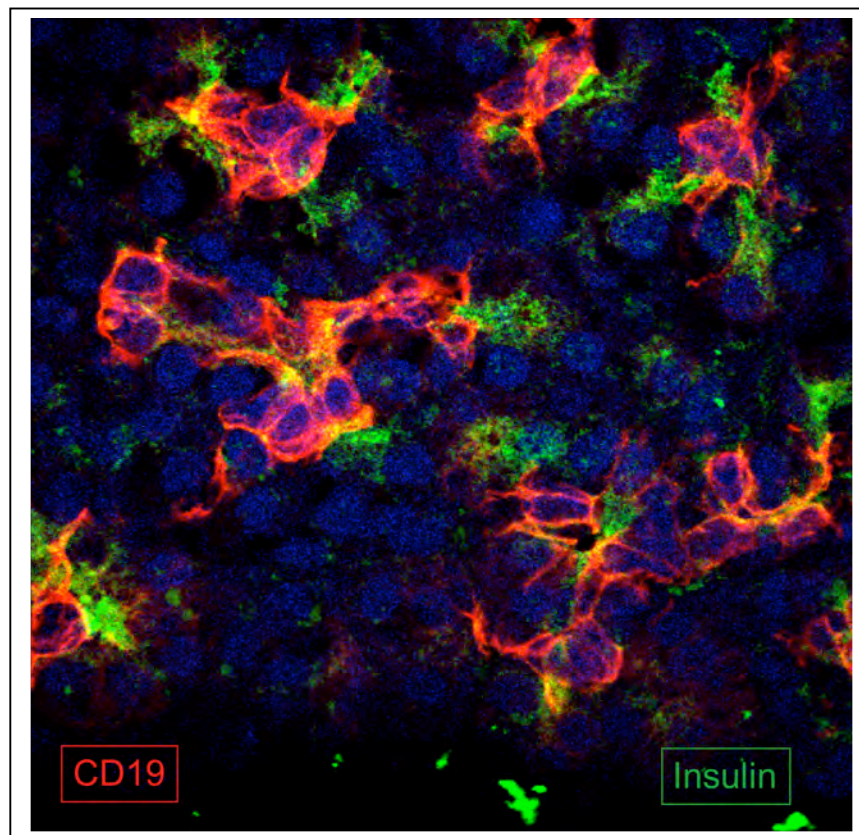


Figure 5



KEY RESEARCH ACCOMPLISHMENTS

- Streptozotocin successfully induces hyperglycemia following administration in rodents as well as in non-human primates.
- Diabetes induction is characterized by the establishment of a hyperglycemic status that, in non-human primates, requires exogenous insulin treatment to prevent fatal cheto-acidosis. Following streptozotocin administration, a drastic reduction of endogenous circulating C-peptide is observed, and histologic analysis of the pancreas shows virtual absence of insulin positive cells. Even in those monkeys where low but detectable C-peptide levels were measured after streptozotocin, histological findings confirmed the destruction of insulin positive cells, and in vivo functional studies suggested that any residual insulin producing mass is not responsive. We consequently observed that hyperglycemia and diabetes management were not influenced by the residual C-peptide amounts.
- No report of a spontaneous remission of the hyperglycaemic status in diabetic monkeys even months after streptozotocin administration was found. It appears therefore that in non-human primates, similarly to rodents, exogenous insulin itself does not trigger a healing process of the pancreatic beta cell mass, even in the absence of an autoimmune contest, thus after chemically-induced diabetes.
- On the putative source of cells that contribute to reform functional insulin producing tissue in the recipient, quite interesting speculation have been put forwards, however, in our experiments as well in those of other groups no direct evidence that an external, donor origin cell source is really needed, supporting the concept that the pancreatic organ can potentially heal itself. It remains scope for investigations the metabolic effect of the islet graft, that in addition to providing insulin, essential to maintain the animal alive, may further provide additional factors able to affect/trigger the recovery of endocrine function. In the absence of the autoimmune mechanisms (no evidence of autoimmunity is accrued in streptozotocin diabetic monkeys), our experiments suggest that diabetic monkeys that receive xenogeneic islet support with sustained detectable biological activity for sometime, they may recover a self-driven endocrine function. Such function is evidenced not only by stepwise increasing levels of endogenous C-peptide, but also by amelioration of the metabolic control, not simply explained by the effect of the graft. In this contest, the xenotransplantation model offers the advantage to allow discernment between graft and endogenous insulin production. In several monkey recipients, following transplantation we observed an increased in endogenous C-peptide levels, however only two recipients maintained graft function long enough and endogenous C-peptide substantial recovery occurred relatively late over time, consistent with the kinetics of recovery described in rodent models.
- This recovery of the endogenous insulin production was associated with quite peculiar histological features of the pancreatic tissue. We found a generalized augmented proliferative activity in the pancreatic tissue, spread throughout the organ and associated to fibroblast positive, ductal and endothelial phenotype, but not specifically to insulin positive cells. We also observed that in monkeys that exhibited endogenous production of C-peptide, pancreatic ducts were hypertrophic. Interestingly, substantial numbers of ductal cells (CK19+) showed also co-staining with proinsulin. More close analysis of the pancreas in the monkey that presented double CK19-proinsulin phenotype, indicated the presence of small size insulin positive cell aggregates. Such clusters appeared not even partially damaged, and differed morphologically from those that clearly showed regressed signs of injury, commonly associated with undamaged alpha cells (glucagon positive cells). Moreover proinsulin positive aggregates were often found not in correspondence of glucagon positive cells suggesting that they were not part of pre-existing islet of Langerhans.
- Since such effects were not found in monkeys that did not receive a transplant, and monkeys recipient of non functional graft, but partially present in monkeys with longer survival of the graft, we hypothesize that the transplantation may play a role. Whether a functional graft is exerting an effect on recovery, it remains unclear but in this line there are reports in human clinical islet allografts that indicate an amelioration of the metabolic conditions, often accompanied by increased endogenous C-peptide production, in patients that received a transplant, even if the graft was rejected or insufficient to establish normoglycemia.

CONCLUSION

Overall our data suggest that the monkey pancreas retains the ability to recover even after a chemical damage that specifically destroys the islet beta cells inducing a irreversible status of hyperglycemia. Such ability seems to be associated with activation of proliferative events in the pancreatic organ, and hypertrophy of the ductal cells. Double phenotype, epithelial and endocrine on the same pancreatic cells further indicate that a direct relation there exist between these two cell types. Recovery of the insulin producing function, in association with the histological findings may provide indirect supportive evidence that epithelial/ductal cells play a role in the generation of insulin producing beta cells. The metabolic enhancement achieved by even partially functioning islet transplantations compared to mere exogenous insulin administration may contribute to promote return of endogenous production of C-peptide and it is desirable to further investigate the multiple biological events that accompany engraftment. Further studies will clarify whether the reparative events involve ex-novo generation of endocrine tissue from pancreatic precursor cells, or, alternatively it involves the recovery of previously damaged cells. In both cases, considering that monkeys are the closest animal species to humans, useful information can be accrued on the dynamic properties of the endocrine pancreas in men.

APPENDICES

Wang Z, Zhu T, Rehman KK, Bertera S, Zhang J, Chen C, Papworth G, Watkins S, **Trucco M**, Robbins PD, Li J, Xiao X: Widespread and stable pancreatic gene transfer by AAV vectors via different routes. **Diabetes** 55:875, 2006.

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INTRODUCTION:

Lesions of the endocrine pancreas, as they occur in Type 1 Diabetes (T1D), were historically considered to be permanent and irreversible since patients, once endogenous insulin production is blunt, require hormone therapy for life. More recently it was proposed that islet beta cells actually retain the ability to heal from an injury, and to proliferate and multiply from precursors into mature beta cells during adult life, in a way similar to that during embryonic development. It was also proposed that possible reparative events affecting the beta cell mass normally occur but in diabetes are opposed by the destructive mechanisms that cause diabetes in first place such as the autoimmune attack (1,2).

In mouse models, evidence was presented that adult animals retain the ability to expand the beta cell mass as a response to various triggers. However, it remains largely unclear through which molecular and cellular mechanism(s) reestablishment of the beta cell mass takes place. Furthermore it was shown in the diabetic prone NOD-mouse that, even after establishment of the clinical diabetic status, it is still possible to recover endogenous pancreatic insulin production. This can be achieved by successfully combining strategies aimed at blocking the autoimmune attack with the use of non-diabetogenic immunosuppressive drugs. Normally an islet transplantation was used in parallel to supply beta cell function for the relatively long periods required to recover beta cell function (3-6).

A spontaneous recovery of the pancreatic beta cell function is also normally reported in patients diagnosed with autoimmune T1D, the so-called “honeymoon” period. Although the honeymoon may vary in duration quite dramatically from one patient to the next, there are also anecdotal cases of complete and permanent recovery. Following diagnosis of T1D and initiation of exogenous insulin treatment these individuals experienced a return of endogenous insulin production, characterized by increased C-peptide secretion, and reduction in the titre of circulating islet auto-antibodies, confirming the return of the immunologic tolerance and a consequent recovery from islet cell destruction (7). Although it is unquestionably proven that some beta cells are still present and able to produce insulin years after the clinical onset of diabetes (8,9), a steady recovery from the diabetic status is quite unique and is worth further investigations. At any rate, the potential for the pancreatic organ to recover substantial endocrine function is quite fascinating and should be exploited for possible clinical applications.

To this aim, the observation that only a limited islet mass is actively engaged in supplying insulin to maintain normoglycemia at a give time point is remarkable. This information seems to indicate that the critical mass required to synthesize and release insulin sufficient for the body's normal needs is far less than that produced by the entire beta cell pool of a healthy pancreas. This observation implicates that even a relatively limited quantity of insulin producing tissue should exert clinically evident effects in patients.

Non-human primates are relevant animal models for pre-clinical studies in general and, in particular, for xenotransplantation experiments. Monkeys and humans present strong similarities although significant metabolic differences do exist. It is therefore of great value to investigate the potentiality of non-human primate pancreatic tissue to resume endogenous insulin production following its destruction, for a better understanding of the human behaviour as well.

In monkeys, a permanent diabetic status can be induced by total pancreatectomy, a major surgical procedure usually not devoid of technical difficulties or, alternatively, by chemical destruction of the insulin producing cells by means of streptozotocin, a potent toxic agent that specifically target the pancreatic beta cells (10).

Our experimental protocol for pig islet xenotransplantation into monkeys involved induction of diabetes in the recipients by streptozotocin, intra-portal transplantation of porcine islets after recipient immunologic pre-conditioning, completed by an adequate non-diabetogenic immunosuppressive therapy. While the majority of recipients exhibited a transitory islet graft function and were sacrificed early after transplantation, in some recipients' islet graft function (indirectly assessed by measurable levels of porcine C-peptide) lasted for months (11). At a certain point in time, concurrently to a gradual reduction of the C-peptide (porcine) graft output, we observed a stepwise increased in endogenous C-peptide (monkey) levels. Metabolic clinical improvement and peculiar histological features of the pancreatic tissue were also found associated to this change. To note, no

spontaneous recovery of endogenous function was observed in transplanted monkeys with transient function of the graft or diabetic monkeys maintained under exogenous insulin for up to 1 year.

Our data provide evidence that the pancreatic tissue is able to re-establish endogenous insulin production after chemically induced beta cell specific injury. Additional investigation is required to understand whether conditions such as improved glucose metabolic control and an appropriate immunosuppressive regimen, in association with functional islet transplantation could play a role in beta cell rescue or regeneration, and if so, through which mechanism(s) (12).

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BODY:

[Our first quarterly scientific progress report \(06/01/08 – 08/31/08\) for the third year of this project, detailed the following steps forward in reaching the aims of our study.](#)

Until a few years ago, lesions of the endocrine pancreas, as they occur in type 1 diabetes, were thought to be permanent and irreversible since diabetic patients require hormone replacement therapy for life (1). Despite the clinical evolution of the disease, it is still unknown whether the islet beta cells preserve, at least in part, the ability to heal from an injury (2). Moreover, it is not clear whether in type 1 diabetes a possible recovery of the beta cell mass is contrasted by the destructive mechanisms that cause diabetes in the first place (3).

In mouse models, evidence has accrued that adult animals retain the ability to expand their beta cell mass after stimulation with a variety of triggers (4-7). It has also been shown that in non-obese diabetic (NOD) mice, strategies involving reversal of the autoimmune attack were key to allow recovery of endogenous insulin production even after diabetes onset (8-12). It remains largely uncertain through which molecular and cellular mechanisms the reparative process works, i.e. if new beta cells are formed via self-proliferation or originate from pancreatic precursors as they do during embryonic development (2).

In humans, the ability of post-natal pancreas to expand beta cell mass after injury is still debated (13-15). Spontaneous recovery of the beta cell function has been reported in only a few cases of patients previously diagnosed with type 1 diabetes. These individuals experienced a return to endogenous insulin production after diagnosis and initiation of exogenous insulin treatment. Concurrently, the humoral signs of autoimmunity (i.e., autoantibody positivity) eventually vanished (16-18).

It has been reported that in humans some beta cells are still present even years after the clinical onset of diabetes (19,20). We also know that in the healthy pancreas only a limited islet mass is actively engaged in supplying insulin to maintain normoglycemia, meaning that the critical mass that needs to be functional at a given time point is far less than that of the entire pancreatic beta cell pool (21). This observation may entail that replacement of relatively small numbers of insulin producing cells should exert substantial effects in patients. The potential for the pancreatic organ to recover the endocrine function is quite fascinating and it is the object of intense study for possible clinical applications.

Non-human primates are important animal models for pre-clinical studies and, in particular, for xenotransplantation experiments. From a physiological point of view, monkeys and humans present strong similarities even though the monkey does not show signs of spontaneous autoimmune diabetes (22). Nonetheless a permanent diabetic status can be induced by total pancreatectomy or by chemical destruction of the beta cells with streptozotocin (STZ) (23). It is therefore of great value to investigate the potential of non-human primate pancreatic tissue to recover endogenous insulin production after injury.

Our experimental protocol involved induction of diabetes in eleven monkeys by high dose of STZ followed, in eight of them, by intra-portal pig islet transplantation. In the pig-to monkey model, endogenous primate C-peptide can be easily distinguished from donor pig C-peptide. No spontaneous recovery of endogenous function was observed in any of the STZ-diabetic monkeys that were not transplanted (n=3) and in those that experienced early graft failure (n=6). In two diabetic recipients in whom pig islet graft functioned for a few weeks, we observed instead increasing endogenous C-peptide levels paralleled by clinical improvement, associated to supportive histological and molecular findings. Interestingly, exceptionally long graft survival time was not associated with recovery of endogenous beta cell function (our unpublished data and 24).

Results.

Recovery of endogenous C-peptide in two STZ-diabetic islet recipients

All monkeys became hyperglycemic within 48 hours and required exogenous insulin following STZ injection. A lack of C-peptide increase during IVGTT was recorded in all STZ treated monkeys (data not shown). C-peptide was under detection levels (<0.16 nmol/L) in all animals using a chemiluminiscent method. Using commercially available RIA kits, C-peptide concentrations were above detection levels (>0.03 nmol/L) in the majority of the monkeys however showed a reduction of at least 75% compared to the pre-STZ values (Figure 1A). Monkeys 5204 and 4804 (monkeys with recovered function) showed levels below detection using both methods for more than two months.

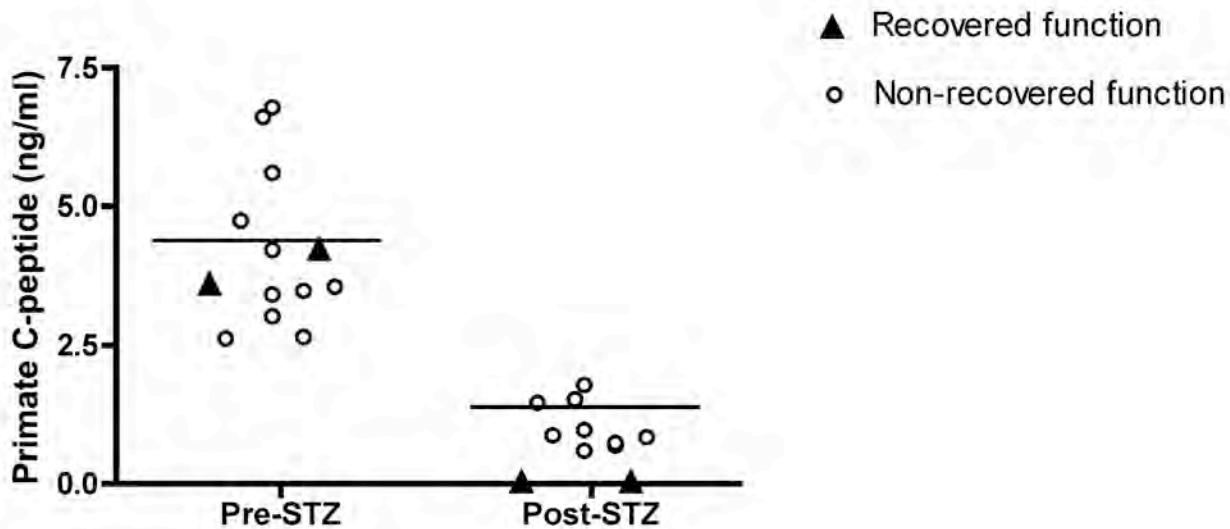


Figure 1A: Endogenous C-peptide levels before and after STZ treatment. Close triangles indicate the values of C-peptide in the two monkeys that recovered autologous insulin production.

Diabetic monkeys that did not undergo transplantation and islet recipients with early graft loss (undetectable porcine C-peptide or detection for less than 2 weeks) showed no significant increase of the autologous C-peptide over the post-STZ basal levels (**Figure 1B**) for more than one year and exogenous insulin requirements remained unchanged (data not shown). The pancreatic tissue of STZ treated monkeys showed islets characterized by almost complete absence of insulin positive cells. In two monkeys (M4804 and M5204), however, a gradual recovery of basal endogenous C-peptide occurred over time (**Figure 1B**).

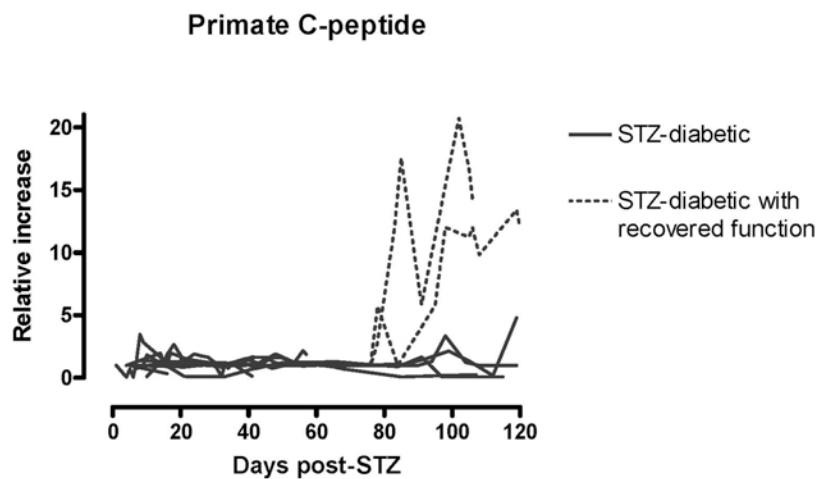


Figure 1B: Relative increase of endogenous C-peptide over basal post-STZ values. Close lines indicate STZ-diabetic monkeys that were not transplanted (n=3) as well as STZ-diabetic recipients that experienced early graft loss (n=6). Dotted lines represent the monkeys with recovered beta cell function (n=2).

This increase was noted after a period of three weeks of islet graft function evidenced by detectable porcine C-peptide levels. Interestingly, the two curves representing endogenous Vs porcine C-peptide followed an opposite trend (Figure 2A,C). It appears therefore that graft failure marks a switch in insulin production from the graft to the endogenous pancreas. Graft failure was not followed by a return to severe hyperglycemia and to higher exogenous insulin requirements possibly because of the autologous insulin production (Figure 2B,D). In addition, IVGTT showed that stimulated endogenous C-peptide response became positive with time in both monkeys, further confirming the progressive recovery of the pancreatic function (Figure 2E,F). In contrast, in all other recipients following islet graft failure, glycemia and insulin requirements returned to pre-transplantation levels (data not shown). Finally, in monkeys with a long-term graft function (3 and 12 months), recovery of endogenous insulin production was not at all observed (our unpublished data).

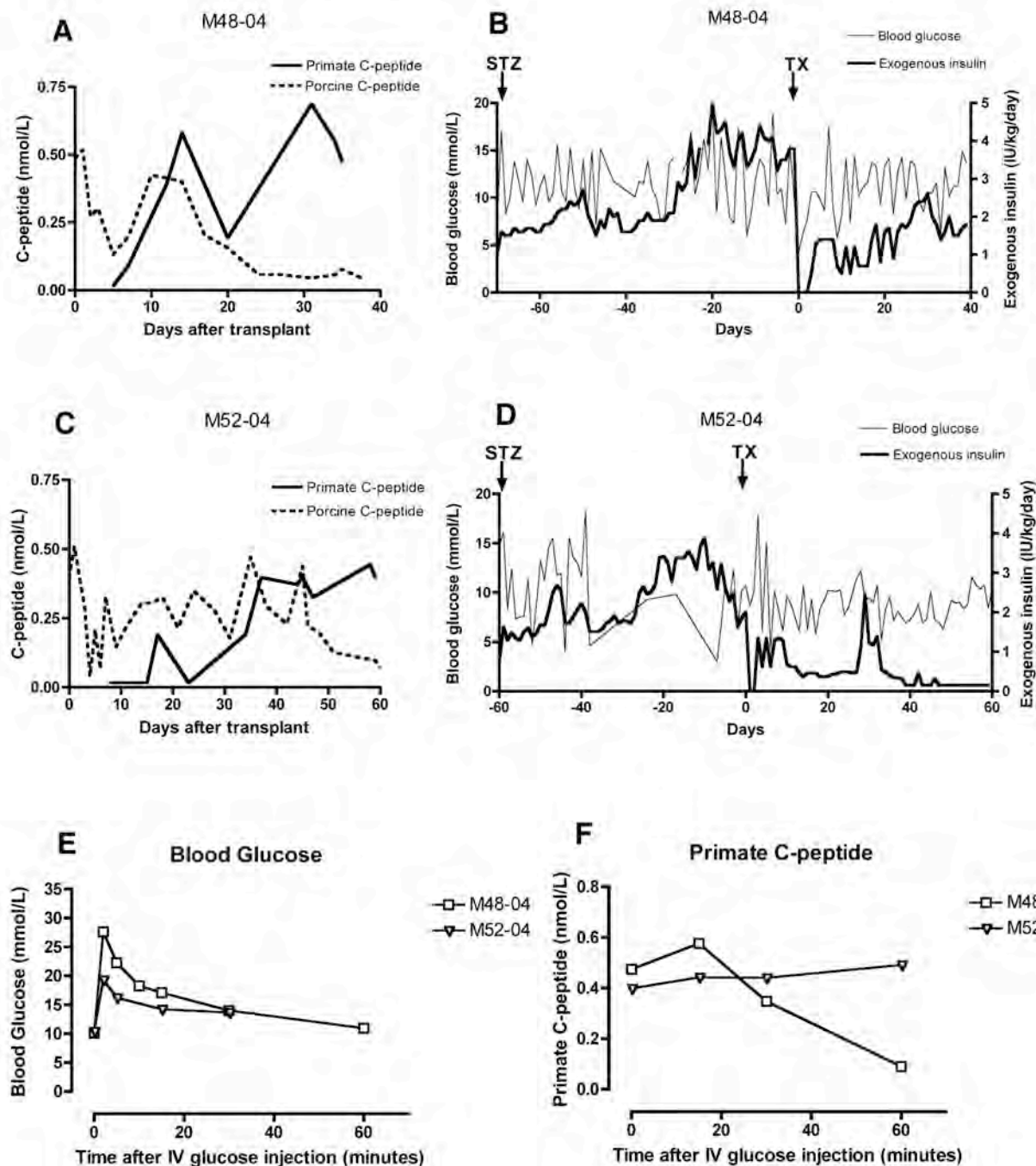


Figure 2: Metabolic profile of two monkeys recipient of islet grafts recovering endogenous C-peptide. A and C: Graft (porcine) and endogenous (primate) C-peptide levels. (B and D): Blood glucose and exogenous insulin administration. E and F: Improved endogenous beta cell response to glucose stimulation overtime in monkey M4804 and M5204. Blood glucose levels (E) and endogenous C-peptide (F) during IVGTT at day 36 (M4808) and day 59 (M5204) following transplantation.

Proinsulin/glucagon immunostaining

The pancreas of all the monkeys was analysed for the presence of proinsulin positive cells. Pre-existing islets in diabetic monkeys were individuated by glucagon immunostaining.

The two monkeys that re-established endogenous C-peptide production showed several proinsulin positive cells grouped in small aggregates or scattered as single cells throughout the pancreas. They were not part of previously existing islets as demonstrated by the absence of neighbouring glucagon⁺ cells (Figure 3).

The frequency of proinsulin positive cell clusters with area equal or smaller than $30 \mu\text{m}^2$ (cell clusters not included in the islets existing before STZ treatment or in the ducts) in the monkeys with recovered endogenous insulin production was higher than in STZ-diabetic controls, with respectively 0.23 and 0.18% positive area/whole section versus 0.05 ± 0.0004 in 4 diabetics, however less than in the pancreas of 3 non diabetic monkeys ($0.30 \pm 0.01\%$).

Proinsulin positive cells also co-stained with PDX-1. Double alpha and beta phenotype (glucagon⁺ and proinsulin⁺) was never found (Figure 3A) in any of the groups of animals analysed

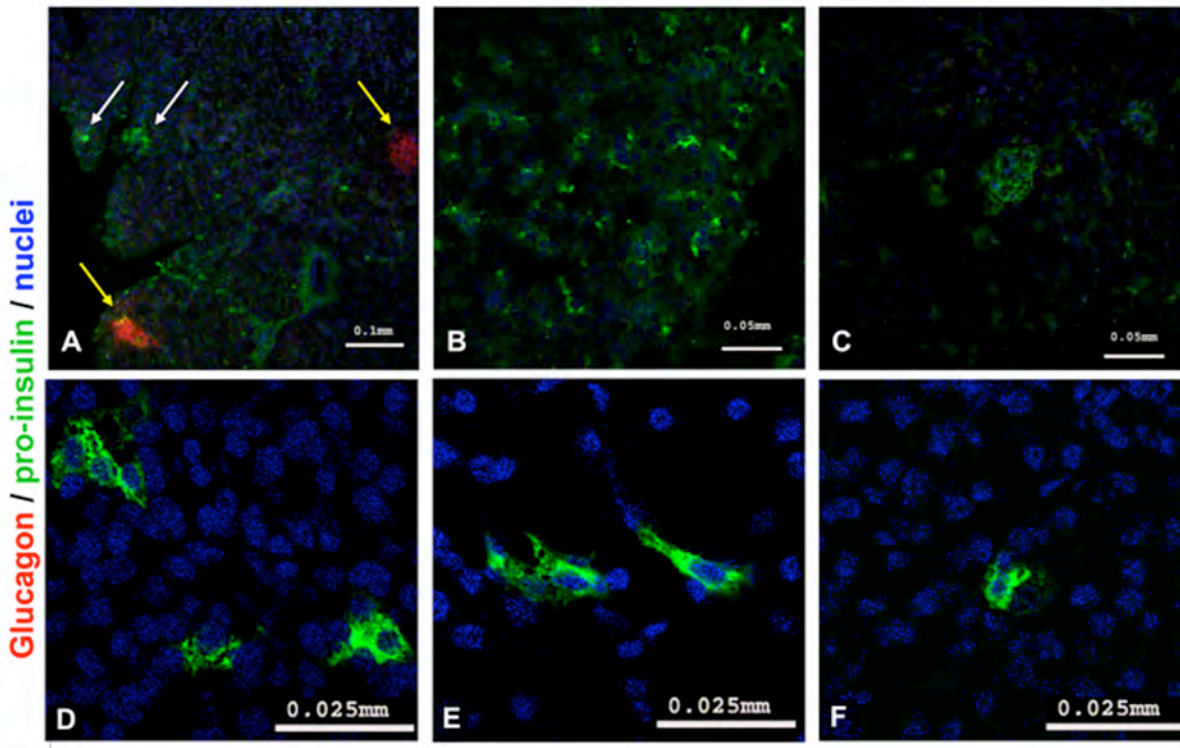


Figure 3: Proinsulin positive cells in monkeys with recovered endogenous beta cell function. Proinsulin positive cells in the pancreas are organized in small aggregates (A) or scattered as single cells (B) outside pre-existing islets characterized by glucagon positive staining. White arrows: proinsulin positive cells. Yellow arrows: pre-existing islets. (C-F): different magnifications. Pictures are representative of both monkeys with recovered endogenous beta cell function.

Ductal cells co-stain with proinsulin and PDX-1 in monkeys with recovered function

Anti-CK19 antibody staining was used to identify the ductal/epithelial cell compartment in the pancreatic tissue. Interestingly, a subpopulation of CK19 cells co-stained with proinsulin in the parenchyma (Figure 4A-D) and in the ducts (Figure 4E-H) only in the two monkeys with recovered function. The pancreas of the two monkeys with recovered endogenous beta cell function showed stronger expression of CK19 cells in comparison to non-diabetic and STZ-diabetic transplanted and non transplanted controls.

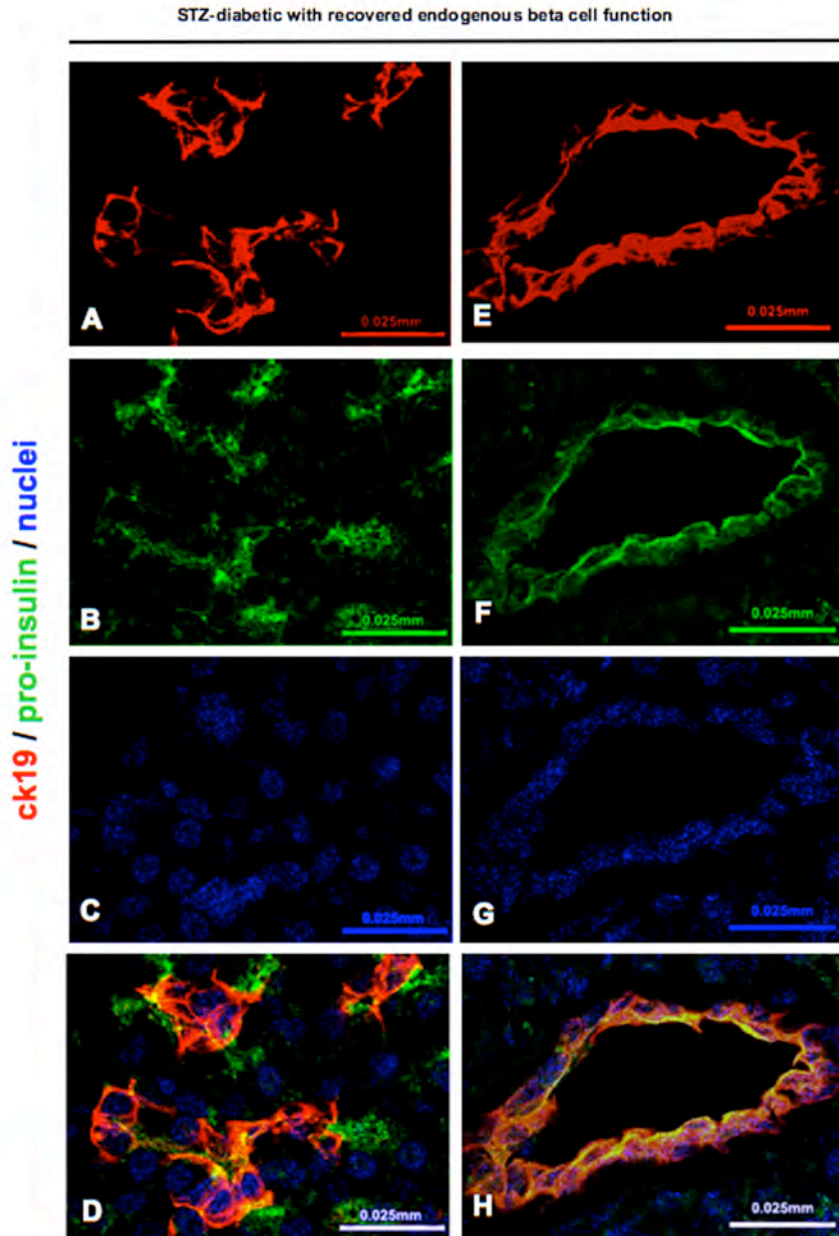


Figure 4: Presence of double phenotype CK19/proinsulin in monkeys with recovered endogenous beta cell function. A,B,C, and D: Monkeys with recovered endogenous beta cell function show co-expression of CK19 with proinsulin (yellow). E,F,G, and H: Detail of a pancreatic duct.

Additionally, as shown in [Figure 5](#), the monkeys with recovered beta cell function presented double $PDX-1^+/CK19^+$ and $PDX-1^+/proinsulin^+$ staining. To note, $PDX-1^+$ cells are found in STZ-diabetic control monkeys but they do not co-localize with CK19 ([Figure 5B](#)) and with proinsulin (data not shown).

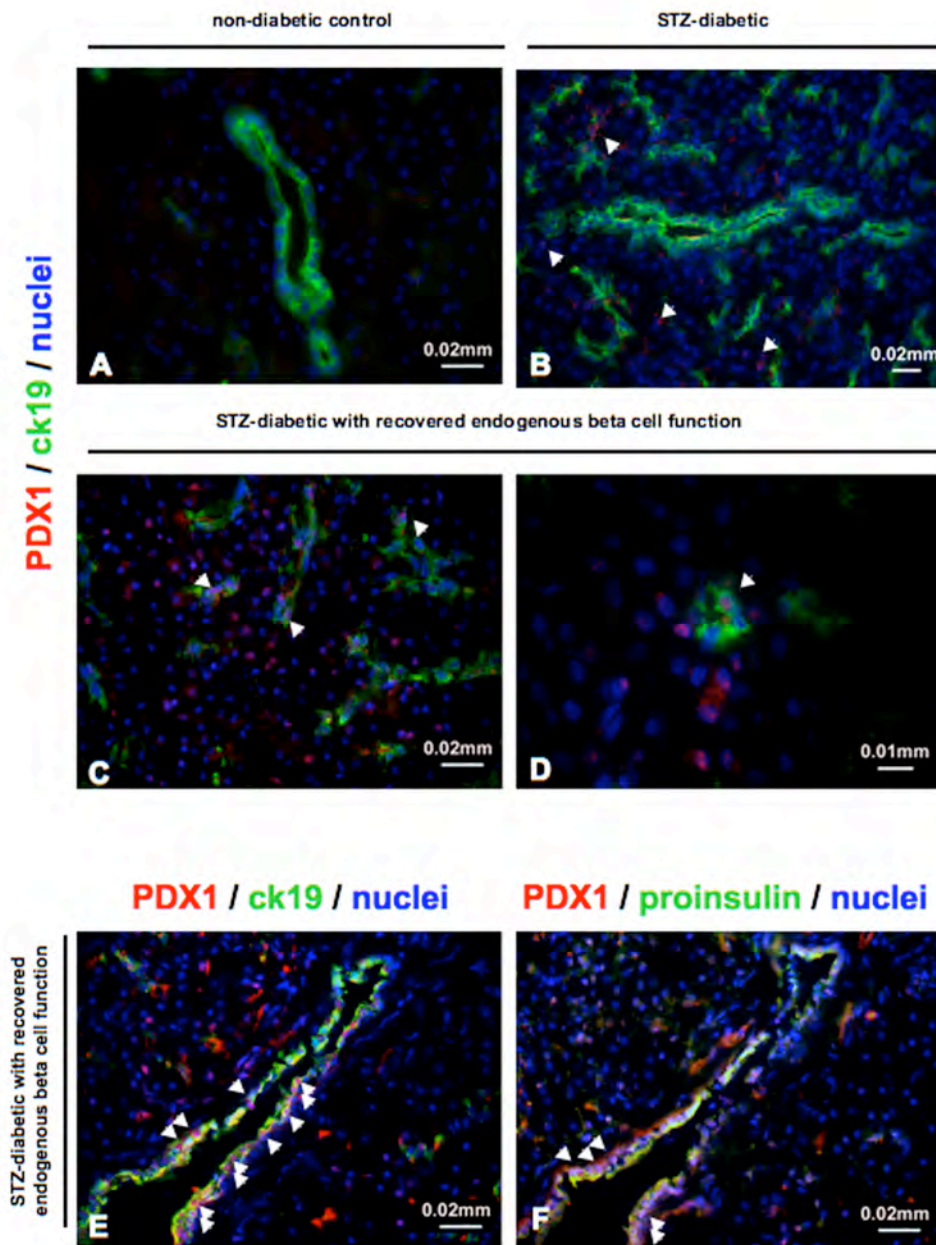


Figure 5: PDX-1 co-expresses with CK19 in the pancreas of monkeys with recovered function. CK19 and PDX-1 do not co-stain in the pancreas of non-diabetic healthy monkeys (A); PDX-1 positive cells are found scattered throughout the pancreas of STZ-diabetic monkeys, but do not co-localize with CK19 (B); CK19 and PDX-1 co-localization shown in the pancreas of a monkey with recovered beta cell function (C with detail in D). E and F: pancreatic consecutive sections M5204 (monkey with recovered beta cell function) showing CK19⁺/PDX-1⁺ (E) and CK19⁺/proinsulin⁺ (F) cells respectively. Arrows show PDX-1⁺ cells in B, double positive PDX-1⁺/CK19⁺ in C, D and E and PDX-1⁺/proinsulin⁺ in F.

Proliferative activity in the pancreas: Ki67 immunostaining

Anti-Ki67 antibody was used as a nuclear marker of active cell proliferation.

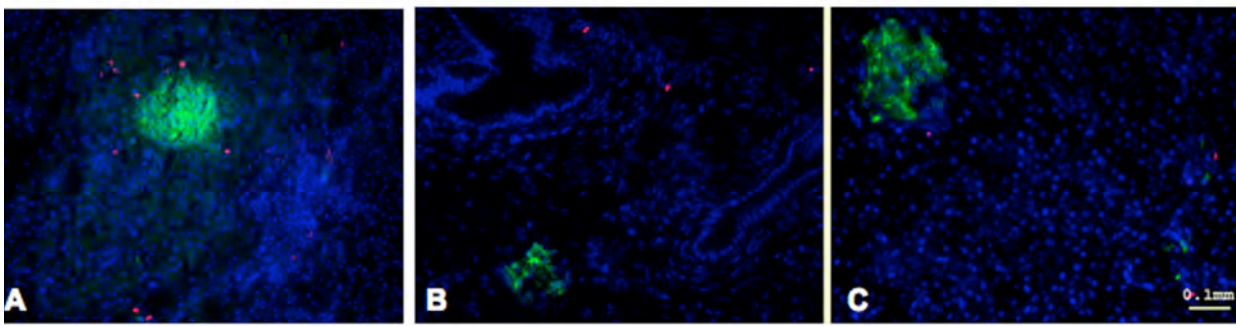
Positive cells were observed in pancreatic sections of the monkeys that showed return of endogenous C-peptide production (Figure 6A,D,E and F). Ki67⁺ cells co-stained with the ductal marker CK19 or with fibroblasts (Figure 6D,E), but not with proinsulin⁺ cells (Figure 6F). Pancreatic sections of non-diabetic and STZ-diabetic control monkeys contained occasional Ki67⁺ cells (Figure 6B,C).

ki67 / glucagon / nuclei

STZ-diabetic with recovered endogenous
beta cell function

STZ-diabetic

non-diabetic control



ki67 / fibroblast / nuclei

ki67 / ck19 / nuclei

ki67 / pro-insulin / nuclei

STZ-diabetic with recovered endogenous beta cell function

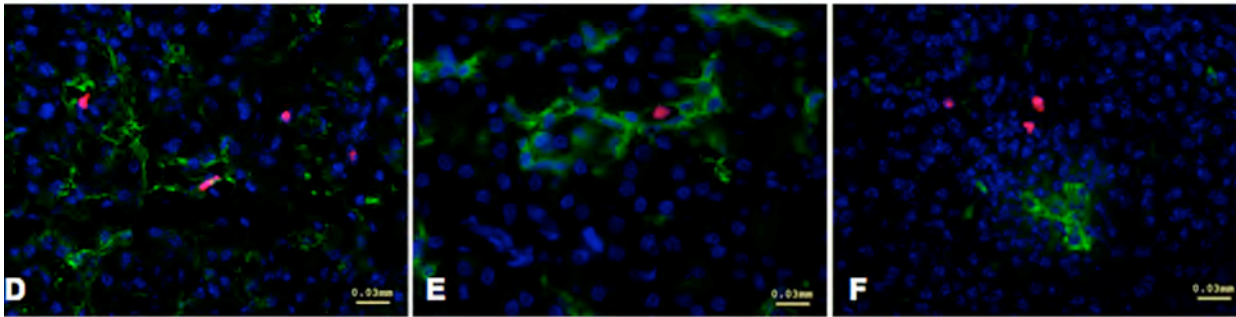


Figure 6: Proliferative activity in the pancreas. Ki67 staining is increased in pancreas of STZ-diabetic monkeys with recovered endogenous beta cell function (A) in comparison to STZ-diabetic monkeys (B) and non-diabetic control (C). In STZ-diabetic monkeys with recovered endogenous beta cell function (D-F) Ki67 co-stains with fibroblast marker (D) and CK19 (E) but not with insulin (F).

Peculiar GLUT2 immunostaining in the pancreas of the monkeys with recovered function

GLUT2 expression was studied as an additional beta cell specific marker to characterize the beta cell population of the two monkeys that rescued insulin production. In these monkeys, GLUT2 co-stained with glucagon in islets devoid of the beta cells due to STZ. (Figure 7A,D). GLUT2⁺/glucagon⁺ cells were not found in STZ-diabetic monkeys (Figure 7C,F) and in non-diabetic control monkeys, where GLUT2 was only found in proinsulin⁺ cells within the islets (Figure 7B,E).

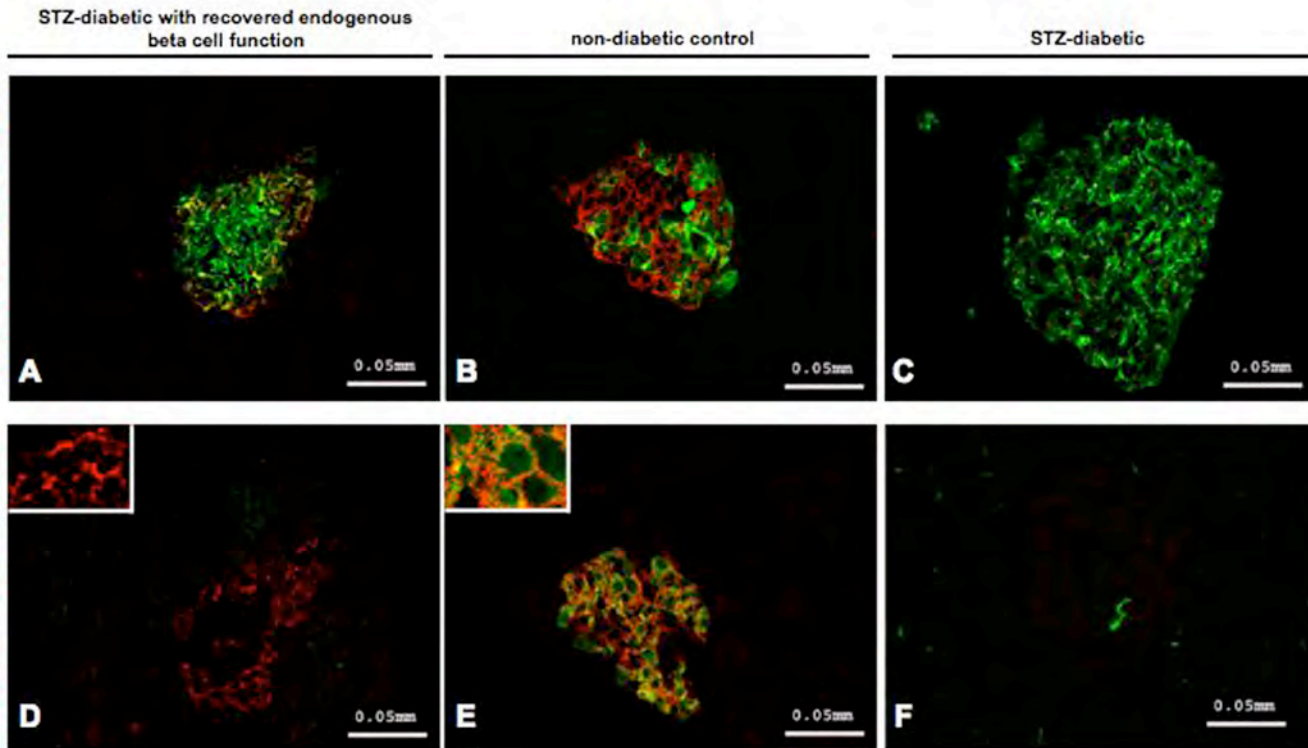


Figure 7: Presence of double phenotype GLUT2/glucagon in monkeys with recovered endogenous beta cell function. Monkeys with recovered endogenous beta cell function show co-expression of glucose transporter GLUT2 with glucagon (A) and not with insulin (D) within pre-existing islets damaged by STZ. B and E: non-diabetic monkey. C and F: STZ-diabetic monkey. Double phenotypes GLUT2/glucagon and GLUT2/insulin stain yellow.

Analysis of endocrine markers by qRT-PCR

The expression of selected islet-specific nuclear transcription factors was also analyzed in transplanted and non transplanted monkeys (34). Gene expression in the two monkeys with recovered endogenous beta cell function was compared to non-diabetic and STZ- diabetic controls. qRT-PCR of NKX6.1, Pax6, ISL1, Foxa2, Beta2NeuroD, and PDX-1 showed mRNA levels of the same order of magnitude in all monkeys, with only individual variability (Figure 8A-F). When the expression of each gene was normalized against insulin, in the regenerating monkeys such factors resulted consistently upregulated (Figure 8G). Figure 8H shows PDX-1 normalized against GAPDH in two untransplanted STZ-treated diabetic monkeys with short (17 days) and long (424 day) diabetes follow-up, in the two monkeys with rescued beta cell function and in non-diabetic controls (mean of 4 different animals). PDX-1 expression is markedly decreased in the first days after STZ in comparison to long-standing diabetic, recovered and non diabetic monkeys.

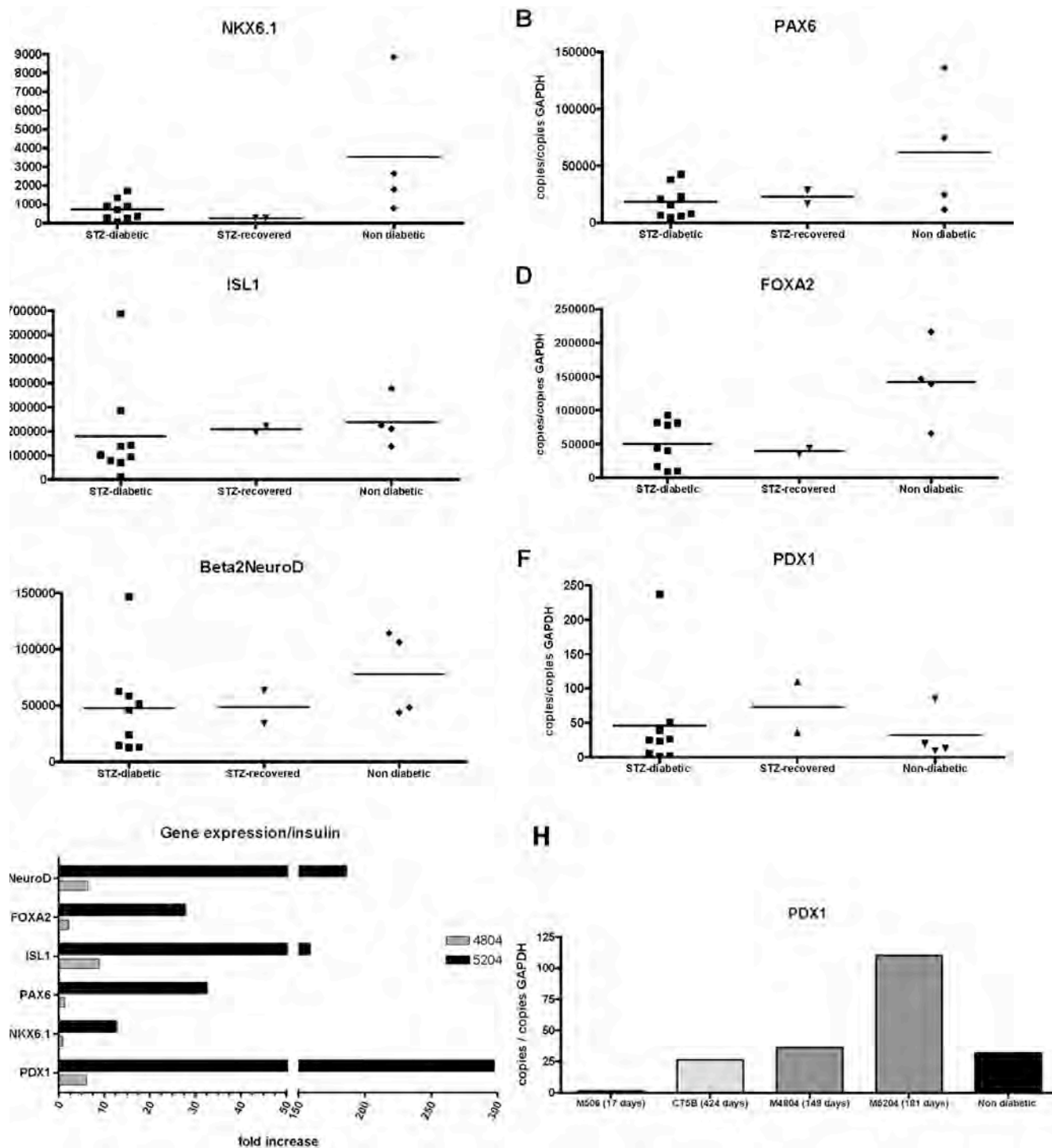


Figure 8: qRT-PCR analysis of endocrine precursor markers. A-E: mRNA expression of the nuclear transcription factors PDX-1, NKX6.1, PAX6, ISL1, FOXA2, Beta2NeuroD in STZ-diabetic monkeys (n=9), monkeys with recovered endogenous C-peptide (n=2) and non-diabetic controls (n=4). Copies are normalized against the housekeeping gene GAPDH. Normalization against HPRT showed no substantial differences. **G:** normalization of the nuclear transcription factors against insulin in the two monkeys with recovered beta cell function. Expression levels relative to insulin are consistently upregulated. Values represent fold increase in relation to normal non-diabetic pancreatic tissue (mean of n=4 monkeys). **H:** PDX-1 expression decreases in short and long standing non transplanted diabetic monkeys Vs monkeys with recovered beta cell function. Non diabetic controls: mean of 4 different monkeys.

Conclusions

Very little is known on the ability of adult human pancreatic beta cells to regenerate. It is unclear whether during adult life the endocrine pancreatic cells proliferate, or whether non-endocrine cells are recruited to make beta cells, and what is the appropriate stimulation that might trigger an increase of the beta cell number (35,36). In rodents regenerative properties of the pancreas have been unveiled zeroing in beta cell proliferation (7) and the ductal cells as the source of the newly generated insulin secreting cells (6), however in humans there is no clear similar indication. The autoimmune process that causes type 1 diabetes in first place appears to be also responsible for halting potential attempts at restoring insulin production in diabetic patients (2). Nonetheless it is yet unclear whether, even in the absence of the immune attack, the pancreatic beta cell function can recover efficiently (37). Anecdotic human reports describing return to a normoglycemic status in patients diagnosed and treated for type 1 diabetes seem to prove that pancreas functional properties can be re-established concurrently with disappearance of autoimmunity signs (16-19).

We present here the case of two non-human primates rendered diabetic with STZ and so with virtually no endogenous residual beta cell mass. These monkeys were clinically diabetic for more than two months before transplantation. With the failure of pig islet graft, a few weeks after transplantation, these two monkeys progressively regained endogenous insulin production and showed improved metabolic profiles.. At the time these monkeys were sacrificed, endogenous C-peptide levels were higher than after STZ and associated to lower insulin requirements, however they were still below the normal range of a non-diabetic cynomolgous monkey, (22). Evaluation of the residual beta cell mass by IVGTT was also consistent with a progressive improvement of the endogenous insulin production. Longer follow-up may have provided further information on the extent of the recovery. Interestingly these two monkeys were the youngest in the study.

Another interesting factor that characterizes the clinical course of these two monkeys is that, despite graft insulin production, blood glucose levels were persistently higher than in the normal physiologic range. Prolonged mild hyperglycemia may have contributed to increase the beta cell mass, as previously reported (38,39). In line with this observation, in the six monkeys with short graft function and consequent severe hyperglycemia and those that returned to stable normoglycemia after transplantation (our unpublished data, and 24), no recovery of the endogenous function was seen.

Histological examination of the pancreas of these two monkeys showed proinsulin positive cells, mostly scattered as single cells or organized in small clusters, apparently not part of pre-existing islets but often associated to ducts. The frequency of proinsulin positive cells not associated to islets was higher in the monkeys with regenerated beta cell function compared to diabetic controls but lower than in non-diabetic monkeys. This observation may fuel the hypothesis that these proinsulin positive cells are the result of a phenomenon of degranulation-regranulation, similar to the one described by Sherry and colleagues in the autoimmune NOD mouse model (40). However, if regranulation was the main mechanism of recovery of the beta cell mass also in our STZ model, entailing that STZ did not actually killed the beta cells, this would not explain why the number of proinsulin positive cells associated to the damaged islets (thus near large glucagon positive aggregates) did not actually increase and that PDX-1 expression is markedly low early after STZ administration. Additionally, the presence of proinsulin positive cells expressing CK19 within and outside the ducts further foster the prospect that beta cells may have formed *ex novo*. This is in line with the notion that, during pancreatic organogenesis, endocrine progenitors arise from the duct pancreatic epithelium, and with the observation that ductal cells are involved in beta cell neogenesis during adult life (6,41,42).

Another interesting feature of the pancreas of the two monkeys with recovered beta cell function was the presence of cells with a double glucagon⁺/GLUT2⁺ immunostaining in the pre-existing islets, i.e. islets devoid of beta cells. Lack of evidence for co-expression glucagon/proinsulin suggests that these cells are unlikely committed to become beta cells. On the other hand, the double glucagon⁺/GLUT2⁺ cells could recall the phase of embryonic development where GLUT2 is expressed temporarily in pancreatic non beta cells, likely acting as a signal for further development (43,44).

To better characterize the beta cell recovery, we analyzed the expression of selected islet nuclear transcription factors activated during pancreatic ontogenesis. These genes are also persistently transcribed in the adult beta cell, where they are involved in the maintenance of the beta cell phenotype. Interestingly, qRT-PCR analysis revealed that betaNeuroD, ISL1, Foxa2 and Pax6, NKX6.1 and PDX-1 are transcribed in the same order of magnitude in the pancreas of all monkeys analysed. However, when normalized against insulin, their expression resulted to be upregulated. This is particularly interesting for PDX-1 and NKX6.1, whose expression is restricted to the beta cells during the adult life. Ngn3, a master gene regulating early pancreatic

endocrine lineage commitment and expressed only transiently during fetal life, was never detected in the samples, suggesting that if beta cell “precursors” were present they would not fully recapitulate the embryonic development. These observations, together with the finding of CK19⁺/PDX-1⁺/proinsulin⁺ cells in the monkeys with recovered endocrine function, suggest that, at least in our model, recovered beta cell mass may have originated from resident pancreatic cells with a ductal phenotype. Interestingly, single PDX-1⁺ cells were also found in STZ-diabetic control monkeys, although they did not co-localize with CK19 (Figure 5B) or proinsulin (data not shown). Damage secondary to STZ may be itself a trigger for pancreatic regenerative responses, however such condition appears unable in itself to sustain sufficient beta cell recovery, unless additional stimuli, e.g., a “danger” signal possibly due to islet graft failure (45), occur. We then hypothesize that graft failure, i.e., islet graft beta cell dysfunction and death, may foster regenerating signals also described in other forms of pancreatic injury (5). Our hypothesis is consistent with reports indicating that clinical islet allograft patients experiencing graft loss can exhibit detectable C-peptide levels and they have a better management of the diabetic status (46,47). Noteworthy, the immunosuppressive protocol used in our study did not include sirolimus and tacrolimus, both known to abolish beta cell regeneration in mouse models (7). Nonetheless the effect of islet graft evolution as a putative stimulator of beta cell recovery remains to be properly demonstrated.

12. The plans for the next quarter.

Overall our data suggest that the monkey pancreas retains a limited ability to recover beta cell function after induction of a diabetic status. Our observations do not unquestionably prove that insulin is produced by newly generated rather than recovered pre-existing beta cells. However, the presence of small aggregates of insulin positive cells outside the pre-existing islets, the absence of proliferating beta cells (Ki67⁺/proinsulin⁺), the double phenotype CK19⁺/PDX-1⁺ and CK19⁺/proinsulin⁺, the up-regulation of transcription factors typically expressed during beta cell differentiation, support the first option. The additional stimulating effect of the injury induced by islet graft loss may play a crucial role but needs further investigation.

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[In our second quarterly scientific progress report \(09/01/08 – 11/30/08\), we presented our evidence that CD46 transgenic pigs are better donors for islet xenotransplants in non-human primates.](#)

Until a few years ago, lesions of the endocrine pancreas, as they occur in type 1 diabetes, were thought to be permanent and irreversible since diabetic patients require hormone replacement therapy for life (1). Despite the clinical evolution of the disease, it is still unknown whether the islet beta cells preserve, at least in part, the ability to heal from an injury (2). Moreover, it is not clear whether in type 1 diabetes a possible recovery of the beta cell mass is contrasted by the destructive mechanisms that cause diabetes in the first place (3).

In mouse models, evidence has accrued that adult animals retain the ability to expand their beta cell mass after stimulation with a variety of triggers (4-7). It has also been shown that in non-obese diabetic (NOD) mice, strategies involving reversal of the autoimmune attack were key to allow recovery of endogenous insulin production even after diabetes onset (8-12). It remains largely uncertain through which molecular and cellular mechanisms the reparative process works, i.e. if new beta cells are formed via self-proliferation or originate from pancreatic precursors as they do during embryonic development (2).

In humans, the ability of post-natal pancreas to expand beta cell mass after injury is still debated (13-15). Spontaneous recovery of the beta cell function has been reported in only a few cases of patients previously diagnosed with type 1 diabetes. These individuals experienced a return to endogenous insulin production after diagnosis and initiation of exogenous insulin treatment. Concurrently, the humoral signs of autoimmunity (i.e., autoantibody positivity) eventually vanished (16-18).

It has been reported that in humans some beta cells are still present even years after the clinical onset of diabetes (19,20). We also know that in the healthy pancreas only a limited islet mass is actively engaged in supplying insulin to maintain normoglycemia, meaning that the critical mass that needs to be functional at a given time point is far less than that of the entire pancreatic beta cell pool (21). This observation may entail that replacement of relatively small numbers of insulin producing cells should exert substantial effects in patients. The potential for the pancreatic organ to recover the endocrine function is quite fascinating and it is the object of intense study for possible clinical applications.

Non-human primates are important animal models for pre-clinical studies and, in particular, for xenotransplantation experiments. From a physiological point of view, monkeys and humans present strong similarities even though the monkey does not show signs of spontaneous autoimmune diabetes (22). Nonetheless a permanent diabetic status can be induced by total pancreatectomy or by chemical destruction of the beta cells with streptozotocin (STZ) (23). It is therefore of great value to investigate the potential of non-human primate pancreatic tissue to recover endogenous insulin production after injury.

Isolated adult porcine islets have been shown to have minimal expression of the galactose α -1,3-galactose (Gal) epitope, therefore not inducing hyperacute rejection from binding of natural anti-Gal antibodies. The contaminating cells, however, are α -1,3-galactose positive. Also, a major proportion of islets transplanted into the portal vein is prevented to engraft by nonspecific inflammatory phenomena, described as the instant blood-mediated inflammatory reaction (IBMIR). Activation of complement cascades, through binding of antibodies to Gal and/or nonGal epitopes or unknown alternative pathways, is a key effector of this inflammatory response causing islet disruption. Therefore we hypothesized that the use of islets transgenic for human CD46 (hCD46+, membrane co-factor protein - MCP), a complement regulatory protein, would allow increased numbers of islets to survive the IBMIR, engraft and restore normoglycemia in cynomolgus monkeys with chemically induced diabetes. Human-CD46+ porcine islets demonstrated long-term function without the need for excessive immunosuppressive therapy, thereby preventing protocol-related morbidity and mortality in monkey recipients.

Diabetes was induced and confirmed in 9 monkey recipients with high dose streptozotocin (**Fig. 1**).

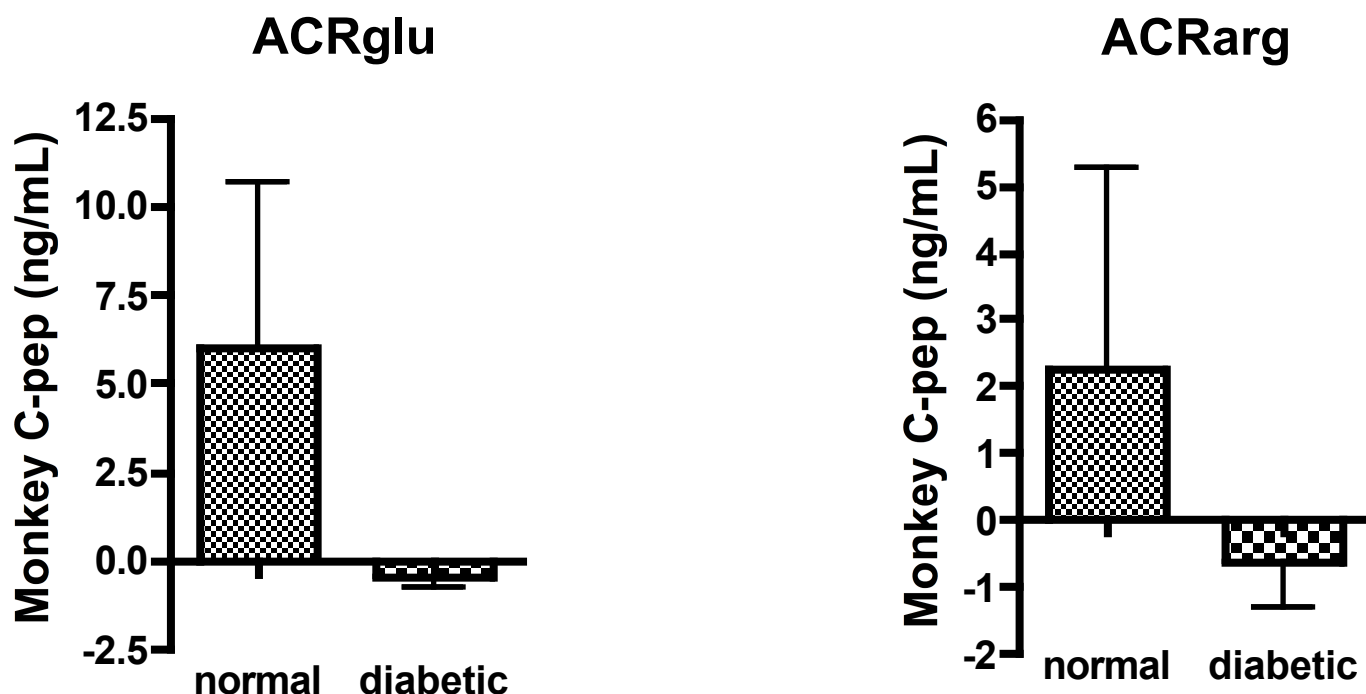


Figure 1

Group A recipients (n=4) were transplanted with either wild-type porcine islets (n=2) or islets isolated from Gal-transferase knock-out (GT-KO) pigs (n=2), in numbers of 80,000 to 100,000 IEQ/kg of body weight. Group B recipients (n=5) received equal numbers of islets from hCD46+ pigs. Two group B animals were retransplanted with 100,000 IEQ/kg after 49 and 91 days, respectively. Immunosuppression was identical for both groups and consisted of anti-thymocyte globulin for induction, and anti-CD154 monoclonal antibodies and mycophenolate mofetil (MMF) (**Fig. 2**). Monkeys were followed until loss of graft function, or up to 3 months after transplantation, except for 1 group B animal that was allowed >1yr of follow-up.

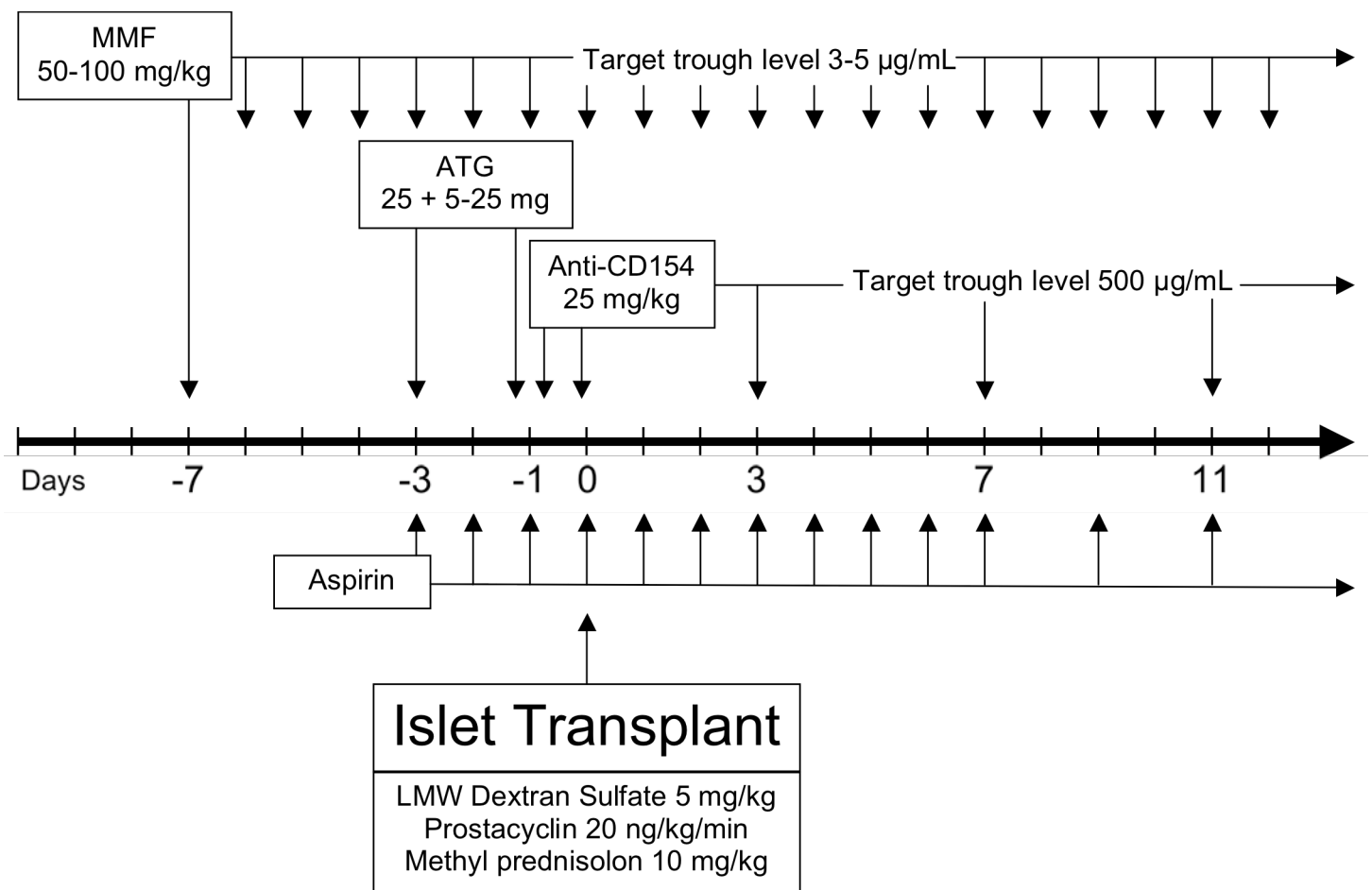


Figure 2

Functional porcine islet survival, determined as detectable porcine C-peptide in combination with a more than 50% reduction of exogenous insulin needs, was achieved in all monkeys. In group A, islet survival lasted for >7, 20, 31, and 46 days. In group B, the use of hCD46+ islets significantly prolonged functional porcine islet survival to the full 3 months or beyond 1yr of follow-up, respectively (log-rank test $P=0.0042$) (**Fig. 3**). Averages of weekly fasting porcine C-peptide levels were 1.10 ± 0.41 ng/mL for group A versus 0.90 ± 0.51 ng/mL for group B (student t -test $P=0.546$). Subsequently, insulin independence was achieved in 3 of 4 Group A monkeys for a period of 5, 17, and 36 days, respectively. Four of 5 group B monkeys became insulin independent for 87, 91 92, and >396 days, respectively. During times of insulin independence, fasting blood glucose values were well controlled (group A: 91 ± 18 mg/dL; group B: 112 ± 22 mg/dL).

Figure 3A.

Fasting blood glucose, exogenous insulin administration and porcine C-peptide in group A monkeys

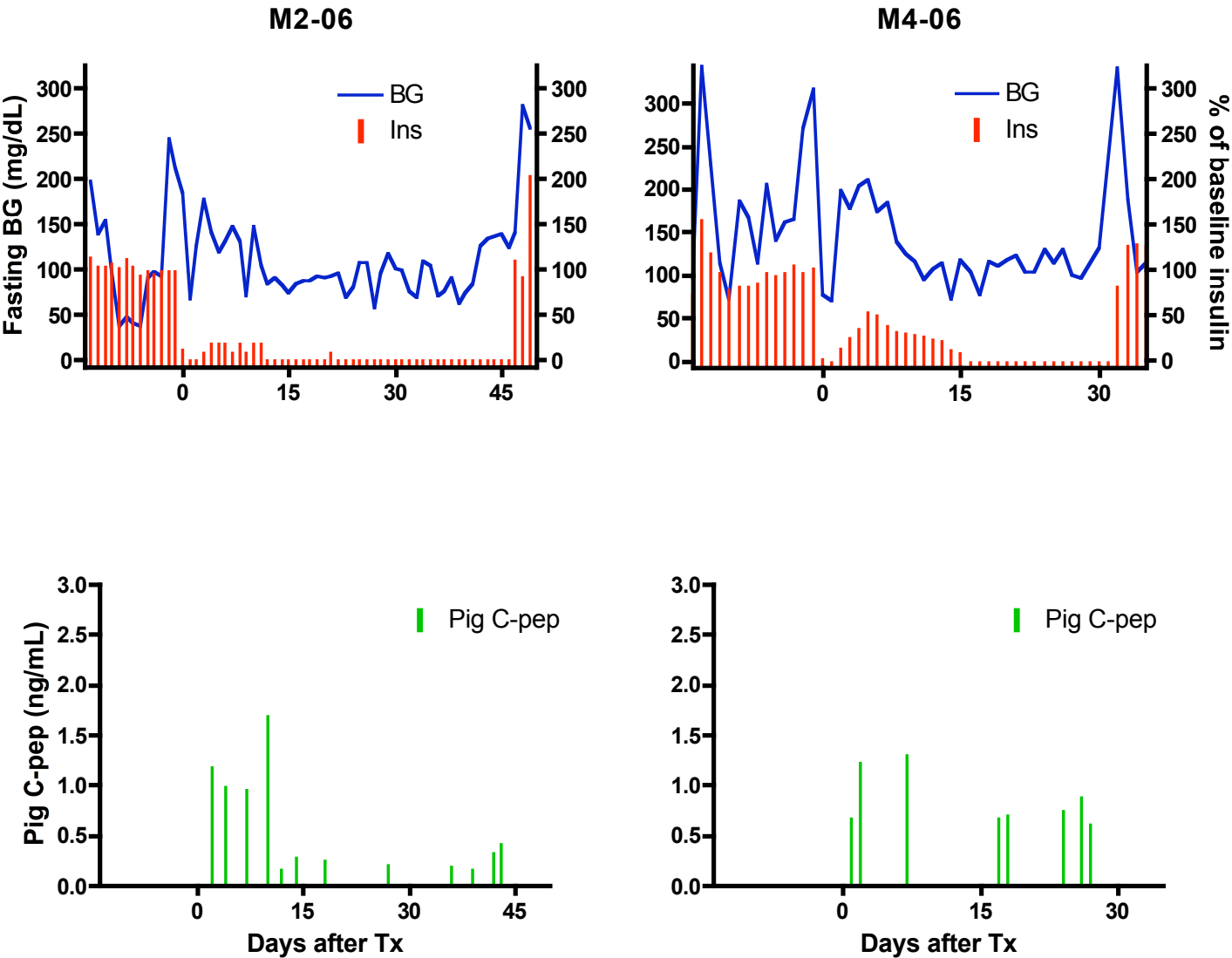
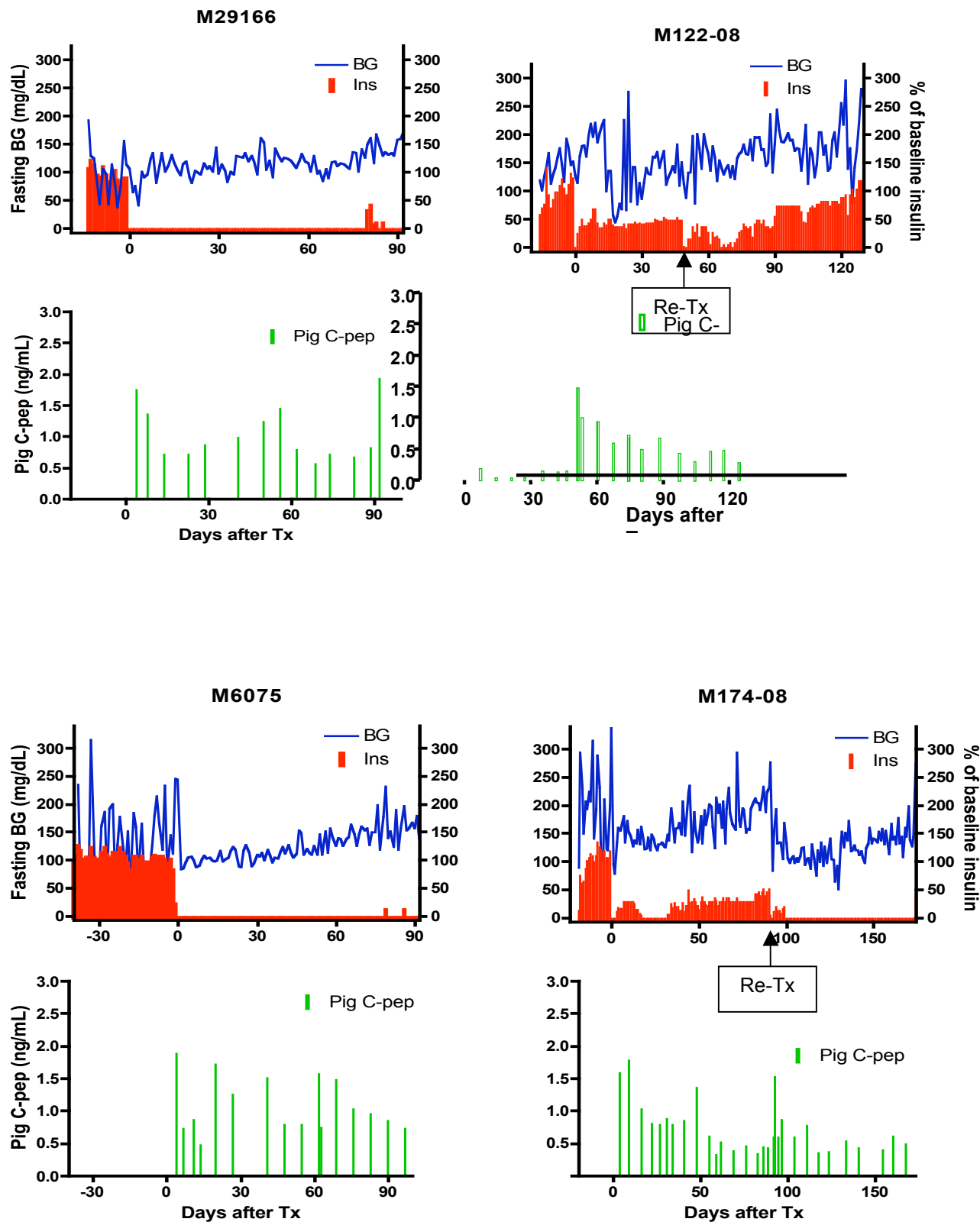
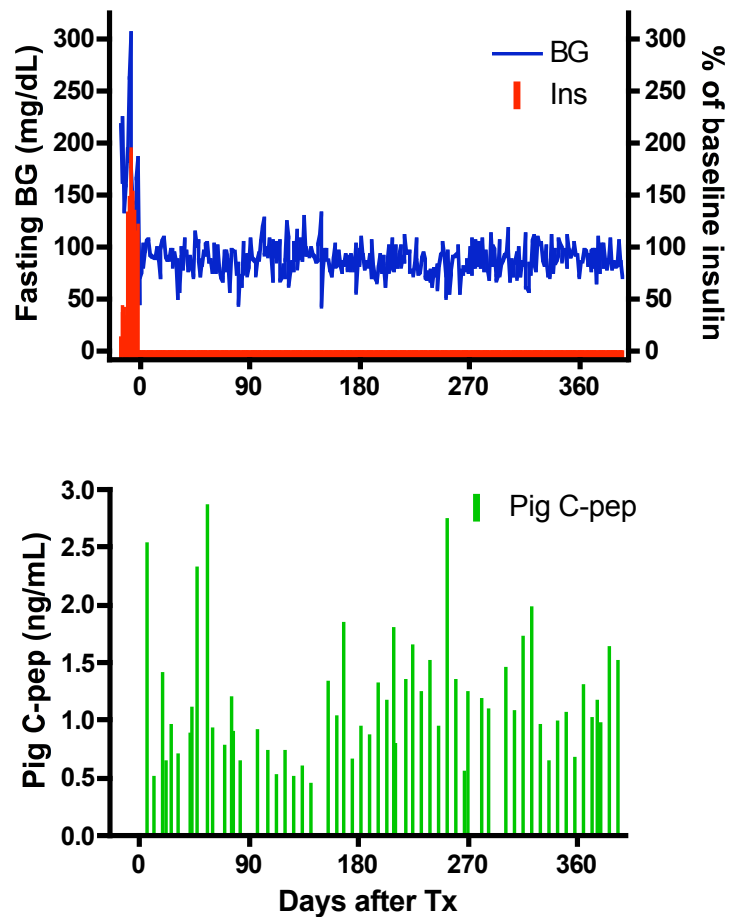


Figure 3B.

Fasting blood glucose, exogenous insulin administration and porcine C-peptide in group B monkeys



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Post-transplant responses to stimulation with intravenous glucose and arginine are shown in **Fig. 4**. A C-peptide response of engrafted pig islets in absence of a response by native beta cells is evident.

Histological evaluation of post-transplant livers revealed many viable porcine islets in Group B animals. T cell infiltration, the dominant way of xeno islet rejection, was successfully prevented, which is confirmative of previous studies in which co-stimulation blockade was applied (24).

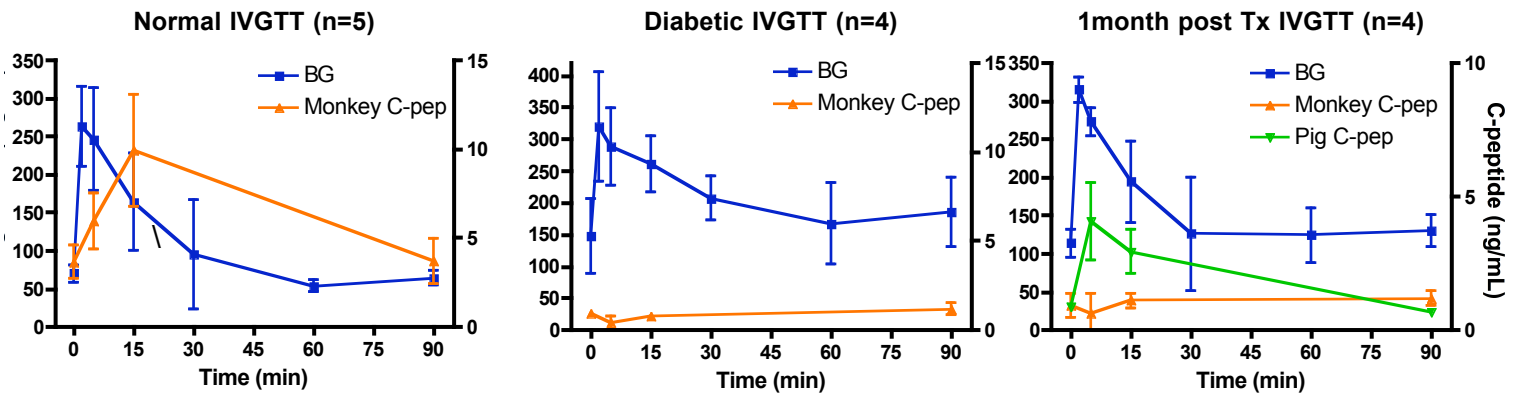
Serum levels of anti-Gal or anti-nonGal antibodies did not significantly increase in group B monkeys. As previously reported, even in the event of cellular rejection, islet xenotransplantation does not induce a humoral immune response detectable in serum (24). Nevertheless, IgM and IgG binding was seen on histological analysis.

Animals remained healthy during the course of the experiments, except one group A monkey that was euthanized 7 days after transplantation with acute gastric dilatation, a rare but not uncommon condition in laboratory animals. Adverse events were minimal, the majority of monkeys maintained or gained weight, and CMV reactivation was not observed; neither clinically nor after PCR amplification.

Our results demonstrate the advantage of the transgenic expression of human CD46, a complement regulatory protein, on porcine islets if transplanted into diabetic, immunosuppressed nonhuman primates (NHP). With a limited immunosuppressive protocol, normoglycemia was maintained for 3 months (or beyond 1yr) in 4 of 5 transplanted monkeys, compared to maximum 36d in the absence of the transgene.

Figure 4.

Intravenous glucose tolerance tests (IVGTT) in group B monkeys before STZ (Normal), after STZ (Diabetic), and $\pm$ 1 month after transplantation if normoglycemic. Additional data obtained during IVGTT and arginine stimulation tests (AST) are presented in Supplementary Table 3 online.



CD46 or MCP is well characterized as a protein with regulatory properties regarding protecting the host cell against complement attacks initiated by both classical and alternative pathways. Its features were maintained when transgenically expressed on pig cells **Fig. 5**. Human CD46 is compatible with NHP complement systems. In the experiments we herein report, hCD46 on pig islets likely offered protection against complement lysis during inflammation, such as in IBMIR, as well as against humoral rejection by low levels of natural or induced anti non-Gal antibodies. As a result, more islets were able to engraft and could subsequently be protected against cellular rejection by reduced immunosuppression.

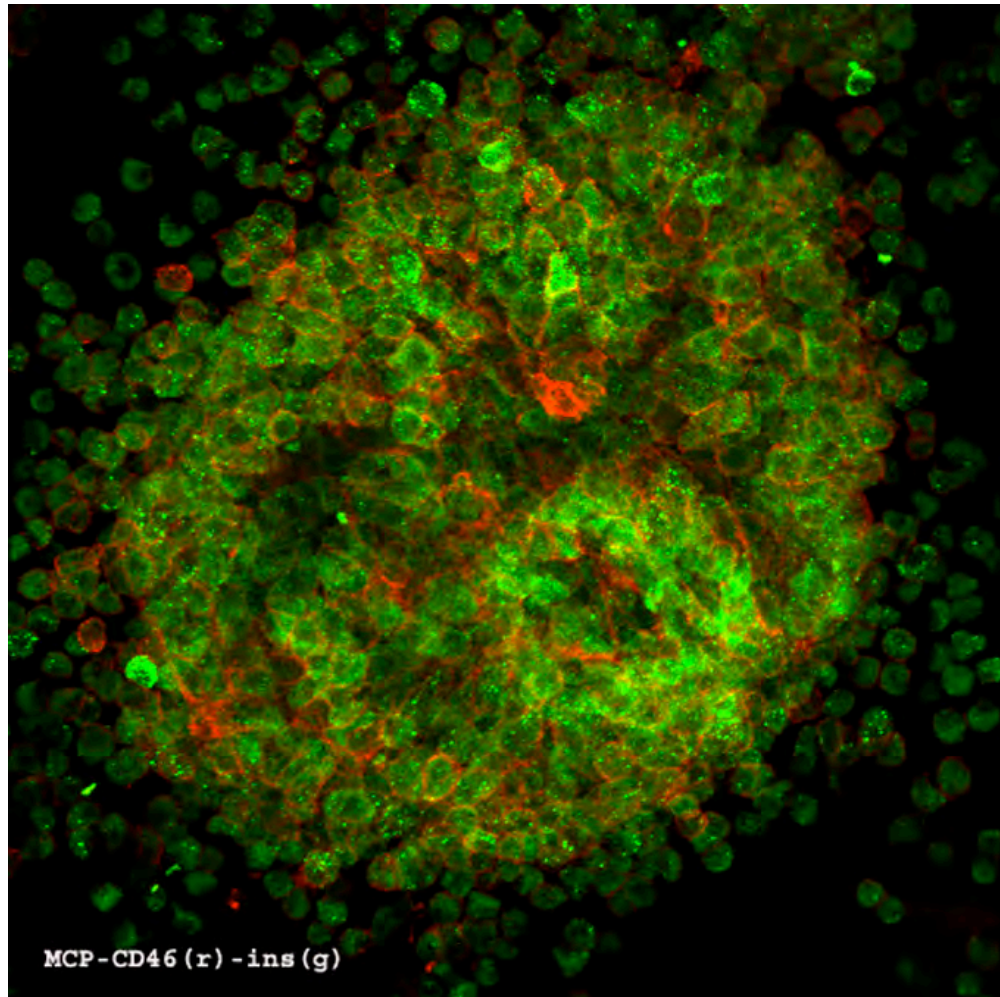
We attribute the low incidence of protocol related adverse events to the use of this very mild immunosuppressive regimen in comparison with other NHP models in which many experiments had to be prematurely terminated. In addition, ganciclovir therapy likely contributed to prevent viral infections, where as aspirin may have effectively prevented thrombo-embolic complications of anti-CD154 treatment.

Despite the expression of hCD46 and the application of an anti-inflammatory and anticoagulant protocol, in our current and previous experiences (23), there is a need for the transplantation of very high numbers of porcine islets to obtain normoglycemia. Possible causes for this observation represent the disadvantages of the islet transplantation technique in clinical islet allo-transplantation, now generally recognized: the failure of many islets to engraft after portal venous injection, and gradual loss of the surviving islet mass due to toxicity of immunosuppressive drugs and metabolic exhaustion, requiring multiple islet injection from 2 to 4 donors. Our study design, without the selection of donor islet preparations after the islet isolation procedure, as sometimes performed in other studies (24), resembles the clinical experience, with monkeys requiring a small dose of exogenous insulin toward the end of follow-up.

In conclusion, our study is the first to show how genetic alterations of donor pigs, tailored to combat the specific inflammatory or immunological hurdles, can contribute to advancing the field of islet xenotransplantation toward clinical application. For this to become reality, however, consistent long-term normoglycemia in NHP models needs to be achieved with immunosuppressive that is safe for patients.

Figure 5.

Expression of human CD46 on isolated porcine islets



12. The plans for the next quarter.

Overall our data suggest that the monkey pancreas retains a limited ability to recover beta cell function after induction of a diabetic status. Our observations do not unquestionably prove that insulin is produced by newly generated rather than recovered pre-existing beta cells. However, the presence of small aggregates of insulin positive cells outside the pre-existing islets, the absence of proliferating beta cells ($Ki67^+$ /proinsulin $^+$), the double phenotype $CK19^+$ /PDX-1 $^+$ and $CK19^+$ /proinsulin $^+$, the up-regulation of transcription factors typically expressed during beta cell differentiation, support the first option. The additional stimulating effect of the injury induced by islet graft loss may play a crucial role but needs further investigation.

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In our third quarterly scientific progress report (12/01/08 – 02/28/09) we then reported the pattern followed by C-peptide during its internalization in human endothelial and vascular smooth muscle cells.

C-peptide is a biologically active peptide

C-peptide is the peptide segment connecting insulin A and B chains, and a product of pro-insulin cleavage in the secretory granules, generated in the pancreatic beta cells as part of the normal insulin production. In healthy individuals, C-peptide is secreted in the peripheral circulation in equimolar amount with insulin, but it is absent in the majority of T1D patients [1]. C-peptide was for long time considered to only possess the biological activity of favoring pro-insulin folding within the beta cells. Recent studies have, however, challenged this classical view by demonstrating that C-peptide is capable of biological effects in many different cell types and importantly reduces the complications associated with T1D when given to patients suffering from small vessel complications [2,5-8]. Previous work from our laboratory has demonstrated a protective effect of physiological concentrations of C-peptide on the vasculature in conditions of hyperglycemia by decreasing secretion of the pro-inflammatory cytokines interleukin(IL)-6, IL-8, and monocyte chemoattractant protein(MCP)-1 from endothelial cells. We also found that C-peptide inhibits expression of the endothelial cell adhesion molecule vascular cellular adhesion molecule(VCAM)-1 and reduces attachment of monocytes to endothelial cells, one of the first step of atherosclerosis plaque formation. These effects were mediated by a reduced activation of the nuclear factor(NF)- κ B pathway by C-peptide, a pathway involved in inflammatory responses [13]. Our group has also shown physiologic concentrations of C-peptide decrease high glucose-induced proliferation of UASMC and this was accompanied by decreased phosphorylation of I κ B and reduced NF- κ B nuclear translocation [15]. Recently, C-peptide has also been shown to exert beneficial effects in endotoxic shock in mice with improved survival of LPS-treated mice, reduction in plasma levels of several pro-inflammatory cytokines, and decrease in overall lung inflammatory response [4]. Another group has demonstrated that C-peptide plays a physiological role in glucose clearance, and release of ATP from red blood cells of diabetic patients [17]. C-peptide was also shown to improve erythrocytes deformability in diabetic subjects [18].

Gap in knowledge

The cell biology of C-peptide is for the most part unknown, in particular regarding the pathway the peptide uses to cross the cellular membrane and possible sub-cellular destinations. A specific C-peptide receptor has not been identified yet, although it is often suggested to be a G-protein-coupled receptor as deduced from effects of pertussis toxin [11,12]. Binding of C-peptide to plasma membranes has been demonstrated in rat pancreatic beta cells [9], human renal tubular cells [10], human fibroblasts and endothelial cells [11]. More recently, C-peptide was shown to cross plasma membranes localizing in the cytoplasm of HEK-293 cells and Swiss 3T3 fibroblasts [12], where it was detected up to 1 h after uptake of the peptide. A nuclear localization of C-peptide in HEK-293 cells and Swiss 3T3 fibroblasts has also been demonstrated by the same group [12]. These findings demonstrate that once internalized in the cytoplasm, C-peptide is not rapidly degraded but remains intact, possibly interacting with sub-cellular components through which it might achieve its cellular effects. The process of internalization from the cell surface and sub-cellular localization of C-peptide in target cells have not yet been investigated. In particular, it is not known whether C-peptide passively diffuses across the cellular membrane or whether it is actively translocated by a specific pathway of internalization, such as endocytosis. In this study, we will specifically investigate the hypothesis that C-peptide internalizes in target cells by following a specific endocytic pathway. By understanding the internalization and signaling mechanisms in its target cells we will not only provide further knowledge of its mechanism(s) of action but also drug targets.

Significance of the study

T1D patients lack physiological levels of insulin and C-peptide in their bloodstream due to the autoimmune destruction of their pancreatic beta cells. T1D patients are also at increased risk to develop both micro- and macro-vascular complications. Unfortunately, the standard of care for T1D patients is solely insulin-replacement therapy. Despite the recently published evidence of the beneficial effect of C-peptide replacement therapy on diabetes-associated vascular complications, C-peptide is not universally prescribed. Our findings will establish basic cell biological mechanisms underlying C-peptide mechanisms of action. Identification of

intracellular pathways of C-peptide activity will allow better understanding of how C-peptide can achieve its effects in target cells and thus further support an international effort to test C-peptide treatment clinically.

Preliminary Studies

Successful completion of this project is based on the availability of custom AlexaFluor-labeled C-peptide probes that we have designed and had synthesized by Molecular Probes (Invitrogen Corporation, Carlsbad, CA). One probe is labeled with the AlexaFluor488 fluorescent dye at the carboxy-terminal end of the C-peptide. A second probe is labeled with the AlexaFluor546 fluorescent dye at the amino-terminal of the C-peptide molecule. As shown below, these two probes showed equally good results in internalization studies and will be used indistinctively except when fluorescent color matters.

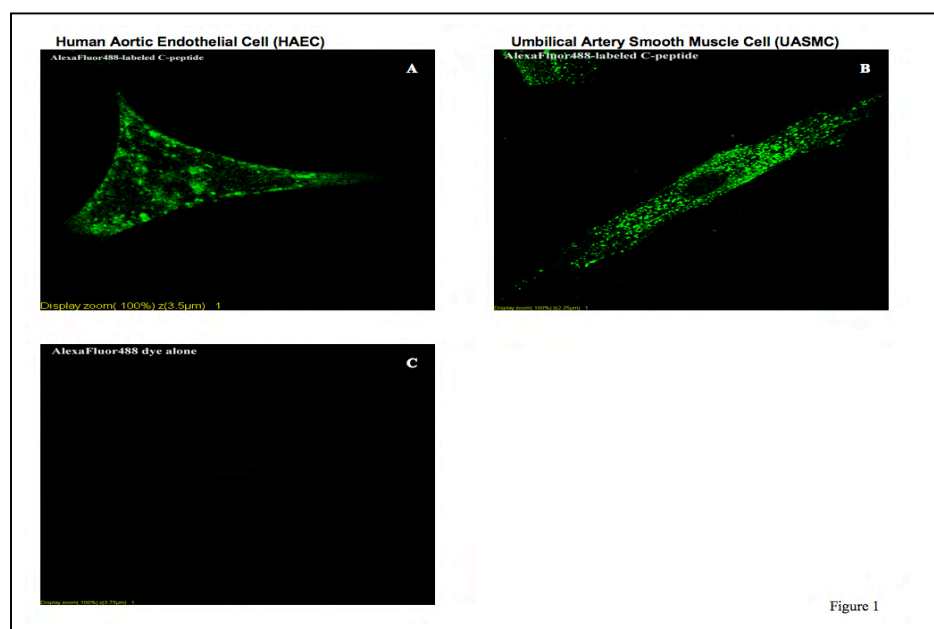


Fig. 1 C-peptide internalizes in HAEC and UASMC as punctate structures. HAEC (A) and UASMC (B) were incubated for 10 min with 1 mmol/l AlexaFluor488-labeled C-peptide at 37°C, washed with medium and imaged by using confocal microscopy. The green punctate-staining corresponds to the C-peptide probe localized at the periphery of the cell and in the cytoplasm. In (C) as a control for the specificity of the staining, HAEC were incubated for 30 min with 1 mmol/l AlexaFluor488 dye at 37°C. Note the absence of green fluorescence staining. The Figure shows a representative z-section across one cell.

C-peptide internalizes in human aortic endothelial cells (HAEC) and umbilical artery smooth muscle cells (UASMC). By using confocal laser-scanning microscopy in a live-cell setting, we found that C-peptide binds to plasma membranes and internalizes in the cytoplasm of HAEC (Figure 1A) and UASMC (Figure 1B). The uptake of C-peptide was minimal after 5 min of incubation at 37°C with 1 μ mol/l of AlexaFluor488-labeled C-peptide probe and began to be clearly visible after 10 min. As a control for specificity of the staining, we incubated the cells with the AlexaFluor488 fluorescent dye alone and this incubation resulted in the absence of staining, as shown in Figure 1C.

Effect of temperature on internalization of AlexaFluor-labeled C-peptide Consistent with the cellular trafficking of proteins in general, the internalization of C-peptide was blocked at 4°C. (Figure 2A). Accordingly, C-peptide internalization slowly recovered when cells that were incubated at 4°C were placed back at 37°C (Figure 2B-C).

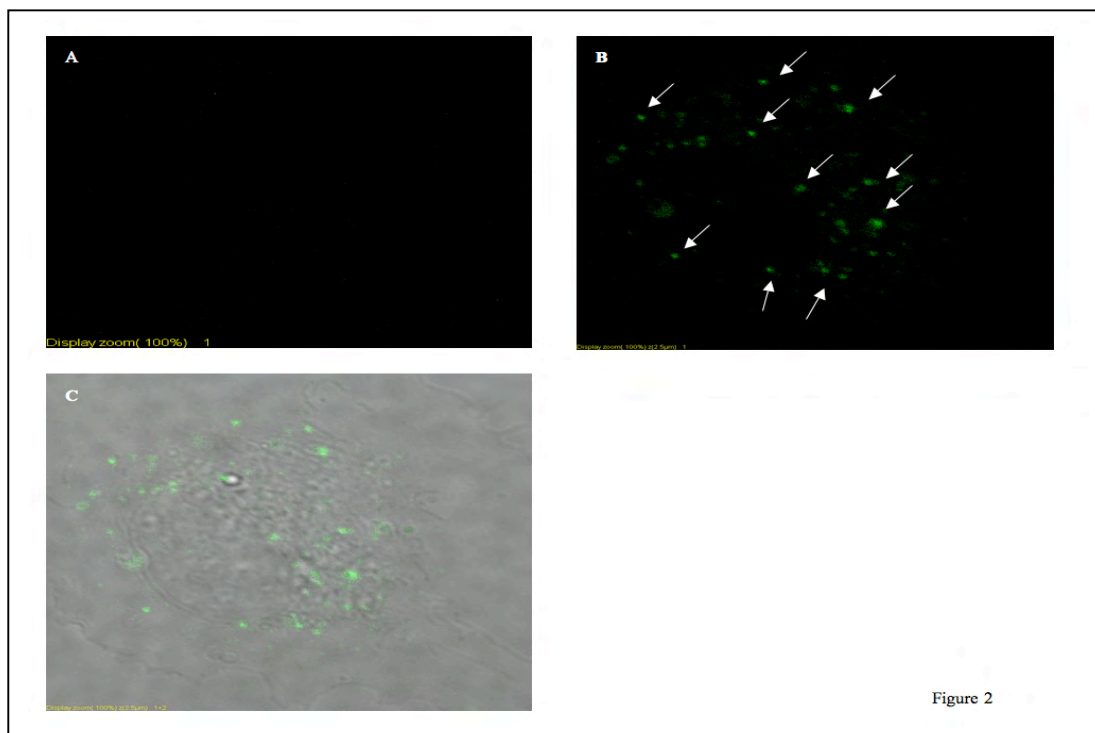


Figure 2

Fig. 2 C-peptide internalization is inhibited at 4°C. In (A) HAEC were incubated for 30 min with 1mmol/l AlexaFluor488-labeled C-peptide at 4°C, washed with medium, and immediately imaged by using confocal microscopy. The figure shows absence of green fluorescence staining inside the cells, consistent with no internalization of the probe. In (B) internalization of the green C-peptide probe slowly recovered when cells were put in the incubator at 37°C after being at 4°C. Arrows show examples of green punctate staining corresponding to the internalized C-peptide probe. In (C) it is shown the differential interference contrast (DIC) confocal imaging of the same z-section as in (B) merged with the fluorescence image to demonstrate localization of the green punctate staining in the cytoplasm. The Figure shows a representative z-section across one endothelial cell.

C-peptide upon internalization co-localizes with early endosomes in HAEC and UASMC. We tested whether C-peptide is directly translocated across the plasma membrane or whether it follows a vesicle-mediated pathway during internalization. In this latter case, the fate of C-peptide upon internalization from the cellular membrane might be to localize in classic endocytic organelles, such as endosomes. To explore this possibility, HAEC and UASMC were transduced with Organelle Lights™ Endosome-GFP reagent, which targets expression of fluorescent Rab5a, an early endosome-specific marker. Cells were then labeled with 1 mmol/l of AlexaFluor546-labeled C-peptide probe at 37°C for 30 min and imaged in a live-cell setting under a confocal microscope. As shown in Figure 3A, early endosomes appear as green punctate structures in the cytoplasm and close to the cellular membrane of an endothelial cell. Figure 3B shows the internalization of AlexaFluor546-labeled C-peptide as red punctate staining inside the cytosol. When the two images were merged, a yellow staining is evident, corresponding to co-localization of the green, early endosomes, with the red, C-peptide (Figure 3C, see arrows). In the majority of cases the red staining corresponding to the internalized C-peptide probe is evidently contained inside the endosome structures, observed as a bright yellow peripheral staining all around the central red staining of the vesicles (Figure 3C-D).

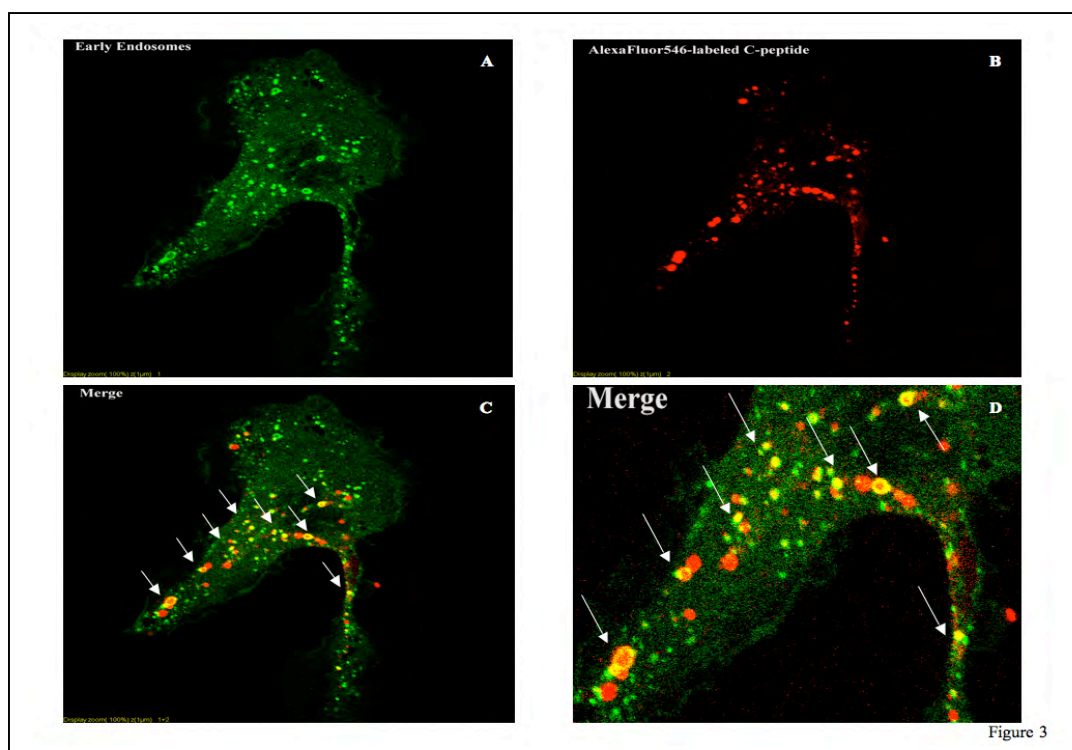


Figure 3

Fig. 3 Internalized AlexaFluor546-labeled C-peptide co-localizes with early endosomes in HAEC. HAEC were transduced with Organelle Lights™ Endosomes-GFP, which induces expression of the fluorescent early endosomal marker Rab5a. Cells were then incubated for 30 min with 1 mmol/l AlexaFluor546-labeled C-peptide at 37°C, washed with medium and imaged by using confocal microscopy in an “open” live-cell setting. In (A) early endosomes identified as green Rab5a staining; in (B) internalization of AlexaFluor546-labeled C-peptide as red vesicles in the cytosol; in (C) co-localization of C-peptide (in red) with early endosomes (in green), shown as yellow staining; in (D) enlarged image of co-localization shown as yellow staining. Arrows are pointing to examples of co-localization. The figure is a representative z-section across one endothelial cell

Immunohistochemistry of C-peptide localization to early endosomes As an additional technique to demonstrate internalization of C-peptide and localization to endocytic organelles, we performed immunofluorescence studies on paraformaldehyde (PFA)-fixed HAEC after exposure to 10 nmol/l of C-peptide for 3 h in the incubator at 37°C. As shown in Figure 4A, C-peptide internalizes in the cytoplasm of HAEC as green punctate structures, the majority of which localize with the red early endosomes (Figure 4B) identified with a monoclonal antibody to the early endosome antigen 1 (EEA1), resulting in a yellow staining (Figure 4C).

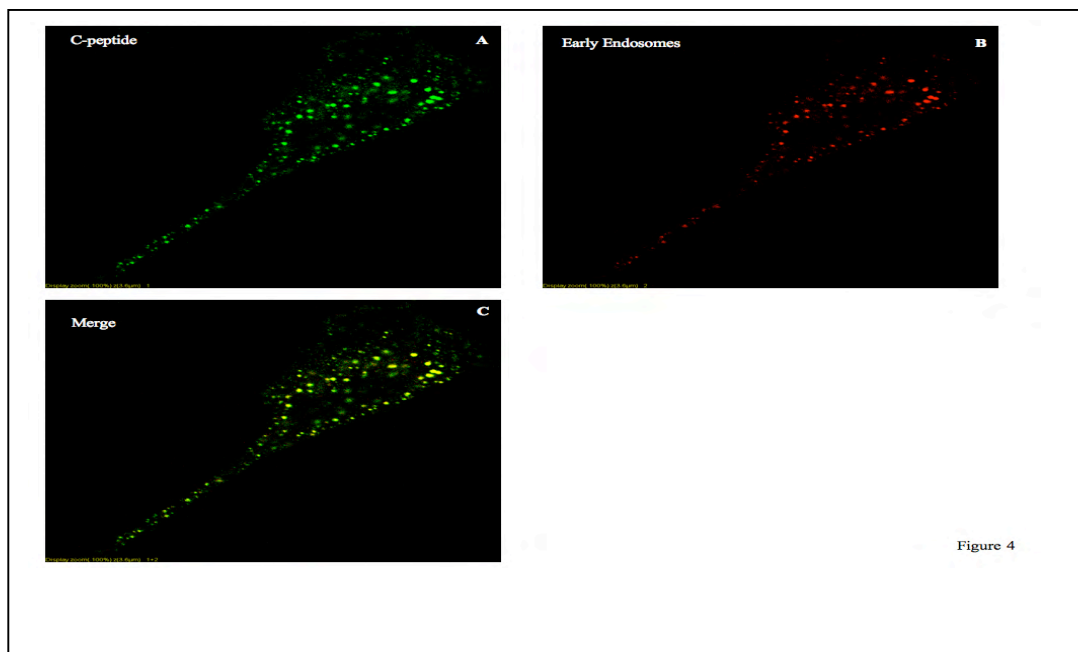


Fig. 4 Immunohistochemistry of C-peptide internalization and localization to early endosomes. HAEC were incubated for 3 hours with 10 nmol/l of C-peptide at 37°C. After fixation with PFA, cells were incubated with rabbit anti-human antibody to C-peptide together with a mouse monoclonal antibody to the early endosomal antigen 1 (EEA1) for 2 hours and then with the following secondary antibodies: AlexaFluor488-conjugated goat anti-rabbit IgG and AlexaFluor594-conjugated donkey anti-mouse IgG for 1 h. Cells were then washed and imaged by using confocal microscopy. In (A) internalized C-peptide as green fluorescence vesicles; in (B) early endosomes stained in red; in (C) merging of the two images showing co-localization of the majority of C-peptide with

The plans for the next quarter

Human C-peptide is a 31 aminoacid long peptide released by pancreatic beta cells in equimolar amounts with insulin in response to elevated blood glucose levels (hyperglycemia). Once secreted in the bloodstream, C-peptide circulates at low nanomolar concentrations in healthy individuals, but it is absent in the majority of Type 1 Diabetes (T1D) patients [1]. In recent years, C-peptide has been shown to exert insulin-independent biological effects on a variety of cells, where it affects activation of several intracellular pathways, such as, but not limited to, those involved in cellular proliferation and inflammation [2-4]. Importantly, C-peptide has been demonstrated to be beneficial when administered as replacement therapy to T1D patients who suffers from diabetic vascular complications, in particular in relation to nephropathy, neuropathy, and augmentation of blood flow [5-8]. How exactly C-peptide achieves its intracellular effects in target cells, however, is still unknown. Data suggest that C-peptide bind to and cross plasma membranes and localizes in the cytoplasm, where it is detected up to 1 h after uptake of the peptide (9-12). What pathway C-peptide uses to cross the cellular membrane and possible sub-cellular destinations have not been investigated prior. In particular, it is not known whether C-peptide passively diffuses across the cellular membrane or whether it is actively translocated by a specific pathway of internalization, such as endocytosis.

The purpose of this study is to investigate the trafficking pathway of C-peptide in target cells upon its binding to the cellular membrane. We hypothesize that C-peptide internalizes in target cells by following a specific endocytic pathway. In this scenario, the fate of C-peptide upon internalization from the cell surface would be to localize in classic endocytic organelles, such as early endosomes, and subsequent traffic to lysosomes for degradation.

We will test this hypothesis by working towards the following specific aims:

Specific Aim 1: To study the time-course of C-peptide internalization in vascular endothelial and smooth muscle cells by employing live-cell confocal imaging. C-peptide internalization will be explored in Human Aortic Endothelial Cells (HAEC) and Umbilical Aortic Smooth Muscle Cells (UASMC), two important targets of C-peptide activity especially in the context of vascular dysfunction leading to vascular complications in T1D [13-16]. We designed two AlexaFluor-labeled C-peptide probes (one labeled with AlexaFluor488 dye and the other with AlexaFluor546 dye) and will use them to study uptake of the fluorescent peptides in a live-cell imaging setting employing confocal laser-scanning microscopy from 5 min up to 1 h from peptide addition. Images will be compared to the ones obtained by using AlexaFluor488 or 546 fluorescent dyes alone.

Specific Aim 2: To investigate co-localization of C-peptide with sub-cellular organelles, such as early endosomes and lysosomes by employing optical and biochemical methods. We will study trafficking of C-peptide to intracellular compartments in HAEC and UASMC by studying co-localization of the AlexaFluor-labeled C-peptide probes to early endosomes and lysosomes visualized with specific fluorescent constructs in a live-cell confocal imaging setting. Immunohistochemistry will also be used as an additional technique. We will then isolate endosomes and lysosomes from control and C-peptide treated cells and analyze C-peptide content in these fractions by Western Blot.

Specific Aim 3: To determine whether C-peptide endocytosis is mediated through a clathrin-coated pit pathway. As an ultimate proof for C-peptide endocytosis in HAEC and UASMC, we will use small interference RNA (siRNA) to silence clathrin heavy chains and block endocytosis. As an alternative method, we will block clathrin-mediated endocytosis by using the selective inhibitor monodansylcadaverina (MDC). Uptake of AlexaFluor-labeled C-peptide will then be visualized by live-cell confocal imaging and compared to not-treated cells.

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In the fourth and final quarterly scientific progress report (3/01/09 – 05/31/09) of Year 03 we now report on the endocytotic pathway C-peptide uses to enter into its target cells.

To complete the studies proposed in the aims for the last quarter, we completed the following experiments:

Uptake of AlexaFluor-labelled C-peptide in the presence of pharmacological compounds.

Cells were pre-treated for 30 min at 37°C, 5% CO₂ with either Monodansylcadaverine (MDC;43 mmol/l), which inhibits the clathrin-coated pit pathway, Filipin (5 mg/ml), which blocks caveolae pathway, Nocodazole (10 mmol/l), which disrupts microtubule assembly, or Cytochalasin D (30 mmol/l), which disrupts actin filaments. The dose and time of treatment for each compound, which showed no toxic effect, was determined experimentally on the basis of previous reports. After 30 min, fluorescent C-peptide was added for 30 min and cells put back in the incubator. Cells were then examined under a confocal microscope. Three independent experiments were performed, with a minimum of 3 MatTek plates for each experiment. The response of cells to each condition was highly consistent with inhibition of C-peptide uptake with MDC and Nocodazole. We therefore analyzed the mean fluorescence per cell in only 3 cells/plate (total of 9 cells) for each condition by using Olympus Fluoview 300 Software. Repeated Measures ANOVA was applied to determine statistical significance.

The conclusion of these experiments can be summarized as follows:

C-peptide uses a clathrin-mediated endocytotic pathway to enter HAEC.

We investigated whether internalization of C-peptide is mediated through clathrin-coated pits or caveolae by using MDC and Filipin. MDC is a pharmacological inhibitor of receptor-mediated endocytosis, while Filipin, which binds cholesterol in the plasma membrane, impairs the invagination and subsequent internalization of caveolae. Under our experimental conditions, pre-treatment of HAEC with MDC (43mmol/l)

for 30 min completely blocked uptake of AlexaFluor488-labelled C-peptide ($p<0.01$ versus control). By contrast, Filipin pre-treatment of HAEC for 30 min (5mg/ml) lacked nearly completely inhibitory effect on fluorescent C-peptide probe internalization (see Figure 4 of the original article Luppi et al., *Diabetologia* 2009 in press).

Role of the cytoskeleton network.

The involvement of the cytoskeleton network in C-peptide endocytosis was studied in HAEC with Cytochalasin D (30mmol/L) and Nocodazole (10mmol/L), which act by inducing depolymerization of actin filaments and microtubules, respectively. As shown in Figure 4, uptake of AlexaFluor488-labelled C-peptide following Cytochalasin D treatment was not significantly different from control cells. Conversely, Nocodazole drastically reduced fluorescent C-peptide uptake ($p<0.01$ versus control) suggesting that entry of C-peptide into HAEC was dependent on microtubule integrity.

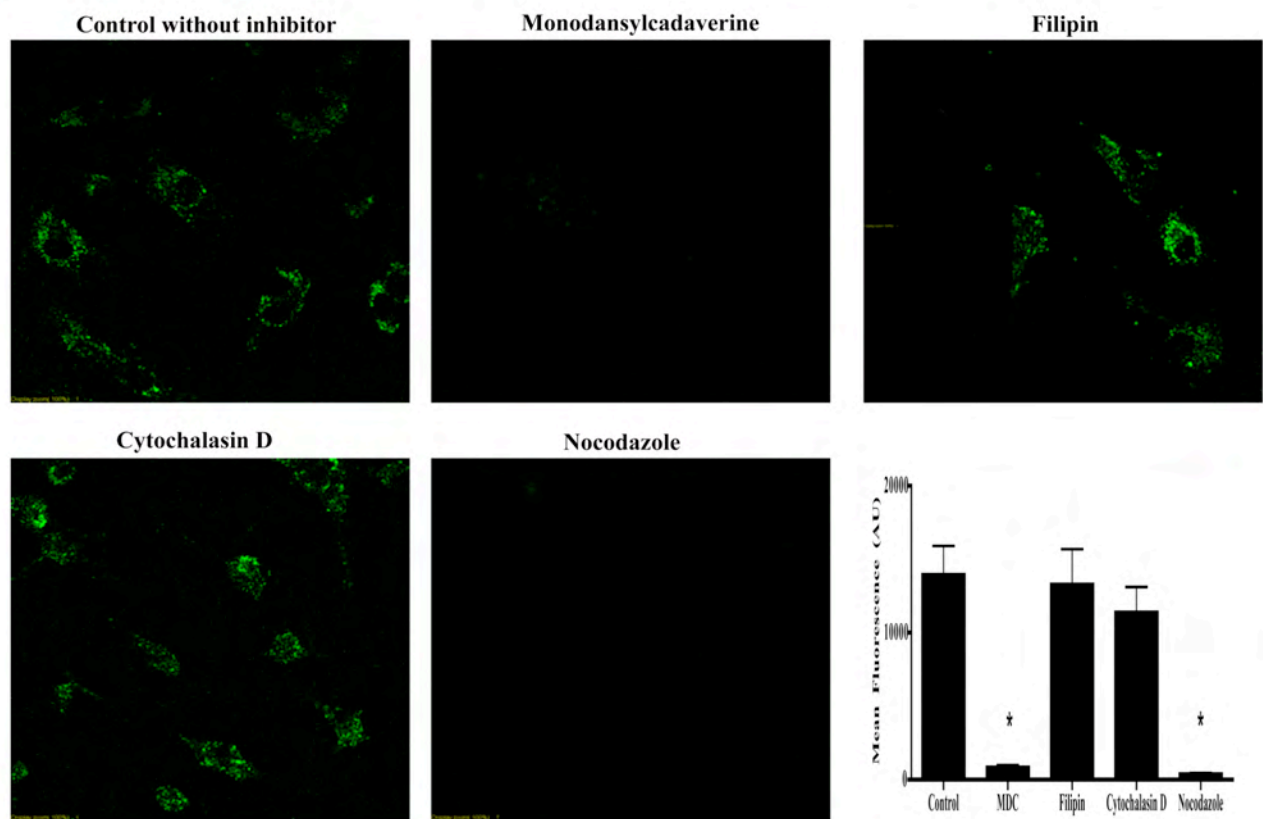


Figure 4

Figure 4. Effect of different pharmacological compounds on C-peptide entry in HAEC Cells were either treated with 1mmol/l AlexaFluor488-labelled C-peptide for 30 min (Control) or pre-treated with the following compounds before adding 1mmol/l AlexaFluor488-labelled C-peptide : Monodansylcadaverine (MDC; 43mmol/l), Filipin (5mg/ml), Cytochalasin D (30mmol/l), and Nocodazole (10mmol/l). As shown, pre-treatment of HAEC with MDC, an inhibitor of clathrin-mediated endocytosis, inhibited entry of the C-peptide probe. Pre-treatment with Nocodazole, which prevent microtubule assembly, also impairs C-peptide internalization. Filipin and Cytochalasin D did not inhibit C-peptide internalization. The Figure shows a representative z-section across some endothelial cells. The bar graph shows the mean fluorescence (AU=Arbitrary Units) of AlexaFluor488-conjugated C-peptide internalization quantitated in each experimental condition. The asterisk shows presence of a significant decrease in uptake of fluorescent C-peptide was detected when HAEC were pre-treated with MDC and Nocodazole ($p<0.01$ versus control cells).

Isolation of endosomes and analysis of C-peptide content.

To identify early endosomes, we followed internalization of AlexaFluor488-conjugated human transferrin, a ligand known to enter the cells by clathrin-mediated endocytosis and a specific marker of early endosomes in the presence of AlexaFluor546-labelled C-peptide in UASMC in the incubator for 10 min. Endosomes were isolated from other sub-cellular compartments by centrifugation and an aliquote of both the pellet and the endosome-containing supernatant used to quantitate AlexaFluor488-transferrin and AlexaFluor546-C-peptide fluorescence spectra by using a scanning spectrofluorimeter. Repeated Measures ANOVA was applied to determine statistical significance. Three independent experiments were performed.

Uptake of AlexaFluor-labelled C-peptide follows transferrin internalization in UASMC.

To isolate early endosomes, we briefly incubated UASMC with AlexaFluor546-labelled C-peptide together with AlexaFluor488-conjugated human transferrin, and then proceeded to cellular fractionation. High-speed centrifugation allows plasma membranes and other cellular compartments to sediment in the pellet, while clathrin-coated pits and early endosomes, which are relatively small molecules (100-200nm), remain in the supernatant. By using a scanning fluorimeter, we quantified the fluorescence present in the endosome-containing supernatant of the homogenized cells. As shown in Figure 8 of the original article (Luppi et al., *Diabetologia* 2009 in press), the mean fraction of the AlexaFluor488-transferrin fluorescence (peak emission at 516), in the endosome-containing supernatant was 0.86 ± 0.06 ($n = 3$). The mean fraction of AlexaFluor546-C-peptide (peak emission at 568), in the endosome-containing supernatant was 0.77 ± 0.08 ($n = 3$). The fraction of each probe in the supernatant versus pellet was significantly different ($p < 0.001$), whereas the fraction of transferrin and C-peptide in the endosomal fraction were statistically indistinguishable ($p > 0.05$). The cell fractionation results further support the internalization of C peptide into early endosomes.

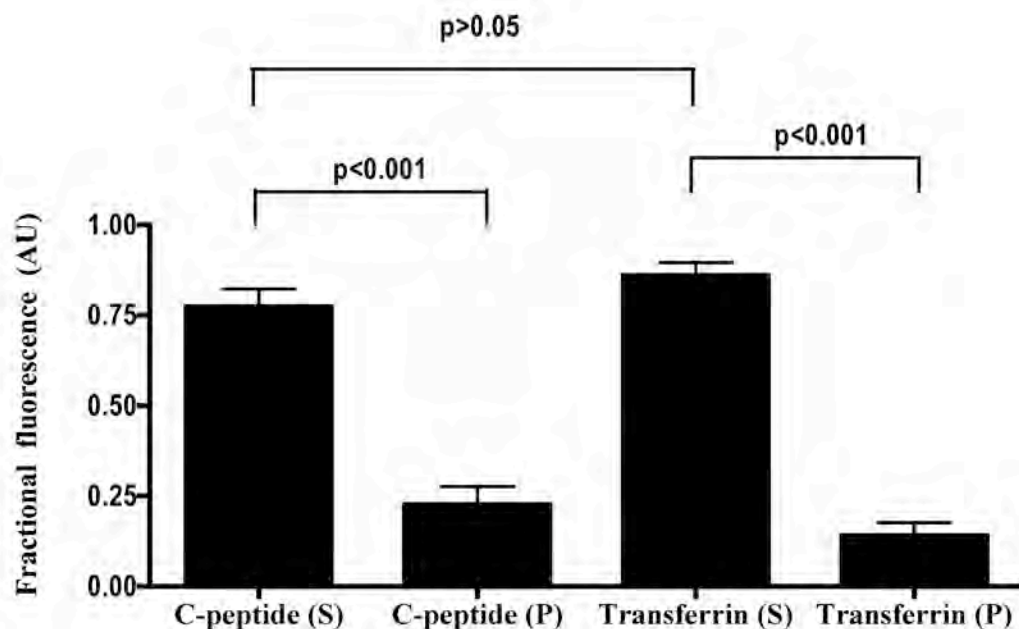


Figure 8

Figure 8 C-peptide co-localizes in isolated early endosomes in UASMC. To identify early endosomes, we studied internalization of AlexaFluor488-conjugated human transferrin, a specific marker of early endosomes, in the presence of AlexaFluor546-labelled C-peptide in UASMC at 37°C, 5% CO₂ for 10 minutes. Endosomes were isolated from other sub-cellular compartments by high speed centrifugation and an aliquote of both the pellet (P) and of the endosome-containing supernatant (S) used to quantitate AlexaFluor488-transferrin and AlexaFluor546-C-peptide fluorescence spectra by using a scanning spectrofluorimeter. The fluorescence of each molecule was expressed as a fraction of the total for each probe (on the Y axis; AU=arbitrary units). The mean fraction of the AlexaFluor488-transferrin fluorescence, in the endosome-containing supernatant was 0.86 ± 0.06 (n = 3). The mean fraction of AlexaFluor546-C-peptide, in the endosome-containing supernatant was 0.77 ± 0.08 (n = 3). The fraction of each probe in the supernatant versus pellet was significantly different ($p < 0.001$), whereas the fraction of transferrin and C-peptide in the endosomal fraction are statistically indistinguishable ($p > 0.05$). The cell fractionation results further support the internalization of C-peptide to early endosomes.

KEY RESEARCH ACCOMPLISHMENTS

- Chemically (i.e., using Streptozotocin) induced diabetes is characterized by the establishment of a hyperglycemic status that, in non-human primates (NHP), requires exogenous insulin treatment to prevent fatal cheto-acidosis. Following streptozotocin administration, a drastic reduction of endogenous circulating C-peptide is observed, and histologic analysis of the pancreas shows virtual absence of insulin positive cells. Even in those monkeys where low but detectable C-peptide levels were measured after streptozotocin, histological findings confirmed the destruction of insulin positive cells, and in vivo functional studies suggested that any residual insulin producing mass is not responsive. We consequently observed that hyperglycemia and diabetes management were not influenced by the residual C-peptide amounts.
- Our results demonstrate the advantage of the Tg expression of hCD46 on adult porcine islets when transplanted intraportally into diabetic, immunosuppressed NHP. In fact, we documented for the first time the survival of a functional islet xenograft for more than one full year (at which time the experiment was electively terminated), in a chemically-diabetic monkey, which was neither limited in diet nor received exogenous insulin injections. hCD46 successfully protected Tg islets against humoral rejection, thus reducing the need for immunosuppression. The avoidance of tacrolimus and sirolimus may have added to the long-term preservation of a functional islet mass. Our moderate immunosuppressive regimen allowed monkeys to remain healthy, in contrast to reports in which adverse events prematurely terminated many experiments.
- In our study, we used market-age hCD46 pigs (not retired breeders) and did not perform any pre-selection of donor islet preparations, in order to model future clinical applications. Despite the expression of hCD46 and the application of an anti-inflammatory and anticoagulant protocol, after intraportal islet Tx early islet loss occurred, suggesting that these maneuvers were insufficient to prevent IBMIR and cell death. Although high numbers of porcine islets were considered necessary, we conclude that only a marginal mass of surviving cells is sufficient to sustain normoglycemia.
- Based on these and on our previous observations on the metabolic compatibility between species, we anticipate that the islet graft mass sufficient to promote normoglycemia would be substantially lower in pig-to-human versus pig-to-NHP.
- No report of a spontaneous remission of the hyperglycaemic status in diabetic monkeys even months after streptozotocin administration was found. It appears therefore that in non-human primates, similarly to rodents, exogenous insulin itself does not trigger a healing process of the pancreatic beta cell mass, even in the absence of a autoimmune contest, after chemically-induced diabetes.
- In the absence of the autoimmune mechanisms (no evidence of autoimmunity is accrued in streptozotocin diabetic monkeys), our experiments suggest that diabetic monkeys that receive xenogeneic islet support with sustained yet limited in time detectable biological activity may recover a self-driven endocrine function. Such function is evidenced not only by stepwise increasing levels of endogenous C-peptide, but also by amelioration of the metabolic control, not simply explained by the effect of the graft. In this contest, the xenotransplantation model offers the advantage to allow discernment between graft and endogenous insulin production. However only two recipients showed an endogenous C-peptide substantial recovery that occurred relatively late over time, consistent with the kinetics of recovery described in rodent models.
- Since such effects were not found in monkeys that did not receive a transplant, and monkeys recipient of non functional graft, but partially present in monkeys with longer survival of the graft, we hypothesize that the transplantation may play a role. Whether a functional graft is exerting an effect on recover, it remains unclear but in this line there are reports in human clinical islet allografts that indicate an amelioration of the metabolic conditions, often accompanied by increased endogenous C-peptide production, in patients that received a transplant, even if the graft was rejected or insufficient to establish normoglycemia.

- This recovery of the endogenous insulin production was associated with quite peculiar histological features of the pancreatic tissue. We found a generalized augmented proliferative activity in the pancreatic tissue, spread throughout the organ and associated to fibroblast positive, ductal and endothelial phenotype, but not specifically to insulin positive cells. We also observed that in monkeys that exhibited endogenous production of C-peptide, pancreatic ducts were hypertrophic. Interestingly, substantial numbers of ductal cells (CK19+) showed also co-staining with proinsulin. More close analysis of the pancreas in the monkey that presented double CK19-proinsulin phenotype, indicated the presence of small size insulin positive cell aggregates. Such clusters appeared not even partially damaged, and differed morphologically from those that clearly showed regressed signs of injury, commonly associated with undamaged alpha cells (glucagon positive cells). Moreover proinsulin positive aggregates were often found not in correspondence of glucagon positive cells suggesting that they were not part of pre-existing islet of Langerhans.
- C-peptide is the peptide segment connecting insulin A and B chains , and a product of pro-insulin cleavage in the secretory granules, generated in pancreatic beta cells as part of normal insulin production. C-peptide was for a long time considered to only possess the biological activity of favoring pro-insulin folding within the beta cells. Recent studies have, however, challenged this classical view by demonstrating that C-peptide is capable of biological effects in many different cell types and importantly reduces the complications associated with type 1 diabetes. Previous work from our laboratory has demonstrated a protective effect of C-peptide on the vasculature in conditions of hyperglycaemia by decreasing the inflammatory damage and nuclear factor(NF)-kB activation.
- The cell biology of C-peptide is for the most part unknown, in particular regarding the pathway the peptide uses to cross the cellular membrane and possible sub-cellular destinations. Our study was focused on the trafficking pathway of C-peptide in target cells upon its binding to cellular membranes.
- The internalization of C-peptide from the plasma membrane to the cytoplasm raises important biological questions. The first question concerns the fate of C-peptide upon internalization. Unique to this work was our finding that C-peptide traffics from the cell surface to early endosomes.
- C-peptide internalization evidently proceeds by clathrin-mediated endocytosis, as it was inhibited by MDC, a pharmacological compound used to inhibit receptor-mediated endocytosis from clathrin-coated pits. Microtubule integrity appears to be important in facilitating the early stages of internalization of C-peptide in HAEC as pre-treatment of cells with Nocodazole, an inhibitor of microtubule assembly, impairs C-peptide endocytosis. Internalization of C-peptide in the low nanomolar range via endocytosis provides indirect evidence for the presence of a specific membrane receptor. Although a putative C-peptide receptor has not been identified, it has been suggested to be a G-protein-coupled receptor as deduced from effects of pertussis toxin.
- Based on our findings, signaling from putative C-peptide-receptor complexes might initiate at the plasma membrane, continue from early endosomes, and terminate at lysosomes. The effect of C-peptide that we observed on the NF-kB pathway in both HAEC and UASMC might thus originate from C-peptide-receptor complex signaling from the endosomes, as it has been demonstrated for certain Toll-like receptor pathways and other inflammatory pathways, which affect activation of the NF-kB pathway from the endosomes.

CONCLUSION

Overall our data suggest that the monkey pancreas retains the ability to recover even after a chemical damage that specifically destroys the islet beta cells inducing a irreversible status of hyperglycemia. Such ability seems to be associated with activation of proliferative events in the pancreatic organ, and hypertrophy of the ductal cells. Double phenotype, epithelial and endocrine on the same pancreatic cells further indicate that a direct relation there exist between these two cell types. Recovery of the insulin producing function, in association with the histological findings may provide indirect supportive evidence that epithelial/ductal cells play a role in the generation of insulin producing beta cells. The metabolic enhancement achieved by even partially functioning islet transplantations compared to mere exogenous insulin administration may contribute to promote return of endogenous production of C-peptide and it is desirable to further investigate the multiple biological events that accompany engraftment.

APPENDICES

Pasquali L, Giannoukakis N, **Trucco M**: Induction of immune tolerance to facilitate beta cell regeneration in type 1 diabetes. **Advanced Drug Delivery Reviews** 60:106, 2008.

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Pearson T, Shultz LD, Lief J, Burzenski L, Gott B, Chase T, Foreman O, Rossini AA, Bottino R, **Trucco M**, Greiner DL: A new immunodeficient hyperglycemic mouse model based on the *Ins2^{Akita}* mutation for analyses of human islet and beta stem and progenitor cell function. **Diabetologia** 51:1449, 2008.

Cifarelli V, Luppi P, Tse HM, He J, Piganelli J, **Trucco M**: Human proinsulin C-peptide reduces high glucose-induced proliferation and NF-kB activation in vascular smooth muscle cells. **Atherosclerosis** 201:248, 2008.

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Luppi P, Geng X, Cifarelli V, Drain P, **Trucco M**: C-peptide is internalized in human endothelial and vascular smooth muscle cells via early endosomes. **Diabetologia**, In press, 2009.

In the first quarterly scientific progress report (6/01/09 – 08/31/09) of Year 04 we now report on the biological properties of the pro-insulin C-peptide, in general, and specifically, on the activation of cell signaling pathway.

Background information

Type 1 diabetes (T1D) is associated with the presence of systemic and cellular inflammation, characterized by elevation of plasma levels of several inflammatory biomarkers, such as interleukin(IL)-6, IL-8, tumor necrosis factor(TNF)-alpha, and C-reactive protein. The vascular endothelium represents a likely target of this inflammatory response by inducing endothelial cell activation, alteration in endothelial cell function, and monocyte adherence eventually leading to overt vascular damage in the later stages of T1D. Indeed, inflammation is now considered a major component in the development of T1D-associated vascular dysfunction, especially the one affecting the small vessels of the eyes, the kidneys, and the peripheral nerves. These microvascular complications lead to visual impairment, renal dysfunction, and sensory loss, all of which seriously affect the quality and the life style of diabetic patients.

C-peptide is the 31 amino acid peptide generated in the secretory granules of pancreatic beta cells as part of the normal insulin biosynthesis. In healthy individuals, C-peptide is secreted in the bloodstream in equimolar amount with insulin and upon internalization in target cells displays multiple biological effects. It has been proposed that C-peptide deficiency may contribute to the development of microvascular complications in T1D patients. In fact, small clinical trials of C-peptide replacement to these patients, together with regular insulin therapy, have demonstrated the beneficial effect of C-peptide in the treatment or prevention of microvascular complications, possibly through a protective, direct, effect on the inflamed, dysfunctional, endothelium.

Although the molecular mechanism(s) of action of C-peptide are not known, C-peptide at physiological concentrations affects activation of a variety of cell signaling pathways and transcription factors. One of these pathways is the one activating the peroxisome proliferator-activated receptor(PPAR)-g, a member of the nuclear receptor superfamily of ligand-activated transcription factors. In addition to its function in adipogenesis, and increasing insulin sensitivity, PPAR-g also plays critical roles in vascular dysfunction by suppressing activation and inflammation of smooth muscle cells, endothelial cells, and monocytes through altering the activity of the nuclear factor(NF)-kB.

Aim of the project

Recently, it has been demonstrated that injections of C-peptide *in vivo* to an animal model of endotoxic shock, up-regulated expression of PPAR-g in the lung and significantly reduced both local and systemic inflammatory responses, with reduction of plasma levels of pro-inflammatory cytokines thus leading to an improved survival of the lipopolysaccharide(LPS)-treated mice. Thus, activation of PPAR-g by natural or synthetic ligands improves vascular dysfunction and is emerging as a new therapeutic approach to treat diseases with a strong inflammatory component, such as T1D.

One component of the inflammatory response in T1D is represented by activation of circulating monocytes. Activation of monocytes, with secretion of pro-inflammatory cytokines, has been recently detected in T1D patients, even at the clinical onset of the disease, and is now considered a critical factor in the development of endothelial dysfunction and vascular compromise in T1D patients.

In this project, we investigated the hypothesis that C-peptide might exert a beneficial effect in T1D by decreasing activation of circulating monocytes of T1D patients and therefore decreasing secretion of harmful inflammatory cytokines that will eventually cause vascular dysfunction. We hypothesized that C-peptide could achieve these anti-inflammatory effects by activating the PPAR- gpathway in monocytes.

Methods

To test this hypothesis, we exposed the human monocytic cell line U-937 to high glucose (30mmol/l), to mimic the diabetic condition, and stimulated with LPS (1ng/ml) to induce cytokine secretion. We then investigated whether the anti-inflammatory effects of physiological levels of C-peptide (1nmol/l) in the LPS-stimulated U-937 cells are associated with up-regulation of PPAR-g by Western blot and immunohistochemistry.

Results

We present here preliminary results showing that C-peptide reduces secretion of inflammatory cytokines and stimulate expression of the anti-inflammatory PPAR-g pathway in U-937 monocytes.

C-peptide reduces MCP-1 secretion in LPS-treated U-937 after 30min

To demonstrate activation of U-937, we stimulated the cells with LPS for 30 min in the presence of high glucose, and measured levels of the inflammatory cytokine monocyte chemoattractant protein(MCP)-1 in the supernatant by ELISA. As expected, we found that LPS stimulated secretion of MCP-1 by U-937 (0.585 ± 0.02 pg/mL) (**Figure 1**). Addition of C-peptide (1nmol/l) to LPS-treated cells, significantly decreased MCP-1 secretion (0.313 ± 0.00 pg/mL) compared to LPS-treated cells in the absence of C-peptide and this difference was statistically significant ($p < 0.01$).

C-peptide decreases LPS-stimulated MCP-1 secretion by U-937

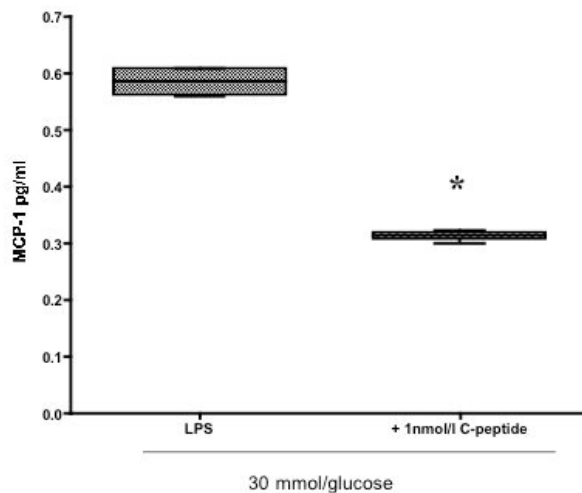


Figure 1. C-peptide decreases LPS-stimulated MCP-1 secretion by U-937 cell. U-937 cells were cultured in high glucose (30 mmol/l glucose) and LPS (1 ng/ml) in presence or absence of C-peptide (1 nmol/l) for 30 min and secretion of MCP-1 in the supernatant fraction was assessed by ELISA. Addition of C-peptide significantly reduced MCP-1 secretion by U-937 monocytes ($p < 0.01$).

C-peptide stimulates expression of PPAR-g in LPS-treated U-937

Treatment of U-937 cells with LPS for 4 hours triggered an increased expression of PPAR-g compared to cells cultured in medium alone, as detected by Western Blot (**Figure 2**). Addition of C-peptide (1nmol/L) to LPS for 4 hours induced a further increase in PPAR-g expression in U-937 cells compared to stimulation with LPS alone (**Figure 2**). In contrast, addition of a randomized version of C-peptide (scrambled C-peptide; 1 nmol/L) to LPS did not significantly modify PPAR-g expression (**Figure 2**).

C-peptide induces PPAR- γ in LPS-stimulated U-937 monocyte

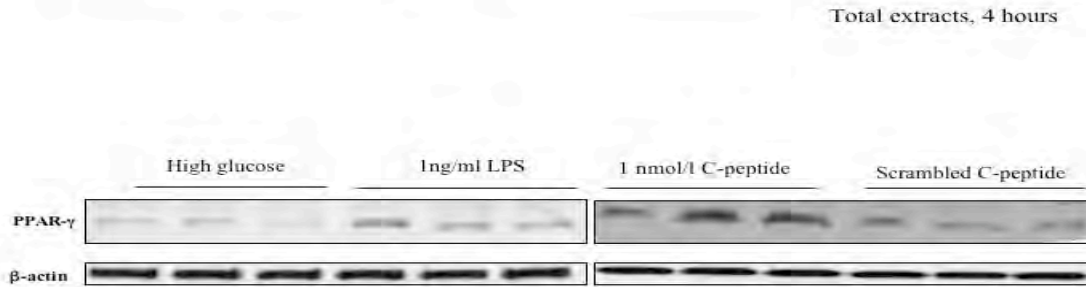
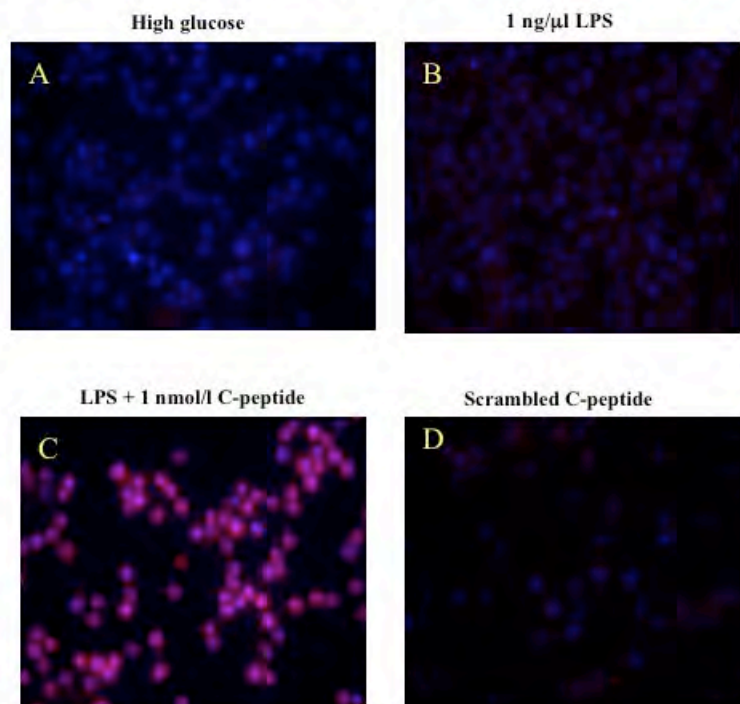


Figure 2. C-peptide stimulates expression of PPAR- γ in U-937 cultured in high glucose. U-937 cells were cultured in high glucose medium (30mmol/L glucose) in the presence or absence of LPS (1ng/mL) for 4 hours. In parallel experiments, either C-peptide (1nmol/L) or scrambled C-peptide (1nmol/L) was added to LPS for 4 hours. Cellular extracts were subjected to a Western immunoblotting to detect PPAR- γ . Membranes were stripped and reprobed for β -actin as a loading control. In cells exposed to LPS for 4 hours there was an increase in PPAR- γ nuclear expression compared with cells in medium alone. A further increase in PPAR- γ nuclear expression was observed in the presence of 1 nmol/L C-peptide. Scrambled C-peptide was used as a control for C-peptide activity.

Immunofluorescence staining of U-937 cells to detect expression of PPAR- γ confirmed that C-peptide treatment of LPS-stimulated cells for 4 hours induces an increase in expression of PPAR- γ in comparison to U-937 cells incubated with LPS alone (**Figure 3**). As expected, addition of scrambled C-peptide did not seem to significantly alter PPAR- γ expression in LPS-treated U-937 cells (**Figure 3**).

C-peptide induces PPAR- γ expression on U-937 monocytes



Blue = DAPI (nuclei); Red = PPAR- γ positive cell

Figure 3. Immunofluorescence staining of PPAR-g in C-peptide stimulated U-937 cells. Images of PPAR-g immunostaining (in red) in U-937 cells exposed for 4 hours to the different conditions stated. DAPI staining was used to stain the nuclei (in blue). C-peptide addition to LPS dramatically increased PPAR-g nuclear expression in U-937 cells as compared with LPS alone.

C-peptide induces expression of the PPAR-g-regulated monocyte differentiation marker CD36

We investigated whether C-peptide is able to induce expression of the scavenger receptor CD36 in the monocyte cell line U-937, as CD36 is an example of a protein product of a well-established PPAR-g-regulated gene. In these experiments, U-937 cells were stimulated with C-peptide (1nmol/L) for 4 and 24 hours and changes in CD36 protein expression was examined by Western blot. Stimulation of U-937 cells for 4 hours with either C-peptide or with the CD36 inducer compound phorbol-12-myristate 13-acetate (PMA), did not cause any significant changes in CD36 expression levels over the constitutive expression detected in medium alone (**Figure 4A**). A dramatic increase in CD36 protein expression was detected after 24 hours of C-peptide or PMA stimulation as compared to basal levels detected in medium alone (**Figure 4B**). In contrast, addition of scrambled C-peptide (1nmol/L) to U-937 cells did not induce any changes in CD36 protein expression both at 4 or 24 hours (**Figure 4A-B**).

Densitometric analysis of CD36 immunoblot demonstrated that CD36 protein levels after either PMA or C-peptide were statistically higher than in U-937 cells in medium alone ($p < 0.01$) (**Figure 4C**).

C-peptide induces CD36 expression in high glucose-treated U-937

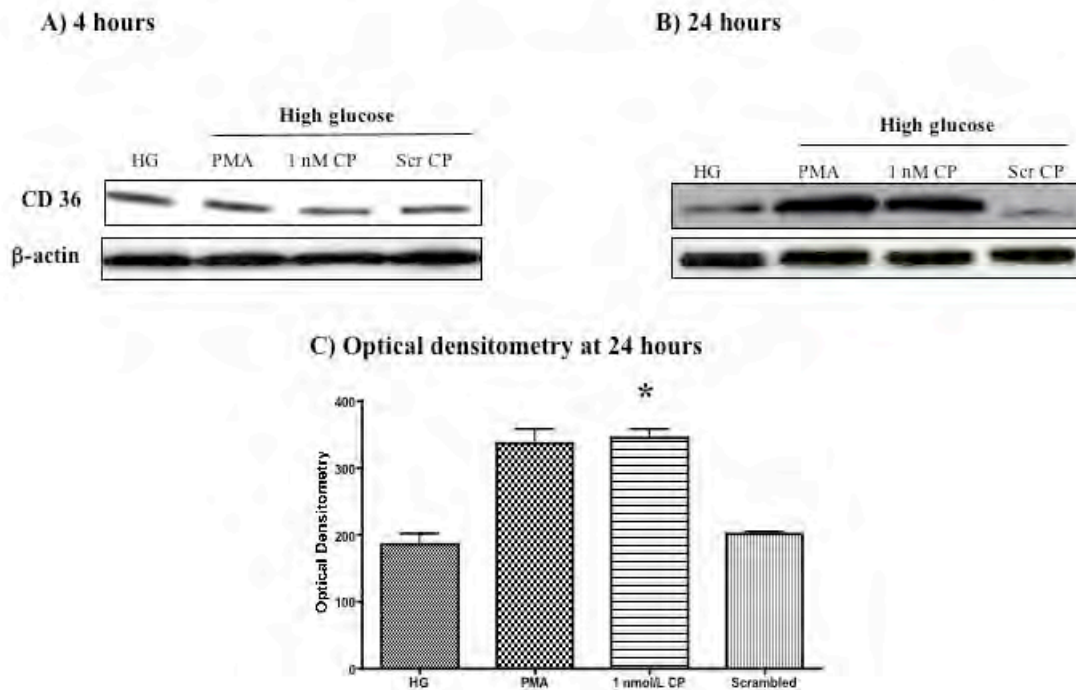


Figure 4. C-peptide increase CD36 protein levels in the monocyte cells U-937. Cells were either cultured in medium added with 30mmol/L glucose or stimulated with phorbol myristate acetate (PMA; 1mM) or C-peptide (1nmol/L) for the indicated times (4A and 4B). Scrambled C-peptide (1nmol/L) was used as a control for C-peptide activity. CD36 protein was detected by immunoblotting. C-peptide induces a dramatic increase in CD36 protein expression after 24 hours incubation (4B and 4C), an effect similar to that induced by PMA. This is a representative blot of three independent experiments.

Conclusions

These preliminary experiments have demonstrated that C-peptide might achieve its anti-inflammatory effects in monocytes by activating the PPAR-gamma pathway and eventually decreasing NF-kB activation in high glucose-exposed U-937 monocytes. Additional experiments are underway to understand the mechanism(s) by which C-peptide affects PPAR-gamma in these cells and therefore modulate inflammatory responses and reduce the risk to develop microvascular complications in T1D patients.

In the second quarterly scientific progress report (9/01/09 – 11/30/09) of Year 04 we now report on the biological properties of the pro-insulin C-peptide, in general, and in particular, on its ability to decrease – once used at physiological concentrations – the expression of NF κ B on target cells.

Background information

Type 1 diabetes (T1D) is a progressive autoimmune disease resulting from destruction of insulin-producing pancreatic beta cell. The subsequent lack of insulin leads to a dramatic elevated blood glucose level (hyperglycemia) treated with long-life exogenous insulin treatment. Different component of the innate immune system, such as natural killer cell (NK) and monocytes are involved in the autoimmune response characteristic of T1D. It has been shown that monocytes accumulate in the pancreas islets of prediabetic BB rats. Furthermore, monocytes are emerging as major component of the generalized inflammatory activity detected at the level of macro- and micro- vasculature of T1D patients, serving as principal reservoir of pro-inflammatory cytokines.

Human C-peptide is a 31 amino acids peptide that is released by pancreatic beta cells in equimolar amounts with insulin in response to hyperglycemia. C-peptide was initially believed to have no biological function apart from its role in insulin biosynthesis. However, in the last two decades, a growing body of evidence has shown that C-peptide might have a physiological role on a variety of cell types, including monocytes. Several studies have shown that C-peptide exerts a beneficial role in the long-term vascular complication in diabetes mellitus patients.

Moreover, it has been shown that C-peptide antagonizes endothelial dysfunction, which is a major sign of vasculature damage inferred by hyperglycemia, by acting on vascular tissues such as endothelium and smooth muscle. We have previously shown that C-peptide exerts anti-inflammatory effects on monocytes, in a scenario of hyperglycemia. However, the intracellular pathways through which C-peptide may exert its anti-inflammatory action in monocytes have not been fully elucidated yet. The purpose of this project is to investigate whether activated monocytes of T1D patients might represent a target for the beneficial activity of C-peptide observed in inflammatory conditions.

Aim of the project

T1D patients suffering from microvascular complications demonstrate the highest level of monocyte activation with involvement of the transcription factor nuclear factor- κ B (NF- κ B) and the mitogen-activated protein kinase MAPK/ERK signaling pathways.

In a previous study, we have shown that C-peptide affects the activity of the transcription factor PPAR- γ (peroxisome-proliferator-activated receptor γ), a ligand dependent nuclear transcription factor, that plays a crucial role in the modulation of lipid and glucose metabolism, inflammation and vascular biology. Moreover, we have also shown that C-peptide affects the expression of PPAR- γ -regulated monocyte differentiation marker CD36, also known as scavenger receptor in LPS-stimulated monocytes.

In these new studies, we exposed human monocytic cell line U-937 to high glucose- in order to mimic the diabetic condition - and stimulated with LPS. We then tested the ability of C-peptide to decrease the level of inflammation in the LPS-activated monocytes.

In this context, we investigated whether the anti-inflammatory effects of C-peptide associated with activation of PPAR- γ is modulated by ERK1/2 MAPK pathway in LPS-stimulated U-937 monocytes.

As additional sign of an ongoing inflammatory response in T1D, we studied the secretion of inflammatory cytokines in activated monocytes. In a previous scientific report, we have already shown that C-peptide reduces secretion of inflammatory cytokine monocyte chemoattractant protein (MCP-1) in the supernatant of LPS-stimulated U-937 monocytes. However, whether the C-peptide effect on cytokine secretion engages the involvement of NF κ B pathway has not been elucidated yet. In this project, we investigated whether C-peptide is able to decrease the expression of NF κ B during combined inflammatory insults such as hyperglycemia and LPS.

We also investigated the ability of C-peptide to induce the expression of PPAR- γ in Human Aortic Endothelial Cell (HAEC) in the scenario of hyperglycemia.

Methods

To test the hypothesis of the involvement of MAPK in the PPAR- γ expression, we exposed the human monocytic cell line U-937 to MAPK and MAPKK inhibitors, UO126 and PD 98059 respectively, and successively U-937 cells were stimulated with LPS (1 ng/ μ l) for 30 min, in presence or absence of C-peptide.

Human aortic endothelial cell (HAEC) were cultured in high glucose (25 mmol/L D-glucose) in presence or absence of C-peptide (0.5-1 nmol/L) for 4 hours. Protein nuclear extracts were then analyzed by Western blotting using anti-PPAR- γ antibody. TNF-alpha was used as positive control. The expression of PPAR- γ in the nuclei of high glucose-stimulated endothelial cells, in the presence of C-peptide, was also investigated using immunofluorescence microscopy.

Results

The following preliminary results show that C-peptide exerts anti-inflammatory effects in the LPS-stimulated U-937 cells through the activation of transcription factor PPAR- γ . Furthermore, the expression of PPAR- γ is increased in human aortic endothelial cell when cells were cultured in high glucose in the presence of C-peptide.

Time-course detection of phosphorylated extracellular signal-regulated kinases 1 and 2 (p-ERK1/2) and total ERK 1/2 in the monocyte cell line U-937 cultured in high glucose

The expression of phosphorylated active form of ERK1/2 (p-ERK1/2) was studied in a time-course experiment after 0-30 min of LPS stimulation, as the temporal dynamics of substrate phosphorylation is usually very rapid. Analysis of p-ERK1/2 by Western blot showed that its expression increased after 5 min to reach a maximum level after 30 min of LPS administration.

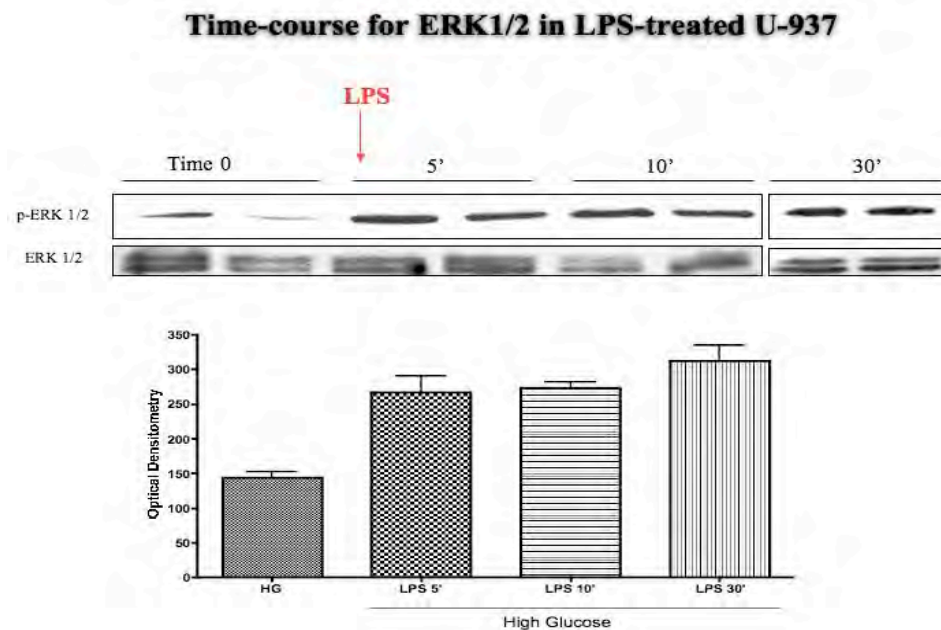


Figure 1. Time-course detection of phosphorylated extracellular signal-regulated kinases 1 and 2 (p-ERK1/2) and total ERK 1/2 in the monocyte cell line U-937. In A) representative radiograph of Western blot analysis of p-ERK1/2 (top panel) and total ERK 1/2 (bottom panel) in LPS-stimulated U-937 cells. In a time-course experiment (0-30min), cells were stimulated with 1ng/mL LPS and total cellular extracts were obtained and p-ERK1/2 and total ERK detected by immunoblotting. There was an increase in p-ERK1/2 expression with a maximum expression reached at 30min LPS-incubation.

Involvement of MAPK and MAPKK in PPAR- γ expression in LPS-stimulated U-937 monocyte

As a further step in understanding whether modulation of p-ERK1/2 activity affects PPAR- γ expression in U-937 cells, we wanted to investigate whether up-regulation of PPAR- γ expression by C-peptide in LPS-treated U-937 monocyte involved a down-regulation in phosphorylation of ERK1/2. U-937 cells were pre-treated with selective inhibitors of MAPK and MAPKK, before stimulating the cells with LPS in the presence or absence of 1nmol/L C-peptide.

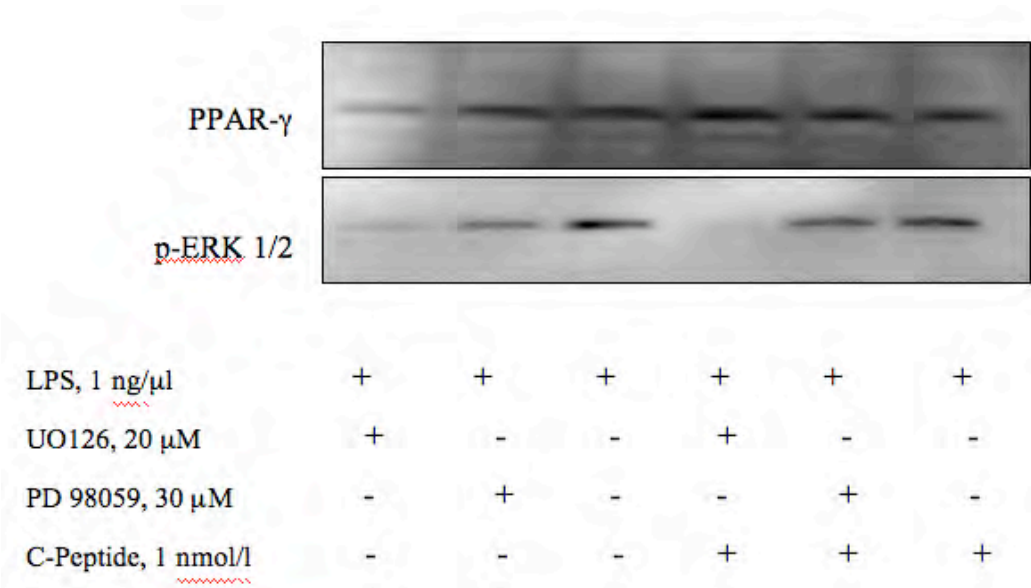


Figure 2. Inhibition of MAPK and MAPKK only partially affects PPAR- γ expression in LPS-stimulated U-937 cells. The involvement of MAPK in the PPAR- γ expression was studied by treating U-937 cells with MAPK and MAPKK inhibitors, UO126 and PD 98059 respectively, and successively stimulating U-937 cells with LPS for 30 min, in presence or absence of 1 nmol/l C-peptide. The same membrane was blotted for both PPAR- γ and p-ERK. PPAR- γ expression was increased in U-937 cells treated with C-peptide for 30 min, even in the presence of the inhibitor UO126 (band #4 in PPAR- γ blot) compared to U-937 cells pre-incubated with UO126 and then stimulated with LPS alone (band #1 in PPAR- γ blot).

NF- κ B p65 nuclear expression is decreased in LPS-stimulated U-937 monocyte in presence of C-peptide.

NK- κ B represents one of the major pathway involved in the inflammatory response and cytokines secretion. We investigated whether C-peptide is able to decrease the expression of p65 sub-unit NK- κ B in the nuclei of monocytes during combined inflammatory insults such as hyperglycemia and LPS.

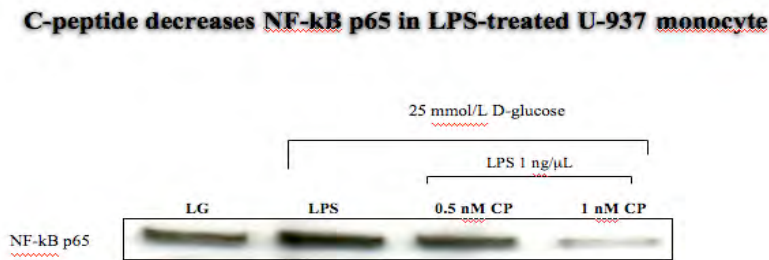


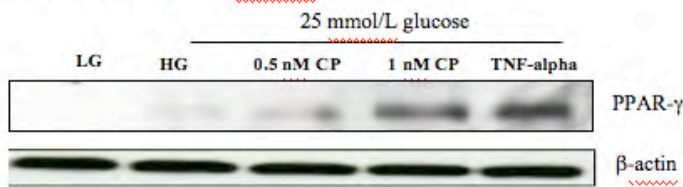
Fig.3 expression of p65 subunit of NF- κ B in LPS-stimulated monocytes is decreased in presence of C-peptide. Representative immunoblot depicting the 65-kDa band of the p65 subunit in LPS-stimulated U-937 monocytes cultured in 5.6 mmol/L (Low glucose; LG) or 25 mmol/L glucose in presence or absence of C-peptide (0.5-1 nmol/L). Cellular nuclear extract were subjected to Western immunoblotting to detect p65 levels using anti- NF- κ B antibody.

C-peptide stimulates expression of PPAR- γ in high glucose-treated human aortic endothelial cell

In order to investigate the hypothesis that C-peptide might exert anti-inflammatory effects on the endothelium of large blood vessels in the scenario of hyperglycaemia, we cultured human aortic endothelial cell (HAEC) in high glucose (25 mmol/L D-glucose), in presence or absence of C-peptide. Treatment of HAEC with C-peptide (1 nmol/L) in presence of high glucose (25 mmol/L D-glucose) triggered an increased expression of PPAR- γ compared to cells cultured in medium alone, as detected by Western blot (**Figure 4a**) and immunofluorescence staining (**Figure 4b**).

C-peptide induces PPAR- γ expression on Aortic Endothelial Cell (HAEC)

A) Western blot of nuclear extracts, 4 h



B) Immunostaining, Blu = DAPI (nuclei); Red = PPAR- γ positive cell

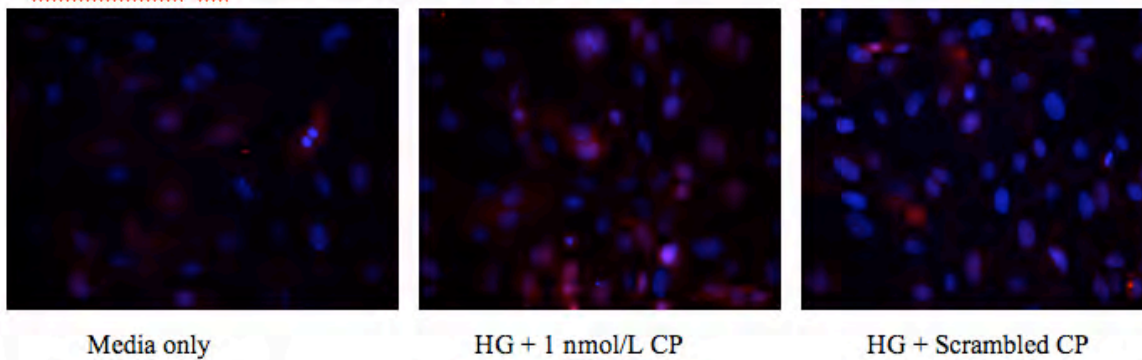


Figure 4. C-peptide stimulates expression of PPAR- γ in high glucose-treated human aortic endothelial cell. Human Aortic Endothelial cells (HAEC) were cultured in high glucose (25 mmol/L D-glucose) in presence or absence of C-peptide (0.5-1 nmol/L). TNF-alpha was used as positive control. **(a)** Western immunoblotting of PPAR- γ expression in nuclear extract of HAEC after 4 hours stimulation with C-peptide. **(b)** Images of PPAR- γ immunostaining (in red) in HAEC exposed to for 4 hours to different conditions stated. DAPI staining was used to stain the nuclei (in blue). C-peptide addition increased PPAR- γ nuclear expression in HAEC as compared to the condition of media alone.

12. Statement of Plans for the Upcoming Research Period

We have shown evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for type 1 diabetes (T1D) patients who suffer from microvascular complications. However, how exactly C-peptide achieves these intracellular effects is still unknown. Based on our previous findings (Luppi P, Geng X, Cifarelli V, Drain P, Trucco M: C-peptide is internalized in human endothelial and vascular smooth muscle cells via early endosomes. *Diabetologia* 52:2218, 2009), it is hypothesized that C peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes. One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization.

Knowledge of how C peptide activates its effector pathways will allow better understanding of how C-peptide achieves its anti-inflammatory effects in endothelial and smooth muscle cells and thus further support an international effort to test C-peptide treatment clinically to T1D patients suffering from vascular complications.

In the third quarterly scientific progress report (1201/09 – 02/28/10) of Year 04 we now report on the biological properties of the pro-insulin C-peptide, in general, and, in particular, on the expression of Bcl-2, a protein able to inhibit apoptosis.

Background information

Type 1 diabetes (T1D) is a progressive autoimmune disease resulting from destruction of insulin-producing pancreatic beta cell. The subsequent lack of insulin leads to a dramatic elevated blood glucose level (hyperglycemia) treated with long-life exogenous insulin treatment. Hyperglycaemia has been recognized to play a crucial role in the development of long-term vascular complications of T1D that affect the eyes, kidneys, peripheral and autonomic nervous system. In the last two decades, the correlation between hyperglycaemia and the occurrence of diabetic complications has been demonstrated by several epidemiologic and clinical studies, suggesting that improved manage of hyperglycaemia may represent a valid therapeutic approach in decreasing the chance of developing vascular complications in diabetic patients.

Human C-peptide is a 31 amino acids peptide that, in healthy individuals, is released by pancreatic beta cells in equimolar amounts with insulin in response to hyperglycemia. T1D patients have diminished levels of C-peptide in the bloodstream. C-peptide was initially believed to have no biological function apart from its role in insulin biosynthesis. However, in the last two decades, a growing body of evidence has shown that C-peptide has indeed a biological role on a variety of cell types, such as erythrocytes, kidney epithelial tubular cells, fibroblasts, endothelial and smooth muscle cells. In addition, C-peptide administration to T1D patients is beneficial in the treatment of nephropathy and neuropathy, two important vascular complications.

Our studies showed that C-peptide antagonizes high glucose-induced endothelial dysfunction by displaying an anti-inflammatory activity directly on endothelial cells. C-peptide reduced high glucose-induced expression of vascular cell adhesion molecule-1 (VCAM-1), and the secretion of macrophage chemoattractant protein-1 (MCP-1) and Interleukin(IL)-8 by modulating nuclear translocation of the transcription factor nuclear factor- κ B (NF- κ B).

To further investigate the effect of C-peptide on high glucose-induced endothelial dysfunction, we focused on studying a possible role of C-peptide on the process of apoptosis of endothelial cells.

Aim of the project

The activation of apoptosis in vascular endothelial cells exposed to high glucose represents an important sign of endothelial dysfunction. Apoptosis is a form of eukaryotic cell death that can be described by morphological and biochemical criteria and it is characterized by membrane blebbing, condensation of cytoplasm and the activation of endogenous and specific proteases. One of the biochemical hallmarks of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability. In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+} dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments. It has been shown that hyperglycaemia can trigger the activation of endogenous endonucleases leading to an enrichment of DNA fragments in the cytoplasm of the apoptotic cell. In this contest, we investigated whether C-peptide might reverse the initiation of DNA fragmentation process in endothelial cell exposed to high glucose. In this project, we exposed human aortic endothelial cells (HAEC) to high glucose in order to mimic the diabetic condition. We then tested the ability of C-peptide to decrease the level of apoptosis.

In addition to that, we were interested in investigating the regulatory machinery necessary for the activation of the apoptotic process. It is well established that the receipt of pro-apoptotic signals and the withdrawal of anti-apoptotic (survival) signals in the cell regulate the initiation of the apoptotic mechanism. We investigated the effect of C-peptide on the expression of Bcl-2, a protein located in the outer membranes of mitochondria, which is able to inhibit apoptosis through the induction of cell survival pathway.

Methods

To test the hypothesis that C-peptide might decrease the level of apoptosis inferred by hyperglycaemia, HAEC were cultured in high glucose (25 mmol/L D-glucose) in presence or absence of C-peptide for 48 hours. As additional stimulus of apoptosis, HAEC were exposed to TNF- α (20 ng/ μ l) for 24 h, in presence or absence of C-peptide. Photometric enzyme-immunoassay was used for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleo- somes), such as biomarkers of an ongoing apoptotic process. Cytoplasmic protein extracts were also analyzed by Western blotting using anti-Bcl-2 antibody.

Results

The following preliminary results show that C-peptide is able to decrease the level of apoptosis in HAEC cultured in high glucose after 48 h. Moreover, C-peptide increases the expression of Bcl-2 (survival gene) in HAEC when exposed to TNF- α for 24 h, suggesting that C-peptide might exert its protective effect by activating cell survival pathway in the scenario of endothelial dysfunction.

C-peptide reduces the generation of cytoplasmic histone-associated-DNA fragments in HAEC exposed to high glucose.

HAEC were exposed to high glucose for 48 h at 37°C, in presence or absence of C-peptide. Before and after lysis, cells were centrifuged and cytoplasmic fraction (20 μ l from 200 μ l) was analyzed by using monoclonal antibodies directed against DNA and histones, respectively. This allowed the specific determination of mono- and oligonucleo- somes in the cytoplasmic fraction of cell lysates, such as prove of DNA fragmentation. We detected an increased level of histone-associated-DNA fragments in the cytoplasmic fraction of HAEC exposed to high glucose compared to 5.6 mM glucose condition ($p < 0.0001$). Addition of C-peptide (10nM) drastically reduced the generation of histone-associated-DNA fragments in the cytoplasmic fraction (HG vs C-peptide condition $p < 0.001$) (Figure 1a). We expressed the reduction of apoptosis in percentage that we calculate by comparing the amount of DNA fragments generated in HAEC treated with C-peptide versus the amount of DNA fragments generated by HAEC cultured in high glucose only (figure 1b). We observed a reduction in apoptosis of 38% in the HAEC exposed to C-peptide compared to HAEC cultured in high glucose only.

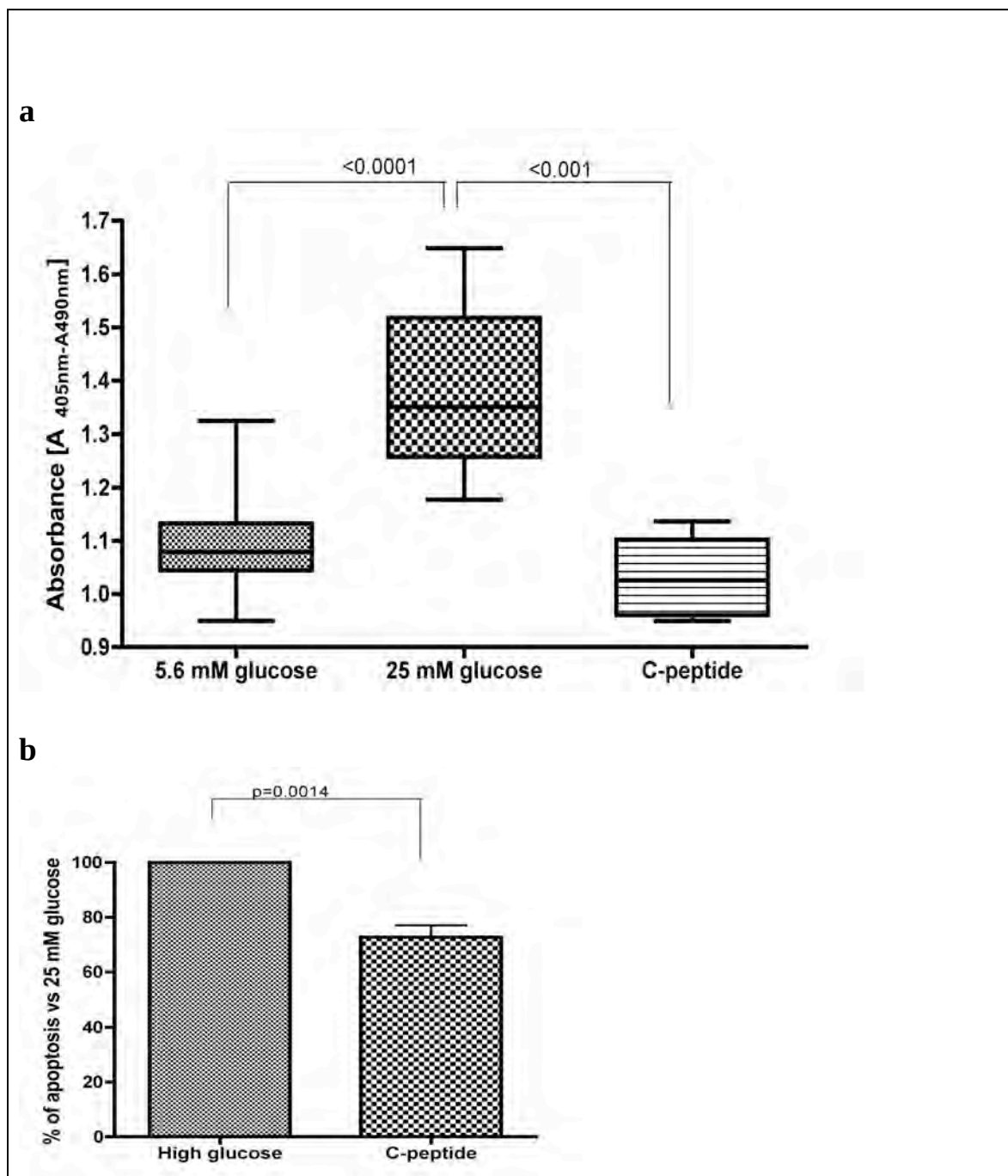


Fig 1. C-peptide reduces the generation of cytoplasmic histone-associated-DNA fragments in HAEC exposed to high glucose. HAEC were exposed to high glucose (HG, 25mM D-glucose) for 48 h at 37 °C, in presence of absence of C-peptide. In **(a)** boxplot graph showing the median values of histone-associated-DNA absorbance measurements generated by analyzing the cytoplasmic fraction of HAEC exposed to high glucose, in presence or absence of C-peptide (high glucose vs. C-peptide in HG, $p < 0.001$). **b** Level of apoptosis in HAEC decreased by C-peptide expressed as per cent versus the appropriate control at 25mM D-glucose ($p = 0.0014$).

Bcl-2 expression is increased in TNF- α stimulated HAEC in presence of C-peptide. We investigated whether the expression of the survival gene Bcl-2 in HAEC is influenced by C-peptide. Bcl-2 exerts a survival function in the apoptotic signaling in response to a wide range of stimuli such as the one inferred by inflammatory cytokines. TNF- α is an inflammatory cytokine and represents an additional trigger of the apoptotic process. HAEC were cultured in high glucose (25mM D-glucose) in T75 cm² flask at 37°C and exposed to TNF- α for 24 h, in presence or absence of C-peptide (10 nM). Cytoplasmic and mitochondrial fractions were separated by re-suspending cells in 10 packed cell volumes of NKM buffer (1 mM TrisHCl, pH 7.4, 0.13 M NaCl, 5 mM KCl, 7.5 mM MgCl₂). Cells were then transferred into a glass homogenizer and incubated on ice. The homogenate was then transferred into a conical centrifuge tube containing 1 packed cell volume of 2 M sucrose solution and mixed gently. Cytoplasmic and mitochondria protein extracts were then collected and analyzed by western blotting. When cell were exposed to TNF- α (20ng/mL), the level of Bcl-2 protein drastically decreased compared to the 5.6mM condition suggesting that TNF- α inhibits the cell survival pathway pushing the cell to commit suicide (apoptosis). On the other hand, addition of C-peptide to HAEC previously exposed to TNF- α , led to an increased expression of Bcl-2 suggesting an activation of the cell survival pathway.

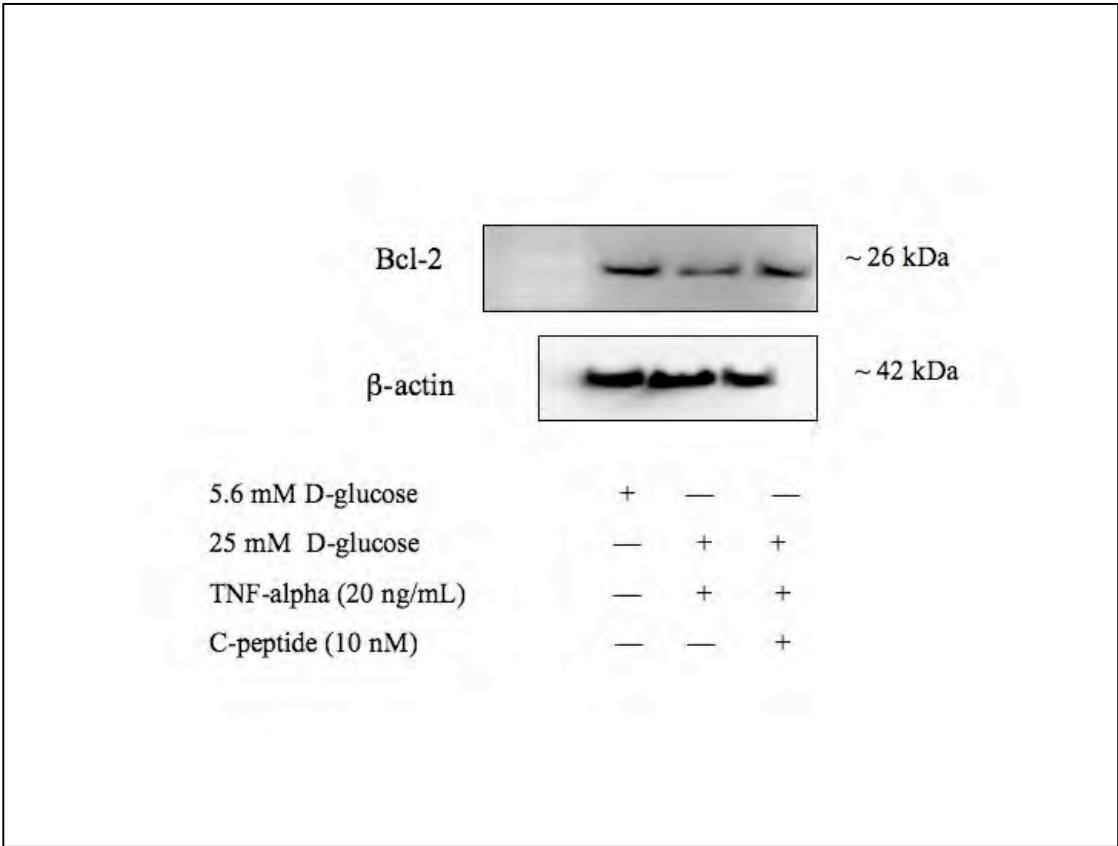


Fig 2. Bcl-2 expression is increased in TNF- α stimulated HAEC in presence of C-peptide. Representative immunoblot depicting the 26-kDa band of the Bcl-2 protein in TNF- α -stimulated HAEC cultured in 5.6mmol/L or 25mmol/L glucose in presence or absence of C-peptide (10nmol/L). Cellular cytoplasmic extracts were subjected to Western immunoblotting to detect Bcl-2 level. When cell were exposed to TNF- α (20ng/mL), the level of Bcl-2 protein drastically decreased compared to the 5.6mM condition suggesting that TNF- α inhibits the cell survival pathway pushing the cell to commit suicide (apoptosis). On the other hand, addition of C-peptide to HAEC previously exposed to TNF- α , led to an increased expression of Bcl-2 suggesting an activation of the cell survival pathway.

12. Statement of Plans for the Upcoming Research Period

We have shown evidence that human C-peptide exerts intracellular effects in a variety of cells and could be of real benefit for type 1 diabetes (T1D) patients who suffer from microvascular complications. However, how exactly C-peptide achieves these intracellular effects is still unknown. Based on our previous findings (Luppi P, Geng X, Cifarelli V, Drain P, Trucco M: C-peptide is internalized in human endothelial and vascular smooth muscle cells via early endosomes. **Diabeteologia** 52:2218, 2009), it is hypothesized that C peptide first binds to its cell surface receptor, then the complex internalizes and signals to effector pathways via endosomes. One major advance in this area would be the identification of the specific C-peptide receptor (CPR) at the level of the cellular membrane and characterization of C-peptide/CPR signaling to effectors upon internalization. Knowledge of how C peptide activates its effector pathways will allow better understanding of how C-peptide achieves its anti-inflammatory effects in endothelial and smooth muscle cells and thus further support an international effort to test C-peptide treatment clinically to T1D patients suffering from vascular complications.

In the fourth and final quarterly scientific progress report (03/01/09 – 05/31/10) of Year 04 we now report on the biological activity of pro-insulin C-peptide in decreasing Caspase-3 expression as a means to activate cell survival pathway and limit endothelial cell dysfunction.

Background information

Type 1 diabetes (T1D) is an autoimmune disease characterized by the disruption of glucose metabolism. T1D results from destruction of insulin-producing pancreatic beta cell. The subsequent lack of insulin leads to a dramatic elevated blood glucose level (hyperglycemia) treated with long-life exogenous insulin treatment.

Hyperglycaemia has been recognized to play a crucial role in the development of long-term vascular complications of T1D that affect the eyes, kidneys, peripheral and autonomic nervous system. In the last two decades, the correlation between hyperglycaemia and the occurrence of diabetic complications has been demonstrated by several epidemiologic and clinical studies, suggesting that improved manage of hyperglycaemia may represent a valid therapeutic approach in decreasing the chance of developing vascular complications in diabetic patients.

Human C-peptide, or connecting peptide, is a 31 amino acids peptide that links the A and B chains of proinsulin and serves to promote the efficient folding, assembly and processing of insulin biosynthesis. In healthy individuals, C-peptide is released by pancreatic beta cells in equimolar amounts with insulin in response to hyperglycemia. T1D patients have diminished levels of C-peptide in the bloodstream. C-peptide was initially believed to have no biological function apart from its role in insulin biosynthesis. However, in the last two decades, a growing body of evidence has shown that C-peptide has indeed a biological role on a variety of cell types, such as erythrocytes, kidney epithelial tubular cells, fibroblasts, endothelial and smooth muscle cells. In addition, C-peptide administration to T1D patients is beneficial in the treatment of nephropathy and neuropathy, two important vascular complications.

Our studies showed that C-peptide antagonizes high glucose-induced endothelial dysfunction by displaying an anti-inflammatory activity directly on endothelial cells. C-peptide reduced high glucose-induced expression of vascular cell adhesion molecule-1 (VCAM-1), and the secretion of macrophage chemoattractant protein-1 (MCP-1) and Interleukin(IL)-8 by modulating nuclear translocation of the transcription factor nuclear factor- κ B (NF- κ B).

To further investigate the effect of C-peptide on high glucose-induced endothelial dysfunction, we focused on studying a possible role of C-peptide on the molecular mechanism of cell apoptosis induced by high glucose in endothelial cells.

Aim of the project

The activation of apoptosis in vascular endothelial cells exposed to high glucose represents an important sign of endothelial dysfunction. Apoptosis is a form of eukaryotic cell death that can be described by morphological and biochemical criteria and it is characterized by membrane blebbing, condensation of cytoplasm and the activation of endogenous and specific proteases. One of the biochemical hallmarks of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability. In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+} dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments. It has been shown that hyperglycaemia can trigger the activation of endogenous endonucleases leading to an enrichment of DNA fragments in the cytoplasm of the apoptotic cell. In this contest, we investigated whether C-peptide might reverse the initiation of DNA fragmentation process in endothelial cell exposed to high glucose. In this project, we exposed human aortic endothelial cells (HAEC) to high glucose in order to mimic the diabetic condition. We then tested the ability of C-peptide to decrease the level of apoptosis.

In addition to that, we were interested in investigating the regulatory machinery necessary for the activation of the apoptotic process. It is well established that the receipt of pro-apoptotic signals and the withdrawal of anti-apoptotic (survival) signals in the cell regulate the initiation of the apoptotic mechanism. We investigated the effect of C-peptide on the expression of Bcl-2, a protein located in the outer membranes of

mitochondria, which is able to inhibit apoptosis through the induction of cell survival pathway. We also studied the expression of Caspase-3 protein, a protease that acts in a cascade triggered by apoptosis signaling.

Methods

To test the hypothesis that C-peptide might decrease the level of apoptosis induced by hyperglycaemia, HAEC were cultured in high glucose (25 mmol/L D-glucose) in presence or absence of C-peptide for 48 hours. As additional stimulus of apoptosis, HAEC were exposed to TNF- α (20 ng/ μ l) for 24 h, in presence or absence of C-peptide. Photometric enzyme-immunoassay was used for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleo- somes), such as biomarkers of an ongoing apoptotic process.

The activity of Caspase-3 protease was measured in the HAEC cell lysates. Cytoplasmic protein extracts were also analyzed by Western blotting using anti-Bcl-2 and anti-Caspase-3 antibodies.

Results

The following preliminary results show that C-peptide is able to decrease the level of apoptosis in HAEC cultured in high glucose after 48 h. Moreover, C-peptide increases the expression of Bcl-2 (survival gene) in HAEC when exposed to TNF- α for 24 h and decreases Caspase-3 expression, suggesting that C-peptide might exert its protective effect by activating cell survival pathway in the scenario of endothelial dysfunction.

As previously shown, C-peptide reduces the generation of cytoplasmic histone-associated-DNA fragments in HAEC exposed to high glucose.

HAEC were exposed to high glucose for 48 h at 37°C, in presence or absence of C-peptide. Before and after lysis, cells were centrifuged and cytoplasmic fraction (20 μ l from 200 μ l) was analyzed by using monoclonal antibodies directed against DNA and histones, respectively. This allowed the specific determination of mono- and oligonucleo- somes in the cytoplasmic fraction of cell lysates, such as prove of DNA fragmentation. We detected an increased level of histone-associated-DNA fragments in the cytoplasmic fraction of HAEC exposed to high glucose compared to 5.6 mM glucose condition ($p < 0.0001$). Addition of C-peptide (10nM) drastically reduced the generation of histone-associated-DNA fragments in the cytoplasmic fraction (HG vs C-peptide condition $p < 0.001$) (Figure 1a). We expressed the reduction of apoptosis in percentage that we calculate by comparing the amount of DNA fragments generated in HAEC treated with C-peptide versus the amount of DNA fragments generated by HAEC cultured in high glucose only (figure 1b). We observed a reduction in apoptosis of 38% in the HAEC exposed to C-peptide compared to HAEC cultured in high glucose only.

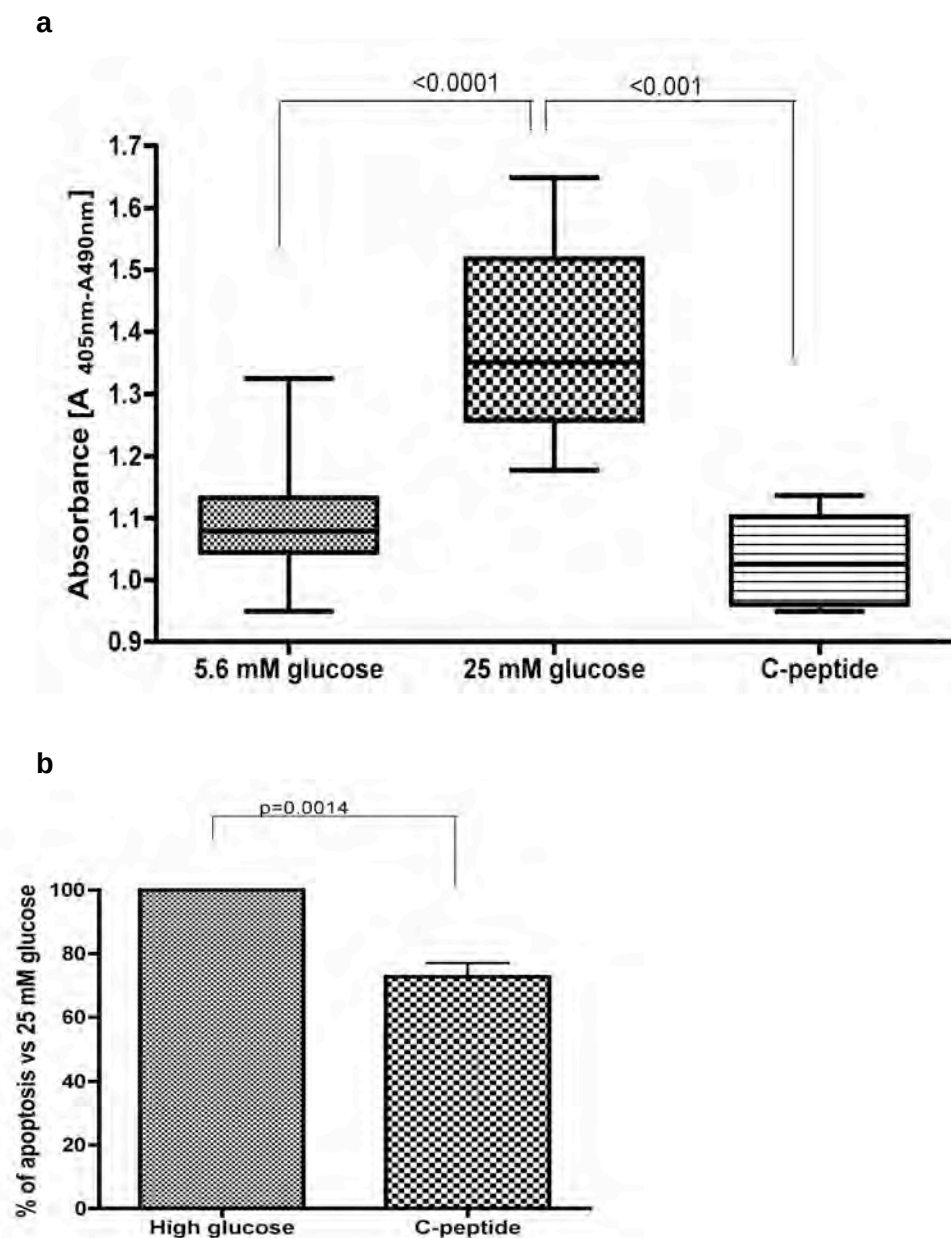


Fig 1. C-peptide reduces the generation of cytoplasmic histone-associated-DNA fragments in HAEC exposed to high glucose. HAEC were exposed to high glucose (HG, 25mM D-glucose) for 48 h at 37 °C, in presence or absence of C-peptide. In **(a)** boxplot graph showing the median values of histone-associated-DNA absorbance measurements generated by analyzing the cytoplasmic fraction of HAEC exposed to high glucose, in presence or absence of C-peptide (high glucose vs. C-peptide in HG, $p<0.001$). **b** Level of apoptosis in HAEC decreased by C-peptide expressed as per cent versus the appropriate control at 25mM D-glucose ($p=0.0014$).

We also can confirm that the activity of Caspase-3 proteases is decreased by C-peptide in HAEC exposed to high glucose

HAEC were seeded in T-75 flasks and treated with high glucose (D-glucose, 25 mM) for 24 h at 37°C, in presence or absence of 10 nM C-peptide. Cells were lysed and exposed to DEVD peptide, which is a specific substrate of caspase-3 protease. To make detection possible, the DEVD substrate is labeled with a fluorescent molecule, 7-amino-4-trifluoromethyl coumarin. Reaction is monitored by using a fluorescent plate reader capable of measuring excitation ~400 nm, and emission at ~505 nm.

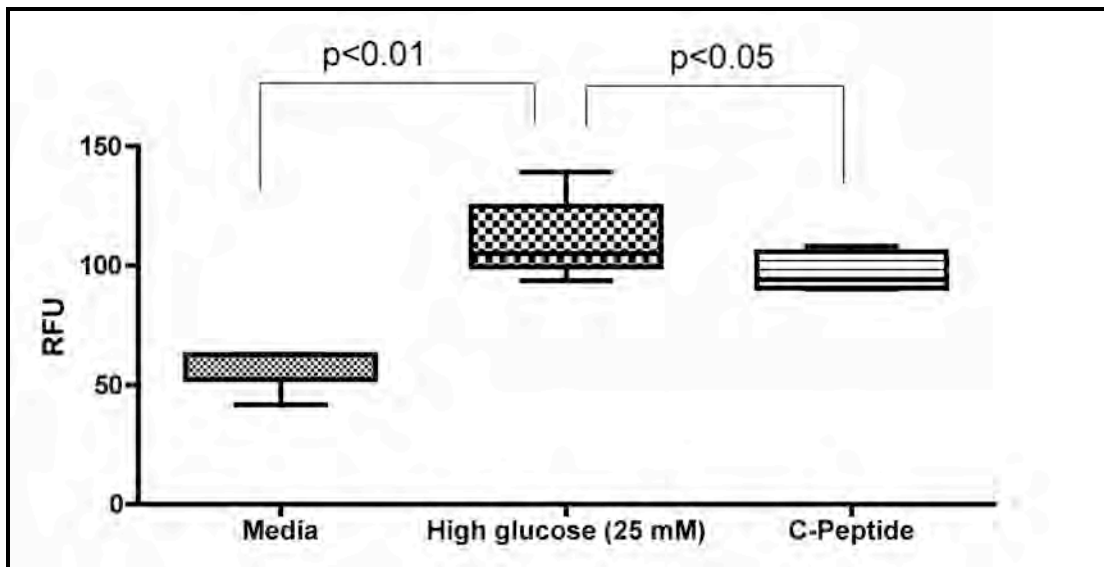


Fig 2. C-peptide decreases the activity of Caspase-3 protease in HAEC exposed to high glucose.

HAEC were exposed to high glucose in presence or absence of 10 nM C-peptide for 24 h at 37°C. The activity of caspase-3 protease is increased in cells exposed to high glucose (25 mM) compared to normal media condition, indicating an ongoing process of cell death (apoptosis). In presence of C-peptide, the activity of caspase-3 protease is decreased showing a protective effect of C-peptide.

Also, C-peptide reduces cell death induced by Tumor Necrosis Factor- α in human aortic endothelial cells.

We investigated the modulation of anti-apoptotic (Bcl-2) and pro-apoptotic genes (caspase-3) in HAEC exposed to TNF- α and hyperglycaemia.

It is well established that a family of intracellular cysteine proteases, known as caspases, represents the effectors of apoptosis. Caspase-3 protease belongs to this family of proteases. On the other hand, Bcl-2 exerts a survival function in the apoptotic signaling in response to a wide range of stimuli such as the one inferred by inflammatory cytokines.

We examined whether the expression of Bcl-2 and caspase-3 in HAEC is influenced by C-peptide. TNF- α is an inflammatory cytokine and represents an additional trigger of the apoptotic process. HAEC were cultured in high glucose (25mM D-glucose) in T75 cm² flask at 37°C and exposed to TNF- α for 24 h, in presence or absence of C-peptide (10 nM). Cytoplasmic and mitochondrial fractions were separated by re-suspending cells in 10 packed cell volumes of NKM buffer (1 mM TrisHCl, pH 7.4, 0.13 M NaCl, 5 mM KCl, 7.5 mM MgCl₂). Cells were then transferred into a glass homogenizer and incubated on ice. The homogenate was transferred into a conical centrifuge tube containing 1 packed cell volume of 2 M sucrose solution and mixed gently. Cytoplasmic and mitochondria protein extracts were collected and analyzed by western blotting. Caspase-3 protein expression is increased in HAEC treated with TNF- α (20ng/mL) compared to normal glucose condition suggesting the on-going activation of the apoptotic process. However, cells treated with C-peptide showed a

lower expression of caspase-3 indicating that C-peptide may protect endothelial cells from apoptotic insults such as TNF- α and high glucose.

Conversely, when cell were exposed to TNF- α , the level of Bcl-2 drastically decreased compared to the 5.6mM condition suggesting that TNF- α inhibits cell survival pathway pushing the cell to commit suicide (apoptosis). On the other hand, addition of C-peptide to HAEC previously exposed to TNF- α , led to an increased expression of Bcl-2 suggesting an activation of the cell survival pathway.

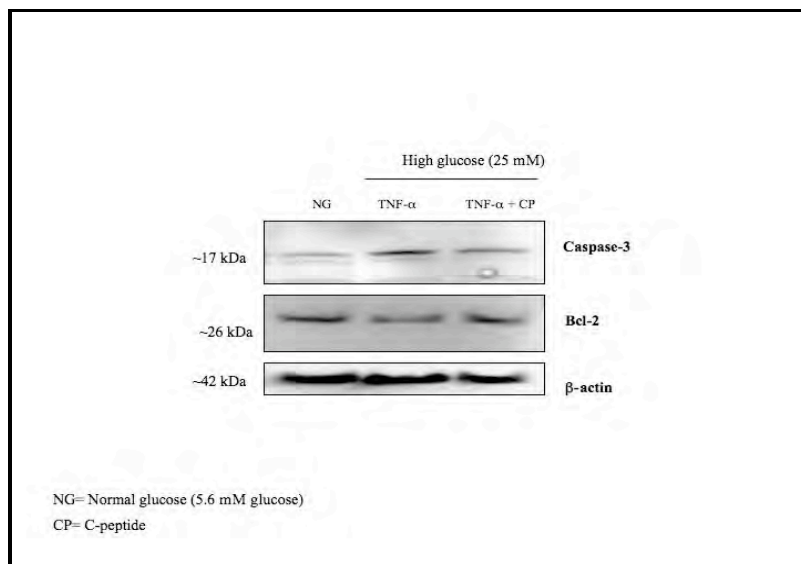


Fig 3. C-peptide reduces cell death induced by Tumor Necrosis Factor- α in human aortic endothelial cells. Representative immunoblot depicting the 17-kDa band of the Caspase-3 protein, the 26-kDa band of the Bcl-2 protein, and β -actin (loading control) in TNF- α -stimulated HAEC cultured in 5.6mmol/L or 25mmol/L glucose in presence or absence of C-peptide (10nmol/L). Caspase-3 expression is increased in HAEC treated with TNF- α (20ng/mL) compared to normal glucose condition suggesting the on-going activation of the apoptotic process. However, cells treated with C-peptide showed a lower expression of Caspase-3 indicating that C-peptide may protect endothelial cells from apoptotic insults. When cell were exposed to TNF- α (20ng/mL), the level of Bcl-2 protein drastically decreased compared to the 5.6mM condition suggesting that TNF- α inhibits the cell survival pathway pushing the cell to commit suicide (apoptosis). On the other hand, addition of C-peptide to HAEC previously exposed to TNF- α , led to an increased expression of Bcl-2 suggesting an activation of the cell survival pathway.

KEY RESEARCH ACCOMPLISHMENTS

- Streptozotocin successfully induces hyperglycemia following administration in rodents as well as in non-human primates.
- Diabetes induction is characterized by the establishment of a hyperglycemic status that, in non-human primates, requires exogenous insulin treatment to prevent fatal cheto-acidosis. Following streptozotocin administration, a drastic reduction of endogenous circulating C-peptide is observed, and histologic analysis of the pancreas shows virtual absence of insulin positive cells. Even in those monkeys where low but detectable C-peptide levels were measured after streptozotocin, histological findings confirmed the destruction of insulin positive cells, and in vivo functional studies suggested that any residual insulin producing mass is not responsive. We consequently observed that hyperglycemia and diabetes management were not influenced by the residual C-peptide amounts.
- No report of a spontaneous remission of the hyperglycaemic status in diabetic monkeys even months after streptozotocin administration was found. It appears therefore that in non-human primates, similarly to rodents, exogenous insulin itself does not trigger a healing process of the pancreatic beta cell mass, even in the absence of an autoimmune contest, thus after chemically-induced diabetes.
- On the putative source of cells that contribute to reform functional insulin producing tissue in the recipient, quite interesting speculation have been put forwards, however, in our experiments as well in those of other groups no direct evidence that an external, donor origin cell source is really needed, supporting the concept that the pancreatic organ can potentially heal itself. It remains scope for investigations the metabolic effect of the islet graft, that in addition to providing insulin, essential to maintain the animal alive, may further provide additional factors able to affect/trigger the recovery of endocrine function. In the absence of the autoimmune mechanisms (no evidence of autoimmunity is accrued in streptozotocin diabetic monkeys), our experiments suggest that diabetic monkeys that receive xenogeneic islet support with sustained detectable biological activity for sometime, they may recover a self-driven endocrine function. Such function is evidenced not only by stepwise increasing levels of endogenous C-peptide, but also by amelioration of the metabolic control, not simply explained by the effect of the graft. In this contest, the xenotransplantation model offers the advantage to allow discernment between graft and endogenous insulin production. In several monkey recipients, following transplantation we observed an increased in endogenous C-peptide levels, however only two recipients maintained graft function long enough and endogenous C-peptide substantial recovery occurred relatively late over time, consistent with the kinetics of recovery described in rodent models.
- This recovery of the endogenous insulin production was associated with quite peculiar histological features of the pancreatic tissue. We found a generalized augmented proliferative activity in the pancreatic tissue, spread throughout the organ and associated to fibroblast positive, ductal and endothelial phenotype, but not specifically to insulin positive cells. We also observed that in monkeys that exhibited endogenous production of C-peptide, pancreatic ducts were hypertrophic. Interestingly, substantial numbers of ductal cells (CK19+) showed also co-staining with proinsulin. More close analysis of the pancreas in the monkey that presented double CK19-proinsulin phenotype, indicated the presence of small size insulin positive cell aggregates. Such clusters appeared not even partially damaged, and differed morphologically from those that clearly showed regressed signs of injury, commonly associated with undamaged alpha cells (glucagon positive cells). Moreover proinsulin positive aggregates were often found not in correspondence of glucagon positive cells suggesting that they were not part of pre-existing islet of Langerhans.
- Since such effects were not found in monkeys that did not receive a transplant, and monkeys recipient of non functional graft, but partially present in monkeys with longer survival of the graft, we hypothesize that the transplantation may play a role. Whether a functional graft is exerting an effect on recovery, it remains unclear but in this line there are reports in human clinical islet allografts that indicate an amelioration of the

metabolic conditions, often accompanied by increased endogenous C-peptide production, in patients that received a transplant, even if the graft was rejected or insufficient to establish normoglycemia.

- Endocrine cells physiologically present in the islets of Langerhans in the pancreas have the capacity to regenerate, even if at a very slow pace. In the case of an abrupt major damage, they are able to more quickly work to replace the lost cells.
- Since the autoimmune reaction systematically limits the physiologic regenerative effort of the endocrine pancreas, the patient still needs daily injections of insulin to control glycemia.
- Injecting insulin, we can control relatively well the glycemia, but we do not prevent sufficiently well the patient complications due to atherosclerotic transformation of the small vessels of the retina, kidney, heart and extremities.
- A possible limit of the insulin injections lies in the absence of C-peptide delivery. The C-peptide is a polypeptide now recognized to have important biological activities able to control atheroma formation.

REPORTABLE OUTCOMES

1. Rood PPM, Bottino R, Balamurugan AN, Fan Y, Cooper DKC, **Trucco M**: Facilitating physiologic self-regeneration: a step beyond islet cell replacement. **Pharmaceutical Research** 23:227, 2006.
2. Rood PPM, Buhler LH, Bottino R, **Trucco M**, Cooper DKC: Pig-to-nonhuman primate islet xenotransplantation: a review of current problems. **Cell Transplantation** 15:89, 2006.
3. **Trucco M**: Is facilitating pancreatic beta cell regeneration a valid option for clinical therapy? **Cell Transplantation** 15:S75, 2006.
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5. Pasquali L, Fan Y, **Trucco M**, Ringquist S: Rehabilitation of adaptive immunity and regeneration of beta-cells. **Trends Biotechnol** 24:516, 2006.
6. Rood PPM, Bottino R, Balamurugan AN, Smetanka CA, Ayares D, Murase N, Cooper DKC, **Trucco M**: Reduction of early graft loss after intraportal porcine islet transplantation in monkeys. **Transplantation** 83:202, 2007.
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8. Casu A, Bottino R, **Trucco M**: The pig as the donor of pancreatic islets for men. **Veterinary Research Communications** 31:27 (Suppl 1), 2007.
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12. Criscimanna A, **Trucco M**: Stem cell replacement therapy in type 1 diabetes. **Ital J Pediatr** 33:254, 2008.
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14. Cifarelli V, Luppi P, Tse HM, He J, Piganelli J, **Trucco M**: Human proinsulin C-peptide reduces high glucose-induced proliferation and NF-kB activation in vascular smooth muscle cells. **Atherosclerosis** 201:248, 2008.
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CONCLUSIONS

Overall our data suggest that the monkey pancreas retains the ability to recover even after a chemical damage that specifically destroys the islet beta cells inducing a irreversible status of hyperglycemia. Such ability seems to be associated with activation of proliferative events in the pancreatic organ, and hypertrophy of the ductal cells. Double phenotype, epithelial and endocrine on the same pancreatic cells further indicate that a direct relation there exist between these two cell types. Recovery of the insulin producing function, in association with the histological findings may provide indirect supportive evidence that epithelial/ductal cells play a role in the generation of insulin producing beta cells. The metabolic enhancement achieved by even partially functioning islet transplantations compared to mere exogenous insulin administration may contribute to promote return of endogenous production of C-peptide and it is desirable to further investigate the multiple biological events that accompany engraftment. Further studies will clarify whether the reparative events involve ex-novo generation of endocrine tissue from pancreatic precursor cells, or, alternatively it involves the recovery of previously damaged cells. In both cases, considering that monkeys are the closest animal species to humans, useful information can be accrued on the dynamic properties of the endocrine pancreas in men.

Based on the presented results, it is then proposed to add to the daily insulin injections C-peptide injections to keep it at physiological concentration. These injections seem to be useful to prevent the tragic complications of type 1 diabetes, allowing a better quality of life in elderly, diabetic patients.

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Highlighted publications are included with this report.

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2008 – 2009

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Expert Review

Facilitating Physiologic Self-Regeneration: A Step Beyond Islet Cell Replacement

Pleunie P. M. Rood,^{1,2} Rita Bottino,¹ A. N. Balamurugan,^{1,2} Yong Fan,¹ David K. C. Cooper,²
and Massimo Trucco^{1,3,4}

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Abstract. Type 1 diabetes (T1D) is an autoimmune disease, the clinical onset of which most frequently presents in children and adolescents who are genetically predisposed. T1D is characterized by specific insulin-producing beta cell destruction. The well-differentiated and specialized islet beta cells seem to physiologically retain the ability to compensate for the cells lost by reproducing themselves, whereas undifferentiated cell sources may help in generating new ones, even while the autoimmune process takes place. Diabetes clinical onset, i.e., establishment of a detectable, chronic hyperglycemia, occurs at a critical stage when autoimmunity, having acted for a while, supersedes the regenerative effort and reduces the number of beta cells below the physiologic threshold at which the produced insulin becomes insufficient for the body's needs. Clinical solutions aimed at avoiding cumbersome daily insulin administrations by the reestablishment of physiologic insulin production, like whole pancreas or pancreatic islet allotransplantation, are limited by the scarcity of pancreas donors and by the toxic effects of the immunosuppressive drugs administered to prevent rejection. However, new accumulating evidence suggests that, once autoimmunity is abrogated, the endocrine pancreas properties may be sufficient to allow the physiological regenerative process to restore endogenous insulin production, even after the disease has become clinically manifest. Knowledge of these properties of the endocrine pancreas suggests the testing of reliable and clinically translatable protocols for obliterating autoimmunity, thus allowing the regeneration of the patient's own endocrine cells. The safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

KEY WORDS: adult stem cells; autoimmunity; beta cell regeneration; islet transplantation; type 1 diabetes.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which autoreactive T cells specifically target and destroy the insulin-producing beta cells of the endocrine pancreas. While the beta cells are selectively destroyed, other nonbeta cells, contained in the islets of Langerhans or in the exocrine pancreas, are left more or less intact (1). In the early 1920s, prior to the discovery of insulin, T1D was almost invariably a fatal disease. With the discovery of insulin, the subcutaneous administration of animal-extracted and subsequently human recombinant insulin became the praxis. Since that time, however, studies have shown that only strict control of glycemic

levels over the years can significantly reduce—but not completely revert—the incidence of diabetic complications (2–6). As a result, T1D still contributes to the high rate of cardiovascular, microvascular, neuropathic, and retinopathic diseases experienced by our population (7).

Despite marked progress achieved with structural modification, better formulation, and improved mode of administration of insulin, which offer more precise management of glucohomeostasis, diabetics must monitor their blood glucose levels several times a day to determine the appropriate quantity of insulin that needs to be injected. Strict glycemic control entails a sustained effort that a patient must make over many decades, frequently beginning in childhood. Uncontrollable hyperglycemia and/or the peril of hypoglycemia—both potentially life-threatening conditions—impose severe limitations on lifestyle, as well as health care of patients. Taking into consideration the many variables that may affect glucose regulation in an individual, like hormonal changes, quantity and composition of food intake, different basal metabolism, and even psychological stress, good metabolic control is difficult to achieve even by diligent patients. Thus, insulin replacement therapy alone does not completely protect these individuals from severe consequences,

¹ Division of Immunogenetics, Department of Pediatrics, University of Pittsburgh School of Medicine, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania, USA.

² Department of Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, USA.

³ Rangos Research Center, Children's Hospital of Pittsburgh, 3460 5th Ave, Pittsburgh, Pennsylvania 15213-3205, USA.

⁴ To whom correspondence should be addressed. (e-mail: mnt@pitt.edu)

suggesting that more appropriate treatments are needed to get closer to a cure for T1D (8).

Transplantation of the whole pancreas, usually coupled with kidney transplantation, has been considered as one possible therapeutic option. It requires, however, major surgery and, as with any other cell or solid organ allotransplantation, lifelong suppression of the immune system of the recipient through administration of immunosuppressive drugs at doses that are frequently associated with toxic effects. This approach has been used almost exclusively in patients with complicated diabetes (9). In the case of simultaneous pancreas and kidney transplantation, it has been reported that recipients experience benefits in terms of life expectancy (10). Pancreas transplantation alone is proposed for a more limited cohort of diabetic patients: adults with frequent unpredictable hypoglycemic events and overall difficult glycemic control. However, whole pancreas transplantation is not considered as a treatment for diabetic children because of the severe secondary effects of immunosuppression and a low survival rate when compared with the survival of waiting-list patients receiving conventional insulin therapy, mainly associated with surgical complications and infections (11–16).

In contrast, improved protocols for the transplantation of pancreatic islets have provided new hope for the treatment of T1D (15–17). Islet cells are the defective cell population that diabetic individuals need; their transplantation overcomes the need for management of the acinar tissue of the pancreas and the consequent exocrine secretion typical of whole pancreas implants, which is often the main cause of complications (17). Islet transplantation can be carried out under local anesthesia using a relatively simple and low-risk procedure that a larger number of potential recipients can tolerate safely. In light of the proven feasibility of islet transplantation in animals and autotransplantations to prevent diabetes in patients in whom the pancreas had to be surgically removed, approximately 750 patients with T1D received allogeneic islet transplants between 1974 and 2000 (15).

The main reason for such a limited number was the rather poor outcome of this intervention until 5 years ago when the Edmonton group reinvigorated this field by reporting their experience treating seven patients consecutively who became insulin-independent (18). Insulin independence was the result of a successful approach that involved the use of a larger islet mass (obtained combining two to three donor islet transplantations), the employment of freshly isolated islets (using media devoid of xenogeneic proteins and processed shortly after organ harvesting), and a new steroid-free immunosuppressive “cocktail” (18). Other transplant centers around the world have now repeated this exciting observation and have obtained initial success rates of as high as 50–80% in terms of providing insulin independence during the first year (19). However, the initial enthusiasm has been tempered by follow-up studies in which a gradual loss of islet function with time has been observed (20); the percentage of insulin-free patients decreased to less than 10% after 5 years (21). The reasons for immediate or late failure are not completely clear (22–24). The procedure of isolation *per se* contributes to impair the quality of the islets and may constitute the basis for the major problems encountered after grafting in the liver of the diabetic recipient (25–29).

The endocrine pancreas represents approximately 1–2% of the entire pancreatic mass and constitutes a pellet of tissue of no more than 2–5 ml. Such an islet-enriched cell suspension can be implanted via intraportal injection into the recipient's liver, where transplanted islets lodge in the hepatic capillary sinusoids where they are abundantly exposed to portal blood. In light of long-term islet survival in animal studies, the liver has been favored as the best site for islet engraftment in clinical trials. However, the liver site is not ideal for the islets and presents important disadvantages. A thrombotic/inflammatory reaction is elicited when islets come into direct contact with the recipient's blood. The detrimental effects of this instant blood-mediated inflammatory reaction, observed in particular when the islets are transplanted intraportally, seem to provide an additional explanation for the relatively low success rate of clinical islet transplantation (30). Also, steatosis of the hepatocytes surrounding the islet graft has been documented relatively soon after transplantation (23,31).

Finally, the immunosuppressive drugs necessary to avoid recurrence of autoimmunity and allorejection are quite toxic, not only to the kidney of the recipient but also to the transplanted beta cells themselves that eventually die demanding additional transplantations (32–34). The number of available donor organs will continue to limit the number of diabetic patients who can be treated even in the event that transplantation-based approaches, coupled with clinically more acceptable immunosuppressive protocols, prove superior in the reestablishment of long-term euglycemia, reduced incidence of T1D complications, as well as overall improved patient health (7,35).

Vigorous research is being performed to improve this situation. Islet allotransplantation has recently been achieved from single deceased obese donors in all eight T1D recipients (36) and from a single living donor where islets, obtained by distal pancreatectomy, were donated from a mother to her diabetic daughter (37). However, diabetes in this latter case was the iatrogenic result of treatment for chronic pancreatitis; it is well known that islet autotransplantation following total pancreatectomy to treat chronic pancreatitis frequently results in long-term prevention of diabetes, persisting for more than 13 years of posttransplantation (38). Longitudinal studies performed over the next several years will indicate the success of these procedures and whether they meet the long-term metabolic needs of the transplanted individuals.

These results illustrate the urgent need for exploration of additional avenues to realize the goal of curing T1D. For example, regeneration of endocrine pancreas function has been documented after partial pancreatectomy, and in streptozotocin (STZ)-treated animal models, including mice (39) and rats (40–42), and there are sporadic reports involving spontaneous remission in T1D patients (15,43), as well as evidence for islet neogenesis in nondiabetic obese adult individuals (44). The potential of the pancreas to heal itself seems to be more efficient once autoimmunity is controlled (45). Recent research efforts involving adult stem cells and gene therapy continue to show great potential in animal models. Combining these independent efforts into a unified approach for treating T1D is the challenge awaiting us in our effort to cure this chronic disease.

TYPE 1 DIABETES IS AN AUTOIMMUNE DISEASE

In 1993, the paper of Lampeter *et al.* (46) reported the first unquestionable evidence that also in humans, T1D can be transferred by bone marrow (BM) cells. T1D was observed in a woman, aged 29, 4 years after transplantation of BM from her HLA-identical brother with T1D. At diagnosis of diabetes, the recipient was positive for high-titer islet cell antibodies (ICA), whereas she had been ICA-negative before transplantation. Chromosomal analyses verified that all circulating leukocytes were of male donor type. This further confirmed the autoimmune nature of the disease fulfilling the first requirement proposed by Bach (1) to reach this conclusion. The other three criteria are as follows: (1) the disease course can be slowed or prevented by immunosuppressive therapy; (2) the disease is associated with manifestations of humoral or cell-mediated autoimmunity directed against the target organ; and (3) the disease can be experimentally induced by sensitization against an autoantigen present in the target organ. All these characteristics defining an autoimmune disease find their origin in abnormalities of the physiologic process that brings the T cells to maturation.

In a healthy individual, the maturation of the T cells, coming from cell precursors present in the BM, takes place in the thymus, where they undergo a positive and a negative selection. Both positive and negative selections depend on interactions between the T-cell receptor (TCR), major histocompatibility complex (MHC) molecule, and antigenic peptide. *Positive selection* occurs as thymic stromal cells bearing MHC molecules, containing self-peptide fragments, engage TCR molecules on the developing thymocytes and direct their continued maturation into functionally mature T cells. T cells with “useless” receptors (i.e., those that cannot bind with sufficient affinity the MHC molecule) are not driven to mature and expand, and these cells eventually die in the thymus. *Negative selection* refers to the set of events that specifically eliminate or alternatively “anergize” potentially autoreactive cells, thereby inducing “tolerance” to self (i.e., self-tolerance). During negative selection, factors such as affinity for self-antigen and antigen load likely influence the final outcome of cell death or clonal anergy. Thus, the peripheral T-cell repertoire of each person (including identical twins) is unique and is a consequence of both the random generation of TCRs in the initial unselected thymocyte pool as well as of positive and negative selection events.

More specifically, peptides from antigens of self-tissues are presented to the various immature double-positive $CD4^+$ and $CD8^+$ T cells entering into the thymus (Fig. 1). T cells that preferentially bind to MHC class II molecules mature into $CD4^+$ cells. The involvement of $CD4^+$ T cells is unquestionably proven to be of primary importance in the etiology of the disease. Class II molecules are heterodimers composed of an alpha and a beta chain that together form the molecule's antigen-combining site. When the TCR has a very low affinity for the MHC molecule/self-peptide complex (in the cartoon presented in Fig. 1, contours of the MHC molecule/self-peptide complex do not fit with the contours of the TCR molecule), the developing T cell does not receive the necessary positive signal to survive and exit the thymus for release into the periphery. However, if the affinity

between the MHC molecule/self-peptide complex and the TCR is too high (in the cartoon presented in Fig. 1, the contours of the MHC molecule/self-peptide complex fit precisely into the contours of the TCR molecule), the T cell undergoes negative selection and dies inside the thymus. In contrast, the T cell that receives a positive survival signal because of the high-affinity interactions between its TCR and the MHC molecule, but shows an affinity that is not further enhanced by the presence of a self-peptide in its groove, so that the negative selection does not take place, matures and enters the circulation to protect the body from foreign (nonself) invaders, with which it is able to efficiently interact.

The immunological basis of T1D can be found in T cells that bind to an MHC molecule unable to properly present self-peptides. These T cells, then, even if potentially autoreactive, are not subjected to negative selection and are free

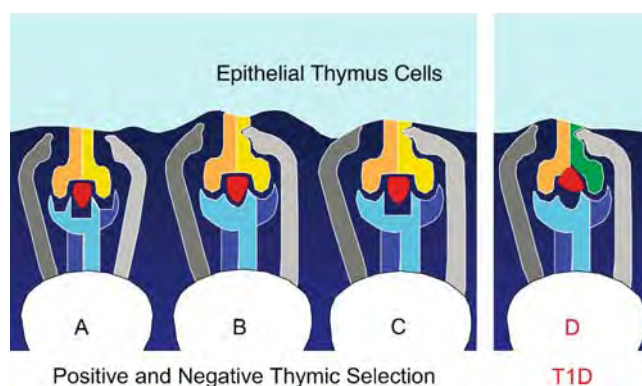


Fig. 1. Theoretical basis of Tian *et al.*'s (62) approach for abrogation of autoimmunity in the nonobese diabetic (NOD) mouse. In the thymus, peptides (in red) from self-antigens are presented to the various (A, B, and C) immature double-positive [$CD8$ (in gray) and $CD4$ (lighter gray)] T cells via the major histocompatibility complex (MHC) molecule. When, as for the A cell, the T-cell receptor (TCR) has a very low affinity for the MHC molecule/self-peptide complex, the developing T cell does not receive the necessary positive signal to survive. If the affinity between the MHC molecule/self-peptide complex and the TCR is too high, as for the B cell, the T cell undergoes negative selection and dies inside the thymus. In contrast, the T cell shown in C receives a positive survival signal because of the high-affinity interactions between its TCR and the MHC molecule, an affinity, however, that is not further enhanced by the presence of a self-peptide in its groove, so that the negative selection does not take place. This T cell matures and enters the circulation to protect the body from foreign (nonself) invaders, with which it is able to efficiently interact. In type 1 diabetes (T1D), the D cell binds to an MHC molecule conferring susceptibility to diabetes. The self-peptide is not presented properly. The T cell, then, even if potentially autoreactive (D has the same TCR as B), is not subjected to negative selection and is free to leave the thymus to circulate in the blood. The approach taken by Tian *et al.* (62) can be illustrated by imagining that the I-Ag⁷ molecule, able to confer susceptibility to the disease carrying a non-Asp-57 beta chain (in green), is supplemented, in the hematopoietic cells of the NOD mouse, with a nondiabetogenic MHC molecule, i.e., an Asp-57-positive beta chain (in yellow), like the one interacting with A, B, or C. Once the cells are returned into the donor, the new MHC molecule (orange and yellow chains) allows the restoration of an efficient negative selection in the thymus (as for B), sufficient to delete autoreactive T cells and consequently to prevent diabetes (108).

to leave the thymus to circulate in the blood. T cells that are potentially reactive to self-antigens, but fail to be deleted inside the thymus, are able to attack tissues of the body expressing these same antigens, generating autoimmunity.

In the late 1980s, in collaboration with Dr. McDevitt, Stanford University, we were able to map and identify the most influential single hereditary susceptibility factor in T1D: a single amino acid of the beta chain of a class II HLA-DQ histocompatibility molecule (47,48). Although T1D is recognized to be a multigenic disease (49), in humans, the principal genetic susceptibility component was proposed to be any allelic form of the HLA-DQ molecule that lacks a charged amino acid at position 57 of its beta chain. Conversely, resistance to disease is associated with the inheritance of HLA-DQ alleles containing a charged amino acid such as aspartic acid, at the same position (Asp-57). Physical explanation of the unusual importance of this particular single amino acid location for the development of the autoimmune characteristics of T1D came with the elucidation of the crystal structure of the HLA-DQ8 molecule, a non-Asp-57 molecule, which confers the highest susceptibility to the disease (50). The most important feature of the susceptibility HLA-DQ8 molecule relevant to diabetes immunology is that its crystal structure is identical to the homologous I-Ag⁷ molecule present in the nonobese diabetic (NOD) mouse (51). This strain of mouse spontaneously develops T1D with etiopathogenetic characteristics very similar to the disease in humans. The peptide-binding site of the majority of human HLA-DQ and murine I-A molecules has an Asp-57 that points into the groove. In these allelic forms, Asp-57 forms an electrostatic salt bridge with the arginine in juxtaposition (i.e., in position 76) of the alpha chain of the molecule (Arg-76), which also points into the groove. HLA-DQ8 and I-Ag⁷ lack Asp-57, and this variation disrupts the electrostatic interaction, leaving the Arg-76 free to interact with the aqueous environment and with any peptide able to lodge inside the binding groove of the molecule (52,53). The absence of Asp-57 allows the binding of peptides that may not find appropriate lodging inside other Asp-57⁺ molecule grooves and may jeopardize an efficient presentation by the histocompatibility molecule to T cells because of incorrectly positioned self-peptides. The susceptibility status can be correlated, in immunological terms, with impaired peptide lodging, impaired peptide presentation to T cells with consequent reduction in positive selection of regulatory T cells, or by the impaired negative selection of self-reactive T cells. Indirect evidence supporting these hypotheses derives from transgenic NOD mice that express class II genes other than I-Ag⁷, which do not develop diabetes (54–57), and from the fact that the transplantation of allogeneic BM from strains that do not spontaneously develop diabetes also prevents the occurrence of diabetes in NOD mice (58–61).

Recently, instead of approaching the problem using an alloreactive BM transplant, with all its inherent severe contraindications [e.g., graft-vs.-host disease (GVHD)], Tian *et al.* (62) transfected *ex vivo* the gene encoding a resistant Asp-57⁺ beta chain into the BM cells isolated from the diabetes-prone NOD mouse itself (Fig. 2). T1D was prevented by the presence of a “diabetes-resistant” MHC molecule at the surface of hematopoietic stem cells (HSCs) of genetically

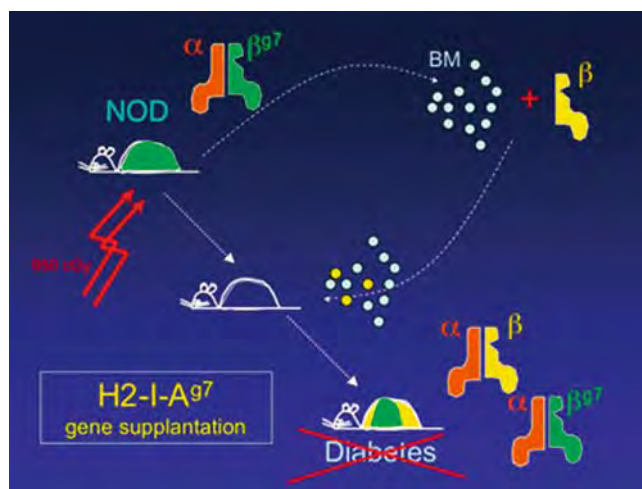


Fig. 2. Schematic representation of Tian *et al.*'s (62) approach for abrogation of autoimmunity in the NOD mouse. NOD bone marrow (BM) stem cells are *ex vivo* transfected with an H-2 I-A beta chain conferring resistance to diabetes (i.e., an Asp-57-positive beta chain, in yellow). The transplanted cells are reinfused in the myeloablated donor that will become a chimera carrying the I-Ag⁷ beta chain (in green) able to confer susceptibility and the resistance beta chain. Because the effect of the resistance molecule is dominant over the other susceptibility molecule, progression to diabetes will be avoided by deleting autoreactive T cells in the repopulated thymus.

susceptible (i.e., carrying a “diabetes-susceptible” allele) NOD mice. The expression of the newly formed diabetes-resistant molecule in the reinfused hematopoietic cells was sufficient to prevent T1D onset in the NOD mouse even in the presence of the native, diabetogenic non-Asp-57, I-Ag⁷ molecule. Mechanistically, the authors suggested a model in which a subset of the engineered BM cells—i.e., hematopoietic precursor cells—migrate, populate the thymus, and become antigen-presenting cells (APCs) involved in the negative selection of thymocytes that would otherwise mature into autoreactive T cells. In fact, diabetes-free NOD mice exhibited neither emergence into the blood stream of T cells capable of responding to putative autoantigens nor the presence of beta-cell-reactive T cells in the pancreatic islets themselves (i.e., no *insulinitis*).

ENDOCRINE PANCREAS REGENERATION PROPERTIES

In both physiologic and pathologic conditions, Lipsett and Finegood (63) attributed the rescue of beta cell mass to increased beta cell replication, increased beta cell size, decreased beta cell death, and the differentiation of possibly existing beta cell progenitors.

In favor of the postulated differentiation of beta cells from progenitor ductal cells is the observation that occasional hormone-positive cells can be found embedded in normal pancreatic ducts (64). The number of these duct-associated endocrine cells increases physiologically as a consequence of severe insulin resistance in obese individuals or during pregnancy (65,66). Similar histological changes are observed under conditions of tissue injury and repair after partial

pancreatectomy, duct ligation, cellophane wrapping of the gland, or IFN- γ overexpression driven by the insulin promoter (67–70). Even then, within the ducts, only a small number of cells become insulin-positive. This suggests that, even if some precursor exists, the process of the formation of endocrine cells in tissues other than islets (i.e., neogenesis) is not a common property of the duct epithelium.

However, alpha and beta cells seem to develop from a possibly common, nonhormone-expressing, yet *Pdx1*-positive, precursor (71). These endocrine progenitors may be located in physically close proximity to the duct but may not actually be components of the ductal epithelium (72). At any rate, these hypothetical precursors are present in extremely small numbers so that lineage analysis becomes very difficult. Considering the lack of known appropriate markers, it becomes even more difficult to quantify their contribution to normal endocrine cell turnover. However, single cell precursors, able to regenerate all kinds of cells present in the islet, have been successfully isolated from both the ducts and the islets themselves (73,74). Thus, the working hypothesis of those who are proposing that pancreatic ductal cells can transiently regain a less differentiated state and then become beta cells seems legitimate (75). Increased metabolic demand and tissue injury seem to be efficient in activating this physiologic process of cellular homeostasis (76).

On this basis, it may also be possible to accommodate the results of Dor *et al.* (77) who proposed instead that new beta cells can arise only from the preexisting beta cells themselves, whether in the normal adult pancreas or after pancreatectomy. As a direct consequence, the number of beta cells should become virtually defined at a certain point in time, and, afterwards, glycemia should be controlled only by that defined cellular pool. The data also argue against the possibility of deriving beta cells from adult stem cells *in vivo*. While the results of Seaberg *et al.* (73) and Suzuki *et al.* (74) do not contest the proven yet limited ability of a beta cell to divide, the failure of Dor *et al.* (77) to observe cells that differentiated from stem or precursor cells might actually be explained by the experiments of Gershengorn *et al.* (78) at the National Institutes of Health that document the possible transition from epithelial-to-mesenchymal (EMT) cells and vice versa. The authors hypothesized that precursor cells could be obtained from insulin-expressing cells that lose their beta-cell identity. After expansion, these cells could potentially be redifferentiated into insulin-expressing beta cells via mesenchymal-to-epithelial transition (MET). Indeed, the authors describe some, although rather limited, success in their *Science* paper.

After several days of culture of human islets in serum-containing medium, adherent cells start to migrate out from the islets and form a monolayer of “fibroblast-like” cells. Gradually, the population of cells down-regulates insulin and other islet-specific protein expression, but increases mesenchymal progenitor cell marker vimentin production. These cells can be passaged more than 30 times and expanded by a factor of $>10^{12}$ *in vitro*. The authors named the cells human islet-derived precursor cells (hIPCs). Interestingly, when serum was taken away from the culture medium, the hIPCs stopped proliferating and formed aggregates of various size. After 2 weeks culture of aggregates under serum-free condition, islet-specific marker expressions, such as insulin and

glucagon, were up-regulated 1000-fold. The authors argued that under the serum-containing culture condition, islet cells undergo EMT and become hIPCs, whereas under the serum-free condition, hIPCs undergo MET and start to differentiate back into islet cells.

If a small number of beta cells can indeed undergo EMT, and dedifferentiate into precursor cells, in Dor *et al.*'s (77) pulse-and-chase labeling system, these cells will still be positively labeled. Whereas Dor *et al.*'s explanation inferred a direct replication of beta cells, Gershengorn *et al.*'s (78) data suggest that beta cells can dedifferentiate into precursor cells, which lose beta-cell-specific marker, while regaining proliferating potential at the same time. Upon proper stimuli, these precursor cells will redifferentiate back into mature beta cells to support islet growth and function.

Further studies are necessary to ultimately define the possible existence and significance of different sources of precursor cells contributing to beta cell regeneration. However, an unconventional type of precursor cell (73,74), possibly located in close proximity and/or inside the endocrine tissue, seems to be present in the pancreas. When metabolic demand increases, these precursors are activated, possibly via various secreted factors that under normal conditions guarantee the cellular homeostasis of the islets of Langerhans.

THE BALANCE BETWEEN AUTOIMMUNITY AND REGENERATIVE ACTIVITY

The physiologic equilibrium between lost and newly generated beta cells can be altered by the action of beta-cell-specific, autoreactive T cells (79). Once the killing activity of activated diabetogenic T-cell clones overcomes the regenerative compensatory activity of the gland, the number of functional beta cells progressively decreases until they become too few to maintain glucohomeostasis in the body. After the clinical onset of the disease, even if the regenerative properties of the pancreas remain functional, the continued presence of autoreactive T cells consistently nullifies the reparative effort. Islet cells transplanted from a healthy monozygotic twin were quickly killed by these same autoreactive T cells present in the body of the genetically identical, diabetic recipient twin (80).

The autoimmune response can be successfully averted in the NOD mouse by the successful induction of mixed allogeneic chimerism. The transplantation of BM from a diabetes-resistant animal into a diabetic recipient following a sublethal dose of total body irradiation (TBI) is sufficient to block and eventually also to reverse the systematic invasion and inflammation of the islets by autoreactive T cells that results in *insulinitis* (58–61).

The allogeneic chimerism induced in prediabetic NOD recipients is multilineage and increases with time: 4 weeks after BM transplantation (BMT), chimerism may reach levels of over 90% (60). In this study, to assess the damage and reparative processes in the pancreata prior to and upon therapeutic intervention, a new morphometric scoring system (called Index *N*) was utilized; this is composed of both the degree of insulinitis, defined by a very detailed scoring system (Fig. 3), and a relative number *A*: the measure of pathology-

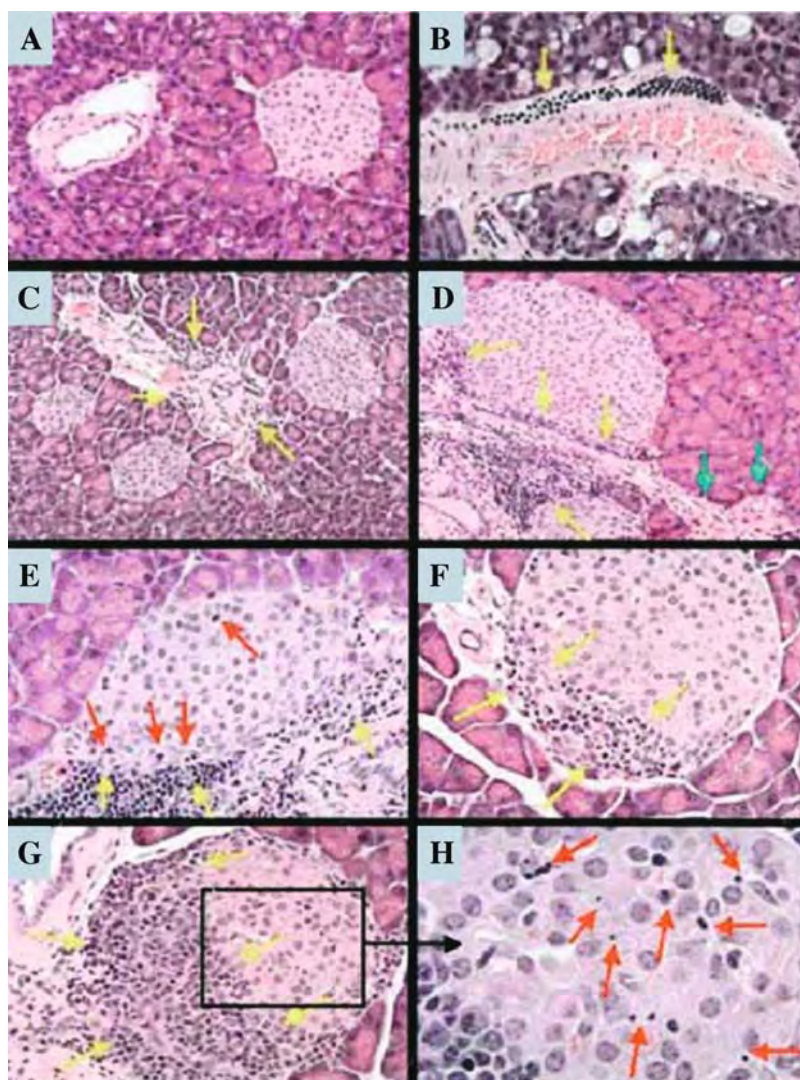


Fig. 3. Scoring of the different stages of destruction of islets of Langerhans during diabetogenesis. Specimens of pancreata from NOD mice of different age were stained with H&E. Magnification for A to G, $\times 400$; for H, $\times 1000$. (A) Score 0: normal pancreatic tissue. Neither morphological abnormalities nor mononuclear cell (MNC) infiltration or retention in the pancreatic vessels are present. (B) Score 1: MNC vascular retention (yellow arrows). No evident pathological features in pancreatic morphology. (C) Score 2: MNC perivascular infiltration (yellow arrows) of the vessels adjacent to the islets; islets maintain a normal morphology. (D) Score 3: MNC infiltration in the periphery of the islets (yellow arrows) and in the perivascular area of the adjacent vessels (compare to intact area distant from the islets, green arrows). (E) Score 4: the insulinitis in the periphery of islets (yellow arrows) is associated with apoptosis (red arrows). (F) Score 5: the infiltration of islets by MNC (yellow arrows) is advanced, but not exceeding one third of islet section. (G) Score 6: more than one third of the endocrine tissue of the islet is infiltrated by the MNC (yellow arrows). This stage of insulinitis is consistently concomitant with extensive apoptosis, presumably of both endocrine and infiltrating cells (H: red arrows) (60).

free area of islet vs. whole pancreatic tissue. The need for this new parameter *A* arose from the observation that in diabetic NOD mice rendered hematopoietic chimeras, a new morphological state of the endocrine pancreas can be recognized. The insulinitis-free state obtained by the abrogation of the autoimmune process must be distinguished from the normal, physiologic condition of the pancreas. The islets in the diabetic chimeric NOD mice, although cleared from insulinitis,

are significantly reduced in size, display an altered morphology, and contain cells, none of which has insulin content. In unmanipulated control NOD mice, Index *N* (insulinitis score/*A*) increased in less than 25 weeks from 0.01 (characteristic of physiological condition) to 0.1 (reflecting the hyperglycemic condition in overtly diabetic mice); at this point, the animal dies. In contrast, the chimeric NOD mice were followed to 32 weeks of age and did not become diabetic (Fig. 4). Fourteen

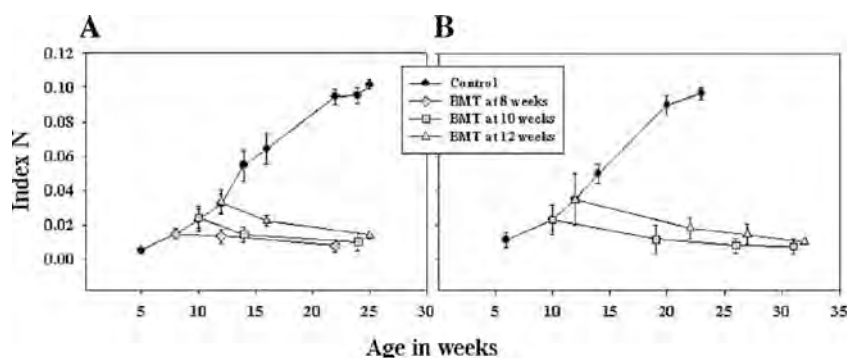


Fig. 4. Chimerism abrogates and reverses destruction of islets of Langerhans in NOD mice prior to the clinical onset of diabetes. NOD mice (8–12 weeks of age) were rendered hematopoietic chimeras by the administration of T-cell-depleted allogeneic BM into recipients conditioned by lethal (A) and nonlethal (B) doses of TBI. Pancreata of these chimeras were evaluated for the degree of endocrine pancreas destruction and graded according to Index N . Gray diamonds, squares, and triangles reflect the kinetics of Index N in mice rendered chimeric at 8, 10, and 12 weeks, respectively. Black circles show progression of islet destruction with age evaluated in unmanipulated control NOD mice. This curve was not extended further because untreated animals did not survive long after reaching an Index N over 0.1 (61).

weeks after BMT, arrest of the destructive processes and total normalization of Index N were observed in all chimeras subjected to nonlethal doses of TBI. Once normalized, Index N remained at a plateau for 14 weeks (length of observation), confirming that normalization of the structure and function of the insulin-secreting tissue in the endogenous pancreas of

chimeric NOD mice was stable and long-lasting (61). To prove that the insulin-producing tissue of the endogenous pancreas can undergo a reparative process, direct detection of proliferating [i.e., bromodeoxyuridine (BrdU)-positive] cells can be performed. In the endogenous endocrine pancreas, and in islet allografts of diabetic experimental mice, some

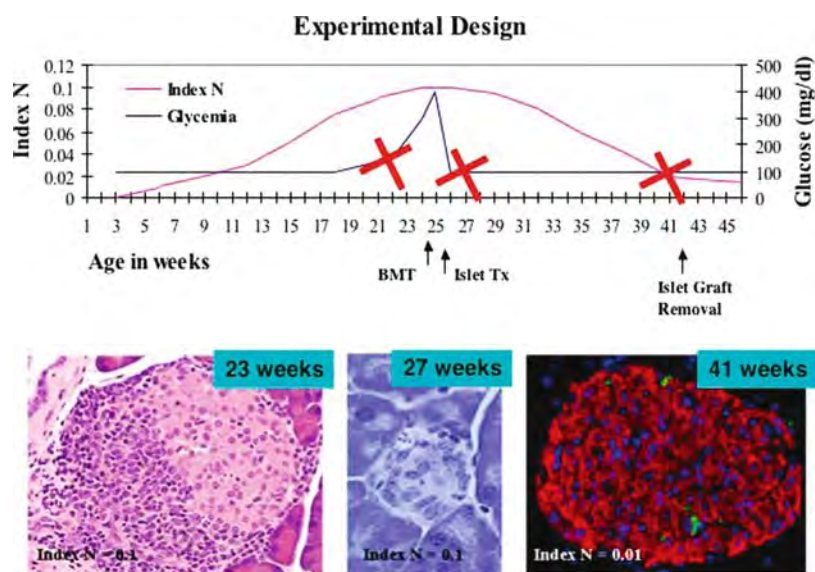
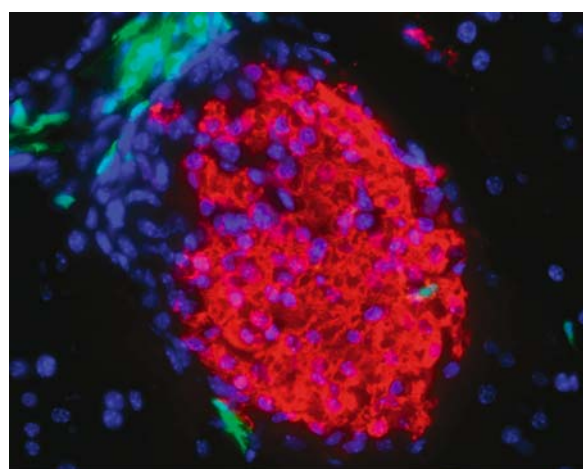
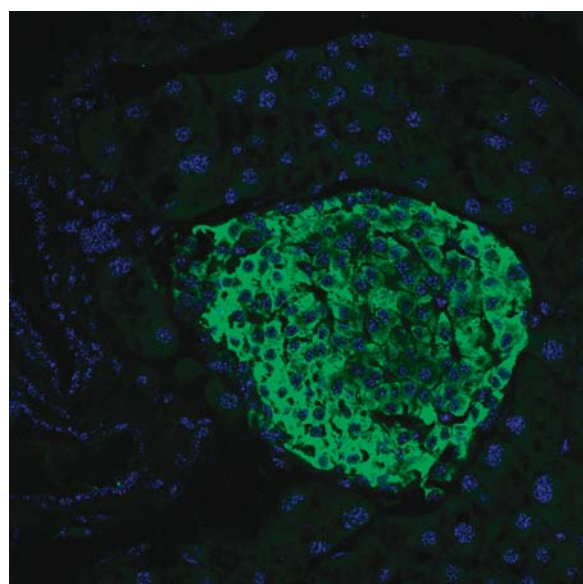
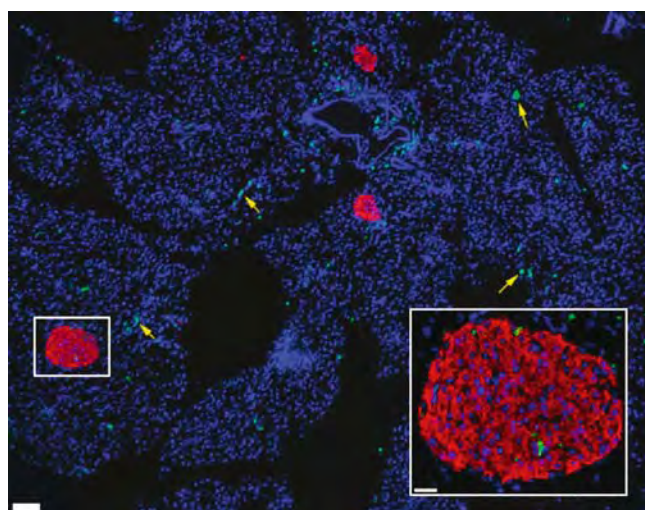


Fig. 5. Schematic representation of the protocol used to test regeneration (or rescue) of the beta cell in diabetic NOD mice. In NOD mice, the infiltration of autoreactive T cells into the islets of Langerhans (resulting in *insulinitis*) begins at around 4 weeks of age. At 20–23 weeks, ~85% of female mice are diabetic; that is, their glycemia is >300 mg/dl. When successfully transplanted with bone marrow (BMT) from a nondiabetes-prone donor and hematopoietic chimerism is established, the NOD mouse no longer shows signs of autoimmune activity. However, whereas there is no more evidence of insulinitis in the endogenous pancreas, there is also no sign of insulin production (no red staining). Three to four months after BMT, new insulin-positive cells (shown in red) are present throughout the endogenous pancreas. Thus, when the islets transplanted under the kidney capsule (to maintain euglycemia until regeneration takes place) are removed by nephrectomy, the mice remain nondiabetic (61). For “Index N ” morphometric scoring system, see (60).



proliferating (i.e., BrdU-positive) cells were also positively stained for insulin, revealing the regenerative capacity of the tissue (61).

Normalization of the endocrine pancreas observed in prediabetic NOD mice could also be achieved in these same

animals after the onset of the overt disease (61). Spontaneously diabetic NOD mice were rendered hematopoietic chimeras by transplanting them with BM from B6-green fluorescent protein (GFP) mice (81). The rationale for the use of GFP-positive BM cells was to track the fate of donor-derived HSCs and to elucidate their possible role in the restoration of the recipient endocrine pancreas. The NOD mice received B6-GFP BM cells along with islet grafts to allow their survival during the time required to reestablish an endogenous insulin production (Fig. 5). These animals became euglycemic within 24 h following transplantation and remained so for the period of observation. After surgical removal of islet-graft-bearing kidneys, performed 17–26 weeks after islet transplantation, the mice remained euglycemic. Direct assessment of the insulin content in the islets from the endogenous pancreases that were harvested from euthanized animals 18 days following nephrectomy revealed insulin-positive beta cells in quantities and morphologies similar to those of the normal mouse pancreas (Fig. 6a). Donor-derived GFP-positive cells were detected in the pancreas, but these cells were considered transient-circulating, mature blood cells or HSCs not directly involved with the restoration of the endocrine pancreas because insulin-positive cells were not GFP-positive too (Fig. 6b) (45,61). It was actually calculated that, in the cured recipient, insulin-producing cells (that were genetically marked to indicate that they are of donor origin) were extremely rare, occurring in 2 out of 100,000 beta cells. These cells may actually be the result of sporadic cell fusion processes (82).

A subject of ongoing debate is whether either or both the transplanted BM and the cotransplanted beta cells are necessary for promoting an efficient regenerative process, independent of their ability to block autoimmunity or preserve euglycemia, respectively. They may, for example, secrete factors that are useful to sustain efficient regeneration. Recent results from the groups of Bignon-Laubert, Baeyens, and Suarez-Pinzon are particularly germane to this issue. In the first case, the ability of *PAX4* to favor regeneration of the endocrine pancreas was proven (83). The combinations of epidermal growth factor (EGF) and leukemia inhibitory factor (LIF) (84), or EGF and gastrin (85), were able to convert *in vitro* exocrine or ductal pancreatic cells, respectively, into insulin-producing cells. Additional factors with a possibly useful activity seem to be those used

Fig. 6. Regeneration (or rescue) of the endogenous pancreas in a diabetic NOD mouse after obliteration of the autoimmune process via allogeneic BMT. (a) The regenerated endocrine tissue of a chimeric NOD mouse becomes evident after ~4 months from the BM transplant and takes the shape of cell agglomerates that resemble but are not identical to islets of a nondiabetic animal. Insulin is in red (61). (b) Comparison between an islet of Langerhans of a nondiabetic B6 mouse (A) with insulin stained green and a newly formed insulin producing cell agglomerate (in red) in the pancreas of a diabetic NOD mouse treated with BM cells from a nondiabetes-prone, B6-GFP-transgenic donor (B). It is possible to observe that the latter does not have the well-organized cell structure of a normal islet, and that the majority of the transplanted BM cells (in green) do not directly participate in the regeneration of the endogenous pancreas: there are no double-positive (orange) cells in the newly formed islets. The donor cells appear to be located close to possibly existing juxta-ductal precursor cells, which may be activated by BM cell-secreted factors (45).

to increase the islet cell mass in transgenic mice or in gene-therapy-treated human islets (86–89). Also, the use of insulin-like growth factor-1 (IGF-1) seems to be useful to promote and/or accelerate islet cell regeneration (90,91), as seems to be the case for glucagon-like peptide 1 (GLP-1), as described by Farilla *et al.* (92).

In the rat, experimental evidence supports the notion that precursor cells in both endocrine and exocrine tissue are not susceptible to damage by STZ; that is, they are not Glut-2-positive. STZ, like alloxan, uses Glut-2 as the receptor to get into the target cells that it eventually kills (40,93,94). Also, even in neonatal STZ-treated rats, a combination of activin A and betacellulin, for example, promoted regeneration of pancreatic beta cells and improved glucose metabolism (41).

ENDOCRINE PANCREAS REGENERATION IN NONHUMAN PRIMATES

As previously anticipated, in the year 2000, the clinical possibility of transplanting islets into the livers of diabetic patients was documented; rejection was avoided, thanks to an immunosuppressive regimen that reduced the use of tacrolimus and sirolimus, removed the use of steroids, and instead used daclizumab, an antibody against the interleukin-2 receptor molecule (18). However, as previously discussed, the Edmonton protocol soon showed its limits. The first limiting factor was the immunosuppressive protocol, which was associated with side effects and allowed this type of transplantation in certain adult recipients only (95). The second limiting factor was the need for more than one islet donor for each recipient.

To respond to the latter limiting aspect of the Edmonton protocol, some groups looked at a theoretically unlimited source of transplantable islets. An unlimited source of islets can be found in animals able to produce insulin very similar to human insulin and in quantities that may satisfy the insulin requirements of an individual of an average body weight. Based on these two parameters, the pig seemed to be the animal of choice. There is only one amino acid difference between human and pig insulin, and the pig is large enough to supply large amounts of donor islets. Pig insulin was successfully used to treat diabetic children for years before recombinant human insulin became available. Also, evidence that pig islets can be used for human transplantation was provided by studies conducted, in particular, in the 1990s in

Sweden (96). This possibility was not further explored when it became clear that the alpha1,3 galactose (alpha1,3Gal) epitopes present on pig tissues were the targets of antibodies, normally found in human serum, that are able to quickly reject xenotransplants. This rapid, deleterious reaction is known as “hyperacute rejection” (HAR). HAR is the major cause of tissue destruction within a few hours after xenotransplantation. The best way to obviate HAR was to work toward the generation of pigs genetically deprived of the activity of the enzyme alpha1,3 galactosyltransferase (alpha1,3GT) and, consequently, free of alpha1,3Gal epitopes at their cell surface (97). In the spring of 2003 (98), our effort of many years (99,100) to generate alpha1,3GT double knockout (DKO) pigs was successfully completed. DKO pigs are better suited as donors for xenotransplantation than their wild-type counterparts because, once their tissues are transplanted into humans or Old World monkeys, they are not targets for a HAR. Adult islet cells from wild-type animals express only low levels of alpha1,3Gal epitopes (101). However, other cells contaminating each preparation used for transplantation do express alpha1,3Gal epitopes at high levels.

Experiments in chemically diabetic (i.e., STZ-treated) monkeys indicated that pig islets can substitute for endogenous islets, producing enough insulin (monitored by pig C-peptide) to control the recipient animal glycemia (102,103). More pertinent to this discussion, however, is the observation that, using a noncalcineurin inhibitor-based immunosuppressive protocol, it has been observed that the monkeys' own pancreatic endocrine tissue is able to regenerate within a period of time similar to that determined for the diabetic mouse. Preliminary studies show that all the insulin-positive and Glut-2-positive cells disappear in the pancreata of monkeys treated with STZ, but insulin-positive and Glut-2-positive cells reappear after 3–4 months of treatment. After STZ treatment, the endocrine pancreas of the monkey was no longer able to produce sufficient quantities of insulin to satisfy the need of the animal, which consequently became diabetic. Monkey C-peptide levels remained <0.5 ng/ml for the entire duration of all experiments in which conventional immunosuppressive cocktails were used, and the arginine stimulation test was always blunted when performed during follow-up. Regenerative properties may have been overpowered by the effects of the diabetogenic calcineurin inhibitors administered to the monkey. Also, regeneration did not spontaneously take place, at

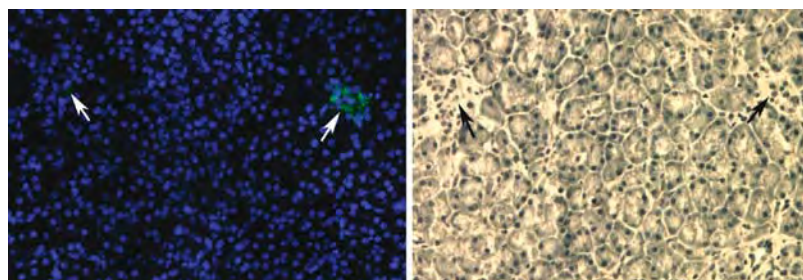


Fig. 7. Newly formed insulin-producing cells in the diabetic monkey. After 3–4 months from STZ injection and diabetes induction, insulin-producing cells are appearing in the monkey endogenous pancreas, eventually forming islet-like conglomerates of insulin-positive cells indicated by the arrows. Immunofluorescence on the left (insulin in green) and H&E on the right, of two consecutive tissue sections (magnification 20 $\times$).

least at a detectable pace, because STZ-treated nontransplanted monkeys continued to need insulin injections after the induction of diabetes. In contrast, in the absence of diabetogenic immunosuppressive agents, using instead an anti-CD154 monoclonal antibody to block the recipient's immune rejection (104), the monkeys transplanted with DKO pig islets not only produced pig C-peptide but eventually (more than 3 months after STZ treatment) recovered the ability to produce monkey C-peptide. New insulin-producing cells are appearing with time in the monkey's endogenous pancreas, eventually forming islet-like conglomerates of cells (Fig. 7; Bottino *et al.*, unpublished observation).

If regeneration can occur not only in rodents but also in the monkeys, we can also expect the endocrine tissue to regenerate in humans once autoimmunity has properly and successfully been abrogated. There is some evidence that supports this expectation.

ENDOCRINE PANCREAS REGENERATION IN HUMANS

A group from Ulm in Germany recently reported the case of a 13-year-old Caucasian boy who, after conventional onset of T1D (i.e., the boy presented with a history of polyuria, polydipsia, weight loss, and serum glucose up to ~500 mg/dl, glucosuria and ketonuria), needed lower and lower insulin doses over time, allowing his physician to completely discontinue insulin therapy after 11 months (43). The authors also reported that, "Without further treatment, HbA_{1c}, and fasting glucose levels remained normal throughout the entire follow up of currently 4.5 years," and that serum autoantibodies to GAD65, IA-2, insulin, and ICA "were initially positive but showed a progressive decline or loss during follow-up." A similar case was recently reported by Rother and Harlan (15).

The main message we draw from all these reports is that within the endocrine pancreas, once the insult of autoimmunity is abrogated, the physiologic process of regeneration can continue efficiently, eventually replenishing the population of insulin-producing cells to a number sufficient to maintain euglycemia, thus curing the diabetic patient. While this process takes place, the recipient's glycemia must be controlled by additional, independent measures. In rodents, the most commonly used technique has been to transplant into the recipient islets from the same BM donor. However, the successful engraftment of the transplanted BM (necessary to abrogate the autoimmunity) and/or islets (necessary to maintain euglycemia) would have to be promoted and maintained without the use of calcineurin inhibitors that will eventually not only kill the autoreactive T cells of the recipient but also limit beta cell neogenesis, thereby undermining the success of the transplant (32–34). The use of these diabetogenic immunosuppressive agents may also interfere with the observed rise of regulatory T cells, a possible explanation for the long-lasting immunoregulatory cell-dominant condition observed in cured animals (105). Adoptive transfer experiments in which both diabetogenic lymphocytes from diabetic NOD mice and splenocytes from treated, long-term diabetes-free NOD mice were transplanted into NOD-*sic* recipients, with no signs of induction of diabetes, support this hypothesis (106,107).

On these bases, it seems that not only in animals, but in humans as well, the abrogation of autoimmunity could allow the physiologic regeneration of insulin-producing beta cells in the host endocrine pancreas, even after the onset of the disease, if a nondiabetogenic immunosuppressive protocol is implemented. These are the premises on which reliable and more clinically translatable alternatives than allogeneic BMT or allogeneic or xenogeneic pancreatic islet transplantations should be found to cure our young diabetic patients.

READJUSTING THE EFFICIENCY OF THE FIRST T CELL SIGNAL

On this basis, it seemed useful to extend the previously described experimental protocol proposed by the Harvard group to implement a new one that is more transferable to clinical trials (108): if Tian *et al.*'s (62) approach to abrogate autoimmunity can facilitate a possible recovery of autologous insulin-producing cells also in the diabetic individual, safe induction of an autoimmunity-free status might become a new promising therapy for T1D. The working hypothesis to be tested considers the use of BM-enriched hematopoietic precursor cells, instead of the nonfractionated BM cell population used by Tian *et al.*, as the recipients of the MHC class II beta chain gene that confers resistance to the disease, to abrogate autoimmunity. Enriched precursors will be more successfully transfected and more easily accepted by the recipient than the total BM cells. Also, differing from Tian *et al.*'s approach, overtly diabetic (rather than prediabetic) individuals would be treated by the reinfusion of transfected BM-enriched precursors. Autoimmunity will be efficiently abrogated if the enriched precursors are able to generate the right derivative cells and in sufficient numbers to efficiently repopulate the thymus, by negatively selecting possibly autoreactive T cell clones and promoting peripheral tolerance mediated by T regulatory cells. In the absence of both autoimmunity and diabetogenic immunosuppressive protocols, by adopting alternative means to correct hyperglycemia, the regenerative property of the autologous endocrine pancreas should repopulate the gland with enough insulin-producing cells to restore euglycemia. Also, to avoid the use of radiation to eliminate the activated T-cell clones present in the diabetic patient, as in Tian *et al.* (62), an antibody-based preconditioning may be used instead. Finally, it should be determined how long after its onset the disease reversal remains possible and the measures to be considered in case this reversal property becomes less efficient with time.

First, all these steps have to be successfully tested in the NOD mouse, as an animal model in which diabetes spontaneously develops as a direct consequence of an autoimmune process, very similar to the one we observe in humans. Then it would be useful to try to reproduce the most promising results in nonhuman primates. Even if nonhuman primates do not spontaneously develop autoimmunity, and consequently do not spontaneously develop T1D, this model can be used for testing the safety of the proposed protocol. The concept that a physiologic regenerative capacity may be present in humans will obtain much more support once it has been demonstrated in a closely related species, such as the monkey.

The proposition of considering the use of this same approach for a possible clinical trial may be complicated by

the presence of more than one susceptibility molecule in humans, i.e., not only the HLA-DQ molecule like in the NOD mouse but also the HLA-DR. However, the efficiency of Tian *et al.*'s gene-based treatment, even in the presence of the native, diabetogenic molecule, may offer solutions also to the problem of dealing with more than one susceptibility molecule (108,109). It is expected that the protective allele will act in an epistatic or dominant manner over the susceptibility allele, also in the case of the DR molecule (109). Thus, to cover all the bases, both a new DQ and a new DR beta chain should be cotransfected into the precursor cells.

For preventing diabetes progression in prediabetic NOD animals by transfected BM precursor cell reconstruction, a combination of AutoMACs with Flow Sorter approaches was used to isolate Sca1-positive, c-Kit-positive, and Lin-negative BM cells for transplantation into myeloablated recipients. The isolation of BM was performed on 8-week-old F1 NOD/NOD H2^b congenic males. F1 donors were chosen to mimic a syngeneic transplant yet with the possibility of recognizing donor from recipient cells in the reconstituted animal. All NOD female recipients remained alive after sublethal radioactive conditioning and showed an evident chimerism in the blood 2 weeks after receiving enriched BM precursor cells (Fan, unpublished observation).

These preliminary experiments were performed using retroviruses carrying only the GFP gene or the GFP gene plus the gene of the I-A beta chain conferring resistance to the disease. However, in light of a possible clinical trial, the use of a retrovirus for performing a successful resistance beta chain transfection, as proposed by Tian *et al.* (62), should be avoided because it is associated with the problem of its preferential insertion in positions of the recipient's cell genome that may facilitate the activation of oncogenes, a problem already sadly encountered in human gene therapy treatments (110). It would be safer to utilize phage integrases (111) to guide the stable and irreversible insertion of DNA at specific locations within the genome to satisfy the need for a safe, yet everlasting, synthesis of the beta chain conferring resistance, even in the offspring of the successfully transfected BM precursor cells.

Some influencing factors of retrovirus activity, besides the preferential position of the insertion in the genome, include the presence of regulatory and bacterial elements in the insertion construct itself and the number of integrated constructs. Efficient, targeted, single-copy integrations would be helpful for the improvement of transgene efficiency. Phage integrases catalyze site-specific, unidirectional recombination between two short *att* recognition sites. Recombination results in integration when the *att* sites are present on two different DNA molecules and in deletion or inversion when the *att* sites are on the same molecule.

By using the integrases, all the insertion sites can be recognized because of their limited number. It is hoped that they will be found in positions that do not alter the activation of any important gene. In the case that possibly dangerous locations are recognized—a case that, statistically, seems to be quite remote—these insertion sites could be obliterated molecularly before transfecting the “therapeutic” genes. This is theoretically possible by culturing *in vitro* for a few divisions the HSC transfected with the corrective recombinant DNA associated with an antibiotic-resistance gene to

select only the “treated” ones. The hope here is that they will remain able to properly repopulate the recipient's BM and thymus, and that they will not result in any additional non-recognized dangerous insertion. However, in practical terms, it can only be concluded that the risk imposed by the use of this new system will dramatically reduce, but not completely exclude, the problems associated with the use of retroviruses.

Irradiation was used in the original experiments of Tian *et al.* (62) for removing activated T cells from the recipient. However, it would be worthwhile to systematically substitute for irradiation different, antibody-based, immunoreductive conditioning protocols. Monoclonal antibodies can be tested as an alternative to, or in association with, the use of Thymoglobulin or Campath. Examples are in protocols originally described by Chatenoud in which the anti-CD3 antibody was successfully used to prevent the onset of the disease in prediabetic NOD mice. It was also possible to reverse recent-onset disease by restoring the lost self-tolerance to beta cell antigens in the same strain of mice (105). Another possibility, proposed by Iwakoshi *et al.* (112) in Worcester, consists of the use of an anti-CD154 antibody. The potentially dangerous thrombogenic characteristics of some anti-CD154 antibodies may not be too worrisome if the treatment can be, as in this case, very limited in time. A third protocol involved the use of an anti-CD20 monoclonal antibody. A chimeric mouse-human immunoglobulin G with this specificity (Rituximab) has shown efficacy in the treatment of some autoimmune diseases (113). More recently, Rituximab has also been successfully used to improve the outcome of allogeneic HSC (e.g., enriched CD34⁺) transplantations into patients who suffered chronic GVHD (114). Its efficacy in inhibiting the activation of a number of T-cell clones in the recipient, by blocking his/her B lymphocyte activity, could be tested here with the aim of preconditioning the recipient before performing transfected BM precursor cell autotransplantation.

REDUCING THE EFFECTS OF SECOND T-CELL SIGNALS

Thymic or central tolerance must be complemented by the peripheral regulation mediated by cell-antigen-specific T cells. Dendritic cells (DCs) are the primary APCs of the immune system that control the activation of naive T cells (115–117). For full activation of naive CD4⁺ T lymphocytes to occur, a second signal is necessary besides the already described presentation of the antigen to the TCR in the context of the MHC class II molecule present at the surface of the DC. Once properly activated, the T cell up-regulates the CD154 molecule (CD40 ligand) at its cell surface, thereby promoting the initiation of the second signal. The interaction of CD154 with the CD40 molecule results in the up-regulation of CD80 and CD86 at the surface of the APC. Up-regulated CD80 and CD86 will engage the CD28 molecule present on the T cell. The full activation of the T cell is the result of this second signal costimulation.

In the absence of the interactions between CD80, CD86, and CD28, the T cell will either enter a state of functional silence, termed anergy, or will be primed for apoptosis, perhaps in a CD95–CD95L (Fas–FasL)-dependent manner (118). Converging lines of evidence indicate that the pheno-

type of the DC surface can play an important role in the development of tolerance to self-antigens, and that it can be manipulated to induce allogeneic as well as autoimmune hyporesponsiveness (119). Phenotypically “immature” DCs, defined by low-level expression of cell surfaces CD40, CD80, and CD86, can elicit host immune suppression in allotransplantation and autoimmunity.

The first use of DC to prevent T1D in NOD mice was documented by Clare-Salzler *et al.* (120), who demonstrated that transfer of pancreatic lymph node DC derived from 8- to 20-week-old NOD mice into prediabetic NOD mice conferred significant protection from T1D, insulinitis, and adoptive transfer of T1D. The latter was possibly because of the presence of regulatory T cells that attenuated these pathologic processes. More recently, Feili-Hariri *et al.* (121,122) have shown prolongation of a diabetes-free state in NOD recipients of BM-derived syngeneic DC. NOD DC exhibits strong immunostimulatory capacity, underlined by hyperactivation of NF-kappa B (123–125). In fact, the inhibition of NF-kappa B, using short, double-stranded transcriptional decoys, renders NOD DC less immunostimulatory. The administration of these engineered DC into NOD prediabetic mice prevents the development of diabetes (126).

A complementary approach is that of engineering DC in a way in which the expression of the costimulatory molecules CD40, CD80, and CD86 only would be suppressed at the cell surface (107). Unlike the intervention on NF-kappa B or the use of anti-CD40L antibodies and CTLA4-Ig, this approach limits the cell population that is targeted because the treatment is performed *ex vivo* and does not involve systemic dissemination of proteins that, in the instance of CTLA4-Ig and anti-CD40L, have exhibited toxic effects (127,128). The *ex vivo* treatment of BM-derived NOD DCs with a mixture of antisense oligodeoxynucleotides (AS-ODN), targeting the CD40, CD80, and CD86 transcripts, confers specific suppression of the respective cell surface proteins (107). A single injection of these engineered DCs into syngeneic prediabetic female NOD mice significantly delays the incidence of T1D and abolishes any sign of insulinitis. More than one injection of AS-ODN-treated DCs maintain the NOD mice diabetes-free indefinitely without affecting the response of T cells to alloantigens. Splenocytes with an increased prevalence of CD4–CD25–CD62L⁺ T cells, from ODN-treated NOD DCs transferred into NODscid recipients, together with splenocytes from a diabetic donor, reduce dramatically the onset of the disease the latter are normally able to induce (107).

The use of AS technology specifically targeting the transcripts of key DC cell surface proteins involved in T-cell activation and regulation could be a useful technique to complement central regulation mediated by a newly populated thymus and might make T1D cell therapy more efficient (45).

INFUSION *IN SITU* OF APPROPRIATE FACTORS ABLE TO SPEED UP THE PHYSIOLOGIC REGENERATIVE PROCESS

The physiologic regenerative potential of the endocrine pancreas seems to be still quite high immediately after (or very close to) the onset of the disease when, in general, there still are some insulin-producing cells able to secrete sufficient insulin to make C-peptide testing possible, i.e., over the

minimum level detectable by the appropriate assays. In the mouse and in the monkey (e.g., cynomolgus), the regenerative process seems to take more than 3 months to substitute enough beta cells to allow the detection of an influence on the control of the glycemia of the animal. Even at this point in time, both of these animals do not yet have perfect control of the glycemia because intravenous glucose tolerance tests are still far from normal. However, this result would constitute already a great advantage for the diabetic patient, even if we do not know for sure whether, at a longer time after clinical onset, the reparative process may still work and at the same speed observed immediately after onset. Preliminary studies in animals seem to indicate that the regenerative process works proportionally more slowly as the time from onset of the disease increases. If eventually the regenerative process ceases to activate, it would be useful to know when that time is, i.e., when the time from onset has become “too long.”

To help the system to activate the regenerative process, or to speed up a possibly very slow physiologic recovery, even after protracted diabetes insulin therapy, it would be useful to test those different factors that have been proven to be efficient in better achieving this goal (84,85). For other factors, like PAX4 (83), HGF (87), IGF-1 (90,91), or GLP-1 (92), the insulin promoter should be used to construct the cassettes eventually introduced into the vector. In a recent study (Wang *et al.*, unpublished data), the capacity of adeno-associated virus (AAV)-mediated pancreatic gene transfer was reexamined using the recently available, novel serotypes of AAV coupled with an improved double-stranded AAV vector DNA cassette, which facilitates rapid and stronger transgene expression (129). The advantage of using AAV vectors consists of their lack of immunogenicity, associated with their limited insertion capabilities, that, particularly in dividing cells, eventually leads to loss of expression of the carried gene. It has been shown that robust and relatively long-term gene transfer can be achieved by these vectors in the vast majority, if not all, of the islets. Gene transfer efficiency and vector distribution in the islets are determined by the choice of AAV serotype vectors, as well as by the delivery methods. The pancreatic exocrine acinar cells are highly susceptible to AAV8 infection. To minimize the unwanted gene transfer to nonendocrine pancreatic and nonpancreatic tissues seen after i.p. or i.v. delivery, we explored a topical route by retrograde delivery into the pancreatic duct, similar to the endoscopic retrograde cholangiopancreatography technique commonly used in patients with pancreatitis. Because the pancreatic beta cell is, by definition, the most important target in gene transfer and therapy for diabetes, we explored the use of the insulin promoter to minimize nonspecific transgene expression in unintended cells, such as the pancreatic acinar cells and those beyond the pancreas. As expected, 2 weeks after delivery of AAV8-insulin-promoter-GFP vector in adult mice, strong GFP expression was readily detected exclusively in the islets, but not in the exocrine acinar cells.

CONCLUSIONS

For decades, efforts have been made to find successful treatments for T1D, such as insulin replacement, pancreas transplantation, and islet transplantation (whether they be

allotransplantation or xenotransplantation). Despite progress in the field of transplantation, this has not yet resulted in a permanent solution.

Rodent studies have given us hope for a new direction: regeneration of the patient's own beta cells. Preliminary studies in primates support anecdotal examples, suggesting that beta cell regeneration might be possible also in humans. If abrogation of autoimmunity can be safely achieved in a diabetic patient with an autotransplant of precursor cells transfected with HLA class II beta chain genes conferring resistance to the disease, while correcting his/her hyperglycemia using conventional insulin administration or an islet allotransplant, *nature* will be left to heal the rest. It should also be possible to speed up the natural process of healing by endoscopic retrograde intraductal delivery of factors known to promote beta cell regeneration. Should this approach work satisfactorily, our young patients will be cured for good, without the need for long-term drug therapies associated with the known troublesome consequences.

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Is Facilitating Pancreatic Beta Cell Regeneration a Valid Option for Clinical Therapy?

Massimo Trucco

Division of Immunogenetics, Department of Pediatrics, Children's Hospital of Pittsburgh,
University of Pittsburgh, Pittsburgh, PA, USA

Type 1 diabetes (T1D) is an autoimmune disease in which the clinical onset most frequently presents in adolescents who are genetically predisposed. There is accumulating evidence that the endocrine pancreas has regenerative properties, that hematopoietic chimerism can abrogate destruction of beta cells in autoimmune diabetes, and that, in this manner, physiologically sufficient endogenous insulin production can be restored in clinically diabetic NOD mice. Recapitulating what also has been seen sporadically in humans, we set out to test reliable and clinically translatable alternatives able to achieve these same goals. Recently, Tian and colleagues demonstrated that T1D can be prevented in genetically susceptible mice by substituting a "diabetes-susceptible" class II MHC beta chain with a "diabetes-resistant" allelic transgene on their hematopoietic stem cells through gene supplantation. The expression of the newly formed diabetes-resistant molecule in the reinfused hematopoietic cells was sufficient to prevent T1D onset even in the presence of the native, diabetogenic molecule. If this approach to obtain autoimmunity abrogation could facilitate a possible recovery of autologous insulin production in diabetic patients, safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

Key words: Autoimmunity; Type 1 diabetes; Beta cell regeneration; Tolerization; Beta cell precursors

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease the clinical onset of which most frequently presents in adolescents who are genetically predisposed (4,7). The disease onset frequently is a totally unexpected, abrupt, frightening event involving families with no previous history of diabetes. From that moment on the patients can only control glycemia with daily injections of recombinant human insulin. The control of glycemia via repetitive insulin administrations, even guided by the most sophisticated schedule, is far from the delicate and fine-tuned control offered by the physiologic secretion of the hormone. The suboptimal control of glycemia seems to be the determining factor, which predisposes to diabetes complications (11,12). Transplantation of whole pancreas or isolated islets offers a therapeutic solution to T1D in which insulin production and secretion are much closer to physiologic conditions (15,33,34,37). However, these two therapeutic approaches suffer from all the drawbacks associated with any allotransplant. In particular, the immunosuppressive regimens necessary for organ or islet acceptance are extremely difficult to

bear for the recipient (35), toxic not only for the beta cells (5,31) but, in the long run, also quite toxic for the kidney, the function of which may eventually become totally impaired (5,31).

BETA CELL REGENERATION AND AUTOIMMUNITY

The case of a 13-year-old Caucasian boy who, after suffering with a conventional T1D onset (i.e., after a history of polyuria, polydipsia, and weight loss, the boy presented with serum glucose up to ~500 mg/dl, glucosuria, and ketonuria), needed less and less insulin doses over time, to ultimately have insulin treatment completely stopped after 11 months of treatment, was recently published by a group from Ulm, Germany (18). The authors also reported that: "Without further treatment, HbA_{1c}, and fasting glucose levels remained normal throughout the entire follow up of currently 4.5 years," and that serum autoantibodies to GAD65, IA-2, insulin, and ICA "were initially positive but showed a progressive decline or loss during follow-up." A similar case was recently reported by David Harlan's group working at the National Institute for Health (35).

It is therefore not surprising to find that in the non-obese diabetic (NOD) mouse the abrogation of autoimmunity is sufficient to promote regeneration or rescue of the insulin-producing beta cells in the host endocrine pancreas, even after the onset of the disease (16,36,48). NOD mice spontaneously develop T1D with etiopathogenetic characteristics very similar to the disease in humans (3).

If the approach to obtain autoimmunity abrogation is safe, it could facilitate a possible recovery of autologous insulin production in already-diabetic patients, and might become the basis for new, less invasive, promising therapies for T1D.

Precursors of a perhaps unconventional type, located both in close proximity and/or inside the endocrine tissue, seem to exist and be activated by increased metabolic demand or by still unknown secreted factors, normally able to accelerate the process that guarantees homeostasis of islets of Langerhans under normal conditions (43). The physiologic equilibrium between lost and newly generated cells is dramatically altered in T1D by the action of beta cell-specific, autoreactive T-cell clones, in instances in which autoimmunity develops (46). Once T-cell-mediated destructive activity overcomes the regenerative compensatory activity of the organ, the number of functional beta cells progressively decreases until they become too few for maintaining homeostasis of the glucose in the body. The time of transition over this metabolic threshold becomes immediately evident with the presentation of the characteristic signs of the clinical onset of T1D. During the disease, even if the regenerative properties of the pancreas remain functional, the continued presence of diabetogenic, autoreactive T cells consistently nullifies the reparative effort (43,46). The fact that these autoreactive T cells remain present in the body of the diabetic patient for a long time is proven by experiments in which healthy islet cells transplanted into syngeneic, long-term diabetic mice or humans were quickly killed by these same autoreactive T cells (38).

The autoimmune response can be successfully averted in the NOD mouse either by directly eliminating the majority of the autoreactive T cells with anti-T cell antibodies (26) or by substituting all or part of the immunocompetent cell repertoire with bone marrow (BM) cells obtained from diabetic-resistant donors (49). We and others (16,21,48) have shown that the successful induction of a mixed allogeneic chimerism obtained after transplanting BM from a diabetes-resistant donor into a diabetic recipient, following a sublethal dose of irradiation, is sufficient to block and eventually also revert the systematic invasion into the islets of autoreactive T cells. This invasion results in insulinitis (i.e., the presence of beta cell-reactive T cells in the pancreatic islets themselves) (Fig. 1). Within the endocrine pancreas, once the

insult of autoimmunity is abrogated, the physiologic process of regeneration can continue efficiently, eventually replenishing the population of insulin-producing cells to a number sufficient to maintain euglycemia, thus curing the diabetic recipient (Fig. 2) (16,43,48). However, while this slow process takes place, the recipient's glycemia must be controlled by additional, independent measures. The most commonly used technique is to transplant islets from the same BM donor under the kidney capsule of the recipient (6,16,48). However, the successful engraftment of the transplanted bone marrow, or the establishment of a steady hematopoietic chimerism, would have to be maintained without the use of immunosuppressive agents (43). These potent drugs may kill not only the still present autoreactive T cells of the recipient, but also the beta cells themselves, thereby defeating the purpose of the transplant (5,31,39). The use of immunosuppressive agents may also interfere with the observed rise of regulatory T cells, a possible explanation for the long-lasting immunoregulatory cell-dominant condition observed in cured animals (9,32,43).

A subject of ongoing debate is whether either or both the transplanted BM and the cotransplanted islets are necessary for promoting an efficient regenerative process, independent of their ability to block autoimmunity or preserve euglycemia, respectively. Strong evidence suggests that the hematopoietic precursors present in the BM cell population do not directly participate in the reparative process of the insulin-producing cell population (Fig. 2) (16,43,48); they might, for example, secrete factors such as glucagon-like peptides, which are useful in order to sustain an efficient regenerative process (13,26,28). In the cured recipient, insulin-producing cells that are genetically marked to indicate they are of donor origin are extremely rare, occurring in no more than 2 out of more than 100,000 beta cells. These cells may actually be the result of sporadic cell fusion processes (19).

SUCCESSFUL APPROACHES TO ABROGATE AUTOIMMUNITY

In order to reduce the complications associated to the radioablation of the BM of the recipient—a procedure difficult to transfer to clinical trials because of all its inherent severe contraindications (e.g., GVHD)—we originally focused on the generation of hematopoietic chimeras in sublethally irradiated recipients as a possibly less dangerous yet still efficient approach to abrogate autoimmunity (49). Donor BM cell number required for the establishment of the chimeric status in the NOD mice was titrated to define a dose-based correlation with the level of the chimerism achieved. NOD mice exhibited not only a higher resistance to allogeneic BM engraftment but also a greater inconsistency in the level of chimerism, induced within one group when

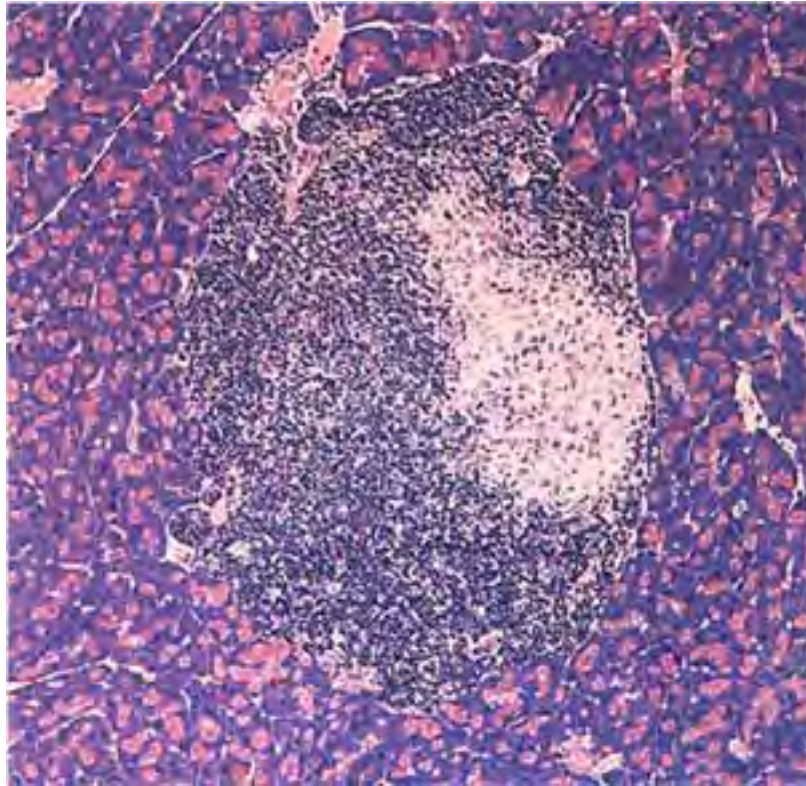


Figure 1. Insulitis: the presence of autoreactive T cells in the islet of pancreas.

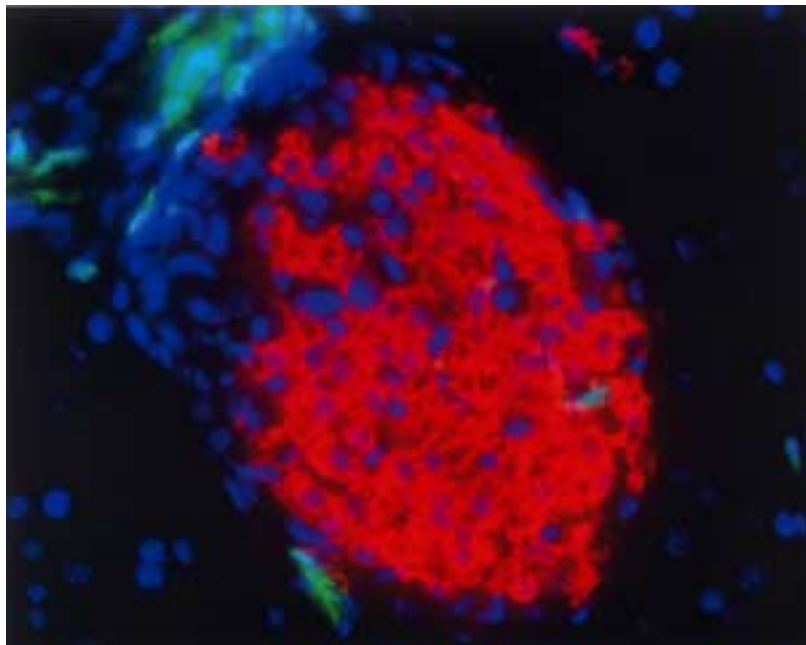


Figure 2. Regeneration of the endogenous pancreas. Using a GFP transgenic mouse donor, it is possible to observe how the transplanted BM cells (green) do not directly participate in the regeneration of the insulin-producing beta cells (red). There are not double-positive (orange) cells in the newly formed islets (17). (Reprinted with permission from *Journal of Clinical Investigation*.)

compared with other autoimmunity-free mouse strains studied (49). Another interesting and distinct feature of the NOD chimerae from other, non-autoimmunity-prone strains of mice that underwent allogeneic BM transplantation (BMT), is the increase of the level of chimerism with time when compared with the initial numbers determined at 4 weeks after BMT (49).

The recovery of the endocrine pancreas was reproducibly observed also in NOD mice treated just after the clinical onset of the disease (48). Diabetic mice that received islet grafts after 3 days of glycemia of >300 mg/dl became euglycemic within 24 h following the transplantation procedure and remained so for the length of observation. After surgical removal of islet graft-bearing kidney, performed at 17–26 weeks after islet transplantation, all mice remained euglycemic for up to 18 days (length of observation). Direct assessment of the insulin content in the endogenous islets was performed by histological evaluation of pancreata harvested from euthanized animals upon termination of the experiments (48,49). Insulin-positive beta cells in these pancreata collected from treated mice about 5 months after the clinical onset of the disease were found in quantities and morphologies similar to those of the normal mouse pancreas (Fig. 3). Allochimerism was multilineage and reached levels over 90% in all animals (49). None of these animals developed clinical signs of GVHD (49). When we used a green fluorescent protein (GFP) transgenic mouse (27) as the BM donor, GFP-positive cells were detected in the pancreas, but these cells did not look as directly involved with the restoration of the endocrine pancreas because both insulin- and GFP-positive cells were not detected (27,48) (Fig. 2). The GFP-positive cells were considered more probably circulating, mature blood cells rather than BM hematopoietic stem cells (HSCs) able to transdifferentiate into insulin-producing beta cells (43).

ALTERNATIVE APPROACHES TO ABROGATE AUTOIMMUNITY

To avoid the possible complications associated with a conventional BMT, we then decided to use the strategy recently proposed by Tian et al. (40), in which T1D can be prevented by substituting a “diabetes-susceptible” class II MHC beta chain with a “diabetes-resistant” allelic transgene on hematopoietic stem cells of genetically susceptible NOD mice through gene supplantation (44).

In the late 1980s, in collaboration with Dr. Hugh McDevitt, Stanford University, we were able to map and identify the most influential single hereditary susceptibility factor in T1D: a single amino acid of the beta chain of the HLA-DQ histocompatibility molecule (25,41). Although T1D is recognized to be a multigenic

disease (10), in humans the principal genetic susceptibility component was proposed to be any allelic form of the HLA-DQ molecule that lacks a charged amino acid at position 57 of its beta chain. Conversely, resistance to disease is associated with the inheritance of HLA-DQ alleles containing a charged amino acid such as aspartic acid, at the same position (Asp-57). Physical explanation of the unusual importance of this particular single amino acid location for the development of the autoimmune characteristics of T1D came with the elucidation of the crystal structure of the HLA-DQ8 molecule, a non-Asp-57 molecule that confers the highest susceptibility to the disease (20). The most important structural features of the susceptibility HLA-DQ8 molecule relevant to diabetes immunology are identical to the homologous I-Ag⁷ molecule present in the diabetes-prone NOD mouse (1). The peptide binding site of the majority of human HLA-DQ and murine I-A molecules have an Asp-57 that points into the groove (24,45). In these allelic forms, Asp-57 forms an electrostatic salt bridge with the arginine in juxtaposition (i.e., in position 76) of the alpha chain of the molecule (Arg-76), which also points into the groove. Both HLA-DQ8 and I-Ag⁷ molecules lack Asp-57 and this variation disrupts the electrostatic interaction, leaving the Arg-76 free to interact with the aqueous environment and with any peptide able to lodge inside the binding groove of the molecule. The absence of Asp-57 allows the binding of peptides that may not find appropriate lodging inside other Asp-57⁺ molecule grooves, and may jeopardize an efficient presentation by the histocompatibility molecule to T cells because of incorrectly positioned self-peptides. The eventually created susceptibility status can be correlated, in immunological terms, with impaired peptide lodging, impaired peptide presentation to T cells, with consequent reduced efficiency on negative selection of self-reactive T-cell clones and positive selection of regulatory T cells (24,44,45). Indirect evidence supporting these hypotheses derives from transgenic NOD mice that express class II genes other than I-Ag⁷, which do not develop diabetes (14,22), and from the fact that transplantation of allogeneic BM from strains that do not spontaneously develop diabetes also prevents the occurrence of diabetes in NOD mice (16,21,48,49).

Instead of performing an alloreactive bone marrow transplant, Tian's approach (40) consisted of the transfection *ex vivo* of the gene encoding a resistant, Asp-57-positive beta chain into the BM cells isolated from the diabetes-prone NOD mouse. The expression of the newly formed diabetes-resistant molecule in the reinfused hematopoietic cells was sufficient to prevent T1D onset in the NOD recipient, even in the presence of the native, diabetogenic, non-Asp-57, Ag⁷ molecule (Fig. 4) (40,44). Mechanistically, the authors suggest a model in

which a subset of the engineered BM cells migrate, populate the thymus, and become efficient antigen-presenting cells involved in the negative selection of thymocytes that would otherwise mature into autoreactive T cells (44). In fact, diabetes-free NOD mice exhibited no emergence into the blood stream of T cells capable of responding to putative autoantigens, nor insulinitis. The mice remained diabetes free even after cyclophosphamide treatment, a maneuver that tests the robustness of a prophylactic antidiabetic therapy (2). Similar experiments performed by Leiter's group (14) in which the T cells from the cured mice were used to transfer diabetes into NOD scid recipient mice seem to indicate, however, that central tolerance was not completely restored because some autoreactive T-cell clones were not deleted; rather, they were still present in the successfully treated recipient. The possible complementary involvement of activated CD4+CD25+ T regulatory (T reg) cells, able to alter the balance of the circulating cytokines and consequently mount an efficient peripheral tolerance, seems to be deserving equal consideration.

If this approach to obtain autoimmunity abrogation could also facilitate a possible recovery of autologous insulin production in the already-diabetic individual,

safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

IMMEDIATE GOALS

With this working hypothesis we intend to test whether: a) the targeting of a diabetes-resistant beta chain gene transfection specifically on enriched precursors cells isolated from BM may improve the procedure outcome; b) nonradiation-based preconditioning approaches would be sufficient to promote engraftment and repopulation capabilities of these engineered cells; c) this approach can enhance thymic repopulation with engineered cells to promote an efficient negative selection of autoreactive T-cell clones and an improved positive selection of T reg cells, to consequently abrogate autoimmunity; d) the abrogation of autoimmunity will allow the rescue of endogenous insulin production even in newly onset diabetic NOD mice.

We have already been able to reproduce the results of Tian and collaborators as far as retroviral vector preparation, transfection, and BM reconstitution in radioablated recipients are concerned. We are now in the process of comparing the experimental protocol proposed by the Harvard group (40) with the new one we intend

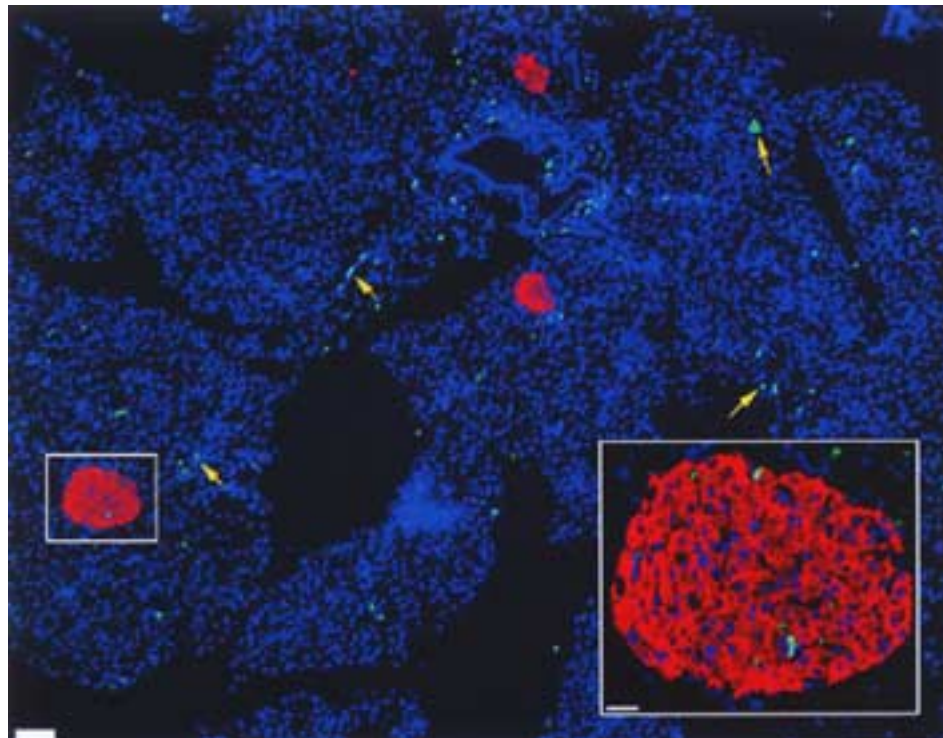


Figure 3. Regeneration (or rescue) of the endogenous pancreas. After 4 months from obliteration of the autoimmune process, via allogeneic bone marrow transplant, we have found clusters of insulin-producing cells (red) resembling pancreatic islets (48). (Reprinted with permission from *Stem Cells*.)

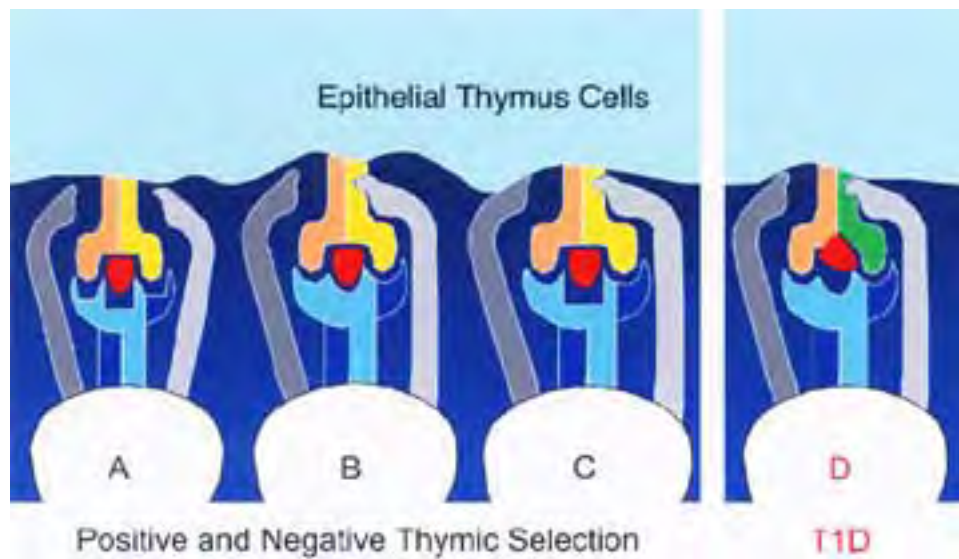


Figure 4. Theoretical basis of Tian's et al.'s (40) approach for autoimmunity abrogation in the NOD mouse. In a healthy individual, the maturation of the T cells, coming from precursors present in the bone marrow, is taking place in the thymus, where they undergo a positive and a negative selection. In the thymus, peptides (in red) from antigens of self-tissues are presented to the various immature double-positive (CD8, in gray, and CD4, lighter gray) T cells (A, B, C) via the MHC molecule. MHC class II molecules are heterodimers composed of an alpha (in orange) and a beta (in yellow) chain that form their antigen combining site. When, like for the A cell, the T-cell receptor (TCR: in blue, alpha chain; in azure, beta chain) has for the MHC molecule/self-peptide complex a very low affinity (in the figure contours of the MHC molecule/self-peptide complex do not fit with the contours of the TCR molecule), the developing T cell does not receive the necessary positive signal to survive and exit the thymus for release into the periphery. However, if the affinity between the MHC molecule/self-peptide complex and the TCR is too high, like for the B cell (in the figure the contours of the MHC molecule/self-peptide complex fit precisely into the contours of the TCR molecule), the T cell undergoes negative selection and dies inside the thymus. The T cell shown in C receives instead a positive survival signal because of the high-affinity interactions between its TCR with the MHC molecule; an affinity, however, that is not further enhanced by the presence of a self-peptide in its groove, so that the negative selection does not take place. This T cell matures and goes in the circulation to protect the body from foreign (non-self) invaders, with which it is able to efficiently interact. The immunological basis of type 1 diabetes is schematically described in T1D. Here the D cell binds to an MHC molecule (orange and green chains) conferring susceptibility to diabetes (like the HLA-DQ8 in humans and the I-Ag⁷ in the mouse), because it does not present the self-peptide properly. The T cell, then, even if potentially autoreactive (D has the same TCR as B), is not subjected to negative selection and is free to leave the thymus to circulate in the blood. T cells that are potentially reactive to self-antigens but failed to be deleted inside the thymus are able to attack tissues of the body expressing these same antigens, generating autoimmunity. The approach taken by Tian and colleagues (40) can be illustrated imagining that the diabetogenic I-Ag⁷ molecule, carrying a non-Asp-57 beta chain (in green like in D), was supplemented, in the hematopoietic cells of the NOD mouse, with a nondiabetogenic MHC molecule, like the one interacting with A, B, or C. The ex vivo transfection of a gene encoding an Asp-57⁺ beta chain (in yellow) into the bone marrow stem cells allowed the reconstruction of an efficient MHC molecule (orange and yellow chains) that, once the cells were returned into the donor, allowed the restoration of an efficient negative selection in the thymus (like for B), sufficient per se to delete autoreactive T cells and consequently to prevent diabetes (44). (Reprinted with permission from *Gene Therapy*.)

to use instead. It must also be proven that the enriched precursors are able to generate new, more efficient APC and in sufficient numbers to efficiently repopulate the thymus and eventually favor peripheral tolerance.

Our first goal certainly is proving that regeneration

(or rescue) of beta cell mass, in the absence of autoimmunity, is a function of the endogenous pancreas we may exploit for possible clinical applications, and that Tian et al.'s approach is useful to this aim. However, the proposition of considering the use of this same ap-

proach for a possible clinical trial of tomorrow may be complicated by the presence in humans of more than one susceptibility molecule [i.e., not only the H2-IA (equivalent to HLA-DQ) like in the NOD mouse, but also the H2-IE (HLA-DR)]. However, the efficiency of Tian et al.'s gene-based treatment, even in the presence of the native diabetogenic molecule, may offer solutions also to the problem of dealing with more than one susceptibility molecule. In fact, as opposed to the HLA-DQ molecule that is composed of a polymorphic alpha chain and polymorphic beta chain, the HLA-DR molecule is formed of a nonpolymorphic alpha chain combined with a polymorphic beta chain (30). The substitution of any HLA-DR beta chain will then be sufficient to create a new DR molecule. The protective molecule may act in

an epistatic or dominant manner over the susceptibility molecule also in the case of the DR alleles.

Our second goal will be to systematically substitute for irradiation different antibody-based, immunoreductive conditioning protocols. We selected three approaches that seem to be giving the most reliable results, with the aim to see whether they may be able to promote engraftment and repopulation capabilities of our engineered BM precursor cells and consequently the abrogation of autoimmunity. The first is the protocol originally described by Lucienne Chatenoud (9), in which the anti-CD3 antibody was successfully used initially to delete the onset of the disease in prediabetic NOD mice. Further expanding the goals of her experiments, Chatenoud was able to reverse recent-onset disease by restoring the

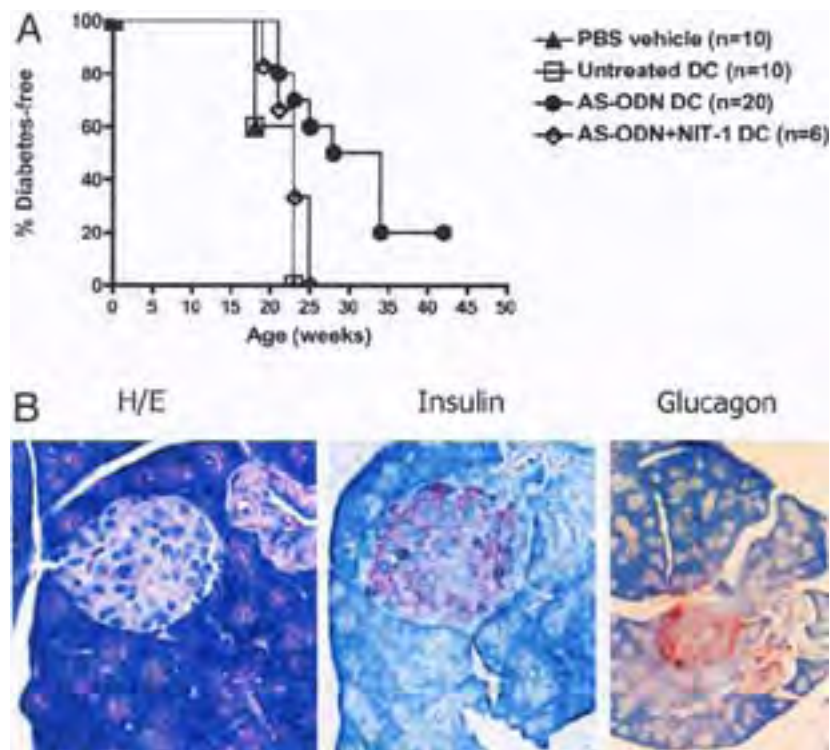


Figure 5. Diabetes incidence in NOD recipients of DC. (A) DC from bone marrow progenitors of 6–8-week-old female NOD mice were propagated in GM-CSF/IL-4 and further treated with AS-ODN with or without NIT-1 lysate as described. PBS-resuspended cells (in 200 μ l; 2×10^6) were injected IP into syngeneic age- and sex-matched NOD recipients. Blood glucose was measured by electronic sampling of tail vein blood beginning at 15 weeks of age. AS-ODN DC: DC treated with a mixture of CD40, CD80, CD86 AS-ODN, each oligo at 3.3 μ M; AS-ODN + NIT1 DC: DC cotreated with the AS-ODN mixture and NIT-1 lysate. $p = 0.012$, AS-ODN DC recipients vs. AS-ODN NIT-1 DC recipients, Kaplan-Meier log rank test. (B) Immunohistochemistry of pancreata from diabetes-free recipients of AS-ODN DC. For immunohistochemistry, the sections were fixed in paraffin, treated to block peroxidase, and incubated with nonfat dried milk. The slides were then incubated with anti-insulin or anti-glucagon Ab followed by an isotype-reactive biotinylated secondary Ab. Avidin-HRP was then added followed by diaminobenzidine after which a brown color could be observed. No evidence of insulinitis was observed with insulin and glucagon readily detectable (23). (Reprinted with permission from *Journal of Immunology*.)

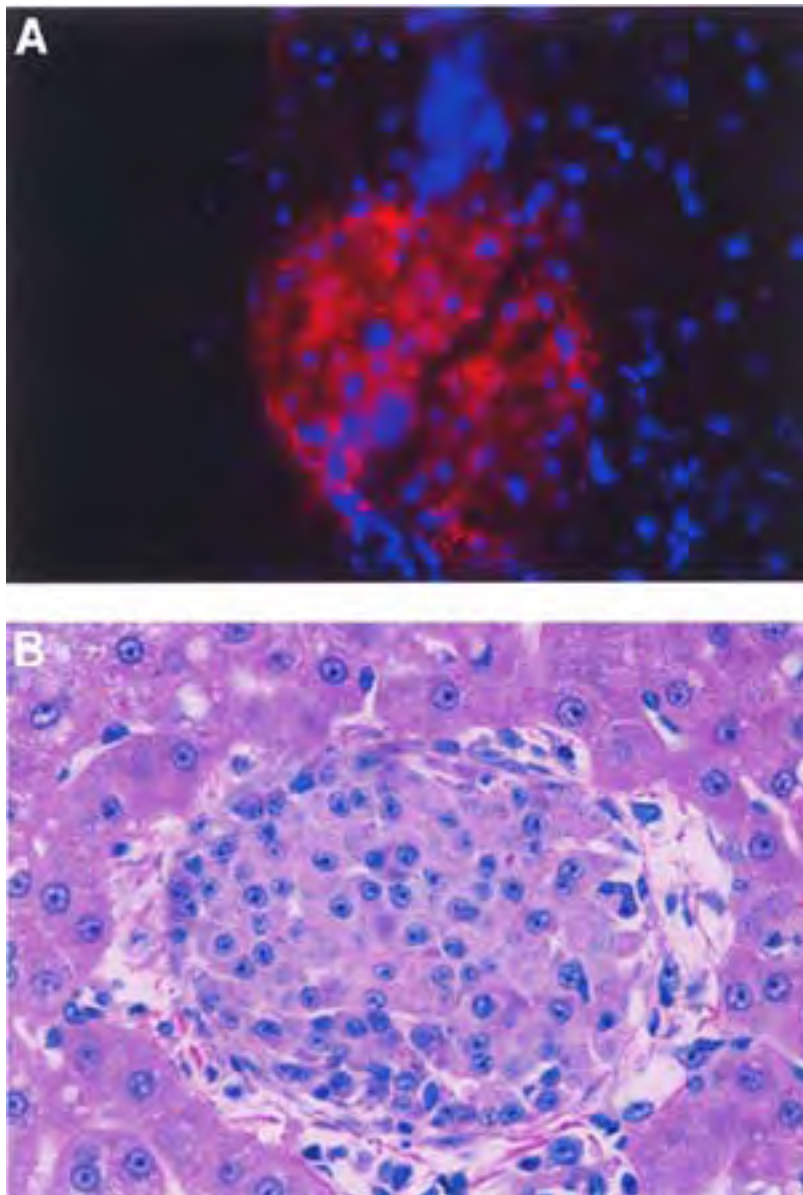


Figure 6. Adult pig islet transplanted into the liver of nonhuman primates. (A) Insulin immunofluorescent staining (red). Nuclear staining with Hoescht (blue). (B) Hematoxylin and eosin staining (40 $\times$).

lost self-tolerance to beta cell antigens in NOD mice. The treatment involved five consecutive doses of anti-CD3 antibody administered within 2–4 weeks (9). The second approach, proposed by Dale Greiner's group in Worcester (17), uses instead the anti-CD154 antibody. This protocol considers one donor-specific transfusion administered before transplant plus a brief course (e.g., four doses of 0.5 mg/mouse, on days –7, –4, 0, and +4 of transplant) of anti-CD154. The third is Megan Sykes's proposed protocol (42) in which anti-CD4 and anti-CD8 monoclonal antibodies are administered on

days –6 and –1 of transplant followed by a 3-Gy total body irradiation and 7-Gy thymic irradiation on day 0. The possible involvement of CD4⁺CD25⁺ T cells in the preservation of tolerance, postulated on the basis of results obtained in cured mice (9,44), is also proposed by Zheng and collaborators (47) to explain the effect of a treatment that involves the administration of rapamycin, agonistic IL-2–Ig fusion protein, and a mutant, antagonist-type IL-15-related cytolytic Ig fusion protein to obtain long-term engraftment and tolerance of allogeneic islets transplanted into overtly diabetic NOD mice. The

CD4+CD25+ T-cell population is also increased once CD40, CD80, and CD86 cell surface molecules are specifically downregulated by ex vivo treatment of NOD mice dendritic cells (DCs) with a mixture of antisense oligonucleotides targeting CD40, CD80, and CD86 primary transcripts (23). The incidence of diabetes is significantly delayed by a single injection of the engineered NOD mouse-derived DCs into syngeneic recipients (Fig. 5) (23). Different quantities of antibodies and injection time schedules will be compared to find optimal conditions for acceptance of transfected, enriched BM precursor cells.

Although we do not have a proven record of our ability to properly use in the mouse model these conditioning protocols, we are obtaining extremely encouraging results (i.e., more than 2 months of pig C-peptide production) (Fig. 6) using the humanized anti-CD154 antibody from Novartis, to immunologically suppress diabetic (i.e., streptozotocin-treated) monkeys, recipients of intrahepatically transplanted, double knock-out (DKO) pig islets that are lacking the α 1-3 galactosyltransferase enzyme (8,29). The dangerously thrombogenic characteristics of the anti-CD154 antibody may not be too worrisome if the treatment can be limited in time. The success of a limited in time, antibody-mediated conditioning will suggest approaches more transferable to humans than lethal irradiation.

CONCLUDING REMARKS

Given that clinical solutions, like whole pancreas or pancreatic islet allotransplantation, are plagued by the paucity of pancreas donors and the toxicity of the immunosuppressive drugs that precludes their implementation in young recipients, relatively simple and possibly safe gene therapy-based approaches may become extremely useful in facilitating new types of clinical interventions.

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Human C-peptide antagonises high glucose-induced endothelial dysfunction through the nuclear factor- κ B pathway

P. Luppi · V. Cifarelli · H. Tse · J. Piganelli · M. Trucco

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Abstract

Aims/hypothesis Endothelial dysfunction in diabetes is predominantly caused by hyperglycaemia leading to vascular complications through overproduction of oxidative stress and activation of the transcription factor nuclear factor- κ B (NF- κ B). Many studies have suggested that decreased circulating levels of C-peptide may play a role in diabetic vascular dysfunction. To date, the possible effects of C-peptide on endothelial cells and intracellular signalling pathways are largely unknown. We therefore investigated the effect of C-peptide on several biochemical markers of endothelial dysfunction in vitro. To gain insights into potential intracellular signalling pathways affected by C-peptide, we tested NF- κ B activation, since it is known that inflammation, secondary to oxidative stress, is a key component of vascular complications and NF- κ B is a redox-dependent transcription factor. **Methods** Human aortic endothelial cells (HAEC) were exposed to 25 mmol/l glucose in the presence of C-peptide (0.5 nmol/l) for 24 h and tested for expression of the gene encoding vascular cell adhesion molecule-1 (VCAM-1) by RT-PCR and flow cytometry. Secretion of IL-8 and monocyte chemoattractant protein-1 (MCP-1) was measured by ELISA. NF- κ B activation was analysed by immunoblotting and ELISA. **Results** Physiological concentrations of C-peptide affect high glucose-induced endothelial dysfunction by: (1) decreasing

VCAM-1 expression and U-937 cell adherence to HAEC; (2) reducing secretion of IL-8 and MCP-1; and (3) suppressing NF- κ B activation.

Conclusions/interpretation During hyperglycaemia, C-peptide directly affects VCAM-1 expression and both MCP-1 and IL-8 HAEC secretion by reducing NF- κ B activation. These effects suggest a physiological anti-inflammatory (and potentially anti-atherogenic) activity of C-peptide on endothelial cells.

Keywords Atherosclerosis · C-peptide · Cytokines · Endothelial cells · Inflammation · Monocytes · NF- κ B · Nuclear factor κ B · Vascular smooth muscle cells

Abbreviations

EBM-2	endothelial basal medium-2
HAEC	human aortic endothelial cells
I κ B	inhibitor κ B
MCP-1	monocyte chemoattractant protein-1
MFI	mean fluorescence intensity
NF- κ B	nuclear factor- κ B
PDTC	pyrrolidine dithiocarbamate
ROS	reactive oxygen species
VCAM-1	vascular cell adhesion molecule-1

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P. Luppi (✉) · V. Cifarelli · H. Tse · J. Piganelli · M. Trucco
Division of Immunogenetics, Department of Pediatrics,
Rangos Research Center, Children's Hospital of Pittsburgh,
3460 Fifth Avenue,
Pittsburgh, PA 15213, USA
e-mail: luppip@pitt.edu

Introduction

Diabetes is a well-established risk factor for vascular diseases. Vascular disease in diabetes originates from common functional and structural changes in the tunica media of small (microangiopathy) as well as large vessels (macroangiopathy). In large vessels, these changes increase the probability of developing atherosclerosis, which is one of the major

complications affecting diabetic patients. As a result of the diabetic state, the vascular compromise at small vessels level principally affects the eye, kidney and both peripheral and autonomic nerves, and this dysfunction contributes significantly to the morbidity associated with diabetes [1].

Diabetes causes vascular compromise secondary to endothelial dysfunction, measured by in vivo studies of flow-mediated vasodilation [2] and increased circulating levels of biochemical markers, such as, but clearly not limited to vascular cell adhesion molecule-1 (VCAM-1) [3, 4]. Generally, VCAM-1 is expressed at a low level on endothelial cells and is upregulated upon cellular activation, such as that observed after exposure to inflammatory stimuli or high glucose [5, 6]. VCAM-1 binds to the leucocyte integrin $\alpha_4\beta_4$ (also called very late antigen-4; CD49d) and has a principal role in the early stages of monocytes adhesion to the vascular endothelium, one of the first steps in atherosclerosis plaque formation. A major hallmark of diabetes is an abnormally elevated blood glucose level, i.e. hyperglycaemia, which has been proposed as one factor causing endothelial dysfunction in diabetes. In endothelial cells, acute and chronic hyperglycaemia works through reactive oxygen species (ROS) production [5, 7, 8] that leads to activation of the transcription factor nuclear factor- κ B (NF- κ B) [5, 9, 10] and ultimately the production of inflammatory mediators [11].

In the unstimulated state, NF- κ B exists in its canonical form as a heterodimer composed of p50 and p65 subunits bound to I κ B. Upon activation, I κ B is phosphorylated and degraded causing the release of p50/p65 components of NF- κ B [12]. The active p50/p65 heterodimer translocates to the nucleus and initiates the transcription of a gamut of genes involved in the inflammatory response, such as those encoding pro-inflammatory cytokines, cell surface adhesion molecules and chemokines, including IL-8 and monocyte chemoattractant protein-1 (MCP-1) [5, 11, 13–15]. IL-8 and MCP-1 production is present in human atherosclerotic plaques [16] and participates in the development of atherosclerosis by recruiting monocytes into the subendothelial cell layer [17].

It has been suggested that proinsulin C-peptide may possess cytoprotective effects on the microvasculature during inflammatory events [18]. In line with this, it has been reported that type 1 diabetic patients with circulating levels of C-peptide closer to the physiological level of 0.5 nmol/l [19] or receiving whole pancreas [20] or allogeneic islet transplantation [21] show a reduced incidence of microvascular complications. The mechanisms able to produce the beneficial effects of C-peptide on vascular dysfunction in diabetes remain largely unknown. One study performed in vivo in a rat inflammatory model of vascular dysfunction showed that a single i.v. dose of C-peptide significantly inhibited leucocyte–endothelium interaction via decreased expression of endothelial cell adhesion molecules [22], a phenomenon

associated with release of nitric oxide [22, 23], which in turn has been shown to inhibit NF- κ B [24]. Similar results were obtained in isolated ischaemic and reperfused rat hearts, where addition of C-peptide attenuated polymorphonuclear cell adherence to the vascular endothelium [25]. To date, no data are available on the effects of C-peptide on human endothelial cells exposed to the damaging insult of hyperglycaemia, a common condition in diabetes.

We therefore initiated a study on the direct effects of C-peptide, testing VCAM-1 expression on the cell surface, monocyte adherence and secretion of IL-8 and MCP-1 by human aortic endothelial cells (HAEC) exposed to short-term high glucose. Since activation of the transcription factor NF- κ B is involved in these pro-inflammatory responses, we also investigated the direct effect of C-peptide on nuclear translocation of the NF- κ B subunits p50/p65 in HAEC. We hypothesised that physiological concentrations of C-peptide protect HAEC from high glucose-induced cellular dysfunction by decreasing NF- κ B activation, thus inhibiting NF- κ B-dependent genes, such as those encoding VCAM-1, IL-8 and MCP-1.

Methods

Cell culture of HAEC HAEC were obtained from Cambrex (Cambrex Bioscience Walkersville, Walkersville, MD, USA) and grown into 75 cm² culture flasks (250,000 per flask) (Corning, Corning, NY, USA) at 37°C, 5% CO₂ and in the presence of endothelial basal medium-2 (EBM-2) supplemented with endothelial growth media SingleQuots (Cambrex). HAEC were used at passages two to six.

Treatment conditions EBM-2 containing 25 mmol/l glucose (Sigma Chemical, St Louis, MO, USA) was used as a high glucose condition in all the experiments, while regular EBM-2, which contains 5.6 mmol/l glucose, was used as normal glucose condition. In all experiments, HAEC were used when having reached an 80% to 90% confluency. On the day of the experiment, cells were washed with fresh EBM-2 and then replaced with EBM-2 containing 25 mmol/l glucose in the presence or absence of physiological concentrations of human C-peptide (0.5 and/or 1 nmol/l) (Sigma Chemical) [26] for 4 to 24 h in an incubator at 37°C and 5% CO₂. As a control for C-peptide activity, C-peptide was heat-inactivated by boiling it for 1 h and then added to the culture. Human recombinant TNF- α (10 ng/ml; R&D Systems, Minneapolis, MN, USA), which activates HAEC, was used as a positive control. The effect of the NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC; 10 μ mol/l; Sigma) was also tested on HAEC in certain experiments. This inhibitor was added to EBM-2 with 25 mmol/l glucose. Separate sets of experiments were performed in which

C-peptide was added to regular EBM-2 containing 5.6 mmol/l glucose.

VCAM-1 detection by RT-PCR HAEC were grown into 75 cm² culture flasks (250,000 per flask; Corning) and exposed to the treatment conditions mentioned above. After 4 and 24 h, cells were trypsinised and frozen at −80°C. RNA extraction was performed using the RiboPure-Blood kit (Ambion, Austin, TX, USA). For RT-PCR, 1 µg RNA was used together with Oligo(d)T (RETROscript; Ambion) and 1 µl of cDNA was used to amplify *VCAM-1*. Human *GAPDH*, 18S ribosomal RNA and β -actin were amplified and served as internal controls [5]. Sequences of the oligonucleotides used to amplify these genes and PCR conditions are reported as [Electronic supplementary material \(ESM\)](#). Three independent experiments were performed. Densitometry was performed with UN-SCAN-IT gel software (Silk Scientific, Orem, UT, USA). Data are expressed as median±SD.

Quantification of VCAM-1 by flow cytometry HAEC (50,000 per well) were maintained in 6-well plates (Corning) until confluent. On the day of the experiment, cells were exposed to the treatment conditions mentioned above for 24 h. Determination of VCAM-1 expression by surface staining was performed on paraformaldehyde-fixed HAEC monolayers following a methodology shown to preserve single cell integrity [27]. A phycoerythrin-conjugated anti-human monoclonal antibody to CD106 (VCAM-1) or corresponding isotype control (BD Pharmingen, San Diego, CA, USA) were used for staining. Cells were run on a Becton Dickinson FACSCalibur and analysed at a later time (Becton Dickinson, San Jose, CA, USA). For a more detailed description of methodology and data analysis, see [ESM](#). Three sets of independent experiments were performed. Within each experiment, each condition was tested in triplicate. Data are expressed as median±SD.

Monocyte adhesion assay HAEC were grown on 48-well plates (12,000 per well; Corning) and exposed to the treatment conditions mentioned above for 4 h. Human monocytic U-937 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown in RPMI 1640 (Cambrex) containing 10% FCS, 100 µl/ml streptomycin, 100 IU/ml penicillin, 250 ng/ml fungizone, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES (all from Gibco Invitrogen, Carlsbad, CA, USA) at 37°C and 5% of CO₂. On the day of the experiment, medium was removed from each well, cells were washed with PBS and fresh medium containing U-937 cells (1×10⁶ cells/ml, 500 µl) was added to each well and incubated for 1 h at room temperature on a rocking plate. Non-adherent U-937 cells were removed and adherent cells fixed in 1% glutaraldehyde.

The number of adherent cells was evaluated by counting three random 40× fields per well by a blinded investigator, avoiding areas of non-confluence and cell clusters. Three experiments were performed. Within each experiment, each condition was tested in triplicate. Results are showed as median±SD.

IL-8 and MCP-1 detection in culture supernatant fraction by ELISA HAEC were maintained in 6-well plates (50,000 per well; Corning) in EBM-2 (Cambrex). On the day of the experiment, cells were exposed to the treatment conditions mentioned above for 4 h. The supernatant fraction was collected and kept at −20°C until tested by ELISA (Quantikine; R&D Systems). Three independent experiments were performed, in which each condition was tested in triplicate. Concentration of the chemokines (pg/ml) was assessed by calculating values according to the values obtained in the standard curve. Results from three separate experiments are shown as median±SD.

NF-κB analysis assays HAEC were cultured in 75 cm² culture flasks (250,000 per flask; Corning) and exposed to the treatment conditions as indicated above. Cells were collected at 4 and 24 h and pretreated with 25 µl of protease inhibitor cocktail (Pierce, Rockford, IL, USA). Nuclear and cytoplasmic fractions were separated using a kit (NE-PER Nuclear and Cytoplasmic Extraction; Pierce). Protein content of the extract was measured using a bicinchoninic acid assay kit (Pierce Biotechnology). For detection of NF-κB p65 subunit by western blot, 10 µg of nuclear protein extracts were used as previously described [28]. Densitometry analysis of the bands was performed with UN-SCAN-IT gel software (Silk Scientific). Activation of the NF-κB p50 subunit was detected on 3 µg of nuclear protein extracts using a kit (EZ-Detect Transcription Factor Kit; Pierce Technology). For each set of data, a minimum of three experiments was performed. Data were averaged and expressed as means±SD.

Statistics Paired *t* test (two-tailed) was used to analyse differences between 5.6 and 25 mmol/l glucose. ANOVA with the Dunnett's post hoc test was used to assess differences between 25 mmol/l glucose, C-peptide and PDTC using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Values of *p*<0.05 were considered to be statistically significant.

Results

C-peptide reduces VCAM-1 on HAEC exposed to high glucose In order to determine the effect of C-peptide on high glucose-stimulated VCAM-1 levels on HAEC, cells

were treated in vitro for 24 h with 25 mmol/l glucose alone or in the presence of C-peptide. High glucose increased *VCAM-1* mRNA expression compared with cells cultured in low glucose (Fig. 1a,b; $p=0.008$). C-peptide added to the high glucose medium inhibited *VCAM-1* mRNA expression as compared with high glucose medium alone (Fig. 1a,b). Although both concentrations of C-peptide (0.5 and 1 nmol/l) decreased *VCAM-1* mRNA expression, statistical significance was reached with 0.5 nmol/l C-peptide only ($p<0.05$). Heat-inactivated C-peptide, used as control, did not have a significant effect on *VCAM-1* expression (Fig. 1b). As expected, the cytokine TNF- α induced a dramatic upregulation of *VCAM-1* expression with a threefold increase in comparison to normal glucose (Fig. 1a,b). The inhibitory effect of C-peptide on high glucose-induced stimulation of *VCAM-1* mRNA in HAEC was observed as early as 4 h incubation (Fig. 2).

We obtained similar data by analysing *VCAM-1* expression on HAEC by flow cytometry. High glucose significantly ($p=0.03$) changed *VCAM-1* levels (average mean fluorescence intensity [MFI] 117.5 ± 25.5 SD) compared with cells

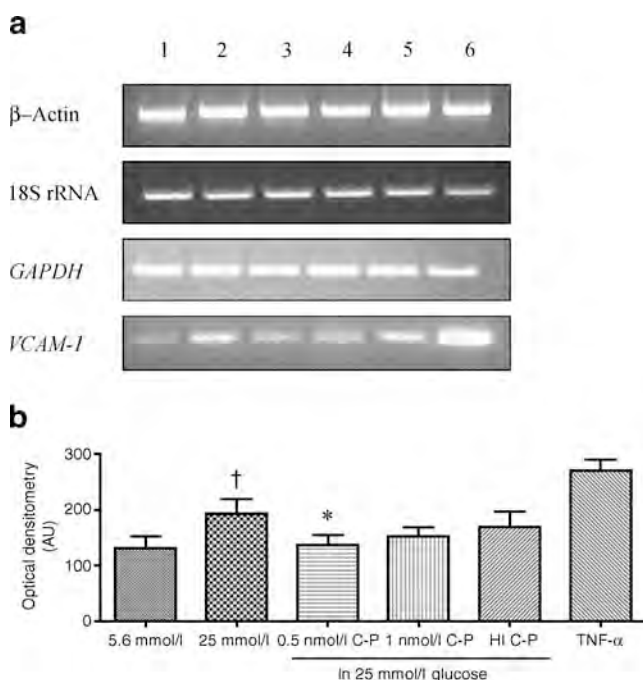


Fig. 1 C-peptide decreases high glucose-stimulated *VCAM-1* mRNA after 24 h. **a** RT-PCR was used to detect *VCAM-1* mRNA in HAEC treated for 24 h with: (1) low glucose (5.6 mmol/l; LG); (2) high glucose (25 mmol/l; HG); (3) HG+0.5 nmol/l C-peptide (C-P); (4) HG+1 nmol/l C-P; (5) HG+1 nmol/l heat-inactivated (HI) C-P; (6) LG+TNF- α 10 ng/ml). *GAPDH*, 18S rRNA and β -actin were used as internal controls. **b** Densitometric analysis of *VCAM-1* mRNA expression. Boxplot graphs showing the median values (limits of the lines are the 5th and 95th centiles of arbitrary units [AU]) of *VCAM-1* mRNA in HAEC ($n=3$ independent experiments). C-peptide decreased high glucose-induced *VCAM-1* mRNA expression compared with high glucose alone. * $p<0.05$, $\dagger p=0.008$ vs 5.6 mmol/l

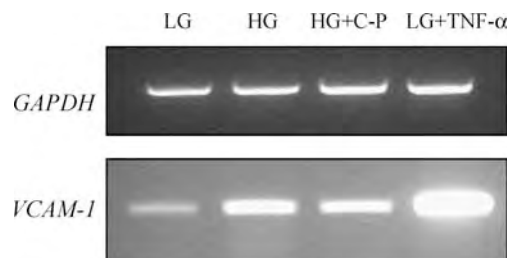


Fig. 2 C-peptide decreases high glucose-stimulated *VCAM-1* mRNA after 4 h. Representative example of RT-PCR to detect *VCAM-1* mRNA expression in HAEC treated for 4 h with: (1) low glucose (LG; 5.6 mmol/l glucose); (2) high glucose (HG; 25 mmol/l glucose); (3) HG+0.5 nmol/l C-peptide (C-P); (4) LG+TNF- α 00 ng/ml). C-peptide reduced high glucose-stimulated *VCAM-1* mRNA in HAEC. *GAPDH* was used as an internal control

in 5.6 mmol/l glucose (average MFI 85.3 ± 2.4 SD) (Fig. 3a). When C-peptide was added to the 25 mmol/l glucose, the cell surface expression of *VCAM-1* decreased (Fig. 3a). Statistical significance was reached with a C-peptide concentration of 0.5 nmol/l (average MFI 80.5 ± 5.5 vs 117.5 ± 25.5 in 25 mmol/l glucose alone; $p<0.01$). The cytokine TNF- α stimulated *VCAM-1* expression (average MFI 305.3 ± 115.5 SD) while heat-inactivated C-peptide did not have a significant effect on *VCAM-1* (average MFI 118.8 ± 23.2 SD).

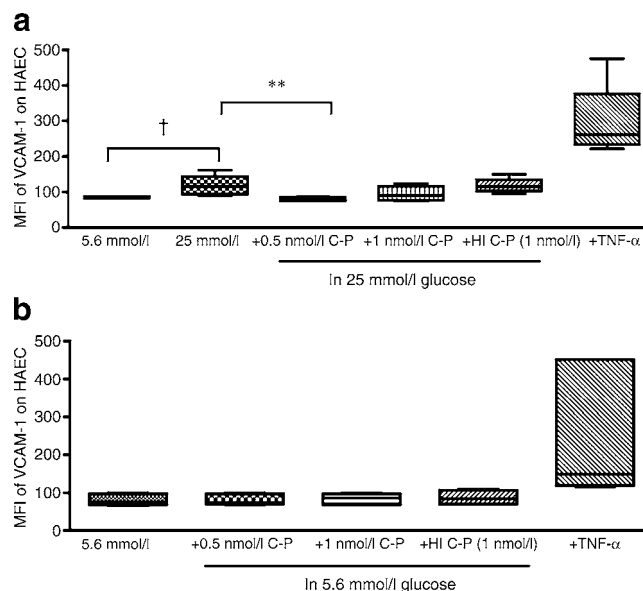


Fig. 3 C-peptide reduces high glucose-stimulated *VCAM-1* protein expression on HAEC. Boxplot graphs showing the median values (limits of the lines are the 5th and 95th centiles) of MFI of *VCAM-1* expression in HAEC exposed to **a** 25 mmol/l glucose or **b** 5.6 mmol/l glucose with and without C-peptide (C-P) for 4 h as determined by flow cytometry ($n=3$ independent experiments). **a** HAEC exposed to 25 mmol/l glucose significantly increased *VCAM-1* expression; $\dagger p=0.03$ vs 5.6 mmol/l. This increase was significantly inhibited by 0.5 nmol/l C-peptide; ** $p<0.01$. No significant changes in *VCAM-1* were observed when C-peptide was added to basal medium containing 5.6 mmol/l glucose (**b**). HI, heat-inactivated

When C-peptide was added to low glucose medium, it did not cause a significant change in VCAM-1 expression (Fig. 3b).

Because C-peptide at a dose of 0.5 nmol/l demonstrated a significant reduction in high glucose-induced VCAM-1 expression, we conducted all further experiments using this dose only.

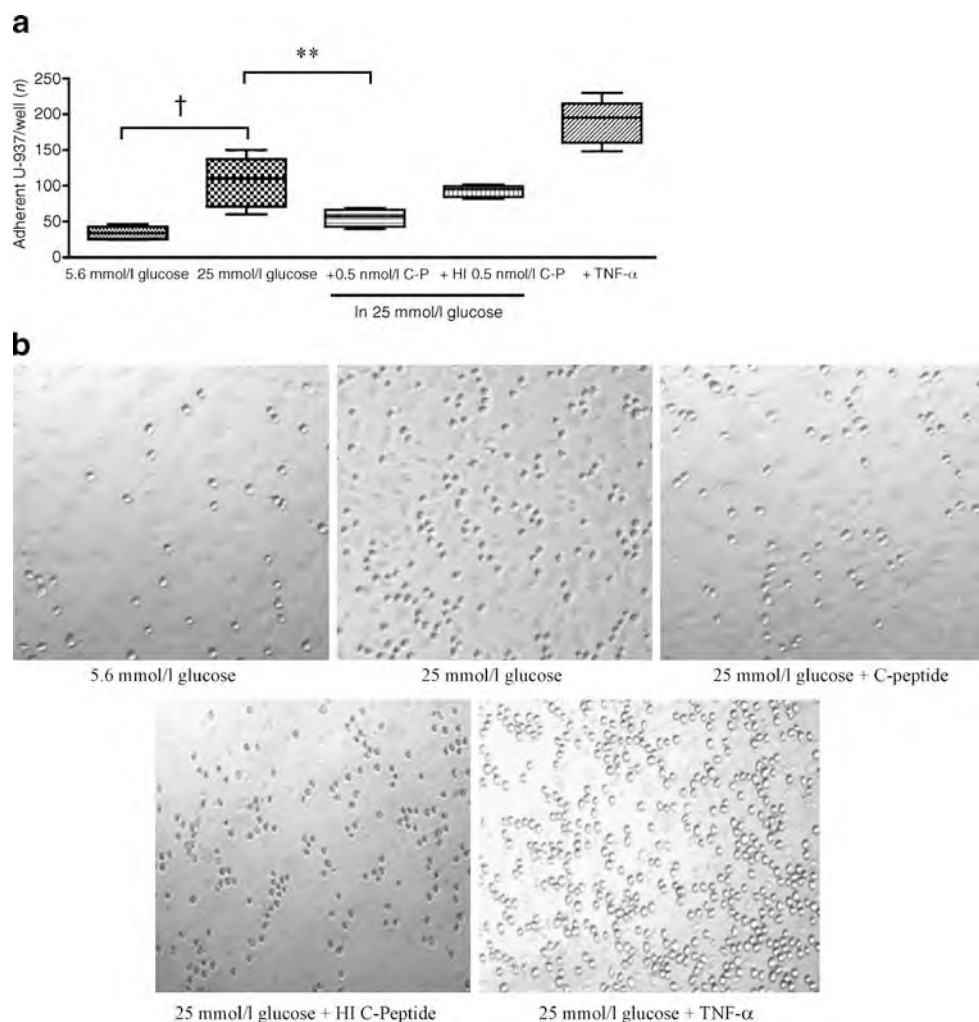
C-peptide inhibits U-937 cells adhesion to endothelial cells To determine whether the C-peptide-induced inhibition of VCAM-1 expression on HAEC was associated with a decrease in U-937 adherence, we assessed U-937 adhesion on HAEC exposed to C-peptide under high glucose conditions. There was a threefold increase in the number of adherent U-937 under high glucose (106 ± 32) as compared with 5.6 mmol/l glucose (34 ± 8 ; $p=0.004$) (Fig. 4a). When C-peptide was added to the high glucose medium, the number of adherent U-937 cells was reduced (56 ± 11 ; $p<0.01$ vs 25 mmol/l glucose; Fig. 4a). Addition of heat-inactivated C-peptide did not significantly alter the number of adherent

cells (93 ± 5) as compared with 25 mmol/l glucose alone. As expected, TNF- α , which activates endothelial cells, produced a fivefold increase in U-937 adherence in comparison with HAEC exposed to normal glucose (Fig. 4a). The adhesion of U-937 to HAEC under the different conditions was also photographed (Fig. 4b).

When we evaluated the adherence of U-937 to HAEC exposed to normal glucose and with C-peptide added, we did not observe a significant change as compared with normal glucose alone (ESM Fig. 1).

C-peptide inhibits IL-8 and MCP-1 secretion by HAEC Another factor possibly affecting U-937 adherence to HAEC in the presence of C-peptide is a downregulation of secreted chemoattractant molecules by endothelial cells. As expected, we found that secretion of IL-8 in the supernatant fraction of high glucose-stimulated HAEC significantly increased (751 ± 99 pg/ml) as compared with normal glucose (592 ± 120 pg/ml; $p=0.04$; Fig. 5a), while addition of C-peptide reduced IL-8 concentrations to levels equivalent to normal

Fig. 4 C-peptide reduces adhesion of U-937 to HAEC in condition of hyperglycaemia. HAEC were cultured in 25 mmol/l glucose in the presence or absence of 0.5 nmol/l of C-peptide (C-P) for 4 h. U-937 were added for 1 h and then counted. **a** Boxplot graphs showing the median values (limits of the lines are 5th and 95th centiles) of number of adherent U-937 per well ($n=3$ sets of independent experiments). High glucose increased the number of adherent U-937; $^\dagger p=0.004$ vs 5.6 mmol/l glucose. Addition of C-peptide reduced the number of adherent U-937; $**p<0.01$ vs 25 mmol/l glucose alone. Heat-inactivated (HI) C-peptide (0.5 nmol/l) did not significantly alter adherence of U-937. TNF- α (10 ng/ml) produced more than a fivefold increase in adherent U-937 in comparison to normal glucose. **b** Photographic view of U-937 adherent to HAEC



glucose ($p<0.05$; Fig. 5b). The heat-inactivated C-peptide control had no significant effect (Fig. 5b). The high glucose-induced IL-8 secretion by HAEC was mediated by NF- κ B activation since treatment with PDTC (10 μ mol/l), an NF- κ B inhibitor, reduced IL-8 secretion to basal levels also detected in normal glucose ($p<0.01$ vs 25 mmol/l glucose; Fig. 5b).

Similarly, when HAEC were stimulated with high glucose for 4 h, secretion of MCP-1 increased (472 ± 89 pg/ml) as compared with normal glucose (329 ± 152 pg/ml; $p=0.01$; Fig. 6a). Addition of C-peptide, decreased MCP-1 concentrations to those found with normal glucose levels ($p<0.05$; Fig. 6b). Here, too, the specific-NF- κ B inhibitor PDTC μ mol/l decreased MCP-1 levels dramatically ($p<0.01$ vs 25 mmol/l glucose; Fig. 6b).

C-peptide added to regular medium containing low glucose failed to significantly modify IL-8 and MCP-1 secretion compared with basal medium without C-peptide (ESM Fig. 2).

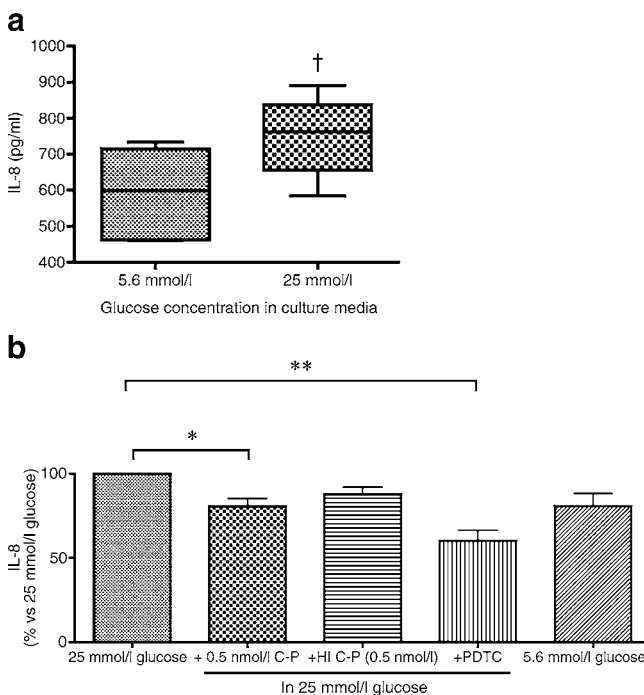


Fig. 5 C-peptide decreases high glucose-stimulated IL-8 secretion by HAEC. HAEC were cultured with high glucose (HG; 25 mmol/l) alone or combined with C-peptide (C-P; 0.5 nmol/l) for 4 h and secretion of IL-8 in the supernatant fraction was assessed by ELISA. **a** Boxplot graph showing the median values (limits of the lines are 5th and 95th centiles) of secreted IL-8 in the supernatant fraction of HAEC exposed to HG compared with low glucose (5.6 mmol/l); $^{\dagger}p=0.04$. **b** Levels of IL-8 in supernatant fraction of C-peptide-treated HAEC expressed as per cent versus the appropriate control at 25 mmol/l. HAEC treated with high glucose+C-peptide showed a decrease in secreted IL-8, approaching levels measured in low glucose; $*p<0.05$ vs high glucose. A significant decrease in secreted IL-8 was also found in the presence of the NF- κ B inhibitor PDTC (10 μ mol/l); $**p<0.01$. The average $\pm$ SD of a set of three independent experiments run in triplicate are shown. HI, heat-inactivated

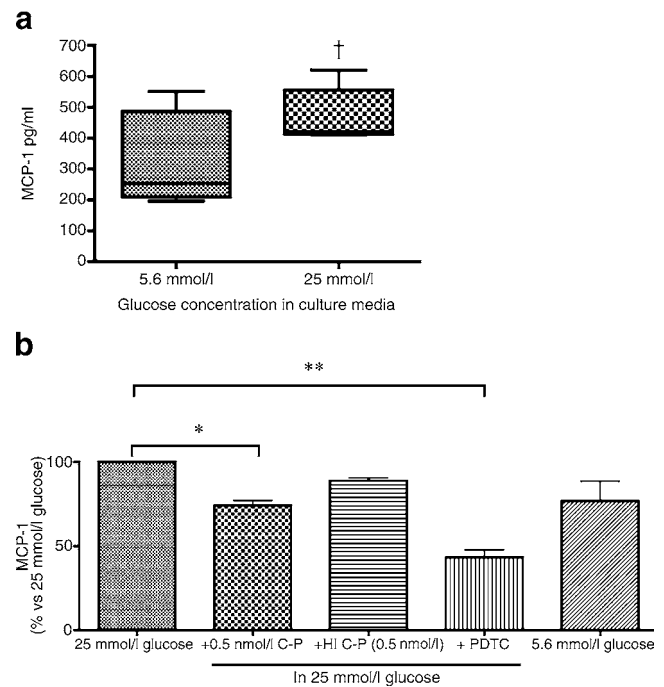


Fig. 6 C-peptide decreases high glucose-stimulated MCP-1 secretion by HAEC. HAEC were cultured with high glucose (HG; 25 mmol/l) alone (**a**) or combined (**b**) with C-peptide (C-P; 0.5 nmol/l) for 4 h and secretion of MCP-1 in the supernatant fraction was assessed by ELISA. **a** Boxplot graph showing the median values (limits of the lines are 5th and 95th centiles) of secreted MCP-1 in the supernatant fraction of HAEC exposed to HG compared with low glucose (5.6 mmol/l); $^{\dagger}p=0.04$. **b** Levels of MCP-1 in supernatant fraction of C-peptide-treated HAEC expressed as per cent versus the appropriate control at 25 mmol/l. When cells were treated with HG+C-peptide the secretion of MCP-1 decreased; $*p<0.05$ vs HG. A dramatic decrease in MCP-1 secretion was obtained with the NF- κ B inhibitor PDTC (10 μ mol/l); $**p<0.01$. The average $\pm$ SD of a set of three independent experiments run in triplicate are shown. HI, heat-inactivated

C-peptide decreases high glucose-induced NF- κ B translocation in HAEC The signal transduction pathway leading to mRNA synthesis of adhesion molecules and chemokines involves activation of NF- κ B. To determine whether C-peptide affected high glucose-induced NF- κ B nuclear translocation in HAEC, immunoblot analysis and NF- κ B-specific ELISAs were performed with nuclear extracts from stimulated-HAEC. As shown in Fig. 7a, exposure of HAEC to 25 mmol/l for 4 h induced an increase in NF- κ B nuclear translocation in comparison to 5.6 mmol/l glucose. A twofold increase in NF- κ B p65 activation was found after 24 h incubation with 25 mmol/l glucose ($p=0.03$ vs 5.6 mmol/l; Fig. 7a). Addition of C-peptide to high glucose decreased NF- κ B p65 nuclear translocation to that found with normal glucose levels, while heat-inactivated C-peptide did not suppress NF- κ B activity (Fig. 7a). Densitometric analysis of this NF- κ B p65 immunoblot demonstrated that C-peptide reduced NF- κ B p65 nuclear translocation by twofold as compared with high glucose alone ($p<0.05$; Fig. 7c).

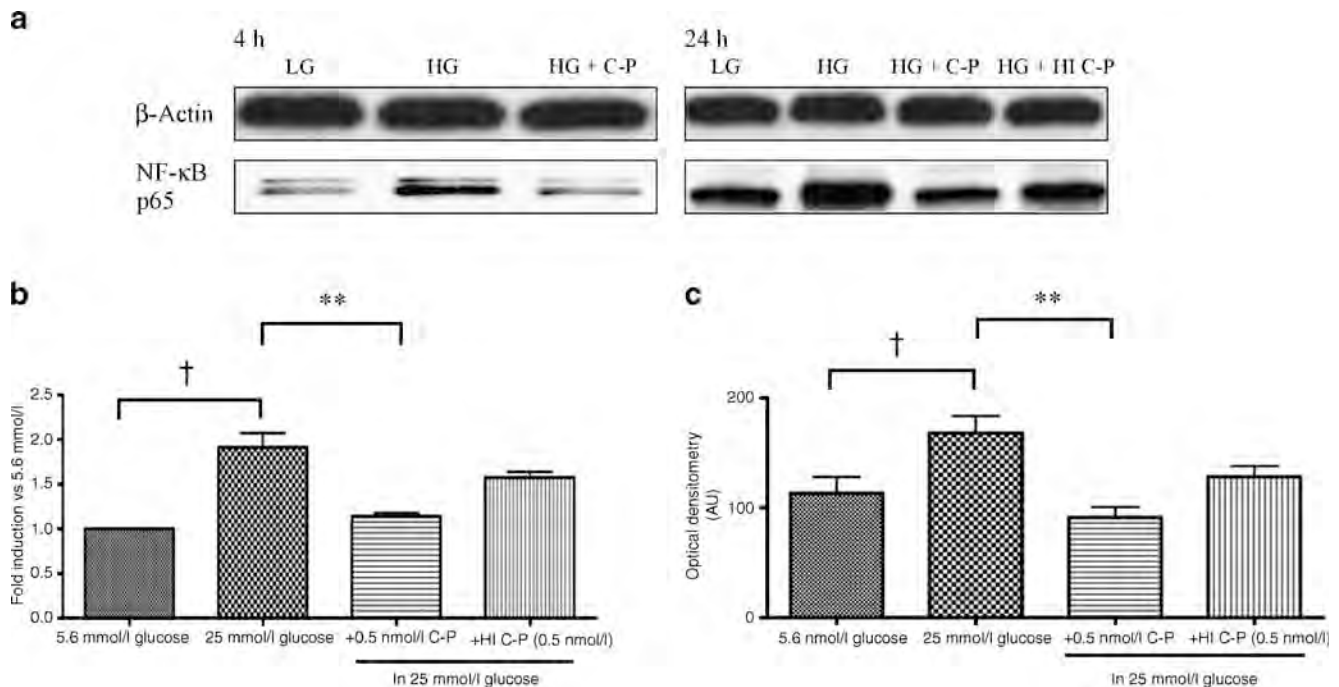


Fig. 7 C-peptide reduces levels of NF- κ B p65/p50 in HAEC cultured in high glucose. HAEC were cultured in low glucose (LG; 5.6 mmol/l) or high glucose (HG; 25 mmol/l) in the presence or absence of 0.5 nmol/l C-peptide (C-P) for 4 and 24 h. Cellular nuclear extracts were subjected to **a** Western immunoblotting to detect the 65 kDa band of the p65 subunit after 4 and 24 h. **b** Detection of the NF- κ B p50 binding activity by ELISA after 24 h. Results are expressed as fold induction of NF- κ B p50 activity in respect to 5.6 mmol/l glucose. In cells exposed to HG there was a twofold increase in NF- κ B p50 nuclear translocation compared with cells in LG; $^{\dagger}p=0.002$. A

decrease in NF- κ B p50 nuclear translocation was observed in the presence of 0.5 nmol/l C-peptide (C-P); $^{**}p<0.01$ vs HG alone. **c** Bar graph showing the densitometric quantification in arbitrary units (AU) of the bands at 24 h. In cells exposed to HG there was a twofold increase in NF- κ B p65 nuclear translocation compared with cells in LG; $^{\dagger}p=0.03$. A decrease in p65 nuclear translocation was observed in the presence of 0.5 nmol/l C-P; $^{*}p<0.05$ vs HG alone. Results are expressed as means $\pm$ SD ($n=3$). Heat-inactivated (HI) C-peptide was used as a control for C-peptide activity in all the experiments

Activation of the NF- κ B p50 subunit in glucose-stimulated HAEC was assessed by ELISA. As shown in Fig. 7b, high glucose significantly induced NF- κ B p50 activity in contrast to normal glucose ($p=0.002$). High glucose-induced NF- κ B p50 binding activity was efficiently ablated by the addition of C-peptide ($p<0.01$).

Discussion

Type 1 diabetes patients have an increased risk of developing atherosclerosis and microvascular complications compared with the non-diabetic population. This risk is in part associated with the difficulty in maintaining euglycaemic conditions even in the context of an appropriate exogenous insulin treatment [29]. Recombinant insulin does not contain C-peptide, a product of insulin protein biosynthesis that exerts beneficial effects on some of the microvascular complications associated with diabetes [30–32].

In this study, we investigated the impact of human C-peptide specifically in the early process of atherogenesis. The few studies available on the topic have tested the effect of C-peptide on low and high glucose-induced proliferative

activities of vascular smooth muscle cells, one major component involved in the formation of atherosclerotic plaque [33, 34]. Here, we wanted to expand upon these studies by evaluating the potential effects of C-peptide on the endothelial cell component of the vessel wall during hyperglycaemia.

The adhesion and migration of circulating monocytes into the subendothelial space is one of the key events in the early stages of atherogenesis [35]. This process is in part regulated by the expression of adhesion molecules, such as VCAM-1, on the surface of endothelial cells [36], and by the release of chemotactic factors, including IL-8 and MCP-1 [17]. We found that in vitro C-peptide exerts an inhibitory effect on high glucose-induced upregulation of the adhesion molecule VCAM-1 on HAEC. C-peptide at the physiological concentration of 0.5 nmol/l reduced high glucose-induced expression of VCAM-1 to basal levels observed under normal glucose conditions. This effect was observed as early as 4 h after C-peptide addition to the high glucose medium and was still detected after 24 h incubation. The decrease in high glucose-induced VCAM-1 expression by C-peptide on HAEC was detected both at the mRNA and protein level. Conversely, when C-peptide was added to the

medium containing normal glucose levels, it failed to significantly reduce VCAM-1 expression. These results are in line with findings from another group demonstrating that C-peptide reduced expression of the adhesion molecules P-selectin and intercellular adhesion molecule-1 on the rat microvascular endothelium during acute endothelial dysfunction *in vivo* [22]. In another model of vascular injury, C-peptide was shown to decrease polymorphonuclear leucocyte infiltration into the myocardium thereby improving cardiac dysfunction [25]. Overall, these data seem to point to an anti-inflammatory effect of C-peptide on the endothelium, especially in conditions of insult. This hypothesis is supported by recent *in vivo* data showing that survival rates of mice following endotoxic shock is improved after C-peptide administration [37]. In these mice, plasma levels of the pro-inflammatory cytokines TNF- α and MCP-1 were also decreased, suggesting a decreased generalised inflammatory response [37]. In the context of type 1 diabetes patients, upregulation of endothelial VCAM-1 and inflammation are early events in the course of the disease [2, 38–40]. These patients are insulin-dependent and take exogenous insulin to manage their blood glucose levels. It might well be that addition of physiological levels of C-peptide to the traditional exogenous insulin therapy could be a means of ‘counteracting’ the insult of high glucose on the endothelial cells of diabetic patients.

Another component of endothelial dysfunction affected by C-peptide is the secretion of IL-8 and MCP-1, chemokines that facilitate leucocyte-endothelial interactions. In support of other investigators [5, 6, 16], we observed an increased secretion of both chemokines in the supernatant fraction of endothelial cells under high glucose. Unique to this study, however, is the finding that C-peptide reduced high glucose-stimulated IL-8 and MCP-1 secretion by endothelial cells to near or below the basal levels measured under normal glucose concentrations. Based on our findings, it seems that C-peptide might exert its most meaningful biological effects on the endothelium in conditions of insult, as C-peptide did not significantly change chemokine secretion by HAEC when added to normal glucose-containing medium. In addition, adhesion of U-937 cells to high glucose-stimulated HAEC decreased after addition of C-peptide, an effect not detected when C-peptide was heat-inactivated. C-peptide at 0.5 nmol/l suppressed U-937 attachment to HAEC exposed to 25 mmol/l glucose by 50% due to a C-peptide-mediated inhibitory effect on VCAM-1, IL-8 and MCP-1 secretion by endothelial cells. Although in this study we focused on the effects of C-peptide on high glucose-induced endothelial dysfunction, another likely cellular target of C-peptide action in diabetes could be the immune cells. Previous studies from our laboratory [41] and others [42] have shown that phenotypic changes suggestive of cellular activation are present in circulating

monocytes of recently diagnosed type 1 diabetes patients. It is tempting to speculate that in conditions of hyperglycaemia and the underlying inflammation typical of diabetes, C-peptide might exert beneficial effects on both endothelial and immune cell dysfunction, thereby decreasing the overall risk of developing vascular lesions. The biological effect of C-peptide on immune cells is currently under investigation in our laboratory.

The mechanisms underlying the effects of C-peptide on the human vasculature, specifically on endothelial cells, are still largely unknown. Nevertheless, the signal transduction pathways that lead to the enhanced expression of genes encoding adhesion molecules and inflammatory cytokine secretion in endothelial cells require translocation of the transcription factor NF- κ B [13]. Therefore, this study investigated C-peptide effects on NF- κ B activation in high glucose-stimulated HAEC. In support of a previous study [5], we confirm that short-term high glucose exposure of endothelial cells stimulates NF- κ B activation. However, our work has moved the paradigm forward by demonstrating that exogenous addition of C-peptide significantly reduced high glucose-induced nuclear translocation of canonical components of NF- κ B, p65 and p50. The suppressive effect on NF- κ B activation and high glucose-induced VCAM-1 expression as well as IL-8 and MCP-1 secretion in HAEC was specific for C-peptide, since heat-inactivated C-peptide was not able to elicit the same phenotype. Although we did not investigate the precise mechanism of action of C-peptide on the inhibition of NF- κ B nuclear translocation in HAEC, evidence of cellular internalisation and binding to intracellular components has been recently demonstrated in Swiss 3 T3 and HEK-293 cells [43]. In the same study, interestingly, C-peptide was also shown to localise within the nuclei [43]. We can therefore speculate that the inhibitory action of C-peptide on NF- κ B activation in HAEC could result from an effect on the phosphorylation of protein substrates in the cytoplasm and/or of a direct interaction of C-peptide with NF- κ B p65/p50 subunits at the nuclear level, preventing DNA binding. In the lung of endotoxin-treated mice, C-peptide inhibited phosphorylation of extracellular signal-regulated kinase-1/2 followed by upregulation of nuclear levels and DNA binding of the nuclear transcription factor peroxisome proliferator-activated receptor- γ , which plays an important role in the modulation of inflammation [37]. Currently, we are exploring which NF- κ B-dependent upstream signalling events are affected by C-peptide in endothelial cells; examples are ROS generation and I κ B kinase, an enzyme that elicits phosphorylation of the cytosolic NF- κ B inhibitor I κ B α . This latter upstream event regulates NF- κ B translocation from the cytoplasm to the nucleus. In vascular smooth muscle cells we found that C-peptide reduced high glucose-induced phosphorylation of I κ B α [44], a pathway likely to be also targeted in HAEC.

Inhibition of NF- κ B would be suggestive of an anti-inflammatory effect of physiological concentrations of C-peptide at the endothelial cell level [19, 22] and would be consistent with a potential anti-atherosclerotic effect in type 1 diabetes. Higher supra-physiological levels of circulating C-peptide, such as those measured in type 2 diabetic patients with the hyperinsulinaemia associated with insulin resistance, might have deleterious effects on the vasculature. This view is supported by Walcher et al. [45], who found that higher concentrations of C-peptide, mimicking those found in the circulation of type 2 diabetic patients, produced maximal stimulation of lymphocyte chemotaxis in vitro. Future studies are required to further elucidate these issues.

Although this evidence in human endothelial cells is reported here for the first time, a protective effect of C-peptide on high glucose-induced vascular dysfunction has been invoked by other groups, who tested the efficacy of C-peptide in small clinical trials of type 1 diabetic patients [30–32, 46]. In addition to endothelial cells, vascular smooth muscle cells also appear to be the target of beneficial effects of C-peptide on the vasculature in conditions of hyperglycaemia [33, 44]. Physiological concentrations of C-peptide attenuate glucose-induced hyperproliferation of vascular smooth muscle cells [33, 44], a phenomenon associated, at least in part, with a specific inhibitory effect on NF- κ B [44].

In conclusion, our findings support the hypothesis that C-peptide has major physiological effects on the inhibition of endothelial dysfunction under high glucose conditions. It does this by interfering with NF- κ B activation and its effect on the reduced production of pro-inflammatory cytokines and chemokines. These findings underscore a role of C-peptide in endothelial cell functions, especially in conditions of diabetic insult to the vasculature. Our results support the idea of prolonged administration of physiological quantities of C-peptide to type 1 diabetes patients in an effort to lessen endothelial dysfunction and complications that may potentially arise during the course of the disease.

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Human proinsulin C-peptide reduces high glucose-induced proliferation and NF- κ B activation in vascular smooth muscle cells

Vincenza Cifarelli¹, Patrizia Luppi^{*,1}, Hubert M. Tse, Jing He, Jon Piganelli, Massimo Trucco

Division of Immunogenetics, Department of Pediatrics, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15213, USA

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Abstract

Excessive proliferation of vascular smooth muscle cells (VSMCs) is one of the primary lesions in atherosclerosis development during diabetes. High glucose triggers VSMC proliferation and initiates activation of the transcription factor nuclear factor (NF)- κ B. Recently, clinical studies have demonstrated that replacement therapy with C-peptide, a cleavage product of insulin, in type 1 diabetic (T1D) patients is beneficial on a variety of diabetes-associated vascular complications. However, the mechanisms underlying the beneficial activity of C-peptide on the vasculature in conditions of hyperglycemia are largely unknown. The effects of C-peptide on the proliferation of human umbilical artery smooth muscle cell (UASMC) and aortic smooth muscle cell (AoSMC) lines cultured under high glucose for 48 h were tested. To gain insights on potential intracellular signaling pathways affected by C-peptide, we analyzed NF- κ B activation in VSMCs since this pathway represents a key mechanism for the accelerated vascular disease observed in diabetes. High glucose conditions (25 mmol/L) stimulated NF- κ B-dependent VSMC proliferation since the addition of two NF- κ B-specific inhibitors, BAY11-7082 and PDTC, prevented proliferation. C-peptide at the physiological concentrations of 0.5 and 1 nmol/L decreased high glucose-induced proliferation of VSMCs that was accompanied by decreased phosphorylation of I κ B and reduced NF- κ B nuclear translocation. These results suggest that in conditions of hyperglycemia C-peptide reduces proliferation of VSMCs and NF- κ B nuclear translocation. In patients with T1D, physiological C-peptide levels may exert beneficial effects on the vasculature that, under high glucose conditions, is subject to progressive dysfunction.

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Keywords: Diabetes; C-Peptide; Smooth muscle cells; Proliferation; NF- κ B

1. Introduction

Patients with type 1 diabetes (T1D) exhibit an increased susceptibility to develop a wide range of vascular complications, including microangiopathy and atherosclerosis, which account for the majority of deaths and disability in diabetic patients [1]. Elevated blood glucose levels (hyperglycemia) are considered one of the major causes of vascular complications in T1D patients [2].

Together with endothelial dysfunction, the proliferation of vascular smooth muscle cells (VSMCs) is one of the characteristic features of human atherosclerosis [3]. Under high

glucose conditions, human, porcine and rat VSMC proliferate [4–6] and migrate from the media to the subendothelial space of the vessel wall where early atherosclerotic lesions are localized [3].

In VSMCs, high glucose initiates the activation of the transcription factor NF- κ B [7], which leads to the transactivation of a number of genes involved in VSMC proliferation [8]. Several studies have pointed to an involvement of the NF- κ B pathway in the process of atherosclerosis by acting at different pathophysiological levels during plaque development. The activated p65 subunit has been found in macrophages, endothelial cells, and VSMCs within human atherosclerotic lesions [9]. Furthermore, administration of anti-sense oligonucleotides to the p65 subunit of NF- κ B blocked human VSMC proliferation [10]. Other studies mostly point to a role of NF- κ B in regulating apoptosis of VSMCs and fine-tuning of the inflammatory response present in the injured vessel wall [11]. Overall, these studies support

* Corresponding author at: Children's Hospital of Pittsburgh, Rangos Research Center, 3460 Fifth Avenue, Pittsburgh, PA 15213, USA.
Tel.: +1 412 692 6570; fax: +1 412 692 5809.

E-mail address: luppi@pitt.edu (P. Luppi).

¹ These two authors contributed equally to this work.

the idea that NF- κ B activation in VSMCs represents a key mechanism for the accelerated vascular disease observed in diabetes. Strategies targeting NF- κ B pathway activation to inhibit VSMC proliferation for the prevention or the treatment of cardiovascular diseases are emerging [12,13].

Despite intensive insulin treatment and well-controlled glucose levels, vascular complications are still common among T1D patients [2]. As well as reduced endogenous insulin, the level of C-peptide is also decreased in the plasma of T1D patients. This peptide is cleaved from proinsulin and released from the pancreas into the circulation in equimolar amounts to insulin. C-peptide was initially believed to have no biological effects apart from its role in insulin biosynthesis. However, recent evidence suggests that C-peptide may have a physiological role on a variety of cell types including the vasculature [14]. Moreover, results from small clinical trials where C-peptide was administered to T1D patients, showed that C-peptide ameliorates renal dysfunction [15], stimulates skeletal muscle microcirculation [16], and improves functional and structural abnormalities in peripheral nerves [15,17]. It was then proposed that C-peptide may represent an important factor in reversing or preventing microvascular damage associated with diabetes. Supporting this hypothesis, was evidence that T1D patients receiving whole pancreas or islet transplantation exhibited improvement in vascular disease in comparison to patients receiving daily insulin injections to control their hyperglycemia [18,19].

Several studies have focused on the direct effects of C-peptide on the vasculature, often with contradicting results. For example, while there seems to be concordance on the vasodilatory properties of C-peptide [20], it is still controversial whether C-peptide exerts pro-atherogenic effects on the vasculature or vice versa [21]. Other observations point instead to a protective role of C-peptide on vascular dysfunction in diabetes. For example, Kobayashi et al. [22] demonstrated that 3 weeks exposure to C-peptide inhibited high glucose-induced hyperproliferation of rat VSMCs. Another group showed that rat C-peptide inhibits leukocyte–microvascular endothelium interaction *in vivo* [23].

This study evaluated (1) the effects of short-term exposure of C-peptide on high glucose-induced proliferation of human VSMCs *in vitro*, and (2) determined whether the NF- κ B pathway is involved in the intracellular signaling events associated with C-peptide in VSMCs, since to date, no data identifies or excludes NF- κ B signaling as a molecular event associated with C-peptide effects on VSMCs under high glucose conditions.

2. Materials and methods

2.1. Cell culture

Human umbilical artery smooth muscle cell (UASMC) and human aortic smooth muscle cell (AoSMC) were obtained from Cambrex (Cambrex Bio Science, Walkersville,

Inc., USA) and grown into 75 cm² cell culture flasks (Corning Incorporated, NY, USA) at 37 °C, 5% CO₂ in the presence of smooth muscle cell basal medium-2 (SMGM-2) (Cambrex Bio Science) additioned with 5% of FBS, 0.1% of antibiotics GA-1000 (gentamicyn, amphotericin B), 0.2% of human basic fibroblastic growth factor (hFGF-b), 0.1% insulin, and 0.1% of human epidermal growth factor (hEGF) (Cambrex Bio Science). Cells were used at passage 4–10 for the experiments.

2.2. Treatment conditions

SMGM-2 containing 25 mmol/L glucose (Sigma Chemical S. Louis, MO, USA) was used as a high glucose condition in all the experiments, while basal SMGM-2, which contains 5.6 mmol/L glucose, was used as normal glucose control. In all experiments, cells were serum-deprived for 24 h with SMGM-2 containing 1% FBS and 0.1% antibiotics GA-1000. Cells were then replaced with SMGM-2 containing 25 mmol/L glucose in the presence or absence of human C-peptide (0.5 and 1 nmol/L) (Sigma Chemical) for 48 h. Basal SMGM-2 containing 5.6 mmol/L glucose was used as control. Insulin was not added to the culture medium. The effects of two different NF- κ B-inhibitors, such as (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile (Bay11-7082; Sigma) (1 μ M) and pyrrolidine dithiocarbamate (PDTc, Sigma) (20 μ M) were also tested in VSMCs. These NF- κ B-inhibitors were added to SMGM-2 with 25 mmol/L glucose for 48 h. Separate sets of experiments were performed in which C-peptide (0.5 and 1 nmol/L) was added to medium containing normal glucose. Purity of C-peptide was $\geq$ 95% as assessed by the manufacturer by HPLC evaluation. C-peptide concentrations were chosen on the basis of previous reports demonstrating that specific binding of C-peptide to human cell membranes reaches full saturation at low physiological concentrations (0.9 nmol/L) [24]. Human scrambled C-peptide (Sigma-Genosys, Texas, USA) was used as a control. The scrambled C-peptide has an identical amino acid composition to that of the human C-peptide, but the sequence was randomized [23]. Scrambled C-peptide was filtered with 0.2 μ m filters (Supor Membrane, Life Sciences, Corning) to decrease any chance of bacterial contamination.

3. Cell proliferation assays

3.1. BrdU measurement of proliferation

Proliferation of VSMCs was evaluated by uptake of bromo-2'-deoxy-uridine (BrdU) using an ELISA kit (Roche Diagnostic, Mannheim, Germany) following manufacturer's instructions. Briefly, UASMC and AoSMC were seeded (6×10^3 cells/100 μ L) in 96-well flat-bottomed plate (FALCON® Becton Dickinson and Company, Franklin Lakes, NJ) and kept them at 37 °C, 5% CO₂ overnight. Cells were then exposed to the indicated treatment conditions in the

presence of 10 $\mu\text{mol/L}$ of BrdU. Each condition was tested in triplicate. Results were expressed as proliferative response (fold induction vs. 5.6 mmol/L) and final data averaged (mean values $\pm$ S.D.).

3.2. Cell proliferation by cell counting

33,000 UASMC/well were seeded in 6-well plates (Corning Incorporated) and left overnight in an incubator at 37 °C, 5% CO₂. Cells were then serum-deprived and exposed to the indicated treatment conditions and then counted using a Neubauer Hemocytometer (Hausser Scientific, Horsham, PA), as previously published [4,25].

3.3. UASMC Ki-67 staining

As another technique to assess VSMC proliferation, we used Ki-67 immunofluorescence staining for proliferating cells. UASMC were seeded into a glass bottom culture dish (35 mm diameter, poly-d-lysine coated) (MatTek Corporation, Ashland, MA) (~10,000 cells/dish) and then cultured following treatment conditions. Cells were permeabilized with 0.02% of Triton-X100 (Sigma), fixed with 2% paraformaldehyde (PFA) (USB Corporation, Cleveland, OH, USA) and stained with a monoclonal mouse anti-human Ki-67 primary antibody overnight (1:100, DakoCytomation, Denmark). A carboxymethylindocyanine 3(Cy3)-conjugated goat anti mouse secondary antibody (1:500, Jackson Immuno Research Lab INC. West Grove, PA) was then added for 1 h. DAPI stain (Molecular Probes) was used to stain the nuclei. Images were recorded with a confocal laser-scanning microscope (Nikon, Eclipse E800, Japan). The number of Ki67⁺ cells was counted in five different fields per dish by one of the investigator blind to the study. A Ki67-labeling index was calculated by dividing the number of Ki67⁺ cells by the total number of cells in each of the 5 fields. An averaged Ki-67-labeling index ($\pm$ S.D.) was then obtained for each section. Four different independent experiments were performed.

4. NF- κ B analysis assays

4.1. Nuclear and cytoplasmic extracts

VSMCs were cultured in SMGM-2 following treatment conditions and then pretreated with 25 μl of protease inhibitor cocktail (Pierce, Rockford, IL). Nuclear and cytoplasmic fractions were separated using NE-PER[®] Nuclear and Cytoplasmic extraction kit (Pierce). Protein concentration was measured using a BCA assay (Pierce).

4.2. Immunoblotting

NF- κ B p65 immunoblots were performed as previously described [26] with 10 μg of nuclear protein extracts. Densitometry was performed with UN-SCAN-IT gel software

(Silk Scientific, Orem, UT). I κ B- α and p-I κ B- α immunoblots were performed with 10 μg of cytoplasmic protein extracts [26]. A minimum of three independent experiments was performed.

4.3. NF- κ B p50 ELISA

Activation of the NF- κ B p50 subunit was detected on 3 μg of nuclear extracts using a EZ-Detect[™] Transcription Factor Kit (Pierce Biotechnology).

4.4. Determination of NF- κ B p65 translocation by immunofluorescence

UASMC were seeded into a glass bottom culture dish (~10,000 cells/dish) (MatTek Corporation). After the indicated treatments, cells were fixed with 2% PFA (USB Corporation), and stained with a rabbit polyclonal primary antibody against NF- κ B p65 (1:150, Santa Cruz Biotechnology, Inc.) at 4 °C overnight. A 3(Cy3)-conjugated goat anti-rabbit secondary antibody (1:500, Jackson Immuno Research Lab Inc.) was then added for 1 h, in the dark, at room temperature. DAPI stain (Molecular Probes) was used to stain the nuclei. Fluorescence staining was evaluated using Nikon, Eclipse E800 epifluorescence microscope connected to a digital camera and interfaced with a computer.

5. Statistical analysis

Results from each experiment were averaged and expressed as mean $\pm$ S.D. Comparisons between 5.6 and 25 mmol/L glucose conditions were performed by paired Student's *t*-test. ANOVA followed by Dunnett's post hoc test was applied to evaluate differences between 25 mmol/L glucose, C-peptide, and NF- κ B inhibitors. A *p*-value of *p* < 0.05 was considered statistically significant.

6. Results

6.1. C-peptide decreases high glucose-induced proliferation of VSMCs

In Fig. 1A, we examined the influence of high glucose on UASMC proliferation by measuring the nuclear incorporation of BrdU (DNA synthesis). We found that exposure of UASMC for 48 h to 25 mmol/L glucose significantly increased BrdU incorporation compared with control cells exposed to 5.6 mmol/L glucose (*p* = 0.002). Administration of C-peptide at a dose of 0.5 (*p* < 0.01), and 1 nmol/L (*p* < 0.01) for 48 h significantly suppressed high glucose-induced increase in BrdU incorporation in UASMC compared to untreated and scrambled C-peptide-treated cells (Fig. 1A). The addition of specific NF- κ B inhibitors PDTC or BAY11-7082 under high glucose conditions (25 mmol/L)

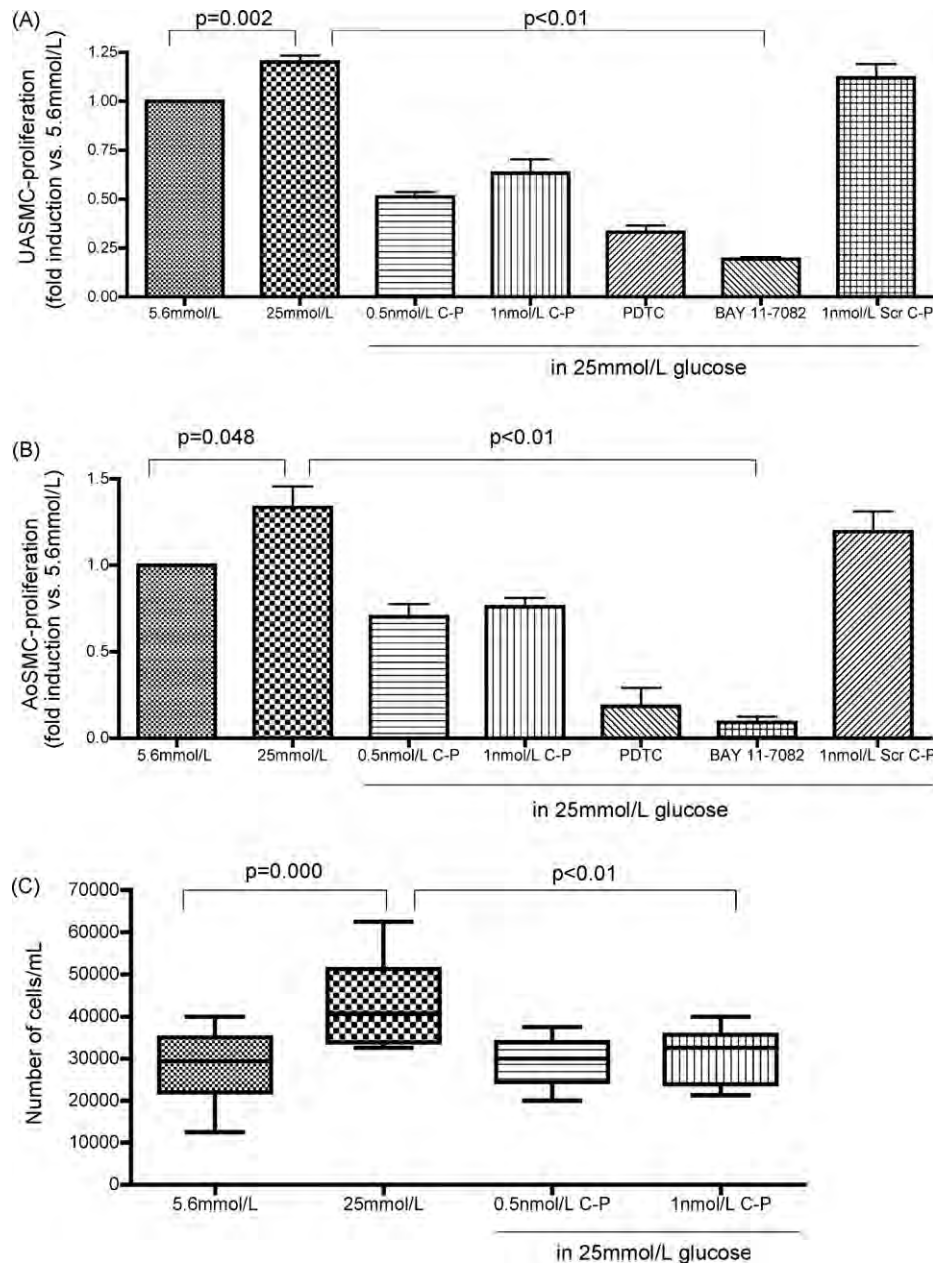


Fig. 1. C-peptide reduces high glucose-induced proliferation of VSMCs. VSMCs were incubated with 25 mmol/L glucose in the presence or in the absence of C-peptide (C-P) for 48 h and assayed for proliferation. In (A), BrdU incorporation shows cellular proliferation in high glucose ($p = 0.002$ vs. 5.6 mmol/L). C-peptide reduced high glucose-induced UASMCM proliferation ($p < 0.01$), while addition of scrambled C-peptide (Scr C-P) did not have any significant effect. Addition of the NF- κ B inhibitors PDTC (20 μ M) and BAY-11078 (1 μ M) also showed a decrease in proliferation ($p < 0.01$ vs. 25 mmol/L glucose). In (B), BrdU incorporation in AoSMCM demonstrates that high glucose-stimulated cellular proliferation ($p = 0.048$ vs. 5.6 mmol/L). C-peptide significantly reduced high glucose-induced UASMCM proliferation ($p < 0.01$ vs. 25 mmol/L), while addition of scrambled C-peptide (Scr C-P) did not have any effect. Addition of PDTC (20 μ M) and BAY-11078 (1 μ M) also showed a decrease in proliferation ($p < 0.01$ vs. 25 mmol/L glucose). Values are mean $\pm$ S.D. of 10 different experiments run in triplicate. In (C), boxplot graphs showing the median values (limits of the lines are 5th and 95th centiles) of number of UASMC counted with a hemocytometer ($n = 10$ experiments) (on the Y-axis). Cell number increased with 25 mmol/L glucose compared to 5.6 mmol/L glucose ($p = 0.000$), while 0.5 and 1 nmol/L C-peptide reduced high glucose-induced UASMC proliferation ($p < 0.01$).

significantly suppressed UASMC proliferation ($p < 0.01$ vs. 25 mmol/L glucose) (Fig. 1A).

We also tested the effect of C-peptide on the proliferative response of human AoSMC. Similarly to UASMC, exposure to 25 mmol/L glucose for 48 h increased cell proliferation compared to cells exposed to 5.6 mmol/L glucose ($p = 0.048$)

(Fig. 1B). We observed a decrease in BrdU incorporation when AoSMC were cultured for 48 h with 25 mmol/L glucose in the presence of 0.5 nmol/L ($p < 0.01$) and 1 nmol/L ($p < 0.01$) C-peptide (Fig. 1B) and the addition of scrambled C-peptide had no effect (Fig. 1B). The NF- κ B inhibitors PDTC or BAY11-7082 added to the medium containing

25 mmol/L glucose significantly inhibited AoSMC proliferation ($p < 0.01$) (Fig. 1A).

The increase in high glucose-induced VSMCs cellular proliferation was also confirmed by counting total cell numbers with a hemocytometer (Fig. 1C). UASMC cultured under high glucose (25 mmol/L) increased in number (42625 ± 9672) compared to normal glucose conditions (5.6 mmol/L) (28000 ± 8337) ($p = 0.000$) (Fig. 1C). The addition of C-peptide at concentrations of 0.5 nmol/L ($p < 0.01$) or 1 nmol/L ($p < 0.01$) to high glucose-treated cells, restored cell numbers to the same level as normal glucose conditions (Fig. 1C).

In contrast, when we cultured UASMCs with regular medium (containing 5.6 mmol/L of glucose) in the presence of C-peptide we detected increased BrdU incorporation as compared to regular medium in the absence of C-peptide ($p < 0.05$) (Fig. 2A). We did not detect a significant increase in BrdU incorporation in AoSMC exposed to regular medium in the presence of C-peptide (Fig. 2B).

6.2. C-peptide decreases the number of Ki67⁺ UASMC exposed to high glucose

As an additional indication of cellular proliferation, immunofluorescence staining of UASMC for the nuclear marker Ki67 was performed under normal (5.6 mmol/L)

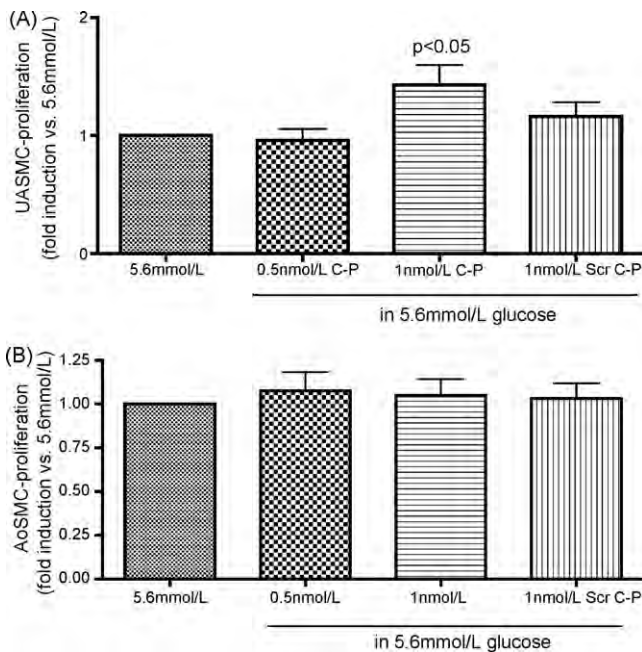


Fig. 2. C-peptide stimulates proliferation of VSMCs under normal glucose. VSMCs were incubated with 5.6 mmol/L in the presence or in the absence of C-peptide (C-P) for 48 h and assayed for proliferation. In (A), BrdU incorporation in UASMC shows increased proliferation in the presence of 1 nmol/L C-peptide ($p < 0.05$ vs. 5.6 mmol/L glucose), while addition of scrambled C-peptide (Scr C-P) did not have any significant effect. In (B), BrdU incorporation in AoSMC shows no significant effect of C-peptide on cellular proliferation under normal glucose conditions. Values are mean $\pm$ S.D. of three different experiments run in triplicate.

and high glucose (25 mmol/L) (Fig. 3A) conditions with or without C-peptide (0.5 nmol/L) treatment. We observed a significant increase in Ki67⁺ cells under high glucose conditions ($p = 0.01$ vs. 5.6 mmol/L), while addition of C-peptide reduced Ki67⁺ cell number ($19.8\% \pm 2.88$) in comparison to cells exposed to 25 mmol/L glucose only ($26.8\% \pm 2.91$) ($p = 0.02$) (Fig. 3A and B). The increase in Ki67⁺ cells from UASMC grown under high glucose conditions was mediated by NF- κ B activation since treatment with PDTC (20 μ M), an NF- κ B inhibitor, reduced the number of Ki67⁺ cells to basal levels detected in normal glucose conditions ($p < 0.01$) (Fig. 3A and B).

6.3. Effect of C-peptide on high glucose-induced NF- κ B activation

To determine if high glucose-induced proliferation of UASMC was associated with activation of the NF- κ B signaling pathway, immunoblot analysis and NF- κ B-specific ELISAs were performed with nuclear extracts from stimulated-UASMC. As shown in Fig. 4A, high glucose (25 mmol/L) induced an increase in NF- κ B p65 nuclear translocation in comparison to normal glucose (5.6 mmol/L) stimulated UASMC. The addition of C-peptide to the high glucose cultures decreased NF- κ B p65 nuclear translocation to normal glucose levels (Fig. 4A), while the addition of scrambled C-peptide did not suppress NF- κ B activity (Fig. 4A). Densitometry of the NF- κ B p65 immunoblot demonstrated that high glucose-stimulated NF- κ B p65 nuclear translocation ($p = 0.038$ vs. normal glucose) and that the addition of C-peptide (1 nmol/L) during high glucose exposure reduced NF- κ B p65 nuclear translocation by 2-fold as compared to high glucose alone ($p < 0.05$) (Fig. 4B). NF- κ B activation was also assessed with a NF- κ B p50-specific ELISA with nuclear extracts from glucose-stimulated UASMCs, as shown in Fig. 4C. High glucose-induced a significant increase in NF- κ B p50 binding activity in contrast to normal glucose ($p = 0.02$) and was efficiently ablated by the addition of C-peptide ($p < 0.05$).

Similar results were obtained with AoSMC, as shown in Fig. 5. While NF- κ B p65 nuclear translocation increased under exposure to high glucose, addition of C-peptide to the high glucose medium reduced NF- κ B activation to basal levels observed under 5.6 mmol/L glucose (Fig. 5).

Nuclear translocation of NF- κ B p65 was also determined by immunofluorescence staining. UASMC cultured in 25 mmol/L glucose resulted in an increase in NF- κ B p65 nuclear translocation as demonstrated by the intense green fluorescence localized in the cell nuclei (Fig. 6) and also from superimposing photomicrographs of DAPI-stained nuclei (blue) (Fig. 6B) with green fluorescence (Fig. 6C). Cells in normal glucose (5.6 mmol/L) retained NF- κ B p65 in the cytoplasm (green fluorescence) with very little staining observed in the nuclei (Fig. 6A). The addition of C-peptide (0.5 nmol/L) to high glucose-treated cells prevented NF- κ B

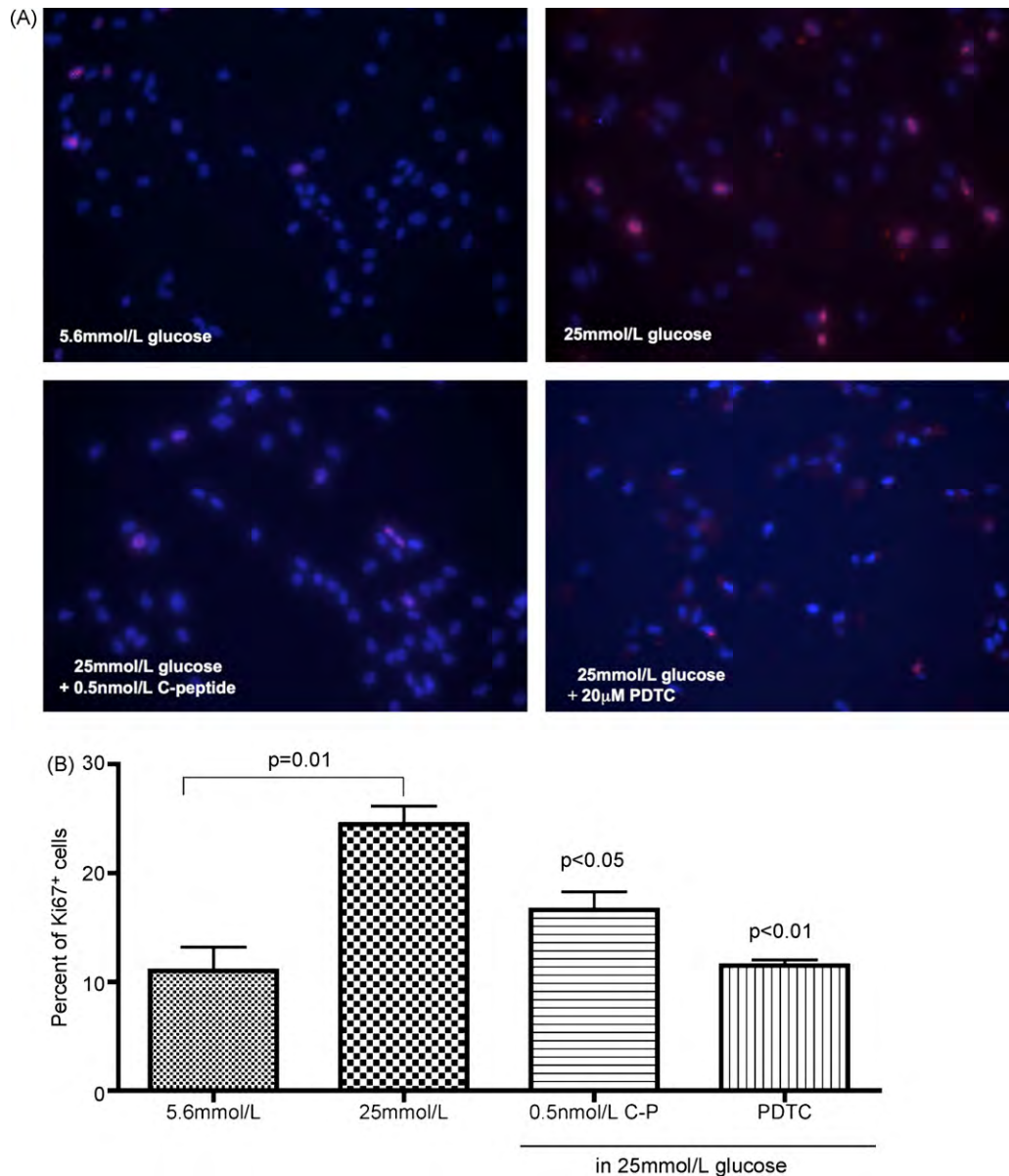


Fig. 3. C-peptide reduces the number of Ki67⁺ cells. In (A), images of Ki-67-immunostaining (in red) in UASMC exposed for 48 h to the different conditions, as stated. DAPI staining was used to stain the nuclei (in blue). C-peptide addition to the high glucose medium reduced Ki67⁺ cell number. In (B), quantitation of Ki67⁺ cells. Bar graph shows percent of Ki-67⁺ cells (mean $\pm$ S.D.) compared to DAPI staining (blue) from five random fields of four independent experiments. Exposure of UASMC to 25 mmol/L glucose increased number of Ki-67⁺ cells compared to normal glucose ($p = 0.01$), while addition of C-peptide significantly reduced the number of Ki-67⁺ proliferating cells ($p < 0.05$ vs. 25 mmol/L glucose). Addition of the NF- κ B inhibitor PDTC to the high glucose medium also reduced the number of Ki-67⁺ cells ($p < 0.01$ vs. 25 mmol/L glucose).

p65 nuclear translocation (Fig. 6A), an effect not observed with scrambled C-peptide.

The mechanism underlying NF- κ B nuclear translocation from the cytoplasm to the nucleus is based on the phosphorylation of I κ B α . We therefore investigated the effects of C-peptide on high glucose-induced phosphorylation of I κ B α by Western blotting on cytoplasmic extracts from UASMC (Fig. 7). As expected, an increase in the level of phosphorylated I κ B α (p-I κ B α) was observed in the cytoplasmic extracts after UASMC treatment (48 h) with 25 mmol/L glucose as compared to UASMC cultured in low glucose (5.6 mmol/L)

(Fig. 7). Addition of C-peptide to the high glucose medium caused a decrease in the level of p-I κ B α as compared to cells exposed to high glucose in the absence of C-peptide (Fig. 7).

7. Discussion

T1D patients have an increased risk of developing atherosclerosis compared to the non-diabetic population with lesions marked by endothelial dysfunction and exacerbated VSMC proliferation. Proliferating VSMCs migrate

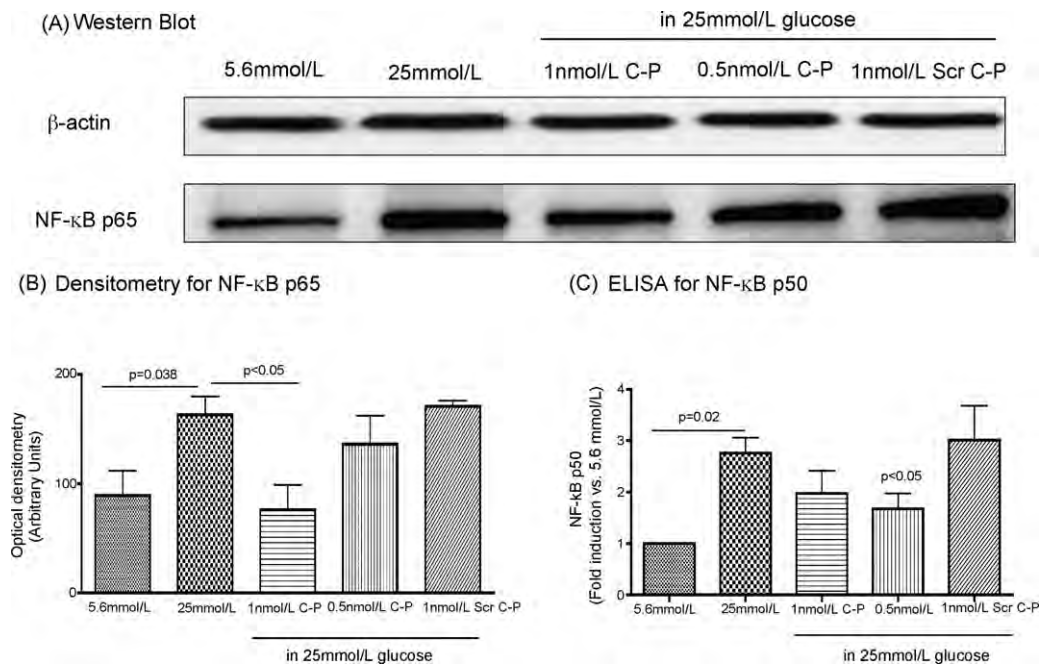


Fig. 4. Expression of p65 subunits of NF- κ B in UASMC cultured in high glucose in the presence of C-peptide. UASMC were cultured in 5.6 mmol/L or 25 mmol/L glucose in the presence or absence of C-peptide (C-P) for 48 h. Cellular nuclear extracts were subjected to Western immunoblotting to detect p65 levels using a specific antibody (1:1,000). Scrambled C-peptide (Scr C-P) was used as control. In (A), representative immunoblot depicting the 65-kDa band of the p65 subunit. To show equal loading of the gel, staining for β -actin is also shown. In (B), bar graph showing the densitometric quantitation of the bands ($n = 4$ different experiments). There is a significant increase in NF- κ B p65 nuclear translocation in cells under 25 mmol/L glucose ($p = 0.038$ vs. 5.6 mmol/L) that is reduced with addition of C-peptide ($p < 0.05$). Results are means $\pm$ S.D. In (C), NF- κ B binding activity of the p50 subunit was examined using a EZ-DetectTM Transcription Factor kit (Pierce Biotechnology). Results were expressed as fold induction of NF- κ B p50 activity respect to control at 5.6 mmol/L. High glucose increased NF- κ B p50 activation as compared to normal glucose ($p = 0.02$). This activation is decreased by addition of C-peptide to the high glucose medium ($p < 0.05$ vs. 25 mmol/L glucose alone). Means $\pm$ S.D. of six independent experiments are shown.

from the media into early atherosclerotic lesions, secrete pro-inflammatory mediators, up-regulate cell adhesion molecules, and promote synthesis of matrix molecules required for the retention of lipoproteins [3]. In advanced human lesions, VSMCs and their secreted product constitutes up to 70–80% of the content of the atherosclerotic plaques. Finally, there is evidence suggesting that VSMCs may also be important for the stability of the atherosclerotic plaque through a formation of a firm fibrous cap [27]. Thus, the involvement of numerous VSMC functional abnormalities in

diabetes, warrant the control of their proliferation to prevent diabetic complications.

In this study, we show that short-term exposure to physiological concentrations of C-peptide inhibited excessive proliferation of UASMC and AoSMC induced by high glucose *in vitro*. Although this evidence in human VSMC is reported here for the first time, a suppressive effect of C-peptide on glucose-induced proliferation of VSMCs has been previously described by Kobayashi et al. in a rat aortic smooth muscle cell line [22]. Our and Kobayashi's results support

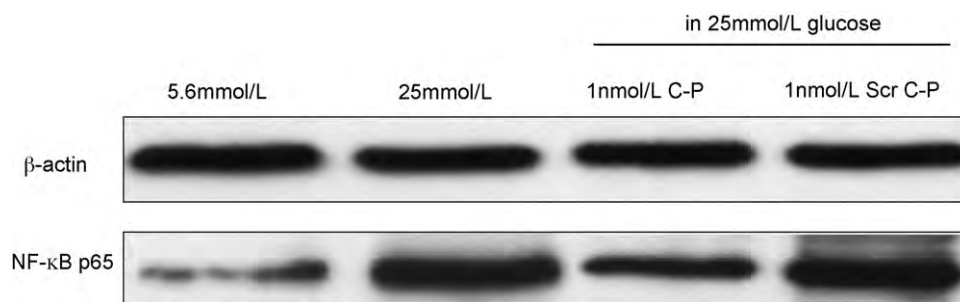


Fig. 5. Expression of p65 subunits of NF- κ B in AoSMC cultured in high glucose in the presence of C-peptide. Representative immunoblot (of three independent experiments) depicting the 65-kDa band of the p65 subunit in AoSMC cultured in 5.6 mmol/L or 25 mmol/L glucose in the presence or absence of C-peptide (C-P) for 48 h. Cellular nuclear extracts were subjected to Western immunoblotting to detect p65 levels using a specific antibody (1:1,000). AoSMC cultured in high glucose in the presence of C-peptide showed a decreased NF- κ B nuclear translocation as compared to high glucose alone. Scrambled C-peptide (Scr C-P) was used as control. To show equal loading of the gel, staining for β -actin is also shown.

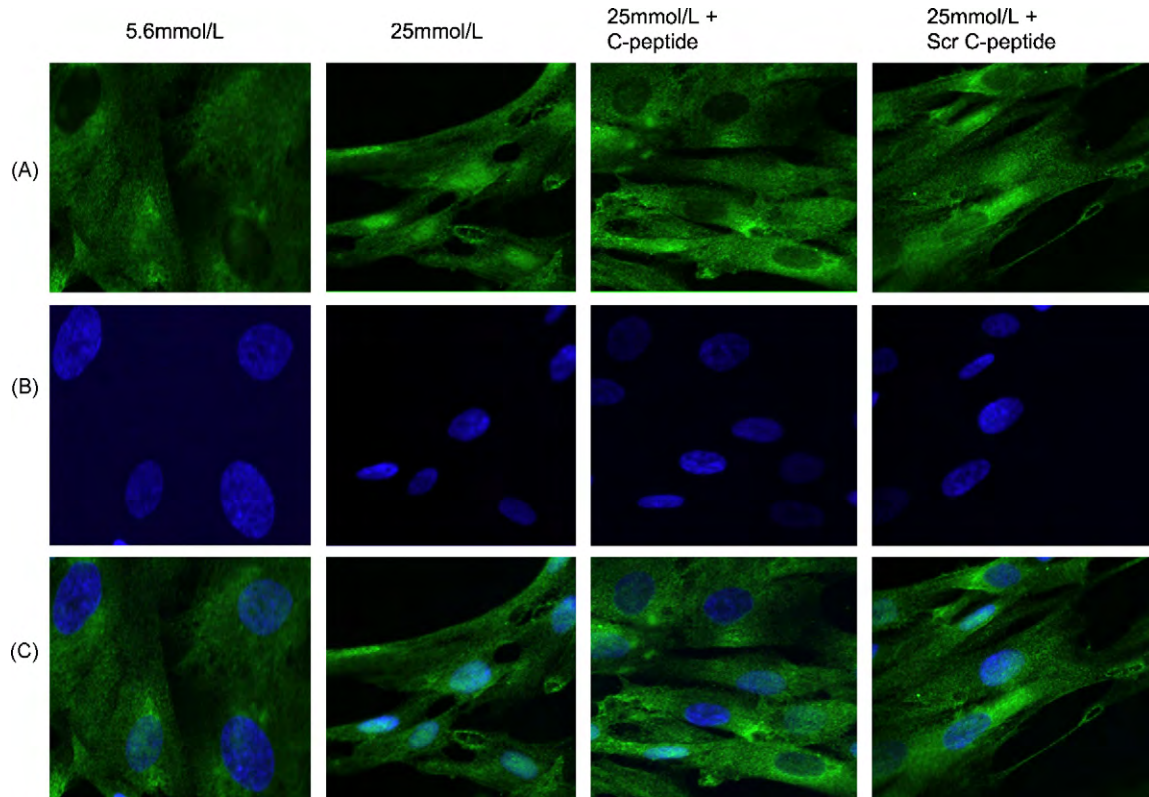


Fig. 6. C-peptide treatment reduces high glucose-induced nuclear translocation of NF- κ B p65 subunit in UASMC. UASMC were serum-starved for 24 h and then treated for 48 h with (I) normal glucose; (II) high glucose; (III) high glucose in the presence of either 0.5 nmol/L C-peptide or 0.5 nmol/L scrambled C-peptide (Scr). In (A), localization of NF- κ B immunostaining using a monoclonal antibody against the p65 subunit (green fluorescence); (B) UASMC nuclei stained with DAPI; (C) composite images generated by superimposing photographs in A and B. As shown in (A), the green fluorescence corresponding to the p65 subunit was localized mostly in correspondence of the nuclei when cells were treated with 25 mmol/L glucose. This was clearly shown by superimposing the DAPI nuclear staining (in B) with the green fluorescence. On the contrary, UASMC treated with C-peptide showed green fluorescence mostly localized in the cytoplasm, rather than in the cell nuclei. Three independent experiments were performed, and one representative photomicrograph sets (30 $\times$) is shown.

the view that physiological concentrations of C-peptide may exert a protective action on VSMCs in conditions of hyperglycemia by targeting the excessive VSMC proliferation. This effect might be specific to conditions of hyperglycemia, as it was not detected under normal glucose. In fact, VSMC cultured in normal glucose in the presence of C-peptide showed an increased proliferation, a result also reported by Walcher et al. [28]. Based on these findings, it is tempting

to speculate that C-peptide effects on proliferative activities of VSMCs *in vitro* is dependent on glucose concentrations in the culture medium, with stimulatory activity under normal glucose and inhibitory one in conditions of hyperglycemia.

Another factor that could affect C-peptide's effect on proliferation of VSMCs *in vitro*, is its concentration in the culture medium. In fact, one possible scenario is that lower, physiological, concentrations of C-peptide produce a beneficial

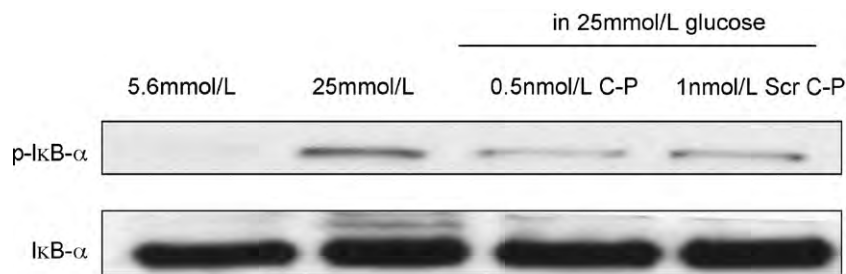


Fig. 7. Inhibitory effect of C-peptide on phosphorylation of I κ B α protein in UASMC. UASMC were serum-starved for 24 h and then treated for 48 h with: (1) 5.6 mmol/L glucose; (2) 25 mmol/L glucose; (3) 25 mmol/L glucose + 0.5 nmol/L C-peptide (C-P); and (4) 25 mmol/L glucose + 1 nmol/L C-P. Cellular cytoplasmic extracts were subjected to Western immunoblotting to detect phosphorylated I κ B α (p-I κ B α) using a specific antibody. In this figure, it is shown a representative immunoblotting depicting decreased phosphorylation of I κ B α (p-I κ B α) with C-peptide. Immunoblot for total I κ B α is also shown. Similar results were obtained in at least two independent experiments.

effect on the vasculature [29], while higher levels of circulating C-peptide, such as those measured in type 2 diabetic patients with hyperinsulinemia associated with insulin resistance, produce deleterious effects on the vasculature. In support of this view is the study by Walcher et al. [21], who found that higher concentrations of C-peptide, mimicking those found in the circulation of type 2 diabetic patients, produced maximal stimulation of lymphocyte chemotaxis *in vitro*. Future studies are required to further elucidate these issues.

The mechanisms underlying the effects of C-peptide on the human vasculature, especially in conditions of hyperglycemia, are still largely unknown. This study investigated C-peptide effects on the NF- κ B pathway, since it is known that NF- κ B activation in VSMCs represents a key mechanism for the accelerated vascular disease observed in diabetes. In support of a previous study [7], we confirm that high glucose stimulates NF- κ B activation in serum-deprived VSMCs, but our work uniquely demonstrates that exogenous addition of C-peptide significantly reduced high glucose-induced nuclear translocation of NF- κ B p65 and p50 in both UASMC and AoSMC. Consistently, C-peptide reduced high glucose-induced phosphorylation of I κ B α in VSMCs, an upstream signaling event that regulates NF- κ B translocation from the cytoplasm to the nucleus.

The suppressive effect of C-peptide on the NF- κ B pathway during conditions of hyperglycemia may represent the underlying mechanism for the inhibitory effect of C-peptide on VSMC proliferation. The involvement of NF- κ B in VSMC proliferation in high glucose conditions is demonstrated by the fact that addition of PDTC and BAY 11-7082, two specific NF- κ B inhibitors, abolished UASMC and AoSMC proliferation *in vitro*. These results are in apparent contrast with the work of Kitazawa et al. [30] who detected a stimulatory activity of C-peptide on the NF- κ B pathway in Swiss 3T3 fibroblasts *in vitro*. The reason for the discrepancy between these two studies could lie on the different experimental design and cell types used. In fact, while Kitazawa et al. investigated fibroblast grown in low glucose conditions, our experiments were carried out in VSMC lines exposed to high glucose.

Identification of signal transduction pathways involved in C-peptide functions on VSMCs may have therapeutic implication for the treatment or the prevention of vascular lesions in diabetic patients. It is well known that vascular dysfunction and generalized monocyte activation are very early phenomenon during the progression of T1D, even in patients with recent onset diabetes and in high-risk individuals in the preclinical phase [31]. Our current study emphasize the idea that C-peptide in replacement doses to T1D patients, who lack endogenous C-peptide and are at risk of developing episodes of hyperglycemia, may be beneficial in the prevention and/or progression of vascular compromise associated with diabetes. A beneficial effect of C-peptide replacement therapy has already been demonstrated by clinical studies on T1D patients where C-peptide has been shown to ameliorate chronic myocardial, renal, and neuronal complications

[15–17]. Furthermore, a physiologic anti-inflammatory effect of C-peptide on glucose-induced endothelial dysfunction has also been demonstrated by our group (Luppi P. et al. unpublished results).

In conclusion, our findings support the hypothesis that C-peptide at physiological concentrations inhibits VSMC proliferation under high glucose conditions likely due to suppression of NF- κ B activation. These findings underscore a role of C-peptide in VSMC functions, especially in conditions of diabetic insult to the vasculature.

Acknowledgements

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Recovery of Endogenous β -Cell Function in Nonhuman Primates After Chemical Diabetes Induction and Islet Transplantation

Rita Bottino,¹ Angela Criscimanna,^{1,2} Anna Casu,¹ Jing He,¹ Dirk J. Van der Windt,¹ William A. Rudert,¹ Carla Giordano,² and Massimo Trucco¹

OBJECTIVE—To describe the ability of nonhuman primate endocrine pancreata to reestablish endogenous insulin production after chemical β -cell destruction.

RESEARCH DESIGN AND METHODS—Eleven monkeys (*Macaca fascicularis*) were rendered diabetic with streptozotocin. Eight diabetic monkeys received intraportal porcine islet transplantation.

RESULTS—Two monkeys transplanted after 75 days of type 1 diabetes showed recovery of endogenous C-peptide production a few weeks after transplantation, concomitant with graft failure. Histological analysis of the pancreas of these monkeys showed insulin-positive cells, single or in small aggregates, scattered in the pancreas and adjacent to ducts. Interestingly, numerous CK19⁺ cells costained with proinsulin and PDX-1 antibodies. Furthermore, the peculiar double phenotype glucagon-positive/GLUT2⁺ was observed. In these monkeys as well as in all others, the original islets showed no insulin staining.

CONCLUSIONS—Our data provide evidence that, in nonhuman primates, the pancreas can reestablish endogenous insulin production after chemical β -cell destruction. This seems to be a nongeneralizable event with only 2 out of 11 monkeys recovering β -cell function. In these two monkeys, younger age and islet graft behavior might have played a role in triggering endogenous β -cell recovery. *Diabetes* 58:442–447, 2009

Until a few years ago, lesions of the endocrine pancreas, as occur in type 1 diabetes, were thought to be permanent and irreversible, since diabetic patients require hormone replacement therapy for life (1). Despite the clinical evolution of the disease, it is still unknown whether the islet β -cells possess, at least in part, the ability to heal from an injury (2).

In mouse models, evidence has accrued that adult animals retain the ability to expand their β -cell mass after stimulation with a variety of triggers (3–8). It remains largely uncertain, however, through which molecular and cellular mechanisms the reparative process works (2).

From the ¹Division of Immunogenetics, Department of Pediatrics, Children's Hospital of Pittsburgh, University of Pittsburgh, Pittsburgh, Pennsylvania; and the ²Division of Endocrinology, Department of Experimental Oncology and Clinical Applications, Università degli Studi di Palermo, Palermo, Italy. Corresponding author: Rita Bottino, rib16@pitt.edu.

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In humans, the ability of the postnatal pancreas to expand the β -cell mass after injury is still debated (9,10). Spontaneous recovery of β -cell function has been reported in only few patients previously diagnosed with type 1 diabetes (11).

Although nonhuman primates do not develop autoimmune diabetes, a permanent diabetes status can be induced by total pancreatectomy or by chemical destruction of the β -cells with streptozotocin (STZ) (12,13).

In this study, a group of 11 monkeys were rendered diabetic by high STZ doses. Eight of them received intraportal pig islet transplantations under an immunosuppressive regimen based on anti-CD154 antibody and mycophenolate mofetil (14).

In two recipients where pig islet grafts functioned for a few weeks and eventually failed, we observed increasing endogenous C-peptide production paralleled by metabolic improvement. We reviewed all metabolic data, did extensive histological analysis, and here report evidence of recovery of endogenous insulin production in these two monkeys.

RESEARCH DESIGN AND METHODS

A total of 15 male Cynomolgus monkeys (*Macaca fascicularis*) (Three Spring Scientific, Perkasie, PA), 2–4 years old and 2.6–4.7 kg (median 3.6 kg), were included in the study.

Four wild-type retired breeder pigs (Wally Whip, Enon Valley, PA) and three GT-DKO pigs (α -1,3-galactosyltransferase KO pigs; Revivicor, Blacksburg, VA) of similar weight were used as sources of pancreata for islet isolation. One wild-type pig was used for two transplants. All animal care procedures were in accordance with the institutional Principles of Laboratory Animal Care.

Induction of diabetes. Diabetes was induced in 11 monkeys with 125–150 mg/kg i.v. of Zanosar STZ (Sicor Pharmaceuticals, Irvine, CA) in a single dose (12).

Islet preparation and transplantation. After pancreatectomy in the anesthetized donor pig, islet isolation was carried out according to a modification of the method described for human islets, optimized for pigs (15). Intraportal injection of islets (an average of 60,000 islet equivalents/kg body wt with a range of 40,000–100,000 islet equivalents/kg) resuspended in plain CMRL-1066 with addition of heparin (70–140 units/kg; Baxter, Deerfield, IL) or dextran sulfate (molecular weight ~5,000 d; 4 mg/kg; Fluka, Buchs, Switzerland) was carried out under general anesthesia of recipients.

Immunosuppressive management in transplanted monkeys. After induction with anti-thymocyte globulin, immunosuppression was maintained with humanized anti-CD154 monoclonal antibody (ABI 793, generously provided by Novartis Pharma, Basel, Switzerland) and mycophenolate mofetil (Roche, Nutley, NJ) (14).

Anticoagulation/antiaggregation/anti-inflammatory treatment was achieved with heparin or dextran sulfate, prostacyclin (GlaxoSmithKline, Research Triangle Park, NC), and aspirin (14).

Diabetes and graft/endogenous β -cell function monitoring. Blood glucose (mmol/l) was measured in whole blood with Free Style (Lifescan). Primate C-peptide (nmol/l) levels were measured by radioimmunoassay (Linco Research, St. Charles, MO). Confirmatory post-STZ C-peptide levels were measured using a chemiluminescent-based technology (Bayer Centaur,

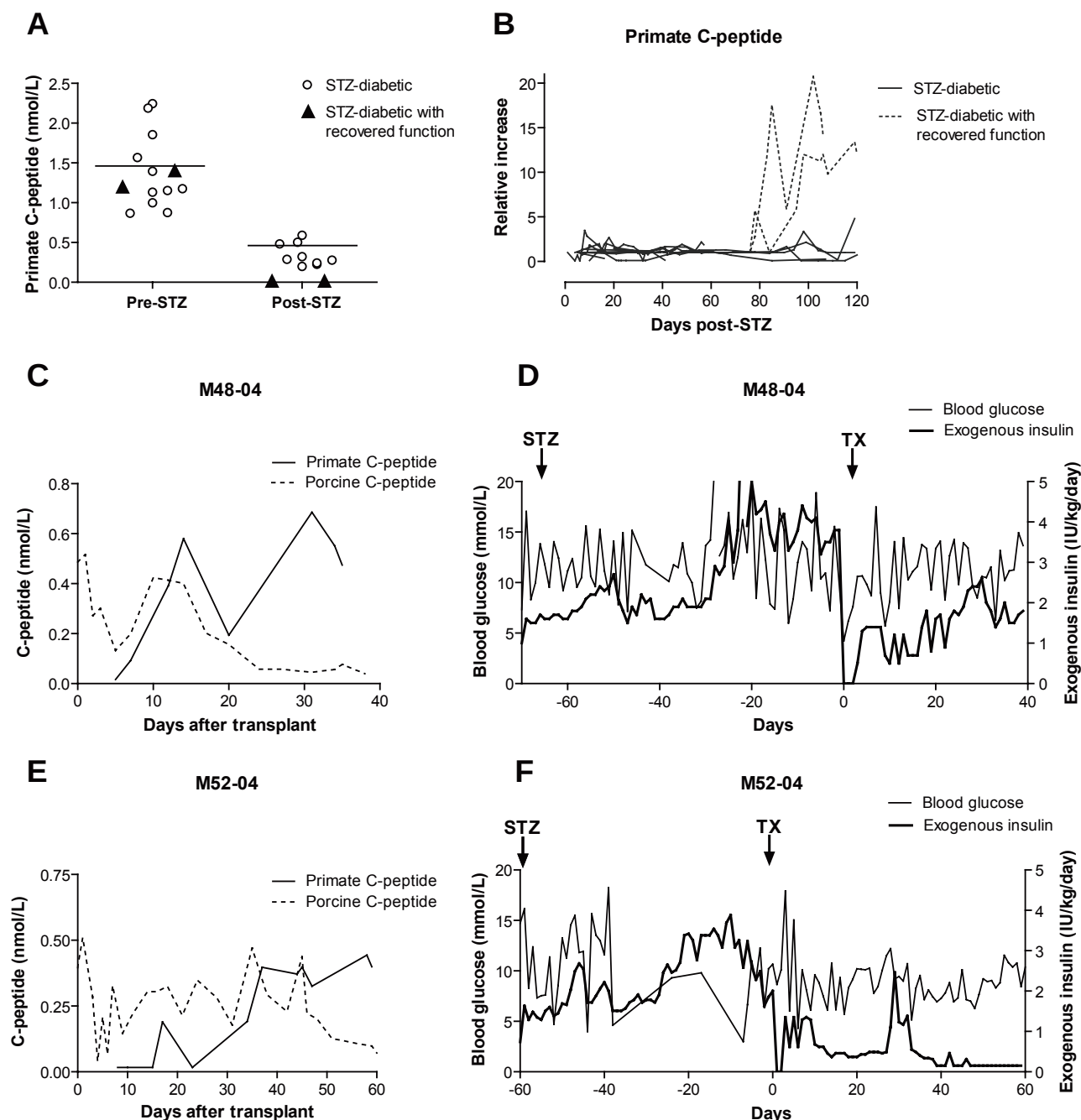


FIG. 1. A: Endogenous C-peptide levels before and after STZ treatment. Closed triangles indicate the values of C-peptide in the two monkeys that recovered autologous insulin production. **B:** Relative increase of endogenous C-peptide over basal post-STZ values. Solid lines indicate STZ-diabetic monkeys that were not transplanted ($n = 3$) as well as STZ-diabetic recipients that experienced early graft loss ($n = 6$). Dotted lines represent the monkeys with recovered β -cell function ($n = 2$). **C–F:** Metabolic profile of the monkeys with recovered endogenous C-peptide (M4804 and M5204). **C** and **E:** Graft (porcine) and endogenous (primate) C-peptide levels. **D** and **F:** Blood glucose and exogenous insulin administration.

Tarrytown, NY). Porcine C-peptide (nmol/l) was detected by radioimmunoassay (Linco Research). Intravenous glucose tolerance tests were carried out before and after transplantation, as described (14).

Fixation and immunostaining of specimens. Monkeys were killed at the time of rejection or when the vascular lines stopped working. Specimens of the pancreatic tissue were obtained, fixed, and analyzed as described in Supplemental Methods (found in an online appendix at <http://dx.doi.org/10.2337/db08-1127>). Morphometric analysis was conducted as per Bouwens and Pipeleers (16).

RESULTS

Recovery of endogenous C-peptide. All monkeys treated with STZ became hyperglycemic (blood glucose

>15 mmol/l) within 48 h and required exogenous insulin. Fasting C-peptide was under detection levels (<0.16 nmol/l) in all treated animals using a chemiluminescent method. Using commercially available ultra-sensitive radioimmunoassay kits, the monkeys showed an overall reduction in C-peptide of at least 75% compared with the pre-STZ values (Fig. 1A). Lack of C-peptide increased 5 min after glucose infusion during an intravenous glucose tolerance test after STZ treatment characterized all treated monkeys. Diabetic monkeys that did not undergo transplantation ($n = 3$) and islet recipients with early graft loss ($n = 6$) (characterized by undetectable or low porcine

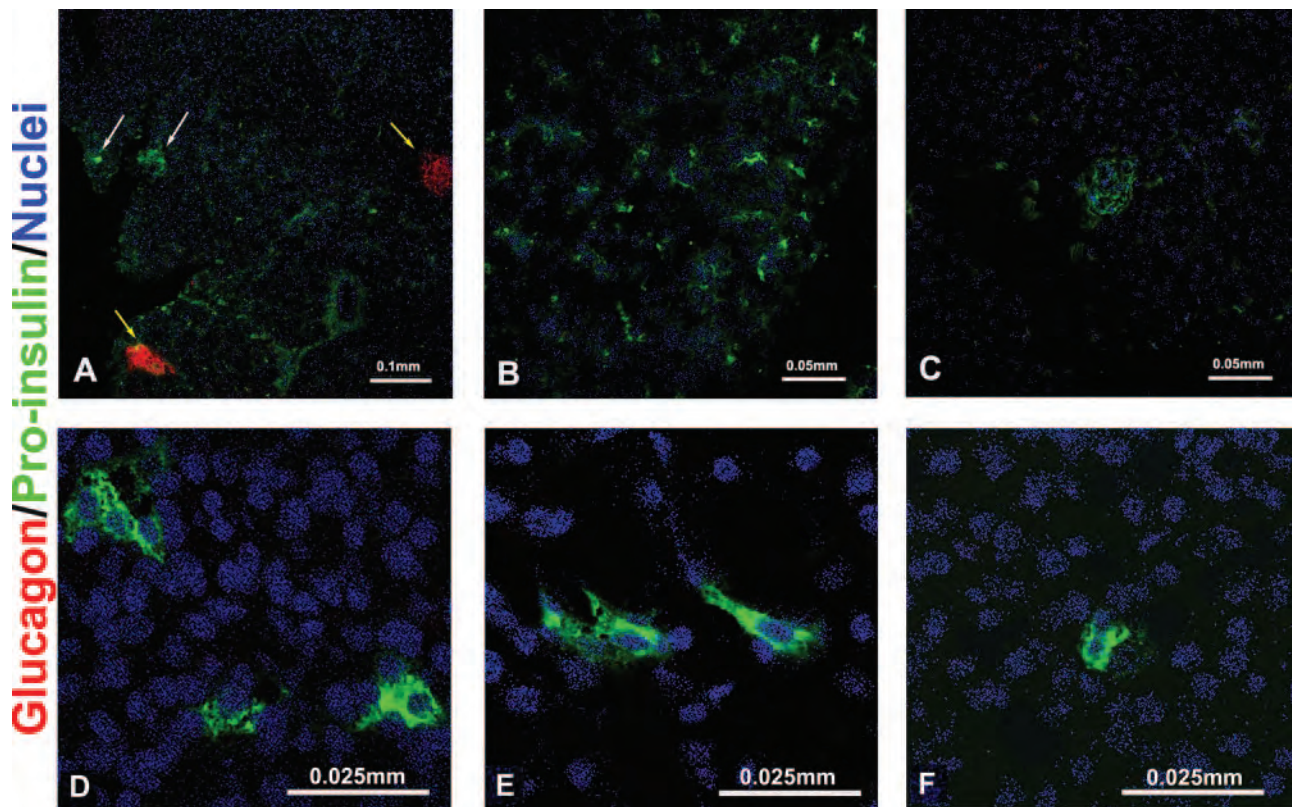


FIG. 2. Proinsulin-positive cells in the monkeys with recovered endogenous β -cell function. *A–C*: Proinsulin-positive cells are organized in small aggregates or scattered as single cells in the pancreas, not associated with glucagon-positive cells. *White arrows*: proinsulin-positive cells. *Yellow arrows*: glucagon-positive cells, presumably islets with damaged β -cells. *D–F*: Higher magnification. Pictures are representative of both monkeys with recovered endogenous β -cell function. (Please see <http://dx.doi.org/10.2337/db08-1127> for a high-quality digital representation of this figure.)

C-peptide levels for <2 weeks) showed no increase of the autologous C-peptide over the post-STZ basal levels (Fig. 1*B*). Exogenous insulin requirements after graft failure or in nontransplanted monkeys remained unchanged over time (data not shown). The pancreatic islets of monkeys with STZ-induced diabetes showed complete absence of insulin immunostaining (Supplemental Fig. 1). Monkeys M4804 and M5204, with C-peptide levels below detection using both detection methods for >2 months after STZ treatment, showed substantial recovery of basal endogenous C-peptide after a period of 3 weeks with islet graft function (Fig. 1*B*). Interestingly, the curves representing endogenous versus porcine C-peptide followed opposite trends (Fig. 1*C* and *E*). Glycemia did not worsen after graft failure, exogenous insulin requirement was lower than before transplantation, possibly because of the autologous insulin production (Fig. 1*D* and *F*), and body weight increased at the pace of healthy monkeys. Several weeks after transplantation, endogenous C-peptide levels were not only detectable but showed a low but measurable response to glucose stimulation (Supplemental Fig. 2). After islet graft failure in all other recipients, glycemia and insulin requirements returned to pretransplantation levels or worsened (data not shown), while endogenous C-peptide did not increase. In monkeys with long-term graft function (3 and 12 months), recovery of endogenous insulin production was not observed (R.B., A.Cr., A.Ca., J.H., D.J.V.d.W., W.A.R., C.G., M.T., unpublished data).

Histological findings. The pancreata of all the monkeys were analyzed for the presence of proinsulin-positive cells. The two monkeys that reestablished endogenous C-peptide production showed several proinsulin-positive cells

grouped in small aggregates, or single cells scattered throughout the pancreas but distant from aggregates of glucagon-positive cells (Fig. 2). We quantified the number of proinsulin-positive cell clusters with area $\leq 30 \mu\text{m}^2$ (i.e., cell clusters not included in the islets of Langerhans existing before STZ treatment nor in the ducts). Proinsulin-positive cell clusters covered 0.23 and 0.18% of the whole section area in M5204 and M4804, respectively. These values were higher than in STZ-diabetic controls ($0.05 \pm 0.0004\%$; $n = 4$ different monkey pancreata) but lower than nondiabetic monkeys ($0.30 \pm 0.01\%$; $n = 3$ monkey pancreata). Proinsulin-positive cells were co-stained with PDX-1 (Fig. 3*N* and Supplemental Fig. 3). Interestingly, anti-CK19 antibody, used to identify the ductal/epithelial cells, was co-stained with proinsulin-positive cells in these monkeys (Fig. 3*A–H*). The pancreata of these monkeys showed stronger CK19 expression in comparison to nondiabetic and STZ-diabetic, transplanted, and nontransplanted controls (Supplemental Fig. 4).

Additionally, the monkeys with recovered β -cell function presented double PDX-1⁺/CK19⁺ staining (Fig. 3*K–M*). PDX-1⁺ cells were also found in STZ-diabetic control monkeys, but they did not co-stain with CK19 (Fig. 3*J*) nor with proinsulin (data not shown). The atypical GLUT2/glucagon phenotype was found in islets devoid of insulin-producing cells in M4804 and M5204 (Fig. 4*A* and *lower panel*), whereas it was not observed in STZ-diabetic (Fig. 4*C*) nor in nondiabetic control monkeys (Fig. 4*B*).

Anti-Ki67 antibody was used as a nuclear marker of active cell proliferation. Anti-Ki67 stained CK19⁺ cells and fibroblasts but not proinsulin-positive cells (data not shown).

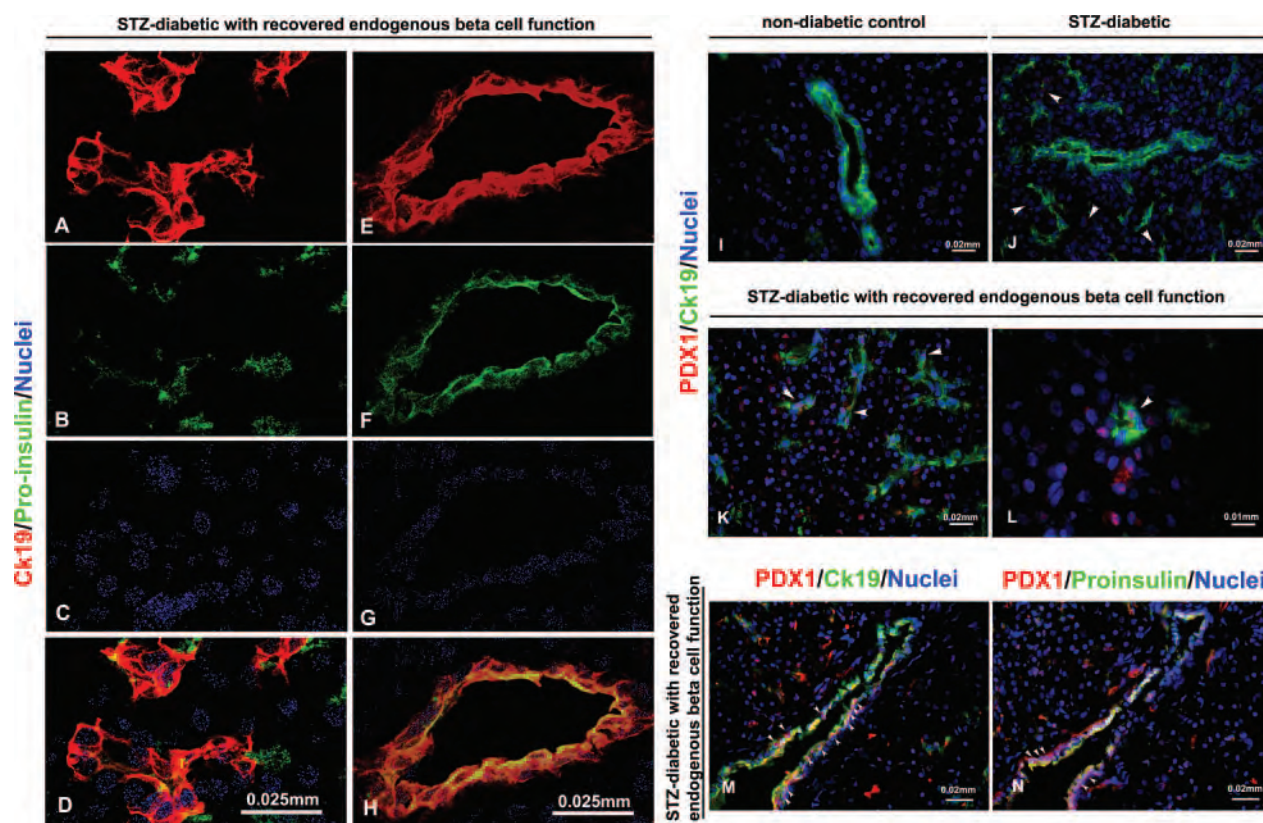


FIG. 3. A–H: Presence of double phenotype CK19/proinsulin in monkeys with recovered endogenous β -cell function. A–D: Monkeys with recovered endogenous β -cell function show co-expression of CK19 with proinsulin (yellow). E–H: Detail of a pancreatic duct. I–N: Double PDX-1⁺/CK19⁺ in the pancreas of monkeys with recovered function (K with detail in L, and M). CK19 and PDX-1 do not co-stain in nondiabetic healthy monkeys (I); PDX-1⁺ cells are found scattered throughout the pancreas of STZ-diabetic monkeys, but do not co-localize with CK19 (J); M and N: consecutive pancreatic sections in M5204 (monkey with recovered β -cell function) showing CK19⁺/PDX-1⁺ (M) and CK19⁺/proinsulin-positive (N) cells, respectively. Arrows show PDX-1⁺ cells in J; double positive PDX-1⁺/CK19⁺ in K, L, and M; and PDX-1⁺/proinsulin-positive in N. (Please see <http://dx.doi.org/10.2337/db08-1127> for a high-quality digital representation of this figure.)

DISCUSSION

In rodents, regenerative properties of β -cells have been unveiled (6,7). In humans, there is no clear similar indication. The autoimmune process that causes type 1 diabetes in the first place might also be responsible for halting potential attempts at restoring insulin production (2). Nonetheless, it is unclear, even in the absence of the immune attack, if the pancreatic β -cell function can recover efficiently (17). Anecdotal reports describing return to a normoglycemic status in patients diagnosed and treated for type 1 diabetes seem to prove that islet function can be reestablished in humans, concurrent with the disappearance of autoimmunity (11). In nonhuman primates used for preclinical investigation of type 1 diabetes treatments, hyperglycemia can be induced by administration of STZ. Once C-peptide is significantly reduced and exogenous insulin administration begins, in contrast to that observed in rodents, spontaneous normoglycemia is believed to be unrecoverable (18).

However, in our hands, two nonhuman primates rendered diabetic with STZ and with virtually no endogenous residual β -cell function for 2 months regained endogenous insulin production concomitant with pig islet graft failure. At the time they were killed, endogenous C-peptide levels were >10 times higher than after STZ treatment with associated lower insulin requirements, despite C-peptide being below the normal range of a nondiabetic cynomolgus monkey (14).

The posttransplantation clinical course of these two monkeys was characterized by glycemic levels persistently

higher than in the normal physiologic range, despite graft insulin production, but well below the range recorded in nontransplanted diabetic monkeys. It is a common notion that chronically elevated blood glucose levels have a negative impact on β -cell function, but it is also known that glucose infusion and mild hyperglycemia may stimulate growth of the β -cell mass. It is unclear whether a threshold for beneficial/toxic effect indeed exists in monkeys and if this effect may have played a role in triggering recovery of insulin production in our monkeys. We observed that in the six monkeys with short graft function and consequent severe hyperglycemia and those that returned to stable normoglycemia after transplantation (R.B., A.Cr., A.Ca., J.H., D.J.V.d.W., W.A.R., C.G., M.T., unpublished data; 19), no recovery of the endogenous function occurred.

Histological examination of the pancreas of these two monkeys showed scattered proinsulin-positive cells, mostly organized as single cells or in small clusters, not associated with glucagon-positive cells, but often to ducts, similar to the ones just recently described (8,20). The frequency of small proinsulin-positive cell aggregates was higher in the monkeys with recovered β -cell function than in diabetic controls but lower than in nondiabetic monkeys. While on one hand we may hypothesize that new cells formed, data do not allow to rule out that proinsulin-positive cells result from a degranulation-regranulation process, as described by Sherry in the autoimmune NOD mouse model (21). However, if reggranulation were the mechanism of recovery of the β -cells, proinsulin-positive

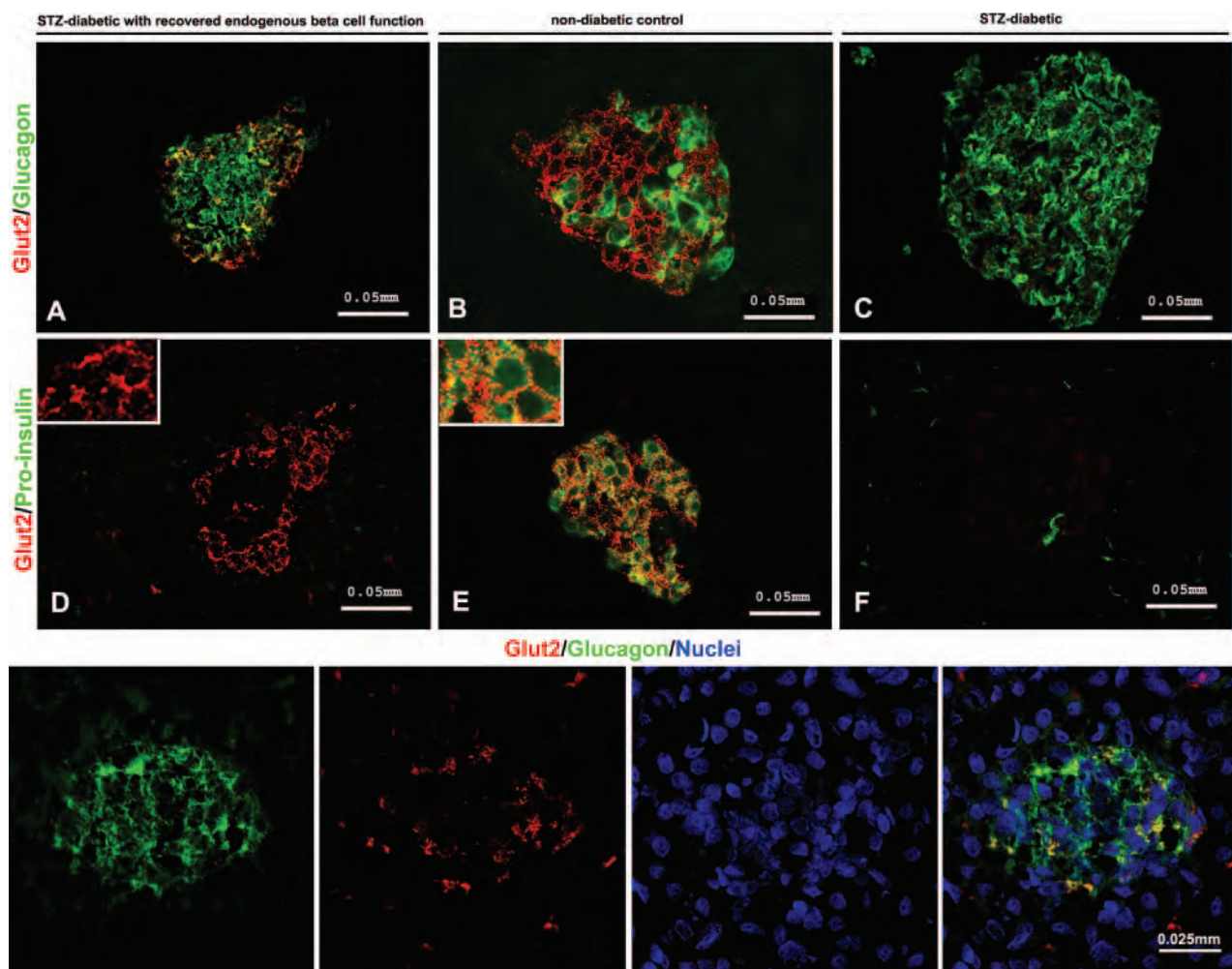


FIG. 4. Double GLUT2⁺/glucagon-positive cells in the pancreas of monkeys with recovered endogenous β -cell function. Co-expression is shown of glucose transporter GLUT2 with glucagon (A) and not with insulin (D) within islets damaged by STZ in M 4804 (monkey with recovered β -cell function). B and E: Nondiabetic monkey islet. C and F: STZ-diabetic monkey islet. Double GLUT2⁺/glucagon-positive and GLUT2⁺/proinsulin-positive cells stain in yellow. Lower panel: GLUT2/glucagon-positive cells in monkey M5204 (higher magnification). (Please see <http://dx.doi.org/10.2337/db08-1127> for a high-quality digital representation of this figure.)

cells associated with the damaged islets (thus near large glucagon-positive cell aggregates) should have been seen.

Interesting features in the pancreas of these monkeys were the presence of proinsulin-positive cells expressing CK19 within and outside the ducts as well as CK19⁺/PDX-1-positive/proinsulin-positive cells, suggesting that β -cells may have formed ex novo from duct progenitors, recapitulating pancreatic organogenesis and neogenesis. In agreement with this observation seems to be the molecular analysis of factors physiologically involved in organogenesis, although the modest numbers of monkey tissue for this study limits the soundness of the conclusion (Supplemental Fig. 5).

An additional characteristic of the pancreas of these monkeys was the presence of glucagon-positive/GLUT2⁺ cells. A lack of evidence for glucagon-positive/proinsulin-positive cells suggests that glucagon-positive/GLUT2⁺ cells were unlikely committed to becoming β -cells, recalling embryonic development phases when GLUT2 is expressed temporarily in pancreatic non- β -cells, likely acting as a signal for further development (22).

The histological findings indicate that damage secondary to STZ may be itself a trigger for pancreatic regenerative responses; however, it seems unable to sustain sufficient β -cell recovery. To explain the phenomenon

observed in these two monkeys, additional stimuli and peculiar conditions must be contemplated. One factor of interest was age: these monkeys were the youngest in the study (38 and 26 months compared with 49 ± 6 months for all others).

The failure of the graft, i.e., islet β -cell dysfunction and death, may also have fostered regenerating signals, as described in other forms of pancreatic injury (23). This would be in line with reports indicating that patients experiencing islet allograft loss can still exhibit detectable C-peptide levels and a better management of the diabetic status (24,25), even if their immunosuppressive regime included sirolimus and tacrolimus, both known to limit β -cell regeneration (7).

Recovery of the β -cell function can occur in nonhuman primates: the mechanisms that lay behind it, however, remains to be demonstrated.

ACKNOWLEDGMENTS

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No potential conflicts of interest relevant to this article were reported.

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Long-Term Controlled Normoglycemia in Diabetic Non-Human Primates After Transplantation with hCD46 Transgenic Porcine Islets

D. J. van der Windt^{a,b}, R. Bottino^a, A. Casu^a,
N. Campanile^{a,c}, C. Smetanka^d, J. He^a,
N. Murase^d, H. Hara^d, S. Ball^e, B. E. Loveland^f,
D. Ayares^e, F. G. Lakkis^{d,g}, D. K. C. Cooper^d
and M. Trucco^{a,*}

^aDivision of Immunogenetics, Department of Pediatrics,
Children's Hospital of Pittsburgh, of UPMC Pittsburgh, PA

^bDepartment of Surgery, Erasmus University Medical
Center Rotterdam, Rotterdam, The Netherlands

^cDépartement de Chirurgie Viscérale, Hôpital
Universitaire de Genève, Genève, Switzerland

^dDepartment of Surgery, Thomas E. Starzl Transplantation
Institute, University of Pittsburgh, Pittsburgh, PA

^eRevivacor Inc., Blacksburg, VA

^fAustin Research Institute, Studley Road, Heidelberg,
Australia

^gDepartment of Immunology, University of Pittsburgh,
Pittsburgh, PA

*Corresponding author: Massimo Trucco, mnt@pitt.edu

Xenotransplantation of porcine islets into diabetic non-human primates is characterized by (i) an initial massive graft loss possibly due to the instant blood-mediated inflammatory reaction and (ii) the requirement of intensive, clinically unfriendly immunosuppressive therapy. We investigated whether the transgenic expression of a human complement-regulatory protein (hCD46) on porcine islets would improve the outcome of islet xenotransplantation in streptozotocin-induced diabetic *Cynomolgus* monkeys. Immunosuppression consisted of thymoglobulin, anti-CD154 mAb for costimulation blockade, and mycophenolate mofetil. Following the transplantation of islets from wild-type pigs ($n = 2$) or from 1,3-galactosyltransferase gene-knockout pigs ($n = 2$), islets survived for a maximum of only 46 days, as evidenced by return to hyperglycemia and the need for exogenous insulin therapy. The transplantation of islets from hCD46 pigs resulted in graft survival and insulin-independent normoglycemia in four of five monkeys for the 3 months follow-up of the experiment. One normalized recipient, selected at random, was followed for >12 months. Inhibition of complement activation by the expression of hCD46 on the pig islets did not substantially reduce the initial loss of islet mass, rather was effective in limiting antibody-mediated rejection. This resulted in a reduced need for immunosuppres-

sion to preserve a sufficient islet mass to maintain normoglycemia long-term.

Key words: Complement regulation, diabetes mellitus, islet xenotransplantation, non-human primate, transgenic pigs

Abbreviations: ACR, acute C-peptide response; alloTx, allotransplantation; AST, arginine stimulation test; ATG, antithymocyte globulin; Gal, galactose α 1,3-galactose; CRP, complement-regulatory protein; GT-KO, α 1,3-galactosyltransferase gene-knockout; HBSS, Hank's balanced salt solution; hCD46, human CD46 transgenic; IBMIR, instant blood-mediated inflammatory reaction; IEQ, islet equivalent; IVGTT, intravenous glucose tolerance test; LMW-DS, low molecular weight dextran sulfate; MMF, mycophenolate mofetil; NHP, non-human primate; PERV, porcine endogenous retrovirus; RIA, radio immuno assay; SI, stimulation index; STZ, streptozotocin; Tg, transgenic; Tx, transplantation; WT, wild-type; xenoTx, xenotransplantation.

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Introduction

Excellent short-term results with islet allotransplantation (alloTx) have been achieved (1). However, the shortage of donor islets, poor long-term outcomes (2) and the risk of allosensitization (3), jeopardizing future kidney transplantation (Tx) in type 1 diabetic patients, have led to active discussions about future directions of this field (4–7). The need for data indicating the efficacy of alternative islet sources in non-human primate (NHP) models has been highlighted (4). Successful xenotransplantation (xenoTx) of porcine insulin-producing cells into diabetic patients could restore physiologic islet function, without the risk of allosensitization (8). Pig insulin has been successfully used for years in treating diabetic patients. Long-term pig islet survival under a limited immunosuppressive protocol would significantly expand the clinical applicability of β -cell replacement therapy for diabetes.

Previously, two groups demonstrated the feasibility of pig islet Tx in NHP (9,10). One group achieved function of adult

islets using a multidrug immunosuppressive protocol that was not considered clinically applicable (9). The other group reached a significant, but poorer, degree of metabolic control using neonatal pig islets and less immunosuppression (10). These results indicate that xenoTx can become a reliable treatment option for diabetes only if new strategies are developed that overcome the xeno-immunologic hurdles and avoid the use of intensive immunosuppression.

Binding of natural and induced antibodies, and activation of complement are part of the xenograft rejection mechanism (11). In addition, complement activation is a key effector mechanism of the instant blood-mediated inflammatory reaction (IBMIR), a nonspecific inflammatory phenomenon occurring immediately after islet Tx into the portal vein that prevents a major proportion of infused islets from engrafting (12,13).

We hypothesized that the use of islets from pigs transgenic (Tg) for human CD46 (hCD46), a complement-regulatory protein (CRP), would allow more islets to survive the IBMIR and complement-mediated rejection, thus more efficiently restoring normoglycemia in Cynomolgus monkeys with chemically induced diabetes. Herein we demonstrate that, although the immediate loss of a significant proportion of transplanted islets could not be prevented, engrafted hCD46 porcine islets can actually function long-term.

Consequently, the applied immunosuppression did not lead to drug-related morbidity and mortality in monkey recipients, advancing the field of islet xenoTx toward clinical application.

Methods

Sources of animals

Two wild-type (WT) female outbred Large White pigs (Wally Whip, Enon Valley, PA), two $\alpha 1,3$ -galactosyltransferase gene-knockout (GT-KO) female pigs (pigs free from galactose $\alpha 1,3$ -galactose (Gal) expression) (Revivicor, Blacksburg, VA) and seven hCD46 Tg female pigs aged 7 months to 2 years (Revivicor), all weighing >180 kg, were used as islet donors. Nine male Cynomolgus monkeys (*Macaca fascicularis*; Three Springs Scientific, Perkasie, PA), 2–5 years of age and weighing 3.7 ± 0.5 kg, were used as islet recipients.

All animal care procedures were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985), and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Production of hCD46 Tg pigs

The hCD46 Tg pigs that were sources of islets were F4 and F5 progeny derived by outcrossing from an hCD46 progenitor line previously described (14). The hCD46 Tg, a minigene under control of its endogenous promoter, was optimized for high ubiquitous expression of this CRP, as demonstrated by robust immunohistochemistry staining in all tissues analyzed, including

all cell types observed in fetal, neonatal and adult pancreas tissues (14), and isolated adult islets (Figure 1B, C).

IVGTT and AST before and after diabetes induction, and after Tx

For blood withdrawal and i.v. drug administration in the monkeys, catheters were inserted into the carotid artery, jugular vein and/or the stomach, and connected through a tether and jacket system to the exterior of the animal cage.

Monkeys underwent intravenous glucose tolerance test (IVGTT) and arginine stimulation test (AST) before induction of diabetes, as previously described (15). The acute C-peptide response after glucose stimulus (ACR_{Glu}) was calculated as the mean of postchallenge C-peptide values obtained at 5 and 15 min minus the corresponding prechallenge value. The response after arginine (ACR_{Arg}) was calculated as the difference between the C-peptide value at 2 min and the baseline value.

Diabetes was induced by the i.v. injection of streptozotocin (STZ, Zanosar, 125–150 mg/kg, but not exceeding 1500 mg/m² to avoid nephrotoxicity; Sico Pharmaceuticals, Irvine, CA). Monkeys were considered diabetic if (i) they had persistent hyperglycemia (>350 mg/dL on at least two occasions, measured twice daily using Freestyle; Abbott Laboratories, Abbott Park, IL), (ii) they required exogenous insulin administration (HumulinR; Eli Lilly, Indianapolis, IN) to prevent ketoacidosis, (iii) baseline primate C-peptide, determined by radioimmuno assay (RIA) (Linco Research, St. Charles, MO), was reduced by >75% after STZ (16), and importantly nonresponsive to IVGTT and AST. If these criteria were not met, even after a second dose of STZ, monkeys were excluded from the study.

IVGTT and AST were also carried out at various time points after Tx to document graft metabolic performance.

Islet isolation and islet quality assurance

Pig pancreata were recovered during a nonsurvival surgical procedure after *in situ* perfusion with cold Hank's balanced salt solution (HBSS) and avoidance of warm ischemia. Organs were transported immediately to the laboratory to begin the isolation procedure. Porcine islets were isolated, purified and cultured as previously reported (17). Following isolation, islets were counted and the number expressed as islet equivalents (IEQs) (17). On the day of islet Tx, after overnight culture, viability and purity were assessed as previously described (17).

For *in vitro* functional studies, islet preparations were subjected to dynamic secretagogue challenges with glucose and theophylline (17). Eluates were collected every minute for measurement of insulin concentration for calculation of a stimulation index (SI) (Table 1). SI indicates the ratio of insulin release induced by high glucose (plus theophylline) over insulin release induced by low glucose.

Islet transplantation and recipient groups

Islets were resuspended in 20 mL CMRL-1066 medium supplemented with low molecular weight dextran sulfate (LMW-DS, 4.5 mg/kg of recipient BW; Fluka, Buchs, Switzerland) for anticoagulation. Under full anesthesia and after laparotomy, Tx was carried out by infusion of the islets into the portal vein by gravity over a period of 5–10 min. Perioperative care consisted of cefazolin for antibiotic and buprenorphine for analgesic treatment.

Monkey islet recipients were divided into two groups. *Group A* recipients ($n = 4$) were transplanted with either WT porcine islets ($n = 2$) or islets isolated from GT-KO pigs ($n = 2$), in numbers of 85 000 to 100 000 IEQ/kg body weight. *Group B* recipients ($n = 5$) received equal numbers of islets from hCD46 pigs. All monkeys were transplanted with islets from a single

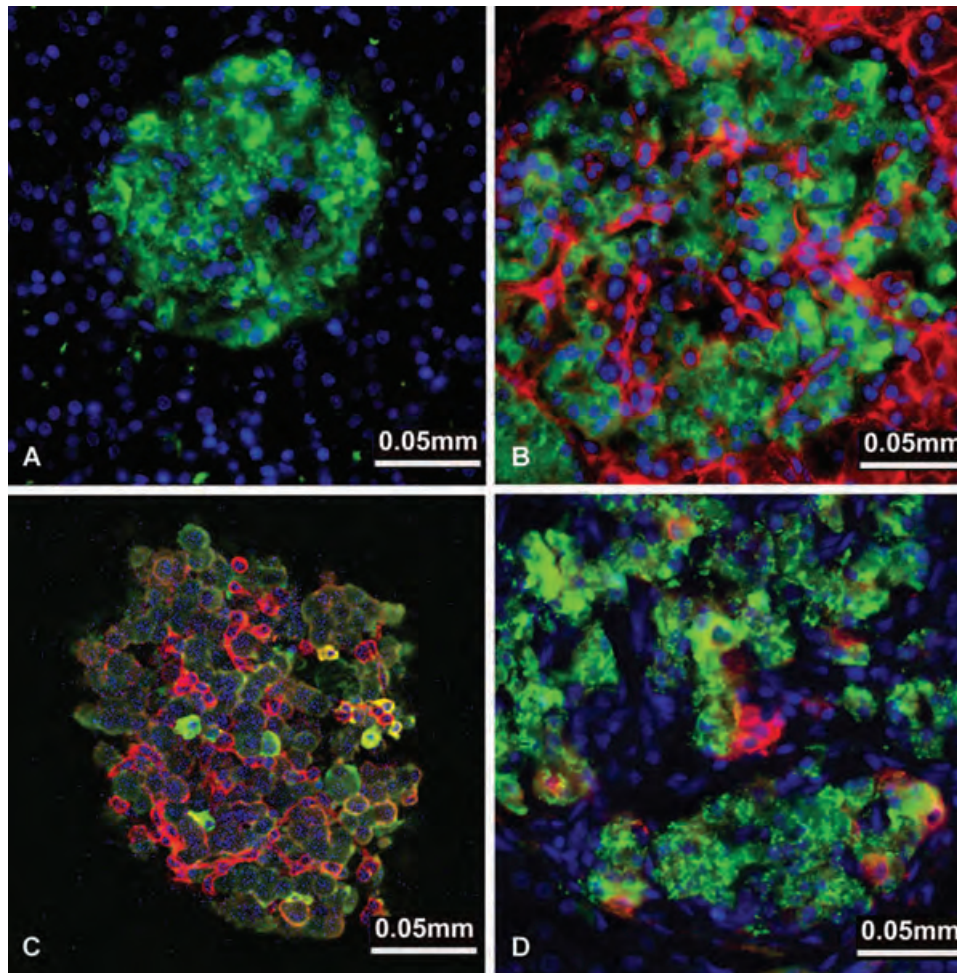


Figure 1. Expression of hCD46. Anti-insulin (green) and anti-hCD46 (red) antibody staining of pig pancreatic tissue and islets anti-nucleus (blue). (A) WT pig pancreas (Group A), (B) hCD46 transgenic pig pancreas (Group B), (C) isolated hCD46 transgenic pig pancreatic islet prior to transplantation and (D) islet in the liver of the monkey recipient one year after transplantation (Group B).

Table 1: Recipient groups, donor and islet characteristics, immunosuppressive drug exposure

Recipient ID	Donor pig	IEQ/kg	Viability (%)	Purity (%)	Stimulation index (high gl/ high gl + theoph)	CD3 T cells on day of Tx (cells/ μ L)	Weekly MMF trough level (μ g/mL)	Weekly anti-CD154 trough level (μ g/mL)
Group A								
M84-05	WT	100 000	90	90	2.4/6.3	75	ND	641
M1-06	GT-KO	85 000	95	85	2.2/8.1	518	3.93 $\pm$ 1.18	1138 $\pm$ 440
M2-06	WT	100 000	85	65	2.6/6.2	315	3.11 $\pm$ 2.31	1021 $\pm$ 489
M4-06	GT-KO	100 000	90	80	4.0/6.1	36	4.58 $\pm$ 1.44	1388 $\pm$ 578
Group B								
M29166	hCD46	90 000	87	90	3.9/5.5	5	2.10 $\pm$ 1.27	1075 $\pm$ 514
M122-08	hCD46	95 000	85	80	2.1/8.7	338	3.31 $\pm$ 1.39	609 $\pm$ 165
	hCD46 ¹	100 000	90	85	2.3/4.8	37		
M6075	hCD46 ²	94 000	95	90	2.3/16.0	608	3.22 $\pm$ 1.41	944 $\pm$ 461
M174-08	hCD46	100 000	90	90	ND	293	4.22 $\pm$ 1.92	663 $\pm$ 184
	hCD46 ³	100 000	90	85	3.0/4.7	23		
M7273	hCD46	100 000	87	90	3.9/5.5	398	2.64 $\pm$ 1.87	744 $\pm$ 415

IEQ = islet equivalent; high gl/high gl + theoph = high glucose/high glucose + theophylline/low glucose; Tx = transplantation; MMF = mycophenolate mofetil; WT = wild-type; GT-KO = α 1,3-galactosyltransferase gene-knockout; ND = not determined.

¹re-Tx on day 49.

²Donor pig was progeny of hCD46 pig crossed with WT pig and was heterozygous for hCD46. All other pigs were homozygous for hCD46.

³re-Tx on day 91.

Table 2: Recipient characteristics

	Body weight pre-STZ/tx/ end follow-up (kg)	STZ dose (mg/kg)	Time between STZ and Tx (days)	Insulin requirements prior to Tx IU/24 h (i.v.)	Off insulin/ duration (days)
Group A					
M84-05	3.6/3.6/3.6	150	15	1.76	Yes/5
M1-06	4.2/3.9/4.0	150	45	3.12	No
M2-06	3.5/3.2/2.8	150	15	3.04	Yes/36
M4-06	4.2/3.9/3.5	125	16	4.4	Yes/17
Group B					
M29166	3.1/3.0/3.2	150	15	2.24	Yes/87
M122-08	3.0/2.8/2.9	125 × 2 doses	17 1st Tx 66 2nd Tx	3.89 pre-Tx (1st) 1.60 pre-Tx (2nd)	No
M6075	4.5/4.3/5.0	150	66	11 SQ	Yes/92
M174-08	3.9/3.7/4.4	125	20 1st Tx 91 2nd Tx	4.88 pre-Tx (1st) 2.4 pre-Tx (2nd)	Yes/91
M7273	3.2/3.0/4.9	150	16	6.56	Yes/396

SQ = subcutaneous.

pig donor. Two Group B monkeys were retransplanted after 49 and 91 days, respectively (Table 1). Table 2 summarizes some additional information on the monkey recipients.

Immunosuppression

Immunosuppressive therapy was identical for both groups (Figure 2). Induction therapy consisted of 25 mg antithymocyte globulin i.v. (ATG, Thymoglobulin; Genzyme, Cambridge, MA) on day -3, followed by 5–25 mg on day -1, depending on efficacy of the initial dose, and aiming for CD3+ T-cell numbers of <500 cells/ μ L whole blood on the day of islet Tx, determined by flow cytometry. One monkey was treated with a single dose of 75 mg ATG on day -1. Immunosuppression was maintained by the oral administration of mycophenolate mofetil (MMF, Cellcept, 50–100 mg/kg/day to obtain trough levels of 3–5 μ g/mL; Roche Pharmaceuticals, Nutley, NJ)

and i.v. injections of anti-CD154 mAb (ABI793, 25 mg/kg on days -1, 0, 3, 7, 11 and 15; generously provided by Novartis Pharma, Basel, Switzerland). Anti-CD154 is a humanized monoclonal antibody cross-reactive with NHP CD154 that inhibits the costimulatory signal between CD40 on the antigen-presenting cell and CD154 on the T helper cell (18). After 15 days, the dose was reduced to weekly injections of 5–15 mg/kg to maintain trough levels of 500 μ g/mL, as measured by quantitative ELISA (19).

We used an anticoagulant, anti-inflammatory treatment protocol developed based on our previous experiences with IBMIR (20), aiming at limiting its detrimental effects during the peri-Tx period (Figure 2). Fifteen minutes before Tx, a bolus of 10 mg/kg methylprednisolone (Solu-Medrol; Pfizer, New York, NY) to reduce inflammation and a bolus of 5 mg/kg LMW-DS for its anticoagulation and anticomplement effects (21) were infused. Subsequently,

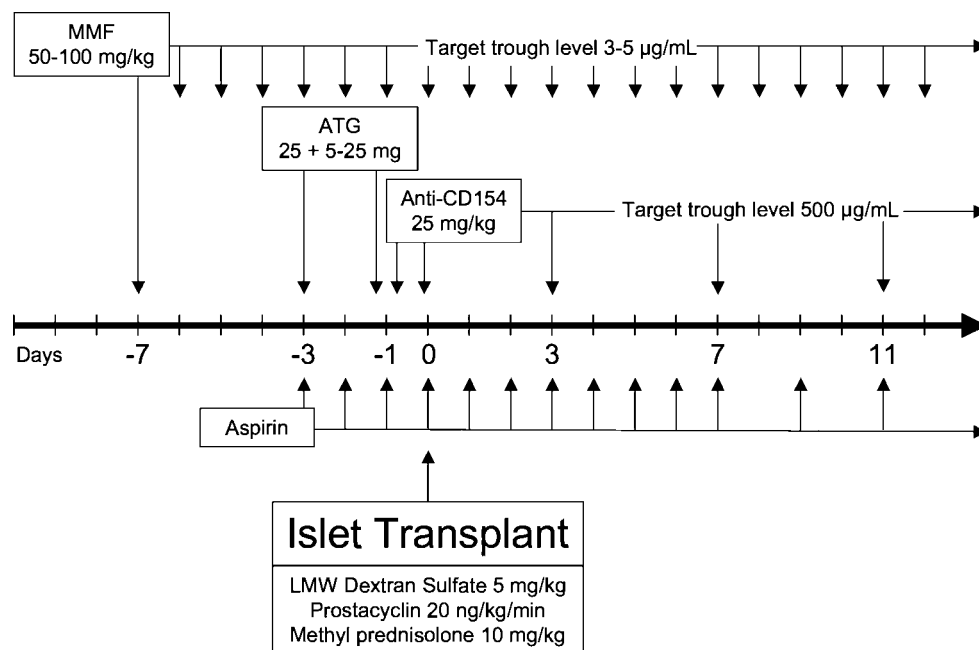


Figure 2. Experimental design. After 14 days, the administration of weekly injections of 5–15 mg/kg anti-CD154 was sufficient to maintain trough levels >500 μ g/mL.

LMW-DS administration was continued at 2 mg/h for 6 h. However, in some cases it was stopped earlier, based on clinical signs of increased bleeding risks (persistent oozing of the laparotomy wound). Prostacyclin (Flolan; GlaxoSmithKline, Philadelphia, PA) was administered i.v. at 20 ng/kg/min beginning 30 min before islet Tx and continued for 6 h for its suppressive effect on endothelial cell activation. From day -7 until 7, monkeys were given aspirin, 81 mg daily, for reduction of both inflammation and platelet aggregation. After 7 days, aspirin dosage was reduced to 81 mg every other day to maintain the antiplatelet effects to prevent the potential thrombotic adverse effects associated with anti-CD154 therapy.

When immunosuppression was started, antiviral prophylaxis consisted of ganciclovir (Cytovene; Roche), administered i.v. at 5 mg/kg/day, and orally at 25 mg/kg twice daily after vascular catheters were removed. Famotidine was given i.v. or orally at 0.25 mg/kg/day for prevention of gastric stress ulceration.

Measures of outcome

Monkeys were followed for 3 months or until graft failure occurred. Functional islet survival was defined as the time after Tx during which fasting porcine C-peptide was detectable, and exogenous insulin requirements were reduced to <50% of pre-Tx levels in order to maintain blood glucose levels <200 mg/dL. Porcine C-peptide can be readily distinguished from primate C-peptide using a porcine-specific RIA (Linco Research). Graft failure was determined by exogenous insulin needs exceeding 50% of baseline for more than 2 consecutive days. In addition, days of complete insulin-independence were recorded.

Histology

At necropsy, sections of the liver and the native pancreas were fixed in 4% paraformaldehyde and frozen. Standard immunofluorescence procedures were applied to cut sections and capture images for qualitative analysis. The primary antibodies were mouse anti-human CD46 (1:100, Thermo Fisher Scientific, Fremont, CA), rabbit anti-human C4d (1:20, EMELCA Bioscience, Bergen op Zoom, The Netherlands), goat anti-human IgG and IgM (1:1000, Kirkegaard & Perry, Gaithersburg, MD), mouse anti-macrophage (1:200, Millipore Corporate, Billerica, MA), rabbit anti-CD97 (1:200, Thermo Scientific, Rockford, IL) and rabbit or mouse anti-insulin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies: goat anti-mouse Cy3, goat anti-rabbit Cy3, donkey anti-goat Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA), donkey anti-rabbit Alexa 488 and goat anti-rabbit or mouse Alexa 488 (1:500, Molecular Probes, Eugene, OR).

In addition, pancreatic and liver tissues were fixed in 10% buffered formalin, and sections were stained with H&E, rabbit anti-proinsulin (1:100, Scytek Laboratories, Logan, UT) and rabbit anti-glucagon antibodies (1:50, Zymed, San Francisco, CA), using standard procedures.

Photographs were taken through a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY). Images were captured by a Photometrics Cool SNAP digital camera (Roper Scientific, Tucson, AZ) and Nikon C1 confocal system at 40× objective lens and analyzed by MetaMorph imaging analysis software (Molecular Devices, Downingtown, PA).

Xenoreactive antibodies

Recipient sera were tested for binding of xenoreactive antibodies to pig cells before Tx and after 1 week, 1 month and at the end of the study according to methods previously described (22). Briefly, 10⁵ wild-type or GT-KO pig target cells were incubated with 5% heat-inactivated recipient serum or FACS buffer (control) for 30 min at 4°C. To prevent nonspecific binding, 10% goat serum was added after washing twice. Detection of IgM or IgG binding was performed by further incubating with FITC-conjugated

goat anti-human IgM (μ chain-specific) and IgG (γ chain-specific) (Invitrogen, Carlsbad, CA) for 30 min at 4°C. Flow cytometric data acquisition was performed with the LSR II (BD Biosciences, San Jose, CA). Binding of IgM and IgG was assessed using relative mean fluorescence intensity (MFI), which was calculated as follows:

Relative MFI = (actual MFI)/(MFI of secondary antibody only, in absence of serum)

Post-Tx relative MFI was compared to levels measured in pretransplant serum.

Statistical analyses

Continuous variables are expressed as mean ± SD, and compared using the Student's *t*-test. The difference in survival between Groups A and B was calculated using the log-rank test. *p*-Values <0.05 were considered to indicate a statistically significant difference. All analyses were performed with GraphPad Prism 4 for Macintosh (GraphPad Software, La Jolla, CA).

Results

Confirmation of diabetes induction

Diabetes was successfully induced in all monkeys. After STZ, for a mean diabetic period of 24 days, continuous i.v. insulin infusion of 1.20 ± 0.41 IU/kg/24 h was required to maintain an average blood glucose level of 186 ± 31 mg/dL and to prevent ketoacidosis. Fasting monkey C-peptide levels were reduced from 4.82 ± 1.66 ng/mL to 1.01 ± 0.20 ng/mL (*n* = 9). Moreover, ACR_{Glu} and ACR_{Arg} became undetectable after STZ in all monkeys, and remained undetectable during follow-up (Figure 3A, B and C). These data indicate that a background level of monkey C-peptide was detectable only with ultrasensitive RIA kits, and that this level failed to increase after physiologic and nonphysiologic stimuli, making it highly unlikely that any native monkey β-cell function was contributing to glucose control, as confirmed by virtually absent insulin staining of postmortem monkey pancreata (Figure 3D).

Outcome of islet transplantation

Functional porcine islet survival, determined by detectable porcine C-peptide in combination with a >50% reduction of exogenous insulin needs, was achieved in all monkeys. In Group A monkeys transplanted with non-Tg islets, islet survival lasted for 7, 20, 31 and 46 days. Insulin-independent normoglycemia was achieved in three of four monkeys for periods of 5, 17 and 36 days, respectively (Figure 4A). In Group B monkeys, the use of hCD46 Tg islets significantly prolonged functional porcine islet survival to the full 3 months of the planned experiment (*p* = 0.0042) or beyond 1 year of follow-up, respectively. This exception was randomly introduced to test the durability of the positive effects of treatment. Four of five Group B monkeys became insulin-independent after Tx for 87, 91, 92 and 396 days, respectively (Figure 4B). One of these four recipients

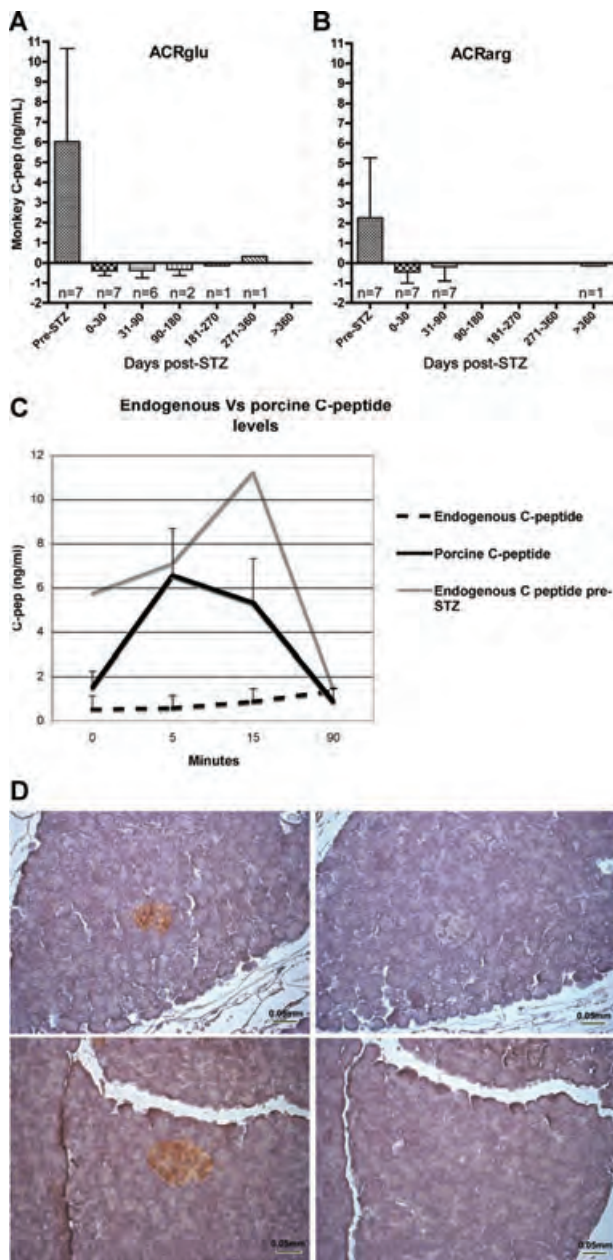


Figure 3. Diabetes induction by i.v. streptozotocin. Absence of an endogenous acute C-peptide response (ACR) after induction of diabetes in monkey recipients, when metabolically challenged with (A) intravenous glucose (glu) or (B) arginine (arg). (C) Endogenous and porcine C-peptide levels during IVGTT in Monkey 7273, the islet recipient followed-up for over 1 year. Data points are mean $\pm$ SD of N = 7 tests. The last test was carried out 3 days prior to euthanasia. (D) Left sets of images: glucagon immunostaining of pancreatic tissue analyzed in Monkey 7273, right panels show insulin immunostaining of consecutive sections.

gradually lost normoglycemia, which was regained after a re-Tx. In the fifth Group B monkey, insulin-independence could not be achieved with two islet infusions. Nevertheless, even in this animal, exogenous insulin needs were reduced >50% for 3 months with detectable porcine C-peptide.

During times of insulin-independence, fasting blood glucose values were well controlled (Group A: 91 ± 18 mg/dL; Group B: 112 ± 22 mg/dL, $p = 0.250$). Post-Tx weekly fasting porcine C-peptide levels were comparable for Groups A and B during the first 45 days (1.10 ± 0.41 ng/mL vs. 1.19 ± 0.88 ng/mL, $p = 0.860$). After 45 days, C-peptide positivity was maintained at 0.87 ± 0.41 ng/mL, but only in Group B recipients. None of the monkeys recovered endogenous β -cell function, evidenced by the absence of primate C-peptide response to arginine and glucose challenge post-STZ (Figure 3).

Post-Tx responses to stimulation with i.v. glucose and arginine showed an evident porcine C-peptide response of engrafted pig islets in Group B, in the absence of a response by native monkey β cells (Figure 5 and Supporting Table 1).

Adverse events

Monkeys remained healthy during the course of each experiment; except one Group A monkey that was euthanized 7 days after Tx with acute gastric dilatation, a not uncommon condition in laboratory animals (23). Adverse events were minimal and the majority of monkeys maintained or gained weight (Table 3). In three Group A monkeys, although free from clinical signs except mild weight loss, reactivated CMV was detectable in serum by PCR. Aspirin may have effectively prevented thromboembolic complications potentially associated with anti-CD154 treatment, except possibly in one case (Table 3).

Post-Tx histology

In Group B monkeys, histologic evaluation of post-Tx livers revealed many viable porcine islets (Figure 6A, B and C) that maintained expression of the Tg even >1 year after Tx (Figure 1D). T-cell infiltration was not observed, confirming previous studies in which costimulation blockade was applied (9,10).

Macrophages and CD97-positive cells were sporadic in both groups (Figure 6H–K). IgG and C4d immunostaining for local antibody binding and complement activation was detected on and around the islet cells in the liver of Group A monkeys; in Group B livers, although IgG was observed, C4d was virtually absent (Figure 6D–G).

Absence of increased in xenoreactive antibody titers

Natural anti-pig (anti-Gal + anti-nonGal) and/or anti-nonGal antibodies were present in monkey sera (mainly of IgM isotype), but the extent of binding to pig PBMC did not

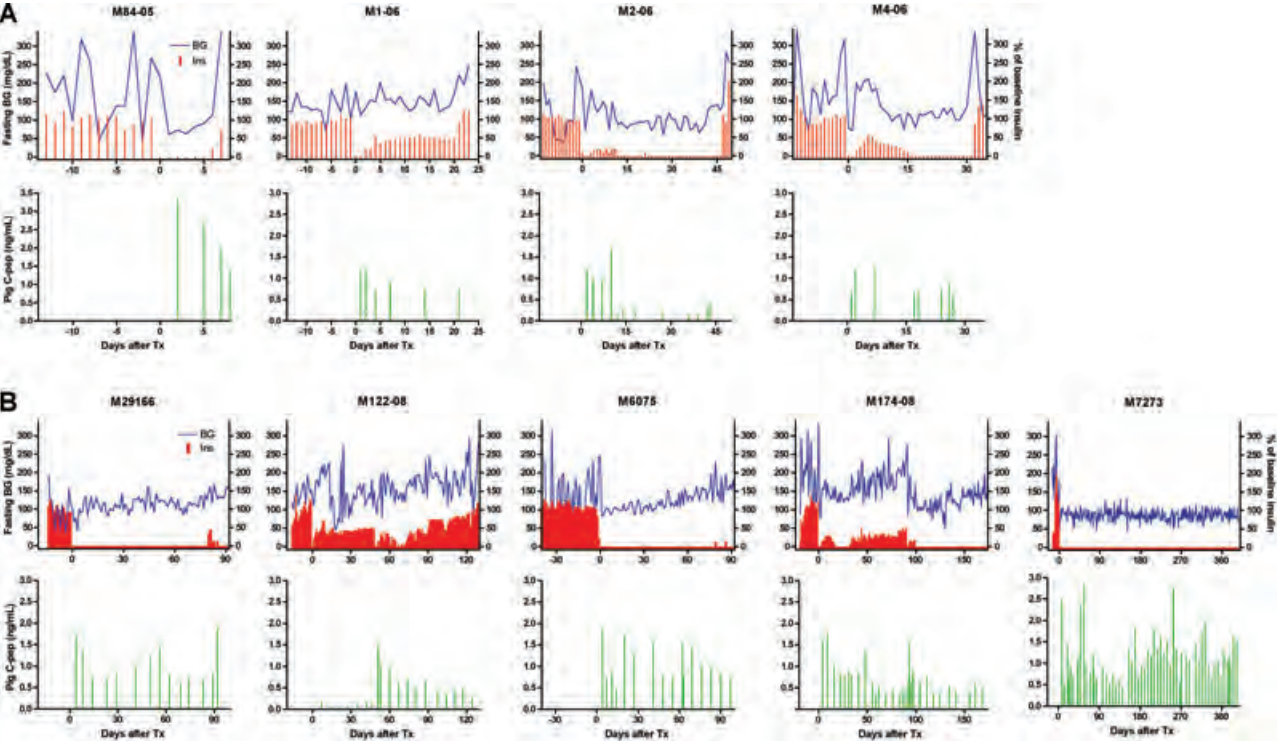


Figure 4. Results of islet xenotransplantation. Fasting blood glucose, exogenous insulin administration as percentage of pretransplant insulin needs, and porcine C-peptide levels in Group A (A) and Group B (B) monkeys. BG, fasting blood glucose; Ins, exogenous insulin.

increase in either Group A or B monkeys after Tx or even after re-Tx (Supporting Figure S1).

when transplanted intraportally into diabetic, immunosuppressed NHP, demonstrated an advantage over not genetically modified WT pig islets.

Discussion

This is the first time in which the survival of a functional islet xenograft was documented for more than 1 year (at which time the experiment was electively terminated) in a chemically diabetic monkey, which was neither limited in diet nor received exogenous insulin injections. Transgenic expression of hCD46 on adult porcine islets,

Encouraging results reported by others were achieved using donor islets from WT pigs, suggesting that natural antibodies against Gal may not be too harmful to islets (9,10), yet their potential role in the context of islet xenoTx has recently been reconsidered (24). Although the levels of antibody binding against pig cells did not increase during our experiments, natural antibodies against Gal and nonGal epitopes were found present. The binding of antibodies was

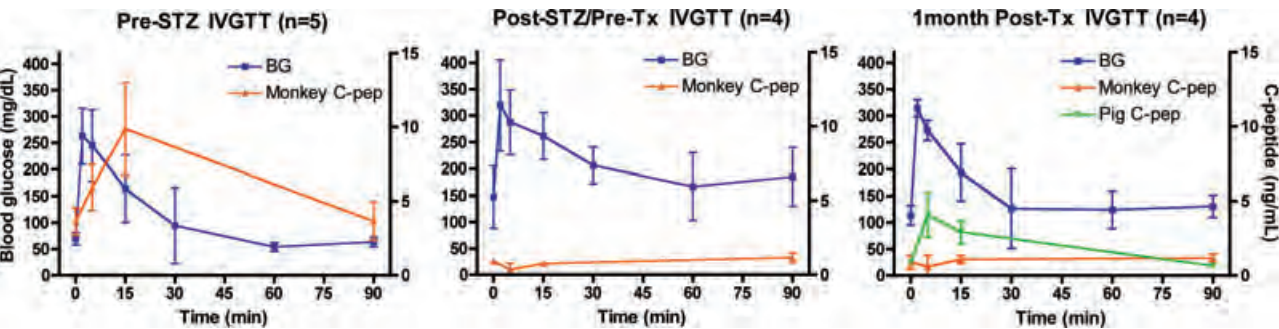


Figure 5. Intravenous glucose tolerance test (IVGTT). IVGTT in Group B monkeys before STZ (Pre-STZ), after STZ (Post-STZ/Pre-Tx) and 1 month after transplantation, if normoglycemic. Additional data obtained during IVGTT and arginine stimulation test (AST) are presented in Supporting Table S1.

Table 3: Development of adverse events, body weight

Recipient ID	Adverse event related to immunosuppression	Adverse event unrelated to immunosuppression	CMV PCR	Final weight as % of pre-Tx
Group A				
M84-05	–	Euthanasia at day 7 for acute gastric dilatation	Positive	100%
M1-06	–	–	Negative	105%
M2-06	Diarrhea requiring reduction of MMF dosage	–	Positive	88%
M4-06	–	–	Positive	90%
Group B				
M29166	Diarrhea with occult blood due to MMF intolerance; MMF discontinued on day 4 At necropsy, microscopic thrombotic lesions in brain and lungs	Mild progressive kidney failure due to STZ	Negative	107%
M122-08	–	–	Negative	107%
M6075	–	–	Negative	116%
M174-08	–	–	Negative	119%
M7273	–	–	Negative	163%

Pre-Tx = Pretransplantation.

observed on both WT and hCD46 islet grafts, whereas activation of complement was only observed on WT islets, suggesting that hCD46 may have exerted a protective role against humoral rejection, possibly reducing the need for immunosuppression. In fact, our immunosuppressive regimen allowed monkeys to remain healthy, in contrast to reports in which adverse events prematurely terminated many experiments (9,10). The avoidance of calcineurin inhibitors may have added to the long-term preservation of a functional islet mass (25).

In our study, we used market-age hCD46 pigs (not retired breeders) and did not perform any preselection of donor islet preparations, to model future clinical applications. Transgenic hCD46 together with an anti-inflammatory and anticoagulant protocol, involving dextran sulfate in the peri-operative Tx period, should have contributed to reduce the damage induced by IBMIR. Nonetheless, in both experimental groups we observed acute early islet loss characterized by C-peptide release and hypoglycemia: C-peptide peaks 1 and 2 h following islet infusion were similar in both groups (data not shown). All together these maneuvers resulted to be insufficient to significantly prevent IBMIR and consequent cell death. Thus, although high numbers of porcine islets turned out to be necessary, we had to conclude that only a marginal mass of surviving cells is sufficient to sustain normoglycemia. Based on these and on our previous observations on the metabolic compatibility between species, we anticipate that the islet graft mass sufficient to promote normoglycemia would be substantially lower in pig-to-human versus pig-to-NHP transplants (15,26).

CD46 is well characterized as a protein with regulatory properties able to protect the host cell against complement-mediated attacks activated via both classical and alternative pathways (27). Its features were maintained when transgenically expressed on pig cells (14,28). Functional complement inhibition activity was demonstrated *in vitro*, whereby both peripheral blood mononuclear cells (PBMCs) and aortic endothelial cells showed significantly greater protection from complement-mediated cytotoxicity than cells from WT or GT-KO pigs (28). Fetal pig islets procured from early lineage progenitors of this same hCD46 Tg line were protected from antibody-mediated destruction in a pig-to-mouse xenograft model (29). Although hCD46 kidneys were resistant to hyperacute rejection when transplanted into baboons (14), Tx of vascularized solid organs from these pig lines could not overcome other xenoTx barriers, such as the occurrence of thrombotic microangiopathy. However, our study seems to indicate that hCD46 expression benefits cell Tx more substantially than organ Tx, especially when cells are transplanted directly into the blood stream.

The small number of animals we were able to study so far is an objective limitation to further speculate on the protective mechanisms of hCD46, but our preclinical study certainly offers the best available contest for testing protocols with potential importance for clinical applications.

In conclusion, our study is the first to show how genetic alterations of donor pigs, tailored to combat complement-mediated xeno-islet injury, contribute to long-term function of islet xenografts. With the development of modified

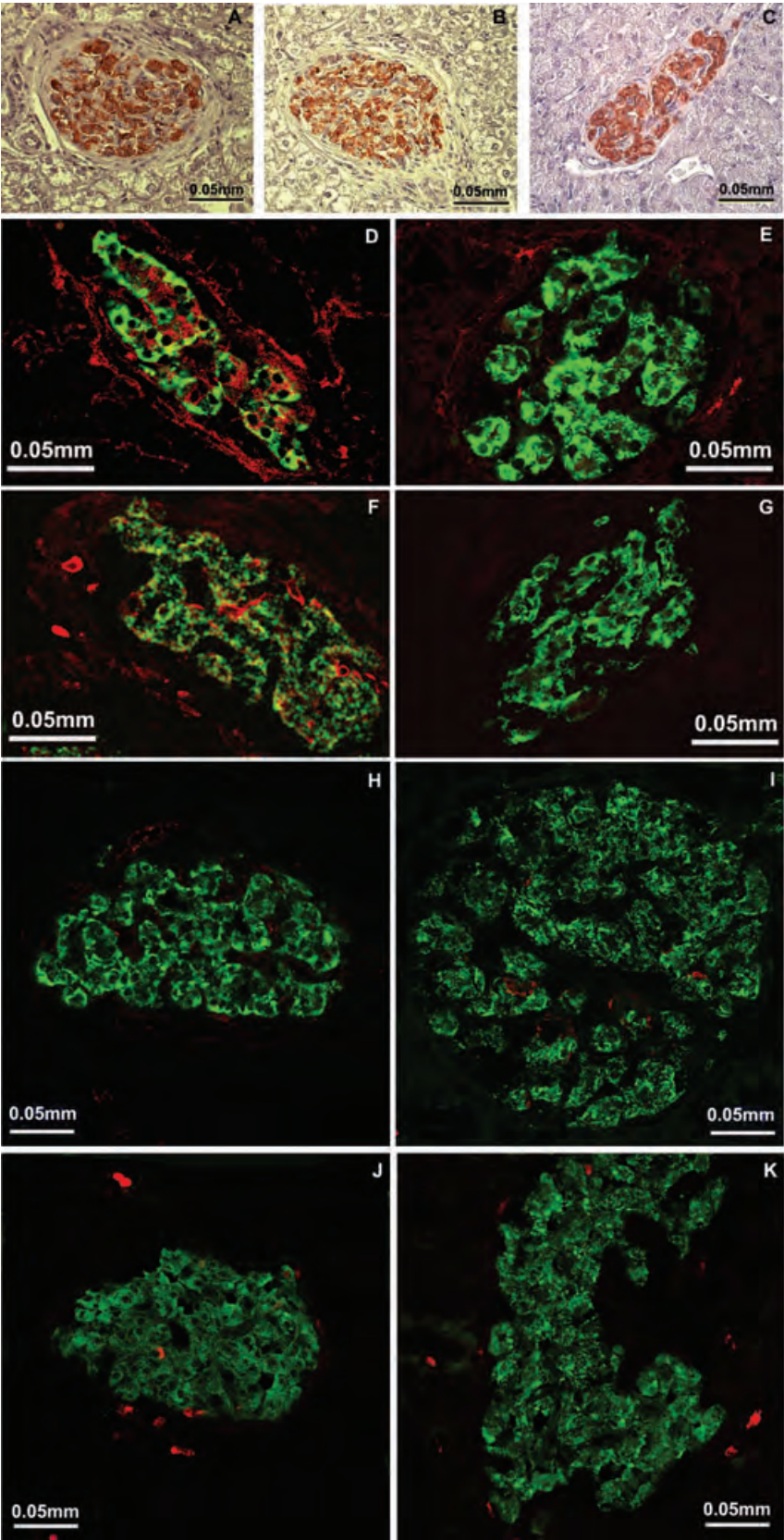


Figure 6. Post-transplant histology. (A, B and C) Insulin immunostaining of liver sections in three monkey recipients after pig hCD46 islet transplantation. A and B: M29166 and M6075 at 3 months after transplantation; C: M7273 >1 year after transplantation. (D–K) Immunofluorescence staining of the liver of monkey recipients following islet transplantation. In all panels insulin is stained green. IgG staining (red) of an islet from a monkey recipient of WT pig islets (Group A) (D) and hCD46 pig islets (Group B) (E). C4d staining (red) of an islet from a monkey recipient WT pig islets (Group A) (F) and hCD46 pig islets (Group B) (G). Macrophage immunostaining and CD97-positive cells in Group A (respectively H, J) and in Group B (I, K).

anti-CD154 antibodies that may prevent the risk of thromboembolism (30,31), or newly generated antibodies with equal efficacy, a safer immunosuppressive regimen might become available for use in human Tx, significantly advancing the field of islet xenoTx toward clinical application. Any hypothetical risk from porcine endogenous retrovirus (PERV) has been largely discounted, as more than a decade of focused research has not demonstrated any *in vivo* transmission of PERV to NHP or human recipients, even after prolonged exposure to porcine tissues (32,33).

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Supporting Information

Additional Supporting Information may be found in the on-line version of this article:

Figure S1. Serum levels of xenoreactive antibodies in Groups A and B monkeys. Antibody levels against WT

and α 1,3-galactosyltransferase gene-knockout (GT-KO) peripheral blood mononuclear cells were determined by flow cytometry before, at 1 week, and at 1 month after islet transplantation, and finally at the time of graft failure or end of follow-up (F/U). No serum antibody sensitization was observed in any monkey recipients. Retransplantation with hCD46 islets in 2 monkeys did not lead to a serum antibody response.

Table S1. Serum acute C-peptide responses (ACR) of pig C-peptide in ng/mL, after metabolic challenges with intravenous glucose (glu) and arginine (arg)

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Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts

K. J. Potter^{a,1}, A. Abedini^{b,1}, P. Marek^c, A. M. Klimek^a, S. Butterworth^d, M. Driscoll^c, R. Baker^e, M. R. Nilsson^f, G. L. Warnock^d, J. Oberholzer^g, S. Bertera^h, M. Trucco^h, G. S. Korbuttⁱ, P. E. Fraser^j, D. P. Raleigh^{c,2}, and C. B. Verchere^{a,d,2}

Departments of ^aPathology and Laboratory Medicine and ^dSurgery, Child and Family Research Institute, University of British Columbia, Vancouver, BC, Canada V5Z 4H4; ^bDivision of Surgical Science, Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, NY 10032; ^cDepartment of Chemistry, State University of New York, Stony Brook, NY 11794-3400; ^eDepartment of Cellular and Physiological Sciences, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada, V6T 1Z3; ^fDepartment of Chemistry, McDaniel College, Westminster, MD 21157; ^gDivision of Transplantation, Department of Surgery, University of Illinois, Chicago, IL 60612; ^hDivision of Immunogenetics, Department of Pediatrics, University of Pittsburgh School of Medicine, Rangos Research Center, Children's Hospital of Pittsburgh, Pittsburgh, PA 15201; ⁱDepartment of Surgery, University of Alberta, Edmonton, AB, Canada, T6G 2E1; ^jDepartment of Medical Biophysics and Centre for Research in Neurodegenerative Diseases, University of Toronto, ON, Canada, M5S 3H2

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Islet transplantation is a promising treatment for diabetes but long-term success is limited by progressive graft loss. Aggregates of the beta cell peptide islet amyloid polypeptide (IAPP) promote beta cell apoptosis and rapid amyloid formation occurs in transplanted islets. Porcine islets are an attractive alternative islet source as they demonstrate long-term graft survival. We compared the capacity of transplanted human and porcine islets to form amyloid as an explanation for differences in graft survival. Human islets were transplanted into streptozotocin-diabetic immune-deficient mice. Amyloid deposition was detectable at 4 weeks posttransplantation and was associated with islet graft failure. More extensive amyloid deposition was observed after 8 weeks. By contrast, no amyloid was detected in transplanted neonatal or adult porcine islets that had maintained normoglycemia for up to 195 days. To determine whether differences in IAPP sequence between humans and pigs could explain differences in amyloid formation and transplant viability, we sequenced porcine IAPP. Porcine IAPP differs from the human sequence at 10 positions and includes substitutions predicted to reduce its amyloidogenicity. Synthetic porcine IAPP was considerably less amyloidogenic than human IAPP as determined by transmission electron microscopy, circular dichroism, and thioflavin T binding. Viability assays indicated that porcine IAPP is significantly less toxic to INS-1 beta cells than human IAPP. Our findings demonstrate that species differences in IAPP sequence can explain the lack of amyloid formation and improved survival of transplanted porcine islets. These data highlight the potential of porcine islet transplantation as a therapeutic approach for human diabetes.

amylin | diabetes | xenotransplantation | pig | islet transplantation

Islet transplantation holds great promise as a treatment for patients with type 1 diabetes. The prospect of better glucose control and fewer complications has considerable appeal compared to traditional glucose monitoring and insulin injection regimens. The long-term success of human islet transplants has been limited, however, with 75% of patients who achieved insulin independence requiring insulin within 2 years posttransplant (1). Although immune rejection of islet allografts certainly plays a role in graft failure, nonimmune-mediated beta cell loss is also likely to play an important role (2–6).

Islet morphology in type 2 diabetes is characterized by progressive apoptotic beta cell loss (7) and the deposition of islet amyloid (8–10). The presence of amyloid in type 2 diabetic humans and in transgenic animal models is associated with beta cell loss and hyperglycemia (9, 11). Human islets transplanted into diabetic, immune-deficient murine recipients rapidly develop amyloid (12). Amyloid is detectable within a few weeks in transplanted human islets, compared to the many months or years thought to be required in type 2 diabetic humans and nonhuman primates. Interestingly, a recent histological study of human islets engrafted into the liver of a diabetic patient found extensive

amyloid deposition within islets, only 5 years following transplantation (13). Transplanted murine islets transgenic for human islet amyloid polypeptide (IAPP) also display progressive deposition of amyloid and gradually lose their capacity to maintain normoglycemia (14). These studies raise the possibility that rapid amyloid formation in transplanted islets may be detrimental to graft function and mass, and may therefore be an unappreciated contributor to islet graft failure.

Islet amyloid forms by aggregation of IAPP (or amylin), a peptide that is produced and secreted by pancreatic beta cells (10). IAPP is released from beta cells in response to glucose and other stimuli that also trigger insulin secretion (15). IAPP is found in the beta cells of all mammals; however, not all species develop islet amyloid. The ability of IAPP to aggregate into amyloid fibrils is dependent upon the primary sequence of the peptide, which has now been determined in a number of species. These fibrils and smaller prefibrillar aggregates are cytotoxic and induce beta cell apoptosis, contributing to beta cell loss in type 2 diabetes. Analysis of the primary sequence of IAPP from mammalian species led to the initial suggestion that the region corresponding to residues 20–29 is a key determinant of its ability to form amyloid (16). In particular, rodent IAPP contains three proline residues in this region. Proline is well known to disrupt β -structure and is not compatible with the cross β -structure of amyloid. Rodent IAPP is soluble, does not form fibrils or prefibrillar aggregates, and unlike human IAPP, is not cytotoxic. More recent investigations have shown that additional regions of the peptide, in particular amino acids 8–20 and 30–37, are also likely to contribute to amyloid formation (17–20).

Xenotransplantation of pancreatic islets, using pigs or other animals as islet donors, has received increasing interest in recent years, given the limited number of human islets available for clinical transplantation. Many factors support the use of pigs as donors in islet xenotransplantation. Neonatal porcine islets show particular promise, as they are easily isolated, are highly resistant to hypoxia and hyperglycemia (21, 22), and have shown remarkable ability to

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¹K.J.P. and A.A. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: draleigh@notes.cc.sunysb.edu or verchere@interchange.ubc.ca.

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expand their beta cell mass following transplantation (23). Notably, porcine islets have been shown to maintain long-term function following intraportal transplantation into nonhuman primates with production of detectable porcine C-peptide and restoration of normoglycemia (24–28). In a limited number of case reports, xenotransplantation of porcine islets into diabetic humans has improved glycemic control (29).

amyloid formation in transplanted pig islets, we cloned and sequenced a porcine IAPP cDNA reverse transcribed using primers based on an EST sequence (BF712755) for porcine hypothalamus and RNA extracted from neonatal porcine islets. The sequence of mature porcine IAPP predicts a 37-amino-acid peptide (Fig. 3) that is well conserved in the N- and C-terminal regions of the molecule, but has unique amino acid differences in the midportion. The predicted sequence (GenBank accession GU396090) matches partial sequences (amino acids 3–34) that are deposited in SwissProt (Q29119) and GenBank (AAB05919.1). Comparison of the porcine and human sequences reveal amino acid differences in 10 positions, 5 of which reside within the 20–29 region thought to be critical for amyloid formation. Noteworthy differences include the substitution of a positively charged arginine at position 20 and a proline at position 29. Overall, porcine IAPP contains six residues that are potentially charged at neutral pH whereas human IAPP contains only three. These sequence differences are predicted to reduce the ability of the peptide to form amyloid as they increase the net charge and reduce the net hydrophobicity (30). This raises the question as to whether porcine IAPP is capable of forming amyloid.

Amyloid formation is a complex process and the kinetics exhibit several distinctive features. A lag phase is observed in which no significant production of amyloid occurs followed by a rapid growth phase leading to a steady state in which soluble peptide is in equilibrium with amyloid fibrils. The ability of porcine IAPP to aggregate and form amyloid fibrils was investigated and compared to its human counterpart at a pH value mimicking that of the extracellular space (pH 7.4). Thioflavin T binds amyloid fibrils formed by aggregation of IAPP but does not bind to preamyloidogenic species and is a useful probe to assess the time course of IAPP aggregation. The experimental curve observed for human IAPP aggregation displayed the characteristic features expected for kinetics of amyloid formation (Fig. 4). At a concentration of 32 μ M human IAPP, a short lag phase was observed in which no significant change in thioflavin-T fluorescence was detected, followed by a rapid rise in fluorescence leading to a plateau phase. These experiments were performed multiple times and similar results were obtained each time. The time for the reaction to reach 50% completion, the t_{50} value, was 862 ± 53 s for the human peptide, where the uncertainty is the apparent standard deviation based on three measurements. The behavior of the porcine peptide was dramatically different. At the same concentration of porcine IAPP, no significant increase in thioflavin-T fluorescence was observed even when the measurements were extended to times much longer than that required for human IAPP to fully convert to amyloid (Fig. 4). Identical results were obtained with independently prepared samples of porcine IAPP on different days.

Amyloid fibrils are rich in β -sheet structure and therefore UV circular dichroism (CD) offers another convenient probe of amyloid formation. CD spectra taken of aliquots removed at the end of the respective thioflavin-T kinetic studies confirm that porcine and human IAPP have very different tendencies to form β -sheet (Fig. 5). The CD spectrum of human IAPP exhibits a strong minimum at 218 nm, a band associated with β -structure that is typical of the CD spectrum of IAPP-derived amyloid. In

contrast, the CD spectrum of porcine IAPP displays a wide band ranging from 208 to 222 nm, indicative of a mixture of helical, random, and β -sheet structures. CD spectra were recorded on independently prepared samples of porcine IAPP on different days and similar results obtained.

Neither CD nor thioflavin-T fluorescence provides direct information about the morphology of any aggregates formed. Consequently, transmission electron microscopy (TEM) was used to image the reaction products. TEM micrographs of human IAPP revealed dense clusters of rod-like fibrils with classic amyloid morphology (Fig. 6). These images are typical of those previously reported for human IAPP under similar conditions (19). A few long fibril-like structures were observed after incubation of 32 μ M porcine IAPP for 24 h, but these fibrils were much fewer in number and appeared dispersed among a mat of smaller amorphous aggregates. Three independently prepared samples of porcine IAPP were imaged and similar TEMs obtained each time. The images displayed in Fig. 6 confirm that porcine IAPP is significantly less amyloidogenic than its human counterpart.

The inability of porcine IAPP to form amyloid may reflect the fact that it cannot form the critical nucleus required to initiate amyloid formation and/or that the sequence is not capable of binding to a nucleus. These two possibilities can be partially distinguished by seeding experiments in which a small amount of preformed human IAPP amyloid fibrils are added to a solution of porcine IAPP. We added porcine IAPP to a small amount of preformed human IAPP amyloid to test the ability of the human peptide to seed porcine IAPP aggregation. Thioflavin-T fluorescence measurements showed that porcine IAPP is incapable of fibril formation even when seeded by human IAPP (Fig. 4B). Although this result may not be particularly surprising given that seeding often occurs with high specificity and even seemingly minor changes in sequence can affect the ability to seed (31, 32), it is entirely consistent with our findings that porcine IAPP is not amyloidogenic.

Differences in Primary Sequence of Porcine and Human IAPP Explain Species Differences in IAPP Cytotoxicity.

Given that synthetic porcine IAPP is much less amyloidogenic than human IAPP, we predicted that it would also be less toxic. The effect of porcine IAPP on beta cell toxicity was next assessed in transformed (INS-1) beta cells by Alamar blue reduction (Fig. 7B). As expected, addition of human IAPP (40 μ M) substantially reduced INS-1 cell viability after 24 h ($12 \pm 5\%$ viability) and further increases in IAPP concentration virtually eliminated any viable cells. In contrast, at the same concentration of porcine IAPP, INS-1 cell viability was significantly greater ($93 \pm 2\%$, $P < 0.001$). To confirm the reduced toxicity of porcine compared to human IAPP, we assessed the induction of apoptosis in INS-1 cells by transferase-mediated dUTP nick-end labeling (TUNEL) staining after 16 h of incubation of cells with either peptide (Fig. 7A). In the presence of 40 μ M human IAPP, the proportion of apoptotic beta cells was $44 \pm 10\%$ compared to only $6 \pm 1\%$ ($P < 0.001$) following incubation in the presence of porcine IAPP. Even in the presence of a higher concentration of IAPP (100 μ M), the proportion of TUNEL-positive beta cells was still much higher when cells were exposed to human ($90 \pm 5\%$) compared to porcine ($14 \pm 3\%$) IAPP ($P < 0.001$). We confirmed these results with porcine IAPP synthesized by two independent sources. Thus, porcine IAPP is much less fibrillogenic and cytotoxic than its human counterpart.

Discussion

IAPP-derived amyloid deposits are hallmarks of islet pathology in type 2 diabetes. Whereas in type 2 diabetes islet amyloid formation takes months or years to develop, islet amyloid has been observed to form rapidly in cultured human islets (33), in human islets transplanted into diabetic murine recipients (34),

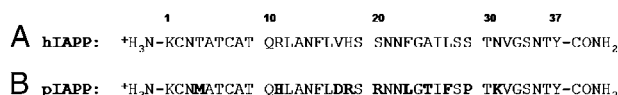


Fig. 3. Primary sequences of human and porcine islet amyloid polypeptide. (A) Human IAPP (hIAPP) sequence. (B) Porcine IAPP (pIAPP) sequence. All peptides have an amidated C terminus and free N terminus with a Cys-2 to Cys-7 disulfide bridge. Residues are numbered according to their position in mature IAPP. Residues differing from the human sequence are indicated in bold.

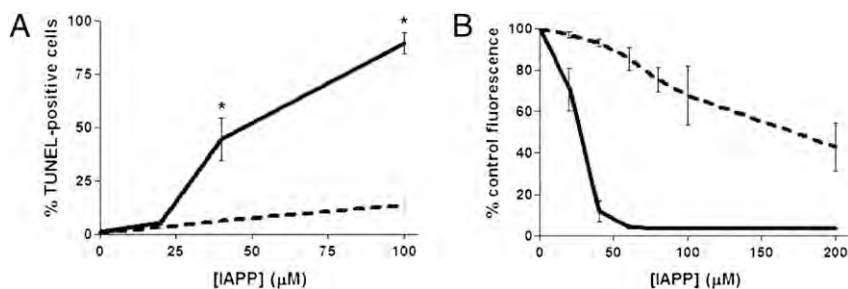


Fig. 7. Porcine IAPP is less cytotoxic than human IAPP. (A) TUNEL staining following 16-h incubation of INS-1 cells in the presence of human or porcine IAPP. *, denotes statistically significant difference from porcine IAPP-treated cells ($P < 0.05$). (B) Alamar blue viability assay following 24-h incubation of INS-1 beta cells in the presence of human or porcine IAPP. The EC_{50} for human IAPP was 27 μ M compared to 172 μ M for porcine IAPP ($P < 0.05$). Solid line, human IAPP; dashed line, porcine IAPP.

second possibility is that beta cell dysfunction is characteristic of both cultured and transplanted islets and may manifest as impaired beta cell prohormone processing. Indeed, we have recently found that islet transplant recipients have impaired proinsulin processing (38) and that impaired processing of the IAPP precursor proIAPP leads to amyloid formation and beta cell death (39).

Given that the number of islet donors is far exceeded by the number of type 1 diabetic patients who could benefit from islet transplantation (40), new sources of transplantable beta cells are sorely needed. Pig islets show great potential in xenotransplantation. Neonatal porcine islets have been shown to maintain normoglycemia in immune-deficient murine recipients for more than 100 days, with no spontaneous failure (41), and similarly adult pig islet autografts were found to function for up to two years (42). Our findings are in keeping with two previous reports that also did not observe evidence of fibrils or islet amyloid in adult porcine pancreas or transplanted pig islets (42, 43).

In summary, our findings point to the importance of the amyloid-forming potential of IAPP and the toxicity of IAPP aggregates as a limiting factor to islet transplant survival. Should rapid formation of amyloid and associated beta cell loss be, as we now suspect, a significant limitation to human islet transplant success, we propose that use of pig islets will circumvent this limitation.

Materials and Methods

Porcine IAPP Sequencing. Primers (Invitrogen) were designed according to EST sequence (Q29119) from porcine hypothalamus homologous to the predicted sequence for mature (amino acids 1–37) porcine IAPP (for details, see *SI Materials and Methods*). Reverse transcription was performed on RNA isolated from neonatal porcine islets using SuperScript First-Strand (Invitrogen). PCR was performed using Accuprime TaqDNA Polymerase High Fidelity (Invitrogen).

Peptide Synthesis and Preparation. Porcine IAPP and porcine IAPP fragments were purchased from Bachem or synthesized in the Raleigh laboratory using methods previously optimized for the synthesis of human IAPP (44, 45). For details, see *SI Materials and Methods*.

Thioflavin-T Fluorescence Assay. Thioflavin-T fluorescence was used to measure the development of structurally ordered fibrils over time. Fluorescence was measured on a Jobin Yvon Horiba Fluorescence Spectrophotometer. The excitation wavelength used was 445 nm and the emission was 485 nm; excitation and emission slits were set at 5 nm. A 1.0-cm cuvette was used. All kinetic experiments were performed by diluting 68 μ L of filtered peptide stock into Tris buffer containing thioflavin T. Final solution conditions were 16 mM Tris HCl and 65 μ M thioflavin T at pH 7.4 (± 0.3). The peptide concentration for kinetic assays was 32 μ M in 2% HFIP at 25 $^{\circ}$ C. All solutions were stirred during fluorescence experiments to maintain solution homogeneity and increase the rate of the reaction to allow data collection in real time. Experiments were repeated multiple times on independently prepared samples of human and porcine IAPP. Similar results were obtained. The uncertainty in the t_{50} value for the human IAPP reaction was estimated as the apparent standard deviation based upon three repeats.

Circular Dichroism. All CD experiments were performed at 25 $^{\circ}$ C on an Aviv 62A DS CD spectrophotometer. A few minutes before data collection, 300 μ L of peptide solution from the kinetic assay was directly pipetted into a 0.1-cm

quartz cuvette. Far-UV CD spectra are the average of five repeats over a range of 190–250 nm. Spectra were recorded at 1-nm intervals with an averaging time of 3 s. Background spectra were subtracted from collected data. The final peptide concentration for all CD experiments was 32 μ M in 2% HFIP and 16 mM Tris HCl. The spectra are the average of three repeats. Spectra were recorded of multiple, independently prepared, samples of porcine IAPP and similar results were obtained each time.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) was performed at the University Life Science Microscopy Center at the State University of New York at Stony Brook. Aliquots (4 μ L) were removed from the end of the thioflavin-T monitored kinetic experiments, placed on a carbon-coated 200-mesh copper grid, and negatively stained with saturated uranyl acetate. Three independently prepared samples of the porcine peptide were studied and the TEM images collected at the end of each independent kinetic run were indistinguishable. Multiple images were collected for each grid and the micrographs displayed in Fig. 6 are representative of the complete grid.

IAPP Cytotoxicity Assays. To assess toxicity of human and porcine IAPP, transformed rat insulinoma (INS-1) beta cells were used (for details, see *SI Materials and Methods*). Porcine and human IAPP were solubilized in RPMI culture media and added directly to cells. After 24 h, Alamar blue (Biosource International) was diluted 10-fold in culture media and cells were incubated for 3 h at 37 $^{\circ}$ C. Fluorescence (excitation 530; emission 590 nm) was measured by a Fluoroskan Ascent plate reader. After 14 h, cells were fixed in 4% paraformaldehyde (20 min), permeabilized with 0.5% Triton X-100 in PBS, and incubated with TUNEL reaction mixture (Roche Diagnostics) for 1 h at 37 $^{\circ}$ C. Cells were costained with Hoechst-33342 for 10 min. Images were taken using an Olympus BX-61 fluorescent microscope. Image quantification was performed using Image Pro-6.2. IAPP was tested at concentrations between 20 and 200 μ M. Experiments were repeated in triplicate.

Immunohistochemistry and Thioflavin S Staining. For double insulin and thioflavin S staining, sections were blocked in PBS containing 2.0% normal goat serum (Vector Laboratories) and incubated with guinea pig anti-insulin antibody (Dako) at a 1:100 dilution in PBS/1% BSA for one hour, followed by incubation with Texas Red-conjugated goat anti-guinea pig antibody (Jackson ImmunoResearch) for 1 h. All steps were performed at room temperature. Slides were then incubated in 0.5% thioflavin S solution for 2 min and rinsed with 70% ethanol.

Human Islet Transplantation. All human islet transplant experiments were performed at the University of British Columbia (UBC) in compliance with institutional guidelines and approved by the UBC Animal Care Committee. Recipient NOD.scid mice were previously rendered hyperglycemic by a single i.p. injection of 180 mg/kg streptozotocin (STZ) (Sigma) in citrate buffer. Recipient mice were transplanted 3–5 days after STZ injection, when blood glucose levels were above 20 mmol/L. Human islets for these studies were isolated from pancreas obtained from 12 cadaveric organ donors by previously described isolation procedures (46). Recipient mice received transplants of 300–500 hand-picked human islets (or $\approx 1,000$ IE) beneath the kidney capsule. Blood glucose was monitored twice weekly. After 4 or 8 weeks, the graft-containing kidney was removed from normoglycemic mice by nephrectomy and the animals allowed to recover, to ensure that normoglycemia was being maintained by the islet graft. Graft failure (return to hyperglycemia) was determined by two consecutive blood glucose measurements above 15 mM.

Neonatal Porcine Islet Isolation and Transplantation. Neonatal porcine islets (NPI) were isolated from 1 to 3 day old Landrace-Yorkshire neonatal pigs (1.5–2.0 kg) using the method developed by Korbitt et al. (23) and cultured for 7–10 days at 37 °C, 20% CO₂ in supplemented Ham's F-10 medium (for details, see *SI Materials and Methods*). A single i.p. injection of STZ (200 mg/kg; Sigma) was administered to 8- to 10-week-old male SCID Biege mice to induce diabetes, and animals were considered to be diabetic after two consecutive blood glucose measurements ≥ 20 mM. Grafts containing a mass of 2,000 NPI were transplanted under the left kidney capsule in confirmed diabetic mice. Animals were monitored weekly for nonfasting blood glucose levels.

Adult Porcine Islet Isolation and Transplantation. Pig pancreata were harvested and islets isolated and transplanted as detailed in *SI Materials and Methods*.

Statistical Analysis. Data are expressed as mean $\pm$ SEM. Statistical analyses were performed using one-way ANOVA followed by a Newman-Keuls post hoc test. $P < 0.05$ was taken as significant. Data are representative of a minimum of three independent experiments performed in triplicate.

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