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Award Number: DAMD17-00-1-0116

TITLE: Endothelial progenitors as vectors for systemic gene therapy of breast cancer

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Birmingham AL 35294-0111

REPORT DATE: August 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-08-2004	Final	10 Jul 00 – 09 Jul 04

4. TITLE AND SUBTITLE
Endothelial progenitors as vectors for systemic gene therapy of breast cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-00-1-0116

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Alabama University at Birmingham
Birmingham AL 35294-0111

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Gene therapy offers a potentially important treatment for breast cancer, however a noted problem with current vector systems is the lack of efficient and targeted systemic delivery to the primary tumor and disseminated metastases. To address this issue we proposed the use of endothelial progenitor cells (EPCs), which have the propensity of homing to sites of neovascularization characteristic of growing tumors. The success of this approach requires efficient isolation of EPCs and subsequent loading of the EPCs with therapeutic modalities. We hypothesized that EPCs can, after intravascular injection, localize into sites of tumor neovascularization and deliver therapeutic payloads. In this regard, the key findings of this research project are that (1) EPCs can be isolated and enriched from fresh human blood, (2) blood-isolated EPCs can be efficiently "loaded" with therapeutic adenovirus (Ad) vectors, (4) the oncolytic Ad vectors are able to amplify their therapeutic payloads within the EPCs, (5) loading EPCs with Ad vectors does not inhibit their intrinsic homing activity, and (6) loaded EPCs deliver the oncolytic Ad vectors that exert an effective anti-tumor activity. Overall, the findings of this project support the application of therapeutic EPCs as cellular vehicles for gene therapy of both local and disseminated breast cancer.

15. SUBJECT TERMS
Endothelial progenitor cells, live-viral vectors, adenovirus

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	71	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

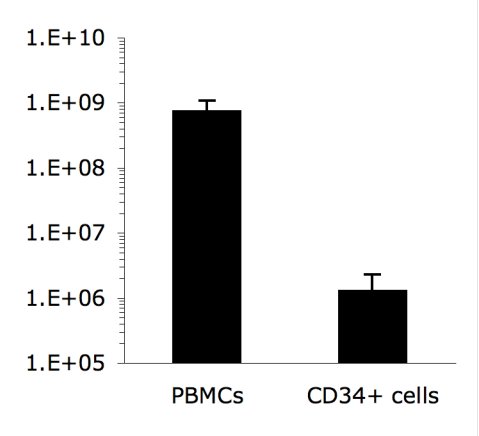
The development of resistance to radiation and chemotherapeutic agents that cause DNA damage is a major problem for the treatment of breast cancer [1], which argues for the development of new therapeutic agents that can either augment the effects of radiation and chemotherapy or that can be applied as an adjunct or alternative treatments. One promising new treatment modality is the application of vector-mediated gene therapy. A noted problem with many vectors, including both viral and non-viral vectors, used for gene therapy is the lack of efficient and targeted delivery to the primary tumor and disseminated metastases. To address this issue, we have proposed the use of $CD34^+$ and/or $Flk\text{-}1^+$ endothelial progenitor cells (EPCs), which have the propensity of homing to sites of neoangiogenesis. Key to the success of this approach is the efficient genetic modification of the EPCs with therapeutic modalities. We have hypothesized that modified EPCs can, after intravascular injection, localize into sites of tumor neovascularization and deliver therapeutic payloads. Within the scope of the proposed work we will test this hypothesis by demonstrating that the EPCs (1) localize to sites of neovascularization in a murine model, (2) express virally-encoded transgenes and (3) effect a therapeutic activity at tumor engraftment sites. Importantly, the natural targeting capacity of EPCs will allow their use vectors for gene therapy of both local and disseminated disease and establish a new paradigm for the treatment of breast cancer.

Body

On 9/14/00 the Principle Investigator responsibilities for this project were reassigned to Dr. Jerry L. Blackwell. During the

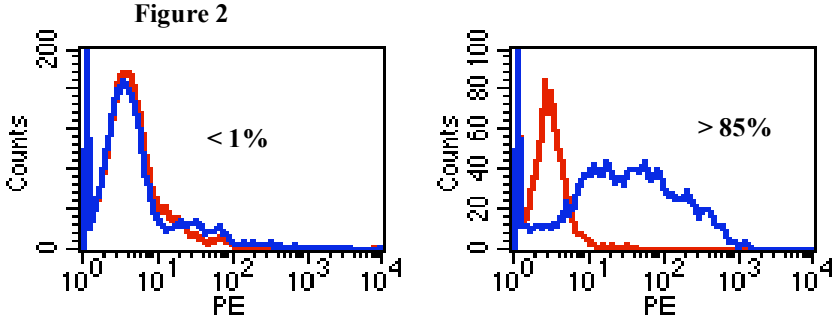
early the early developmental phases of the project I had become very interested in the project [2, 3] and enthusiastically endorsed taking over the project when Dr. Jesus Gomez-Navarro left the University of Alabama and could no longer service the grant. Immediately after taking over the Principle Investigator responsibilities I assigned a highly experienced research assistant, Dr. Hui Li, to work on this project. Dr. Hui had ~50% effort dedicated to this project. Towards the end of 2002, Dr. Yosuke Kawakami, a talented post-doctoral fellow working in the Principle Investgator's lab, also joined the project team. Dr. Kawakami's primary role is to evaluate the *in vivo* translational components of the project and had 25% effort dedicated to the project. During the final reporting period, a pre-doctoral fellow, Ms. Jennifer W McCullars from the UAB Department of Bioengineering, began working on the project at ~50% effort.

As discussed in the 2001 Annual Report, one of our first objectives was to develop a protocol for isolation of blood-derived CD34+ EPCs. We noted at that time that we would need populations of the CD34+ EPCs on a more frequent basis than what was anticipated in the original proposal. For this reason we investigated the use of fresh leukoconcentrates, or "buffy coats", that were readily obtainable from a local community blood bank at a nominal fee (i.e., $25/unit). Over the course of funding period 2-3 of the proposal we obtained blood from 15 different donors. The average number from different donors (n = 15) of total crude PBMCs and enriched CD34+ EPCs are shown in Figure 1. On average we



obtained 7.3 x 10^8 PBMCs and 1.3 x 10^6 CD34+ EPCs from the 200 ml of donor blood. The total number of enriched CD34+ EPCs ranged from 4.5 x 10^5 to 2.2 x 10^6 cells from individual donors. Based on these results we decided that this was valuable resource for the CD34+ cells needed for successful testing of this proposal. Using an immunomagnetic positive selection procedure, we were able to isolate CD34+ EPCs from these PBMCs. Figure 2 shows the purity of CD34+ EPCs before (left) and after (right) isolation from PBMCs. Before purification < 1% of the cells are CD34+, yet after purification >85% of the cells are CD34+. **The ability to obtain a highly enriched CD34+ EPC population from a readily available blood source has been a significant accomplishment in the progression of this project.**

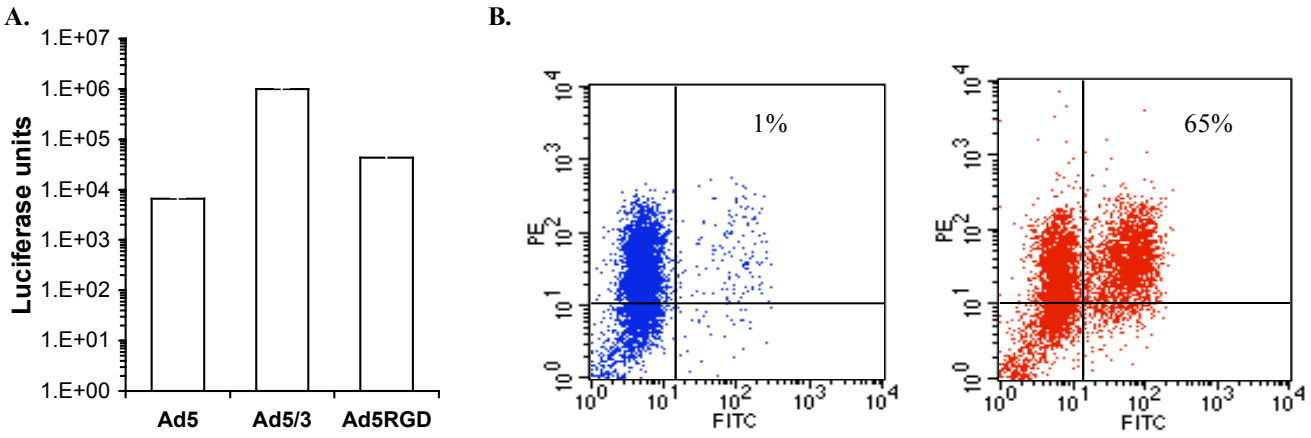
Figure 2



In the original application we had proposed to use herpesvirus vectors for genetic modification of EPCs. However as noted in previous Annual reports, the ability to upscale herpesvirus production proved to be a significant hurdle. Although sufficient amounts of herpesvirus were achievable for pilot *in vitro* experiments, the amounts needed for *in vivo* studies were very difficult to produce. Because of our experience using the adenovirus (Ad) as a gene transfer vector, we began a parallel series of pilot experiments to investigate the use of the Ad vector for gene transfer into CD34+ EPCs. We knew from previous experience that upscaling would not be a problem if the Ad vector could be used for genetic modification of the CD34+ EPCs. In the first pilot experiment we observed that the Ad serotype 5

(Ad5) vector very inefficiently infected CD34+ EPCs, suggesting that CD34+ EPCs express no or low levels of the Ad5 receptor. Next we evaluated gene transfer using two novel Ad5 vectors, Ad5/3 and Ad5RGD, whose cellular receptor tropism has been modified [4-6]. The Ad5/3 vector infects cells through the Ad serotype 3 receptor and the Ad5RGD vector infects cells through RGD-binding integrins, such as the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Figure 3A compares the efficiency of luciferase reporter gene transfer into the CD34+ cells using the Ad5, Ad5/3 and Ad5RGD vectors at equal multiplicities of infection (MOI; MOI = 100). The Ad5/3 vector transduced the CD34+ EPCs >2 log factors better than Ad5. The Ad5RGD vector transduced the CD34+ EPCS ~1 log factor better than Ad5. We next evaluated the frequency of infection using flow cytometric analyses (Figure 3B). As shown in the upper-right quadrants in Figure 3B, approximately 1% of the CD34+ EPCs are infected by Ad5 (left) compared to 65% by Ad5/3 (right). This particular experiment was done using an MOI of 10. Using slightly higher MOIs (i.e., 25-50), we were able to quantitatively infect the entire CD34+ cell population (data not shown). **This series of experiments demonstrated that the tropism-modified Ad5/3 vector is a very efficient alternative gene transfer vector for genetic modification of CD34+ EPCs, which is another significant, novel finding by this project.**

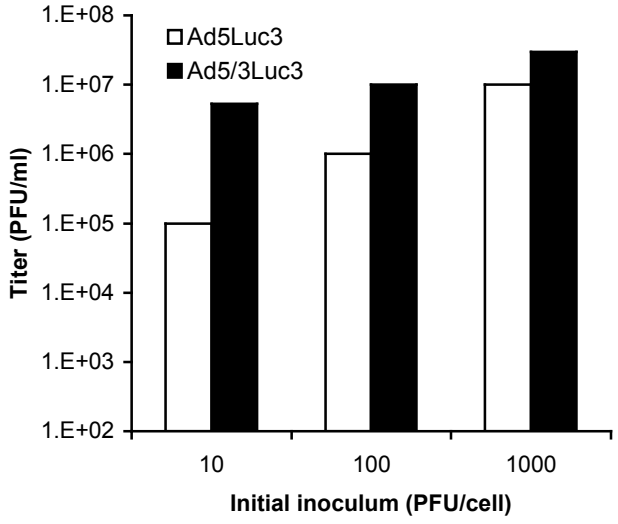
Figure 3



The previous experiments (above) indicated to us at the time that we should further investigate the Ad vector platform for this project. We therefore considered some of the noted advantages over other live-viral vectors that include (i) the ability to easily grow and purify high titered Ad vector preparations, (ii) the Ad vector transduces both dividing and nondividing cells, and (iii) that compared to many other viral and most nonviral vectors, Ad vectors transduce a wide range of cell types. Considering these advantages and the results shown in Figure 3, we decided to further investigate the use of tropism-modified Ad vectors for gene transfer to CD34+ EPCs. In this regard, one extremely promising area in gene therapy research is the use of oncolytic Ad vectors that selectively replicate in tumor tissues. These conditionally-replicative Ad (CRAd) vectors induce oncolysis in tumor tissue, but spare normal tissues [7]. We therefore sought to "load" CD34+ EPCs with tropism-modified CRAd vectors. The hypothesis is that the CD34+ EPCs will deliver their oncolytic payload to the tumor tissue vasculature where the CRAd would subsequently replicate and destroy the tumor blood supply.

For this approach to succeed, it is necessary that the CD34+ EPCs support CRAd replication, which has not been reported to date. To investigate this question, CD34+ EPCs were infected with replication-competent Ad5 or Ad5/3 vectors. Forty-eight hours later, the

Figure 4

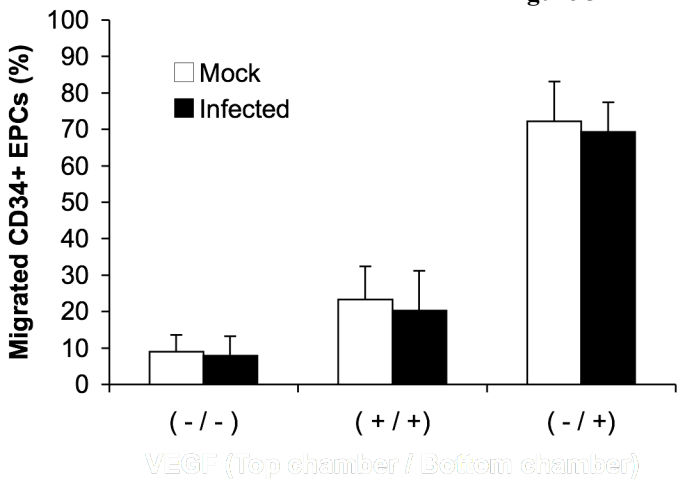


cells were harvested and amounts of *de novo* Ad vector production was measured by plaque assays on HEK293 cells (Figure 4). Infection by both Ad5 and Ad5/3 vectors resulted in *de novo* virus production. As predicted, Ad5/3 infection of CD34+ cells resulted in as much as 1.5 log factors higher titers of virus produced compared to an Ad5 infection of CD34+ cells. **This is the first report of the ability of CD34+ EPCs to support a productive Ad infection and, as such, an important finding by this project. Importantly, this experiment establishes the concept that CD34+ EPCs may be useful vehicles in the application of oncolytic CRAd virotherapy.**

As just described above, one of our downstream goals is to use CD34+ EPCs for delivery of oncolytic CRAd vectors to sites of tumor angiogenesis where the viral vector would subsequently replicate and destroy the tumor blood supply. For such a system to work, the infection of the CD34+ EPCs should not perturb the intrinsic ability to the CD34+ EPCs to home to the tumor in response to angiogenic signals, such as vascular endothelial growth factor (VEGF). We therefore developed an *in vitro* migration model to test whether infection by CRAd vectors affected the ability of the CD34+ EPCs to migrate to VEGF. Briefly, mock- or Ad5/3-infected CD34+ EPCs were placed in the top well of a cell migration chamber. The lower well of the migration chamber contained media supplemented with or without VEGF. Six hours later, the numbers of CD34+ cells that migrated to the lower chamber were determined by a trypan blue exclusion assay. Figure 5 shows the results when VEGF was added neither the top nor bottom well (-/-), both the top and bottom wells (+/+), or only to the bottom well (-/+). In the absence of VEGF in both the top and bottom well (-/-), only a small fraction (<10%) of the CD34+ cell population migrated to the low well.

This is expected since there is no chemoattractant to stimulate migration. When VEGF was present in both the top and bottom wells (+/+), a slightly higher number of cells (~20%) migrated to the lower chamber. A likely explanation is that the VEGF induces the CD34+ cells become more mobile, however in the absence of a gradient between the upper and lower chambers their movement is largely undirected. When VEGF was present only in the lower well (-/+), there is a large increase (i.e. 70-75%) in the numbers of cells that migrate to the lower well. This last result suggests two things: (1) CD34+ cells grown *in vitro* retain that ability to migrate to the chemoattractant, VEGF and (2) infection with the replication-competent Ad5/3 did not significantly alter the ability of the CD34+ cells to migrate (compare Mock to Infected). **Importantly, there results further support the hypothesis that CD34+ EPCs that are loaded with CRAd vectors may be useful as cellular vehicles for targeting tumors that secrete angiogeneic factors like VEGF.**

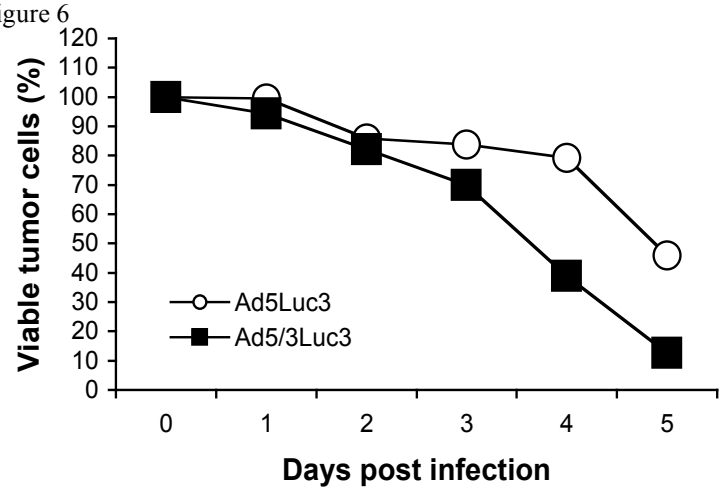
Figure 5



Based on the previous results, we next investigated whether CD34+ EPCs loaded with oncolytic Ad vectors could be used therapeutically to kill tumor cells. To test this question we combined the *in vitro* cell migration model (described above) with an assay for quantifying cell killing. CD34+ EPCs were either mock-infected or infected infected replication-competent Ad5 or Ad5/3 vectors. After washing to remove free virus, the

mock- and CRAd-infected CD34+ EPCs were then placed in the top well of a chemotaxis chamber. MDA-MB-231 breast cancer cells were placed in the bottom well of the chemotaxis chamber. In addition, vascular endothelial growth factor (VEGF) was supplemented to the media in the bottom well of the chemotaxis chamber to simulate the angiogeneic stimulus [8, 9]. The CD34+ EPCs were then allowed to migrate to the bottom chamber for 6 hours, after which the top well of the chemotaxis chamber was removed. Over the course of the next 5 days, the number of tumor cells killed by the release of oncolytic CRAd vectors relative to the uninfected control was determined using an MTT Cell Proliferation assay [10]. The breast cancer tumor cells were efficiently killed in both groups, with the Ad5/3-infected CD34+ EPCs being more effective. Similar results were obtained with three additional breast cancer cell lines as well (i.e., MDA-MB-361, MCF-7 and Hs578T). These results demonstrate that CD34+ EPCs infected with replication-competent Ad vectors are able to migrate to breast tumor cells in response to an angiogenic stimuli and, subsequently, deliver their oncolytic payload to induce an effective anti-tumor effect. To the PIs knowledge this is the first demonstrated use of CD34+ cellular vehicles loaded with a CRAd that elicit a therapeutic effect in breast cancer cells. As such, this a very significant accomplishment for the project and establishes a key proof-of-

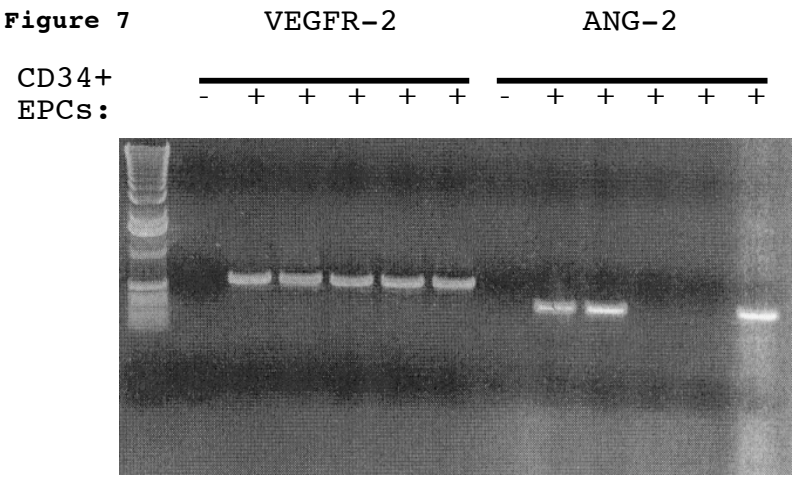
Figure 6



concept for advancing to the next steps in evaluating this approach.

During the final funding period of the project we have sought to establish whether the results we observed *in vitro* thus far translate to an *in vivo* murine tumor model. To establish this point, breast cancer cells were implanted subcutaneously into the flank regions of nude mice. Ten days later, 1 x 10^6 CD34+ EPCs isolated from human blood (as described above) were injected intravenously via the tail vein. Twenty-one days later, VEGF receptor-2 (VEGFR-2) and angiopoictin-2 (ANG-2) mRNA expression in the breast tumor xenografts was analyzed by nested RT-PCR (Figure 7). Both of these proteins have been reported to be expressed by human CD34+ EPCs [9]. We were able to detect VEGFR-2 mRNA expression in 5 of 5 mice and ANG-2 mRNA expression of 3 of 5 mice that received CD34+ EPCs (see lanes labeled (+) in Figure 7). Neither VEGFR-2 nor ANG-2 mRNA expression was detected in any of the breast cancer xenographs from mice that did not receive CD34+ EPCs (see lanes labeled (-) in Figure 7). These results strongly suggest that the CD34+ EPCs migrated and engrafted into the human breast cancer xenografts.

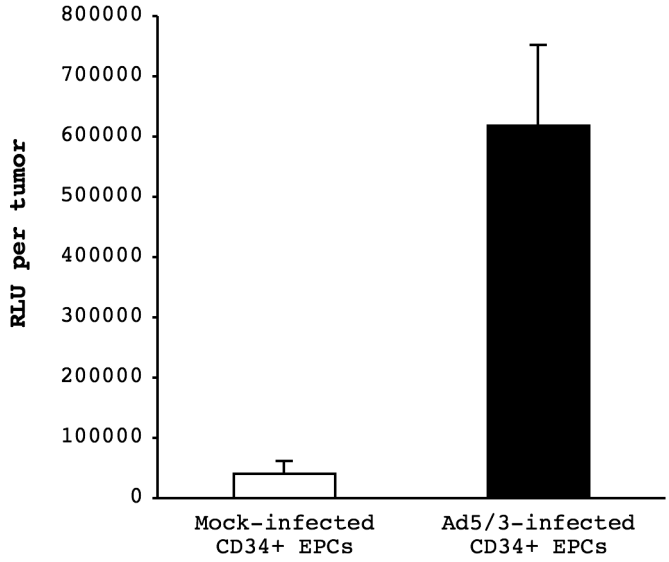
Figure 7



Next, we repeated the previous experiment using CD34+ ECPs that were infected with a non-replicating Ad5/3 vector that expresses the luciferase reporter gene. Three days after injecting tumor-

bearing mice with either mock- or Ad5/3-infected EPCs, the tumors were resected, homogenized and luciferase activity was measured (Figure 8). By correlating the amount of luciferase activity measured in these tumors homogenates to luciferase activity in known numbers of CD34+ EPCs infected *in vitro*, we were able to estimate that ~1 x 10^4 (or ~1%) of the infected CD34+ EPCs homed to the tumors. At later time points (i.e., Days 6 and 14), we were unable to detect luciferase activity in any of the tumors homogenates. We presume that the lack of luciferase activity at the later time points is due to the natural silencing of Ad vectors due to cell turnover. **Nevertheless, these data strongly suggested that at least a fraction of the infected CD34+ EPCs were able to reach the tumor bed, which supported our main hypothesis for applying the CD34+ cells as therapeutic delivery vehicles.**

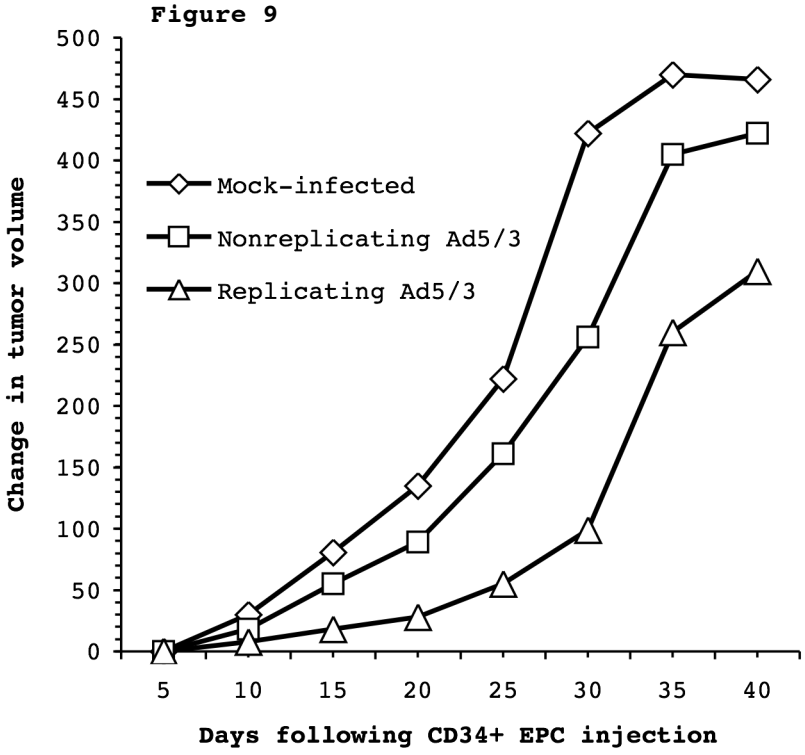
Figure 8



In the most recent series of experiments, CD34+ EPCs were mock-infected or infected *ex vivo* with replication-incompetent Ad5/3 (negative control) or replication-competent Ad5/3 vectors. The hypothesis is that the infected CD34+ EPCs would delivery the oncolytic Ad5/3 vector to the tumor where the virus would subsequently replicate and destroy the tumor cells and/or blood supply feeding the tumor cells. The mock- or Ad-infected CD34+ EPCs were injected intravenously into breast cancer tumor-

bearing mice as described above. Tumor volume (width X length X height) was measured and recorded every other day for 40 days (Figure 9). The graph in Figure 9 shows the tumor volumes every fifth day of the experiment. Although the tumors increased in volume in each of the experimental arms, there was a trend in the replicating Ad5/3 group of slower tumor growth. The effect exerted by the replicating Ad5/3 vector was most noticeable up to the Day 25 time point. At the Day 30 time point the tumors rapidly grew at a rate that was comparable to the Mock- and nonreplicating Ad5/3-infected groups. It is not clear why the tumors in the replicating Ad5/3-infected group began rapidly growing. One possibility is that the rate of tumor cell division exceeded the rate of virus replication and spread. Nevertheless we interpret these results as largely positive because of the amount of control over tumor growth rate exerted by the replicating Ad5/3 virus up to Day 25 of the experiment. We are currently investigating the use of larger numbers of infected CD34+ EPCs at the beginning of the experiment and slower growing tumors. **Overall, although we would have preferred to have seen complete control over tumor growth during the entire experimental period, this is an**

Figure 9



extremely significant result demonstrating the potential utility of CD34+ EPCs as therapeutic vehicles.

Key Research Accomplishments

- Validated protocol development for obtaining a highly enriched $CD34^+$ EPCs preparation from human peripheral blood mononuclear cells
- Established another live-viral vector system for genetic-modification of CD34+ EPCs that has important translational implications
- Demonstrated that tropism-modified Ad vectors can efficiently infect and replicate in CD34+ EPCs with low toxicity
- Demonstrated that CD34+ EPCs loaded with therapeutic agents can efficiently kill cancer tumor cells in an in vitro model
- Showed that the genetic loading with oncolytic Ad vectors does not interfere with CD34+ EPC migration functions in an *in vitro* chemotaxis model
- Showed that CD34+ EPCs are capable of migrating and engrafting into human breast cancer xenografts in an *in vivo* murine model
- Demonstrated CD34+ EPCs are able to carry Ad vectors to the tumor and subsequently the Ad vectors express gene products.

- Developed data suggesting that CD34+ EPCs can deliver replicating Ad5/3 vectors to tumors and the Ad vectors can subsequently exert a partial reduction in the growth rate of the tumors.

Reportable Outcomes

- Invited to present the findings of the current study as an oral presentation at the 2002 American Society of Gene Therapy meeting in Boston MA. The title of the presentation was "CD34+ Endothelial Progenitors as Cellular Vehicles for Oncolytic Adenovirus Anti-Tumor Therapy".

- Invited to present the findings of the current study as an oral presentation at the 2002 Era of Hope DOD Breast Cancer Research Program's Meeting in Orlando FL. The title of the presentation is "CD34+ Endothelial Progenitors as Cellular Vehicles for Gene Therapy of Breast Cancer".

- Manuscript in preparation entitled "Endothelial Progenitors as Cellular Vehicles for Oncolytic Adenovirus Anti-tumor Therapy" for submission to Cancer Research.

- Pilot project funding has been awarded through the UAB Breast Cancer SPORE mechanism based on work supported by this award.

- The "Cell Vehicle Working Group" has been created at the UAB Division of Human Gene Therapy to advance discoveries using this type of vector delivery/targeting system. The Cell Vehicle Working Group meets monthly to discuss project

development and currently includes 6 members, which are Jerry Blackwell (P.I.), David Curiel (Division Director), Yosuke Kawakami (Post doctoral fellow), Laurisa Pereboeva (Group leader), Hui Li (Researh Assistant), and Jill Nagle (Graduate Research Assistant).

- Established a collaboration with an investigator in the UAB Department of Bioengineering for the application of CD34+ EPCs to promote wound healing in patients with pressure ulsers.
- Two manuscripts have been prepared and one is being written that are direct products of this study. The two manuscripts that have been prepared are included in the Appendix.

Conclusions

Important advancements have been made in the development of a readily available source of human CD34+ EPCs, which was a significant problem in the early phases of the project. We can now produce CD34+ EPCs on a regulat basis and more often if needed. In addition to the herpesvirus vector system, we have also established a second live-viral vector system using Ad5/3 for the genetic modification of CD34+ EPCs. A strong argument can be made for using the Ad vector rather than the herpesvirus vector system, which includes both considerations of production and downstream translational issues. The vectors changes allowed us to test the utility of the CD34+ EPCs in a series of *in vitro* and *in vivo* models. The results of these experiments strongly suggest that cellular vehicles are an effective tools for the targeted delivery of novel therapeutics to tumors. This exciting approach has the potential of being developed for a

broad range of both local and disseminated tumors.

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Appendix

IN VIVO EVALUATION OF LOCALLY APPLIED ENDOTHELIAL PROGENITOR CELLS FOR WOUND HEALING

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2

Abstract: Local applications of endothelial progenitor cells (EPCs) were evaluated *in vivo* for improving healing and vascularization of full-thickness wounds. Wounds were created on the dorsum of NZW rabbits and were treated with EPC-seeded scaffolds, non-seeded scaffolds, or no treatment. Histomorphometry was used to calculate epithelialization and contraction rates, and volume fractions of cell nuclei and blood vessels at 2 and 3 weeks. Results showed that local applications of rabbit mononuclear cells led to some improvements in overall healing, particularly in the 3-week animals, with increases in epithelialization, tissue fill, and volume fraction of blood vessels. Increases in vascularization were seen in the outer region of the wound at 2 weeks and in the inner region at 3 weeks. Also, the local application of rabbit cells increased the volume fraction of fibroblasts while decreasing the volume fractions of macrophages and neutrophils. No improvements in healing were seen with local applications of human EPCs, however the human cells did lead to some increases in vascularization, particularly at 3 weeks. Additionally, no significant increases in inflammation were seen with the use of human EPCs in the rabbits. Therefore, local applications of EPCs show promise for improving healing and vascularization in chronic full-thickness wounds.

Key Words: endothelial progenitor cells; wound healing; vasculogenesis; albumin

INTRODUCTION

Chronic wounds, such as pressure ulcers, are difficult to heal and costly to the patient as well as to the healthcare system. For example, spinal cord-injured (SCI) pressure ulcer patients typically have two treatment options: non-surgical treatment with three to six months of bedrest or skin flap surgery followed by six weeks of bedrest. Although surgical treatment often results in less bedrest, the rate of complications and recurrence is high.[1] Furthermore, skin flap surgery adds an estimated $30,000 to the cost of treating a SCI patient.[2]

A non-surgical treatment capable of reducing the time required for healing by half (increasing healing rate by 100%) is desired, making the time to heal comparable to the surgical option. Several non-surgical treatments have been used to improve healing in chronic wounds. These include debridement and wound dressings (polymer films, hydrocolloid dressings, hydrogels, alginates, and saline-soaked gauze).[3] In addition to dressings, topical agents such as growth factors and antibiotics have been applied locally to try to improve healing. However, at this time, none of these treatments have been shown to increase the healing rate by 100%.

Degradable scaffolds are a promising treatment to speed healing for chronic wounds, and there are a number of artificial skin systems clinically available. Scaffolds composed of natural materials (such as fibrin, albumin, and collagen) can be degraded by biofeedback control because the enzymatic degradation is controlled by cellular invasion into the wound as it heals. These degradable scaffolds not only provide a structure to facilitate the ingrowth of tissue and blood vessels, they also have biologic activity and can stimulate cell migration and activity.[4] Adhesive scaffolds, such as fibrin and albumin, can set up *in situ*, preventing the need to suture the scaffold in place. Cellular and tissue ingrowth is important for success of these scaffolds, with the two keys factors being collagen ingrowth and new blood vessel formation to provide sufficient oxygen levels. Angiogenesis (new blood vessel formation from pre-existing vessels) is typically less than half the rate of fibroblast ingrowth and therefore becomes the rate-limiting step. Angiogenesis is also important to support the epidermal layer and is therefore the rate-limiting step for epithelialization as well.[4]

Until recently, revascularization of tissues in adults was thought to be accomplished exclusively by angiogenesis. Neovascularization through differentiated endothelial cells, however, is limited in terms of cellular life span, low proliferative ability, and the inability of endothelial cells to incorporate into remote target sites.[5] However, vasculogenesis, the formation of new blood vessels from bone marrow-derived endothelial progenitor cells (EPCs), is the mechanism responsible for the formation of the primordial vascular plexus during embryogenesis and has been shown to aid in adult revascularization of ischemic tissues.[5] It appears that angiogenesis is most important for minor injuries, while vasculogenesis contributes when extensive vascular regeneration is required.[5]

Circulating EPCs are a subpopulation of CD34+ cells from the bone marrow that mobilize to sites of tissue ischemia, such as vessels during tumor growth, wound healing, skeletal and cardiac ischemia, and corneal neovascularization, where they differentiate into mature endothelial cells and form new vessels.[5-6] These CD34+ EPCs can also be isolated from the peripheral blood, umbilical cord blood, and fetal liver.[7]

The goal of this study was to evaluate the potential of EPCs to be used therapeutically to improve wound healing in chronic wounds, such as pressure ulcers, by promoting vasculogenesis. Specifically, it was hypothesized that applying EPCs locally in an albumin matrix to the wound would enhance wound healing compared to the control by reducing the time required for the EPCs to arrive at the wound site and initiate vasculogenesis.

MATERIALS AND METHODS

Endothelial Progenitor Cells

Human EPCs

Human endothelial progenitor cells were either obtained through the National Disease Research Interchange (Philadelphia, PA) or isolated from donor human blood.[8-11] All protocols for human subjects were approved by the University of Alabama at Birmingham's Institutional Review Board.

For the isolation procedure, human peripheral blood was diluted 1:2 with phosphate buffered saline (PBS) without calcium or magnesium (Cambrex, East Rutherford, NJ). The mixture was layered over Histopaque solution (1.077 g / mL, Sigma, St. Louis, MO), and the peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation (400 g, 20^{o8} cells.

The PBMC were then incubated with blocking reagent (100 µl per 10^8 total cells) for 10 minutes and then labeled with colloidal super-paramagnetic MicroBeads conjugated to monoclonal mouse anti-human CD34 antibody (100 µl per 10^8 total cells) for 30 minutes. The labeled cells are then washed and subsequently applied to a MidiMACS magnetic column (Miltenyi Biotech). After washing the column (3 column volumes), the $CD34^+$-labeled cells are obtained by removing the magnetic field and eluting with PBS/EDTA. Isolated CD34+ cells were then resuspended in M199 media (Sigma) with 20% fetal bovine serum (Hyclone, Logan, UT), 300 mg / mL endothelial cell growth supplement from bovine neural tissue, 100 U / mL penicillin, and 100 mg / mL streptomycin (Sigma).

Rabbit EPCs

Autologous rabbit peripheral blood (20-30 ml) was drawn from the ear vein of the animals on the day prior to surgery. Rabbits have a marker similar to human CD34, however there is currently no available antibody to the rabbit marker for isolating EPCs.[12] Therefore, rabbit PBMC were isolated using the same protocol as for human blood but were not further separated.[12-14] Assuming a similar EPC concentration as in human blood, the rabbit CD34+ cells represent about 1% or less of the PBMC fraction.[8] All animal procedures were performed in compliance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee's guidelines and those outlined in the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals".

Albumin Scaffolds

Albumin gels were made by mixing an albumin protein solution and a poly(ethylene glycol) (PEG) solution to form an adhesive PEG-crosslinked albumin scaffold.[15] The albumin protein solution was prepared by gently dissolving 0.33 g / mL of lyophilized fraction V rabbit serum albumin (Sigma) into a 0.85% NaCl solution until clear. The PEG solution was prepared by solvating 0.10 g / mL of PEG-disuccinimidyl glutarate (molecular weight 10,000 Daltons, SunBio PEG-SHOP, Anyang City, S. Korea) into basic N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Fisher Scientific, Fairlawn, NJ) solution with a pH of 9.2 until clear. Albumin gels were formed by mixing equal amounts of the albumin and PEG solutions using a dual syringe system.

For EPC-seeded scaffolds, isolated EPCs were mixed into the albumin protein solution. Then the albumin and PEG were mixed to form a crosslinked gel containing EPCs evenly distributed throughout.

In Vivo Evaluation of Endothelial Progenitor Cells

Surgical Procedures

The day prior to surgery, the dorsum of each rabbit (female NZW rabbits) was shaved, a depilatory cream was applied to further remove hair, and an antibiotic ointment was applied to reduce irritation. For surgery, animals were anesthetized with ketamine and xylazine administered intramuscularly. Four 4 cm x 4 cm full-thickness wounds extending down to, but not through, the panniculus carnosum muscle were created on the dorsum. The wounds, however, were cut at 3.5 cm x 3.5 cm to allow for expansion after excison. Digital pictures of the wounds were taken to obtain the exact wound dimensions. Each animal had four different wounds: no treatment/control (C), albumin scaffold (A), albumin scaffold + autologous rabbit PBMC (AR), or albumin scaffold + human $CD34^+$ EPCs (AH). For the wounds receiving treatment, two syringes filled with 0.5 mL of albumin solution (or albumin + cells) and PEG solution were applied simultaneously into the wound, and the solutions were mixed. The wound edges were elevated for a few minutes until the scaffold had cured, to prevent loss of scaffold material. For wounds treated with albumin + rabbit EPCs, the scaffolds contained 2×10^7 autologous

PBMC (approximately 2 x 10^5 EPCs). For wounds treated with albumin + human EPCs, the scaffolds contained 2 x 10^5 human $CD34^+$ cells.

The wounds were covered with an occlusive dressing (Tegaderm, Cardinal Health, Dublin, OH). Animals were wrapped with vet wrap and wore rabbit jackets (Alice King Chatham, Hawthorne, CA) to protect the wounds. During weekly assessments, the animals were sedated, and the dressings were removed. Digital photos were taken and a clean dressing was placed on the wounds. Animals were euthanized with an overdose of pentobarbital at the appropriate time (2 or 3 weeks with 5 animals in each group). The wounds were removed and fixed in 10% neutral buffered formalin. Then each wound was embedded in paraffin,Masson's trichrome.

Histomorphometry

Images of the histology slides were captured with an Olympus OLY-750 3CCD camera with Media Cyernetics Pro Series MV capture kit and analyzed using Image-Pro Plus 4.5 software. For epithelialization rate (ER), the lengths of the new epithelial layer on both sides of the wound (Fig. 1) were averaged (EL_1 and EL_2) and converted to mm / wk by dividing by the time (t) interval (Eq. 1),

$$ER = \frac{EL_1 + EL_2}{2t} \quad (1)$$

For contraction rate (CR), the change in wound width was determined from the original (W_o), obtained from the digital photos at the time of surgery, and the new wound width (W_n), measured on the histology slides using hair follicles as a marker for the original wound edge. The wound width was converted to mm / wk from each sound edge by dividing by 2 and by the time interval (Eq. 2).

$$CR = \frac{W_o - W_n}{2t} \quad (2)$$

Overall healing rate (HR) is the sum of CR and ER. Additionally, the ratio of CR to ER was calculated. For tissue fill percentage (TF), the amount of new tissue formed (above the muscle layer and

between the original wound edges) was compared to the total amount of tissue required to completely fill the entire wound.

A stereological point counting method was used to determine the volume fractions of blood vessels and cell nuclei (fibroblasts, macrophages, and neutrophils). Four random areas were used (10x magnification), two from the outer region of the wound (beneath the new epithelium) and two from the inner region of the wound, with a 165-point grid placed over the images. The fraction of grid points on blood vessels or cell nuclei was determined and approximates the volume fractions of these components.[16]

Statistical Analysis

To reduce the influence of animal to animal variability, treated wounds were compared to the controls (no treatment or albumin alone) on the same animal using paired *t* tests. One-tailed tests were used for healing rate parameters, since only increases in HR and ER and decreases in CR were important. Two-tailed tests were used for cell nuclei and blood vessel measurements, since either increases or decreases could be beneficial at different times. Two-tailed paired *t* tests were used to compare the inner and outer regions of the wounds for angiogenesis and cellularity measurements. One-way analysis of variance (ANOVA) was used to compare measurements between the 2- and 3-week time periods. For all data, a p value ≤ 0.05 was used to prove significance.

RESULTS

Epithelialization Rate

At 2 weeks (Tables I and II), there was a significant increase in epithelialization rate (ER) for AR (albumin + autologous rabbit PBMC) compared to A (albumin) (37.4% increase, $p = 0.020$). At 3 weeks, both A and AR increased over C (control), but only A increased significantly (11.5% increase, $p = 0.043$), although AR had the higher percent increase (21.6%). A also gave a significantly higher ER than AH (albumin + human EPCs) at 3 weeks (40.9% increase, $p = 0.041$) and was the only treatment to increase ER significantly between 2 and 3 weeks (56.6% increase, $p = 0.009$). Also at 3 weeks, AH had a lower

ER than C, but was not statistically significant (20.9% decrease, $p = 0.076$). Epithelialization rates generally increased between the 2 and 3-week periods (23.0% average increase, $p = 0.069$).

Contraction Rate

There were no statistically significant differences in contraction rate (CR) between any of the treatments at 2 or 3 weeks (Tables I and II). At 2 weeks, however, AR had a 64% increase in CR compared to C ($p = 0.064$). There were significant increases in CR between 2 and 3 weeks (49.0% average increase, $p = 0.011$), with C increasing the most (81.3% increase, $p = 0.043$).

Healing Rate

At 2 weeks (Tables I and II), AR increased healing rate (HR) significantly over C (30.3% increase, $p = 0.015$). There were no significant differences between treatments at 3 weeks. However, all treatments increased HR between 2 and 3 weeks (42.2% average increase, $p = 0.005$), with the biggest increase for C (58.8% increase, $p = 0.030$).

CR/ER Ratio

At 2 weeks (Tables I and II), there were no statistically significant differences between treatments. Only AH decreased the ratio compared to C. However, the ratio for AH increased significantly between 2 and 3 weeks (137.2% increase, $p = 0.049$) and was 61% higher than C at 3 weeks ($p = 0.086$).

Tissue Fill Percentage

At 2 weeks (Tables I and II), there were no significant differences in tissue fill (TF). However, A and AH both decreased TF compared to C (4.6% decrease, $p = 0.104$ and 2.7% decrease, $p = 0.058$, respectively). At 3 weeks, AR increased TF over A (8.6% increase, $p = 0.021$) and was the best overall treatment. The TF significantly increased between the 2- and 3-week time periods (6.1% average

increase, $p = 0.009$) with AR having the largest increase at 9.5% ($p = 0.014$). AH increased 6.4%, but this was not statistically significantly ($p = 0.108$).

Blood Vessels

In the inner region (Tables III and IV), all treatments had a lower volume fraction of blood vessels (BV_v) compared to C at 2 weeks, but not significantly – with AR having the largest difference (22.6% decrease, $p = 0.099$). There were no significant differences in the inner region at 3 weeks. However, all treatments except C lead to increases in BV_v between the two time periods, though none of the increases were statistically significant.

In the outer region, there were no significant differences in BV_v at either time period. However, at 2 weeks, both A and AR increased BV_v compared to C. At 3 weeks A was the only treatment to increase BV_v compared to C (16.3% increase, $p = 0.086$). A had a significantly higher BV_v in the outer region than the inner region at 3 weeks (16.3% increase, $p = 0.004$), and AR was higher in the outer region at 2 weeks (34.5% increase, $p = 0.060$), though not statistically significant.

Fibroblasts

At 2 weeks (Tables III and IV), in the inner region, the volume fraction of fibroblasts (F_v) was significantly lower for AH when compared to A (29.6% decrease, $p = 0.028$). In the outer region, F_v was significantly higher for A than AR (52.8% increase, $p = 0.003$). A was also greater than C and AH, though not significantly (22.8% increase, $p = 0.077$ and 26.1% increase, $p = 0.065$, respectively). There was a significantly lower (F_v) in the outer region compared to the inner region at 2 weeks for the following treatments: C (33.2% decrease, $p = 0.038$), A (29.1% decrease, $p = 0.006$), and AR (52.7% decrease, $p = 0.005$). At 3 weeks, there were no significant differences between treatments in either region. There were significantly higher F_v at 3 weeks than 2 weeks, for both the inner (99.0% average increase, $p < 0.001$) and outer regions (218.0% average increase, $p < 0.001$).

Macrophages

At 2 weeks (Tables III and IV), in both regions, AH and AR produced the lowest volume fraction of macrophages (M_v). In the outer region, AH was significantly lower than C (41.5% decrease, $p = 0.016$) and also lower than A (31.8% decrease, $p = 0.035$). At 3 weeks, there were no significant differences between any of the treatments in either region. However, there were significant decreases in M_v between 2 and 3 weeks for both the inner (34.8% average decrease, $p = 0.002$) and outer regions (51.0% average decrease, $p < 0.001$).

Neutrophils

At 2 weeks (Tables III and IV), in the inner region, there was a significant increase in volume fraction of neutrophils (N_v) for AH compared to C (28.1% increase, $p = 0.035$). However, AH was significantly lower than C in the outer region (29.5% decrease, $p = 0.036$). Also, in the outer region, A was lower than C (17.9% decrease), though not significantly ($p = 0.072$). C was significantly higher in the outer region (67.0% increase, $p = 0.020$) than in the inner region at week 2. At 3 weeks, there were no significant differences in the inner or outer region, but A was lower than both C and AR in the outer region (38.8% decrease, $p = 0.088$ and 48.2% decrease, $p = 0.099$, respectively). However, all of the treatments significantly decreased N_v between 2 and 3 weeks (67.3% average decrease, $p < 0.001$ for inner region; 72.2% average decrease, $p < 0.001$).

DISCUSSION

The method for seeding EPCs into the scaffolds and the choice of an albumin scaffold were based on previous *in vitro* and *in vivo* studies.[17] Although these cell-seeded scaffolds can be further optimized, this seeding technique with an albumin scaffold worked better than other seeding techniques tried and other EPC-seeded scaffolds such as fibrin.[17]

Although it was hypothesized that applying EPCs directly to the wounds through seeded albumin scaffolds would improve healing rate parameters over the control, it was actually desired that treatments

increase ER while decreasing or maintaining CR relative to the control. Therefore, increases in HR were desired, but with low CR/ER ratios. In general the scaffolds, showed trends toward improving healing rate parameters, though not all of the results were statistically significant. In many of these cases (p values between 0.05 and 0.10), with an n of 5, the test was not sensitive enough to pick up the changes in healing, which had to be about 1.5 times the standard deviation. A larger sample size would have been necessary, increasing the sensitivity of the study, to determine if these improvements could be statistically significant.

Therefore, as hypothesized, AR improved overall HR significantly at 2 weeks. All of the treatments increased HR significantly between 2 and 3 weeks, with C increasing the most. At 3 weeks, AR was still greater than C, but not significantly. While local applications of rabbit PBMC improved HR, human EPCs did not improve HR at either time period. Overall, AR was the best treatment for increasing HR at both time periods, particularly at 2 weeks.

When the healing rates were broken down into epithelialization and contraction rates, it can be seen that some of the increases in overall healing rate were due to higher contraction rates rather than increases in epithelialization. For ER, however, AR again was the best treatment at 2 weeks, higher than C and significantly higher than A. At 3 weeks, A was significantly higher than both C and AH. AR still produced the highest ER at 3 weeks but was not statistically higher than C (21.6% increase, $p = 0.191$). As with overall HR, local application of rabbit PBMC improved ER, but the human EPCs did not. For CR, there were no significant differences between treatments at 2 weeks, but AR and A were about 60% higher than C and 54% higher than AH. Although the treatments significantly increased CR between the time periods, there were no differences between treatments at 3 weeks.

For the ratio of CR to ER, with decreases desired, at 2 weeks AH was the only treatment to decrease the ratio compared to C, though not significantly. However, AH had a significant increase in the ratio between the two time periods, and gave the highest ratio at 3 weeks. For TF, C had higher values than both A and AH at 2 weeks. At 3 weeks, AR had the highest TF and was significantly higher than A.

Looking at all of the healing rate parameters together, as hypothesized, the local EPC treatments showed improvements in healing, particularly in the 3-week period. At 2 weeks, AR had increases in ER and HR, but also increased CR. At 3 weeks, however, AR had increases in ER, HR, and TF (by 21.6%, 9.8%, and 5.2%, respectively) with only a slight increase in CR (5.7%).

It is important to note, however, that the majority of the wounds were completely healed by 3 weeks. Therefore, measurements of contraction and epithelialization would have been underestimated for the 3-week time period. The wounds in the 2-week time period were approximately 63% healed. At the healing rates seen, wounds that healed by 3 weeks probably would have healed at most a few days earlier. Using the actual time period would increase ER, CR, and HR measurements by approximately 5% per day. Measurements made at shorter time periods – 18 or 19 days instead of 21 – are needed to produce more accurate healing rates.

One reason why the local EPCs may not have improved healing as much as expected may be that some of the scaffold and cells were removed during the weekly dressing changes. This problem could be alleviated by leaving the dressings intact for the extent of the study or by reapplying the seeded scaffolds during each dressing change. Based on the healing rate parameters, autologous rabbit PBMC were better at improving healing than human EPCs. However, because the rabbit PBMC fraction contained other cell types in addition to EPCs, it cannot be concluded that the improvements were due solely to EPCs. As reported done in one study, negative selection could be used to remove T-cells, B-cells, and monocytes from the PBMC fraction in order to remove effects of the inflammatory cells on healing.[18]

It was hypothesized that treatment of full-thickness wounds with EPCs would increase revascularization in the wound. More specifically, it was hypothesized that local application of EPCs would increase vessel formation from within the wound versus only from the periphery. At 2 weeks, C gave the highest BV_v in the inner region; in the outer region, A and AR were the best treatments, though these differences were not statistically significant. AH did not improve BV_v in either region. While there were no significant changes between the time periods in either region, all 3 scaffold treatments increased

BV_v in the inner region. At 3 weeks, A was higher than C in the outer region (16.3% increase, $p = 0.086$). A was also significantly higher in the outer than the inner region at 3 weeks.

Therefore, as hypothesized, local applications of EPCs showed improvements in vessel formation compared to the control. However, at 2 weeks, no increases were seen in the inner region. In the 3-week time period, all scaffold treatments improved BV_v in the inner region. Overall, at 2 weeks, AR was the best treatment for increasing BV_v in the outer region. A, AR, AH (for inner) and A (for outer) were the best at increasing vascularization in the 3-week group.

Because there was not as significant an increase in vascularization as expected, the EPCs may have improved healing through other mechanisms. Recent studies suggest that EPCs do play an important role in vasculogenesis, but their function involves more than just blood vessel formation. One study found that EPCs are mobilized to the wound site in response to angiopoietin-1 and stem cell factor, where they secrete other growth factors in a paracrine manner and recruit other cell types to the wound site.[19] Once they are no longer needed, they are cleared from the wound, probably through apoptosis.[19] Additionally, studies have found that there exists two subsets of EPCs. The $CD34^+CD14^-$ subset displays angioblastic characteristics and probably aid in healing by integration into the new vasculature (vasculogenesis). The $CD34^+CD14^+$ subset displays monocytic properties and are more likely promote angiogenesis through the secretion of growth factors and cytokines.[20] Therefore, while EPCs may not lead to significant increases in vascularization, they improve healing through other mechanisms as well.

Fibroblasts are responsible for producing extracellular matrix proteins. Additionally, they secrete growth factors and angiogenic factors that regulate cell proliferation and vascularization,[21] as well as provide the framework for blood vessels. Therefore, increases in the volume fraction of fibroblasts (F_v) were desired. At 2 weeks, A and AR were the best treatments for increasing F_v in the inner region, with A significantly higher than AH. In the outer region, A was best, significantly higher than AR and also higher than C and AH. Also, C, A, and AR produced significantly higher F_v in the inner region than the outer region. At 3 weeks, in the outer area, A and AR once again were the best treatments for increasing

F_v. The increases may have been due to the albumin scaffold alone, though, since AR did not improve F_v over A.

Neutrophils and macrophages are involved in destroying bacteria and phagocytosis of debris.[21] Another goal of the treatments was to shorten the inflammatory process. Both macrophages and neutrophils in the wound are signs are inflammation, with neutrophils more involved in acute inflammation and macrophages in chronic inflammation. Therefore decreases in macrophages and neutrophils, especially in the outer regions of the wounds, were desired.

At 2 weeks, local EPC treatment (AR and AH) decreased M_v in both the inner and outer region. In the outer region, AH was significantly lower than C and also lower than A. At 2 weeks, only AH increased N_v significantly compared to C in the inner region. However, in the outer region, all treatments decreased compared to C, with AH significantly lower and A almost significantly lower. C was significantly higher in the outer region than in the inner region. At 3 weeks, A gave the lowest N_v in the outer region, almost significantly lower than both C and AR.

For all treatments, both M_v and N_v decreased significantly between 2 and 3 weeks. At 3 weeks, there were no major differences in the volume fractions of macrophages or neutrophils for either the inner or outer regions. AR and AH were the best treatments for lowering M_v at 2 weeks in both regions. For N_v, AR was best in the inner region, while AH was best in the outer region. Results from these volume fraction measurements show that there were no significant increases in inflammation caused by using foreign human EPCs in the rabbits. Additionally, there were no significant increases in inflammation for the rabbit PBMC, even though the PBMC fraction contained inflammatory cells. Comparing all cell nuclei measurements, AR was the best treatment, particularly in the outer region, for increasing fibroblasts while decreasing both macrophages and neutrophils.

While the results of this study showed that EPCs were generally successful at improving healing in full-thickness wounds compared to the control, not all of the improvements were statistically significant. Again, increasing the number of animals in the study would be necessary to prove that these

trends are statistically significant. Additionally, it would have been helpful to see the effects of the treatments at earlier time periods, such as 1 week as well as before there was complete healing at 3 weeks.

Another area of concern is the number of EPCs used during treatment. Studies suggest that the number of cells needed to improve revascularization is much higher than the amount of EPCs available in circulation. For example, one study suggested that as many as 0.5-2.0 x 10^4 EPCs per gram of body weight would be needed to revascularize an ischemic limb.[22] While this number may be an overestimate for the smaller areas of wound healing, increasing the number of EPCs used in the treatments could improve vascularization. EPC expansion could be accomplished (1) *in vivo* using drugs or growth factors involved in EPC mobilization, such as GM-CSF, VEGF, angiopoietin-1, or statins;[7] (2) *in vitro* by expanding isolated cell populations in culture;[20] or (3) using gene therapy to overexpress growth factors involved in EPC mobilization, such as VEGF.[23]

Also, in future studies it is probably worth separating the rabbit cells through negative selection to remove T-cells, B-cells, and monocytes.[18] While rabbit PBMC tended to be the best treatment, it cannot be concluded that the positive effects were solely the result of EPCs since the rabbit PBMC fraction contains many other cell populations that may have aiding in healing, particularly some of the inflammatory cells. Removing the inflammatory cells may give a better indication of the role of EPCs in improving healing. In clinical application, however, any benefit gained by further separating out the cells will have to be weighed by the additional time and cost associated with these steps. Future studies should also look at optimizing the matrix for EPC seeding through the addition of growth factors or cytokines or by improving the matrix materials. In addition to local applications of EPCs, future studies should look at the effects of systemic injections of EPCs on wound healing, as well as the combination of local and systemic treatment.

CONCLUSIONS

Applications of EPCs were applied locally to full-thickness wounds through seeded albumin scaffolds and were evaluated for improvements in healing and vascularization at 2 and 3 weeks. The local

EPC treatments improved healing rate parameters mostly in the 3-week animals, with AR being the best treatment for increasing ER, HR, and TF. Local applications of EPCs increased vascularization in the outer region at 2 weeks (AR) and the inner region at 3 weeks (AR and AH). However, the albumin alone (A) lead to similar increases. For all cellular measurements, AR was the best treatment, particularly in the outer region, for increasing F_v while decreasing M_v and N_v. Overall, the local EPC treatment was most influential during the 3-week period, with AR being the best overall treatment for improving healing rate and increasing vascularization.

Local treatment with autologous rabbit PBMC (AR) was better at improving healing than the human EPCs (AH). While the improvement may be due to the use of rabbit EPCs, it may also be due in part to other cell types in the PBMC fraction. The improvement in healing seen with the rabbit cells may also be due to secretion of growth factors and recruitment of other cell types in addition to increases in vascularization.

An important finding from the animal study was that no significant increases in inflammation or adverse reactions were seen from using human cells in the rabbits. This finding suggests that, clinically, using donor EPCs rather than autologous cells could be advantageous for many groups of patients with poor quality or quantity of EPCs (such as those with cardiovascular disease, diabetes, and increasing age) and could improve healing without adverse reactions.

This material is based upon work supported under a National Science Foundation Graduate Research Fellowship and by the Centers for Disease Control through NCIPC.

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FIGURE CAPTION

Figure 1. Image of a hematoxylin and eosin-stained histology slide. Vertical lines mark the original wound edges as determined by hair follicles. ER was determined by measuring the average length of the new epithelial layer between the lines. CR was determined by measuring the width of the wound between the lines compared to the original wound width. HR was the combination of epithelialization and contraction.

Table I
Healing Rate Parameter Measurements at 2 Weeks

	Control	Albumin	Albumin + Rabbit PBMC	Albumin + Human EPC
ER (mm/wk)	1.18 ± 0.34	0.99 ± 0.26	1.36 ± 0.23	1.18 ± 0.36
CR (mm/wk)	2.03 ± 1.26	3.18 ± 1.98	3.33 ± 0.82	2.11 ± 2.14
HR (mm/wk)	3.20 ± 0.99	4.17 ± 2.17	4.17 ± 0.78	3.29 ± 2.24
CR/ER Ratio	2.08 ± 1.75	3.00 ± 1.79	2.52 ± 0.85	1.77 ± 2.18
TF (%)	86.39 ± 2.66	82.39 ± 4.84	85.89 ± 5.17	84.02 ± 1.10

Data shown are mean ± standard deviation.

Table II
Healing Rate Parameter Measurements at 3 Weeks

	Control	Albumin	Albumin + Rabbit PBMC	Albumin + Human EPC
ER (mm/wk)	1.39 ± 0.31	1.55 ± 0.34	1.69 ± 0.68	1.10 ± 0.63
CR (mm/wk)	3.68 ± 1.42	3.75 ± 1.21	3.89 ± 0.68	3.80 ± 0.49
HR (mm/wk)	5.08 ± 1.65	5.30 ± 1.51	5.58 ± 1.05	4.91 ± 0.91
CR/ER Ratio	2.61 ± 0.67	2.40 ± 0.59	2.58 ± 0.98	4.20 ± 1.90
TF (%)	89.39 ± 7.15	86.59 ± 9.17	94.05 ± 4.38	89.40 ± 8.88

Data shown are mean ± standard deviation.

Table III
Volume Fraction Measurements at 2 Weeks

	Control	Albumin	Albumin + Rabbit PBMC	Albumin + Human EPC
BV_v - inner	4.30 ± 0.66	3.27 ± 0.89	3.33 ± 0.91	3.09 ± 1.14
BV_v - outer	3.70 ± 0.84	4.30 ± 1.29	4.48 ± 0.78	3.82 ± 1.15
F_v - inner	23.33 ± 8.86	28.48 ± 2.64	27.94 ± 5.04	20.06 ± 7.49
F_v - outer	15.58 ± 4.34	20.18 ± 3.45	13.21 ± 5.54	14.91 ± 4.75
M_v - inner	1.29 ± 1.46	1.33 ± 0.51	0.85 ± 0.25	1.03 ± 0.35
M_v - outer	1.76 ± 0.54	1.51 ± 0.30	1.33 ± 0.59	1.03 ± 0.41
N_v - inner	3.45 ± 1.26	3.76 ± 0.79	3.52 ± 0.85	4.42 ± 1.20
N_v - outer	5.76 ± 1.05	4.73 ± 1.33	4.97 ± 2.20	4.06 ± 1.82

Data shown are mean ± standard deviation.

Table IV
Volume Fraction Measurements at 3 Weeks

	Control	Albumin	Albumin + Rabbit PBMC	Albumin + Human EPC
BV_v - inner	3.70 ± 1.71	4.12 ± 0.79	4.18 ± 2.00	4.24 ± 1.29
BV_v - outer	4.12 ± 0.95	4.79 ± 0.92	3.52 ± 1.15	4.24 ± 1.50
F_v - inner	52.00 ± 4.60	50.06 ± 7.38	49.45 ± 3.73	44.85 ± 8.10
F_v - outer	48.18 ± 6.27	51.45 ± 7.44	51.27 ± 6.50	47.64 ± 5.32
M_v - inner	0.67 ± 0.25	0.85 ± 0.75	0.73 ± 0.17	0.61 ± 0.30
M_v - outer	0.73 ± 0.35	0.48 ± 0.17	0.61 ± 0.30	0.79 ± 0.35
N_v - inner	1.21 ± 0.37	1.33 ± 1.02	1.64 ± 0.46	1.27 ± 0.14
N_v - outer	1.39 ± 0.46	0.85 ± 0.14	1.33 ± 0.46	1.64 ± 0.66

Data shown are mean ± standard deviation.

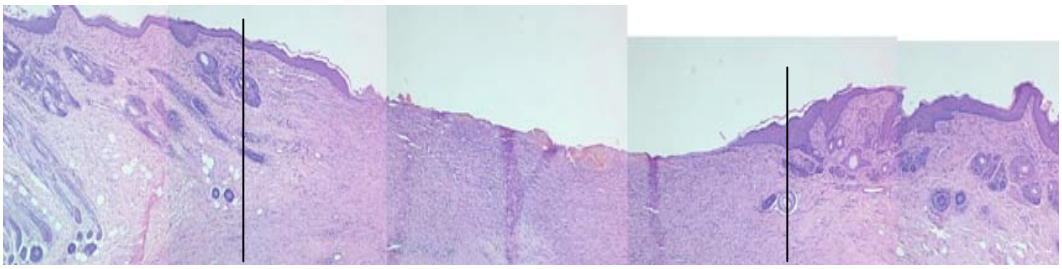

Figure 1

Systemic Endothelial Progenitor Cells for Wound Healing

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ABSTRACT

Previous research has studied the effects of local applications of endothelial progenitor cells for accelerating wound healing. The goal of this study was to evaluate the therapeutic potential of systemic injections of endothelial progenitor cells for improving the healing of chronic wounds and increasing revascularization through vasculogenesis. Additionally, the combination of systemic and local treatments was considered. Full-thickness wounds were created on the dorsum of rabbits and were treated with no treatment, injection only, injection plus albumin scaffold, or injection plus albumin scaffold seeded with endothelial progenitor cells. Results showed that the injection of cells were generally successful at improving healing by increasing epithelialization and overall healing rates compared to the control, particularly at 2 weeks. The injections combined with local cell-seeded scaffolds further improved healing by decreasing contraction rates. It appeared that the injections were most influential during the first 2 weeks of healing. While treatments did lead to some increases in vascularization, improvements in healing may have been due to secretion of growth factors and recruitment of other cells by the injected cells.

INTRODUCTION

Revascularization of chronic wounds, such as pressure ulcers, is an essential part of the healing process since nutrients and oxygen are supplied to the new tissue through the blood vessels. Oxygen is needed for protein production, collagen synthesis, and the destruction of bacteria.[1] Therefore, sufficient blood supply is critical to the production of healthy new tissue and necessary to heal a wound. Fibroblasts in the wound migrate up to 200 μm per day, but will only produce collagen if close enough to a blood vessel to provide sufficient oxygen levels.[2] However, the migration rate of blood vessels into the wound is only 50-70 μm per day.[2] Therefore, it appears that blood supply is a rate-limiting step and increasing revascularization should improve both the rate and completeness of healing in chronic wounds.

Previous wound healing studies have attempted to improve revascularization through angiogenesis—the formation of new blood vessels from the pre-existing network of vessels.[3] A number of angiogenic growth factors and cytokines have been used in wounds to increase blood vessel formation. However, angiogenesis is limited in that mature endothelial cells are already fully differentiated, have a limited life span and proliferative ability, and have a reduced ability to incorporate into remote sites of ischemia.[4]

Once thought to exist exclusively during embryogenesis, recent studies have shown that vasculogenesis is also an important mechanism for revascularization of adult tissues. Vasculogenesis is defined as the formation of vessels from bone marrow-derived endothelial progenitor cells, or EPCs.[4,5] EPCs, isolated from bone marrow, peripheral blood, or umbilical cord blood, have the ability to home to sites of tissue damage in the body, differentiate into mature endothelial cells, and incorporate into new vessel structures.[6] EPCs are released from the bone marrow in response to local levels of angiogenic growth factors or ischemia and are attracted to regions of tissue hypoxia, such as wounds.[7] In addition to physical integration, EPCs have been shown to promote revascularization through the secretion of angiogenic growth factors and recruitment of other cells.[7]

Studies of EPCs have shown that vasculogenesis contributes to neovascularization of developing tumors, cardiac and skeletal ischemia, corneal revascularization, and wound healing.[8] Research in mice suggest that transplanted EPCs possesses therapeutic potential for improving healing in full-thickness wounds.[9,10]

In a previous study, EPCs were seeded into degradable albumin scaffolds and applied directly to wounds in a rabbit model.[11] Results showed that local applications of cells to full-thickness wounds lead to some improvements in overall healing, particularly toward the end of healing at 3 weeks. Increases in epithelialization, tissue fill percentage, and the volume fraction of blood vessels were seen. Additionally, at 2 weeks, some increases in blood vessel formation were seen in the outer region of the wounds.

The goal of this study was to evaluate the potential of EPCs to be used therapeutically to improve healing in chronic wounds through the use of systemic injections, based on the ability of EPCs to home to ischemic sites. There is evidence to suggest that removal and re-injection of isolated EPCs into the bloodstream could increase the concentration of circulating EPCs.[11] Also, the combination of systemic EPC injections with local EPC treatment was evaluated. Specifically, it was hypothesized that injecting EPCs systemically into the bloodstream would enhance wound healing compared to the control by increasing the number of cells which home to the wound and participate in vasculogenesis. Additionally, it was hypothesized that combining the injection with a local application of EPCs seeded into an albumin matrix would be the best method for improving healing, by both increasing the number of EPCs in the blood traveling to the wound site and by reducing the amount of time required for the EPCs to initiate vasculogenesis at the wound site.

MATERIALS AND METHODS

Endothelial Progenitor Cells

Human endothelial progenitor cells were either obtained through the National Disease Research Interchange (Philadelphia, PA) or isolated from donor human blood.[5,12-14] All protocols for human subjects were approved by the University of Alabama at Birmingham's Institutional Review Board.

For the isolation procedure, human peripheral blood was diluted 1:2 with phosphate buffered saline (PBS) without calcium or magnesium (Cambrex, East Rutherford, NJ). The mixture was layered over Histopaque solution (1.077 g/mL, Sigma, St. Louis, MO), and cells were separated by density gradient centrifugation (400 g, 20°C, 30 min.). Peripheral blood mononuclear cells (PBMC) were harvested, washed twice with PBS, and resuspended in PBS to a final volume of 300 µL per 10^8 cells.

$CD34^+$ EPCs were isolated from the PBMC fraction using magnetic microbeads coated with an antibody specific for the human CD34 marker. To the PBMC suspension, 100 µL FcR blocking reagent (human IgG, Miltenyi Biotech, Auburn, CA) was added for every 10^8 cells to inhibit unspecific binding of the microbeads to non-target cells. $CD34^+$ cells were then labeled by adding 100 µL of CD34 Magnetic Microbeads (Miltenyi Biotech) per 10^8 cells and incubating at 4°C for 30 minutes. The cells were passed through the MidiMACS magnetic column (Miltenyi Biotech) to separate the labeled cells, and the $CD34^+$ EPCs were removed from the column using the supplied plunger. Isolated EPCs were then resuspended in M199 media (Sigma) with 20% fetal bovine serum (Hyclone, Logan, UT), 300 mg/mL endothelial cell growth supplement from bovine neural tissue, 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma).

Autologous rabbit peripheral blood (20-30 mL) was drawn from the ear vein of the animals on the day prior to surgery. Rabbits have a marker similar to human CD34, however there is currently no available antibody to the rabbit marker for isolating EPCs.[15] Therefore, rabbit PBMC were isolated using the same protocol as for human blood but were not further separated.[15-17] Assuming a similar EPC concentration as in human blood, the rabbit EPCs represent about 1% or less of the PBMC fraction.[5] All animal procedures were performed in compliance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee's guidelines and those outlined in the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals".

Albumin Scaffolds

Albumin gels were made by mixing an albumin protein solution and a poly(ethylene glycol) (PEG) solution to form an adhesive PEG-crosslinked albumin scaffold.[18] The albumin protein solution was prepared by gently dissolving 0.33 g/mL of lyophilized fraction V rabbit serum albumin (Sigma) into a 0.85% NaCl solution until clear. The PEG solution was prepared by solvating 0.10 g/mL of PEG-disuccinimidyl glutarate (molecular weight 10,000 Daltons, SunBio PEG-SHOP, Anyang City, S. Korea) into basic N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Fisher Scientific, Fairlawn, NJ) solution with a pH of 9.2 until clear. Albumin gels were formed by mixing equal amounts of the albumin and PEG solutions using a dual syringe system.

For EPC-seeded scaffolds, isolated EPCs were mixed into the albumin protein solution. Then the albumin and PEG were mixed to form a crosslinked gel containing EPCs evenly distributed throughout.

In Vivo Evaluation of Endothelial Progenitor Cells

The day prior to surgery, the dorsum of each rabbit (female NZW rabbits) was shaved, a depilatory cream was applied to further remove hair, and an antibiotic ointment was applied to reduce irritation. For surgery, animals were anesthetized with ketamine and xylazine administered intramuscularly. Four 4 cm x 4 cm full-thickness wounds extending down to, but not through, the panniculus carnosum muscle were created on the dorsum. The wounds, however, were cut at 3.5 cm x 3.5 cm to allow for expansion after excision. Digital pictures of the wounds were taken to obtain the exact wound dimensions. Each animal had four different wound treatments: injection only (I), albumin scaffold + injection (AI), albumin scaffold + autologous rabbit PBMC + injection (ARI), or albumin scaffold + human $CD34^+$ EPCs + injection (AHI). For the wounds receiving topical treatment, two syringes filled with 0.5 mL of albumin solution (or albumin + cells) and PEG solution were applied simultaneously into the wound, and the solutions were mixed. The wound edges were elevated for a few minutes until the scaffold had cured, to prevent loss of scaffold material. For wounds treated with albumin + rabbit EPCs, the scaffolds contained 2×10^7 autologous PBMC (approximately 2×10^5 EPCs). For wounds treated with albumin + human EPCs, the scaffolds contained 2×10^5 human $CD34^+$ cells. Additionally, animals

received a systemic injection of autologous rabbit PBMC (approximately 2 x 10^7 cells) through the ear vein immediately following surgery. Control wounds (C, no treatment) were created on additional animals which did not receive injections.

The wounds were covered with an occlusive dressing (Tegaderm, Cardinal Health, Dublin, OH). Animals were wrapped with vet wrap and wore rabbit jackets (Alice King Chatham, Hawthorne, CA) to protect the wounds. During weekly assessments, the animals were sedated, and the dressings were removed. Digital photos were taken and a clean dressing was placed on the wounds. Animals were euthanized with an overdose of pentobarbital at the appropriate time (2 or 3 weeks with 5 animals in each group). The wounds were removed and fixed in 10% neutral buffered formalin. Then each wound was embedded in paraffin, and sections (5 μm thick) were made from the center of the wound. Sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome.

Images of the histology slides were captured with an Olympus OLY-750 3CCD camera with Media Cyernetics Pro Series MV capture kit and analyzed using Image-Pro Plus 4.5 software. For epithelialization rate (ER), the lengths of the new epithelial layer on both sides of the wound (Figure 1) were averaged (EL_1 and EL_2) and converted to mm / wk by dividing by the time (t) interval (Equation 1),

$$ER = \frac{EL_1 + EL_2}{2t} \quad (1)$$

For contraction rate (CR), the change in wound width was determined from the original (W_o), obtained from the digital photos at the time of surgery, and the new wound width (W_n), measured on the histology slides using hair follicles as a marker for the original wound edge. The wound width was converted to mm / wk from each sound edge by dividing by 2 and by the time interval (Equation 2).

$$CR = \frac{W_o - W_n}{2t} \quad (2)$$

Overall healing rate (HR) is the sum of CR and ER. Additionally, the ratio of CR to ER was calculated. For tissue fill percentage (TF), the amount of new tissue formed (above the muscle layer and

between the original wound edges) was compared to the total amount of tissue required to completely fill the entire wound.

A stereological point counting method was used to determine the volume fractions of blood vessels and cell nuclei (fibroblasts, macrophages, and neutrophils). Four random areas were used (10x magnification), two from the outer region of the wound (beneath the new epithelium) and two from the inner region of the wound, with a 165-point grid placed over the images. The fraction of grid points on blood vessels or cell nuclei was determined and approximates the volume fractions of these components.[19]

Statistical Analysis

To reduce the influence of animal to animal variability, treated wounds were compared on the same animal using paired *t*-tests. One-tailed tests were used for healing rate parameters, since only increases in HR and ER and decreases in CR were important. Two-tailed tests were used for cell nuclei and blood vessel measurements, since either increases or decreases could be beneficial at different times. Two-tailed paired *t*-tests were used to compare the inner and outer regions of the wounds for angiogenesis and cellularity measurements. Also, *t*-tests assuming equal variance were used to compare the treated wounds to the control. One-way analysis of variance (ANOVA) was used to compare measurements between the 2- and 3-week time periods. For all data, a P value ≤ 0.05 was used to prove significance.

RESULTS

Epithelialization, Contraction, and Healing Rates

For ER, there were no significant differences between the five treatments at either 2 or 3 weeks (Tables 1 and 2). Additionally, there were no differences between the two time periods. However, AI (albumin + injection) produced a significantly lower CR than I (injection alone) at both 2 weeks (47.4% decrease, P = 0.044) and 3 weeks (38.3% decrease, P = 0.042). Again, there were no differences in CR between the 2- and 3-week periods. Looking at the ratio of CR to ER, there were no differences between treatments at 2 weeks, but again, AI lead to a significantly lower ratio than I at 3 weeks (46.3% decrease,

P = 0.042). Overall, there was a significant increase in the CR/ER ratio between the time periods (54.6% average increase, P = 0.026). At 2 weeks (Table 1), AI gave significantly lower HR than the injection alone (I, 37.6% decrease, P = 0.045), but there were no significant differences between the treatments at 3 weeks (Table 2). The control (C) lead to higher HR at 3 weeks than at 2 weeks (58.8% increase, P = 0.030).

Tissue Fill Percentage

At 2 weeks (Table 1), I produced significantly lower TF than C (6.3% decrease, P = 0.028) and ARI (7.9% decrease, P = 0.050). There were no differences between treatments at 3 weeks (Table 2). Overall, TF increased between 2 and 3 weeks (6.0% average increase, P = 0.037) with I and AHI increasing the most (13.6% increase, P = 0.010 and 6.7% increase, P = 0.040, respectively).

Blood Vessels

At 2 weeks (Table 3), AHI lead to a significantly higher volume fraction of blood vessels (BV_v) than C in the inner region (29.8% increase, P = 0.036). In the outer region, ARI was significantly lower than I (22.9% decrease, P = 0.035). There were no differences between treatments in either region at 3 weeks (Table 4) or between the two regions at either time period. However, there was a decrease in BV_v between the 2- and 3-week period in the inner region (21.5% decrease, P = 0.015), with the biggest change for AHI (34.8% decrease, P = 0.030).

Fibroblasts

At 2 weeks (Table 3), there were no significant differences in the volume fraction of fibroblasts (F_v) between treatments in either region. At 3 weeks (Table 4), ARI produced significantly lower F_v in the inner region that AI (9.1% decrease, P = 0.026). Between the two time periods, F_v increased significantly for all treatments in both the inner (115.1% average increase, $P < 0.001$) and outer regions (166.3% average increase, $P < 0.001$). Comparing the inner and outer regions of the wound, there was significantly

lower F_v in the outer regions at both 2 weeks (26.1% average decrease, P = 0.001) and 3 week s (8.8% average decrease, P = 0.004).

Macrophages

At 2 weeks, there was a significant increase in the volume fraction of macrophages (M_v) for AI compared to I in the inner region (100.0% increase, P = 0.045). There were no significant differences in the outer region at 2 weeks or in either region at 3 weeks. However, there were significant decreases in the M_v between 2 and 3 weeks for both the inner region (43.0% average decrease, P = 0.020) and the outer region (50.1% average decrease, P = 0.004). Overall, there the outer region produced significantly higher M_v than the inner region (2.0% average increase, P < 0.001).

Neutrophils

At 2 weeks (Table 3), AHI lead to a significantly lower volume fraction of neutrophils (N_v) than C (26.4% decrease, P = 0.023) in the outer region. There were no differences between treatments at 3 weeks (Table 4). However, there were significant decreases in N_v between the 2- and 3-week periods for both the inner (69.8% average decrease, P = 0.001) and outer regions (75.0% average decrease, P = 0.001). Overall, the outer region produced higher N_v than the inner region (7.8% average increase, P < 0.001). Compared to the non-injected animals, there was a lower N_v in the inner region for the injected animals at 2 weeks (16.1% average decrease, P = 0.018).

DISCUSSION

The method for seeding EPCs into the scaffolds and the choice of an albumin scaffold were based on previous *in vitro* and *in vivo* studies.[11] Although these cell-seeded scaffolds can be further optimized, this seeding technique with an albumin scaffold worked better than other seeding techniques tried and other EPC-seeded scaffolds, such as fibrin. Results from the injected animals in this study can also be compared to animals in these previous studies that received only local EPC treatment.[11]

During this study, there was some concern about what effect removing EPCs from the blood and re-injecting them back in would have on the concentration of cells in circulation. Therefore, in 2 animals, the concentration of EPCs in the peripheral blood was measured throughout treatment on the day prior to surgery, at the time of surgery, and on day 5 following surgery. Results showed that, in the animal receiving an injection, the concentration of EPCs in the blood increased by 140% by day 5 following surgery. However, the animal without an injection did not see an increase in EPC concentration following surgery. This preliminary data suggests that the injections increased number of circulating EPCs. Thus, the injections may have increased the number EPCs arriving at the wound site, thereby improving overall healing.

It was hypothesized that systemic injections of EPCs would improve healing in full-thickness wounds and that the combination of injections with local EPC treatment would be the best method for improving healing. However, it was actually desired that treatments increase ER while decreasing or maintaining CR relative to the control. Therefore, increases in HR were desired, but with low CR/ER ratios. In general, the results from the animal study generally showed trends toward improving healing rate parameters, though not all of the results were significant. In many of the cases (P values between 0.05 and 0.10), with an n of 5, the test was not sensitive enough to pick up the changes in healing, which had to be about 1.5 times the standard deviation. A larger sample size would have been necessary, increasing the sensitivity of the study, to determine if these improvements could be statistically significant.

As hypothesized, injections of EPCs were able to improve the healing rate, in some cases. This was most evident in the second week, particularly for the control versus injection alone (C vs I). Additionally, the injection alone improved overall HR better than any of the treatments in which injection was combined with a local scaffold. At three weeks, HR for C caught up to the injected, although it was still higher than any of the albumin scaffolds. Data from the previous study, however, showed that local treatment with rabbit PBMC improved HR better than the local treatment combined with injection (ARI); ARI decreased HR by 66.1% compared to local treatment alone ($P = 0.043$).

When the healing rates were broken down into epithelialization and contraction rates, it can be seen that some of the increases in overall healing rate were due to higher contraction rates rather than increases in epithelialization. The injections had the most influence on ER during the first two weeks, with the most significant effect for the injection alone. All treatments with injections increased ER compared to the control at 2 weeks, though not significantly. For the third week the effect of the injections appeared to have diminished with the control wounds catching up. Compared to the previous study, the injections had the most influence at the 2-week period, while the local EPC scaffolds alone had the most positive effect during the 3-week period.

For CR, the injections combined with local treatment had the most significant influence in the first two weeks, with the most significant decrease for ARI. Interestingly, the injections alone increased the CR, which was significantly greater than AI at both 2 and 3 weeks and also greater than C at both time periods. During the 3-week period, the injections combined with the scaffolds still produced the lowest CR, with AI being the best treatment.

For the ratio of CR to ER, with decreases desired, injections helped in the first 2 weeks for all albumin scaffolds, with ARI being the lowest. At 3 weeks, the injection in combination with scaffolds were still better than the injection alone, with ARI and AHI lower than I and AI significantly lower than I. AI was also almost significantly lower than C (26.8% decrease, $P = 0.066$).

For TF, ARI was the best treatment at 2 weeks, significantly better than I and also better than AI. Also, C was significantly better than I. At 3-weeks, I was best and had a significantly higher TF than AI. AI was almost significantly lower than AHI (4.3% decrease, $P= 0.064$).

Looking at all of the healing rate parameters together, at 2 weeks the injection alone was best for increasing ER and HR, while the combination of local and systemic treatment was best at decreasing CR and the CR/ER ratio. While the injection alone increased ER, it also increased CR. Overall, ARI was the best treatment at 2 weeks, increasing ER and TF (by 9.3% and 1.7% respectively) while decreasing CR and CR/ER ratio (by 39.9% and 51.0% respectively). In general, the hypothesis was true that the

combined treatment would be best at improving healing rate parameters for the 2-week time period, although many of the differences were not big enough to be statistically significant.

It is also important to note, however, that the majority of the wounds were completely healed by 3 weeks. Therefore, measurements of contraction and epithelialization would have been underestimated for the 3-week time period. The wounds in the 2-week time period were approximately 63% healed. At the healing rates seen, wounds that healed by 3 weeks probably would have healed at most a few days earlier. Using the actual time period would increase ER, CR, and HR measurements by approximately 5% per day. Measurements made at shorter time periods – 18 or 19 days instead of 21 – would be needed to produce more accurate calculations.

The injections had the effect of improving healing sooner than expected, mostly during the first 2 weeks. It was thought that the injected cells would take at least a week to arrive at the wound site and have a more pronounced effect in the later time period. One study found EPCs incorporated into vessels in hindlimb ischemia models from 1-6 weeks after injection.[5] However, periods of shorter than 1 week were not studied. It is possible that the EPCs reached the wound site sooner than anticipated. Data from animals in a preliminary study seemed to corroborate this by showing EPCs incorporated into blood vessels at the wound site by day 3 or 4.[11]

Based on the measurements for healing rate parameters, autologous rabbit PBMC (ARI) were better at decreasing contraction and the CR/ER ratio, while the human EPCs (AHI) were better at increasing overall HR, particularly at 2 weeks. However, it can not be concluded that the improvements seen with ARI or any of the injections were due only to rabbit EPCs, since the PBMC fraction also contained other cell types. EPC purity should probably be increased in future studies by using negative selection to remove other cell types from the PBMC fraction, especially inflammatory cells such as T-cells, B-cell, and monocytes.[20] It would also be helpful to look at using human EPCs for both local and systemic treatments.

In addition to improving healing rates, it was hypothesized that injections of EPCs would increase vascularization in the wounds. More specifically, it was thought that the injected EPCs would increase

vascularization in the outer regions of the wound, while injections combined with local EPC-seeded scaffolds would increase blood vessel formation in both the inner and outer regions. At 2 weeks, AHI lead to a significant increase in BV_v compared to C in the inner region. In the outer region at 2 weeks, ARI was the lowest, significantly lower than I. The injection alone increased BV_v slightly over C in both regions. BV_v for the injected treatments decreased significantly between the 2 and 3-week periods in the inner region, particularly AHI. At 3 weeks, there were no significant differences in either region. Compared to non-injected animals from the previous study, the injected treatments lead to a significantly lower BV_v in the inner region at 3 weeks (16.6% decrease, $P = 0.024$). In the inner region, AHI was 44.6% higher than albumin + human EPCs with no injection at 2 weeks ($P = 0.005$). In the outer region, AI was 26.4% lower than albumin with no injection 3 weeks ($P = 0.037$).

As hypothesized, the injection of EPCs increased vascularization in the outer region of the wound compared to C at 2 weeks. Also, the injection combined with the local human EPCs (AHI) increased blood vessel formation in the inner region at 2 weeks. However, no improvements were seen in either region at 3 weeks. Overall, at 2 weeks, the injection alone was the best treatment for increasing vascularization in both regions, though AHI was best in the inner region.

The injected animals had increases in vessel density earlier which could have led to improved healing in the first 2 weeks. The decreases in vascularization at 3 weeks with the combination of local and systemic treatment are likely due to vascular regression that occurs at the completion of the healing process. During the remodeling phase of healing, endothelial cells undergo apoptosis to remove any redundant blood vessels.[21] It is also believed that when EPCs complete their function, they apoptose.[22]

Because there was not as significant an increase in vascularization as anticipated, the EPCs may have improved healing through other mechanisms. Recent studies suggest that EPCs do play an important role in vasculogenesis, but their function involves much more than blood vessel formation. One study found that local EPCs are responsible for secreting growth factors and recruiting other cells necessary for vessel formation. The study also found that EPCs are mobilized to the wound site in response to angiopoietin-1 and stem cell factor where they secrete other growth factors in a paracrine manner and

recruit other cell types to the wound site.[22] Once they are no longer needed, they are cleared from the wound, probably through apoptosis.[22] Additionally, studies have found that there exists two subsets of EPCs – one which aids healing by physical incorporation into the vasculature and one which aids through the secretion of angiogenic factors.[7] Therefore, while EPCs may not have lead to significant increases in vascularization, they may have improved healing through other mechanisms.

Fibroblasts are responsible for producing extracellular matrix proteins. Additionally, they secrete growth factors and angiogenic factors that regulate cell proliferation and vascularization,[23] as well as provide the framework for blood vessels. Therefore, increases in the volume fraction of fibroblasts (F_v) were desired. At 2 weeks, there were no differences between any of the treatments in either region, although both ARI and AHI produced significantly higher F_v in the inner region than in the outer region. At 3 weeks, AI was the best treatment in the inner region, significantly higher than ARI, but there were no differences in the outer region. Again, the inner region produced higher F_v than the outer region, especially for AI.

Neutrophils and macrophages are involved in destroying bacteria and phagocytosis of debris.[23] Another goal of the treatments was to shorten the inflammatory process. Both macrophages and neutrophils in the wound are signs are inflammation, with neutrophils more involved in acute inflammation and macrophages in chronic inflammation. Therefore decreases in macrophages and neutrophils, especially in the outer regions of the wounds, were desired.

At 2 weeks, all injections in combination with scaffold increased M_v over I in the inner region (AI significantly), with AI and AHI also higher than C. However, all treatments decreased compared to C in the outer region, with ARI almost significantly lower than C (34.7% decrease, $P = 0.066$). Again, M_v decreased significantly between the time periods in both regions. Compared to the non-injected treatments, at 2 weeks, ARI produced a higher M_v in the inner region (36.1% increase, $P = 0.019$).

At 2 weeks, both AI and AHI increased N_v in the inner region compared to C. All scaffold treatments combined with the injection decreased N_v in the outer region, AHI significantly. Also, AI was significantly lower in the outer region than in the inner region. Although there were significant decreases

for all treatments between the 2 and 3-week time periods, there were no differences between any of the treatments at week 3.

For all treatments, the volume fractions of both macrophages and neutrophils decreased significantly between 2 and 3 weeks. At 3 weeks, there were no major differences in the volume fractions of macrophages or neutrophils for either the inner or outer regions. Results from these volume fraction measurements show that there were no significant increases in inflammation caused by using foreign human EPCs in the rabbits. The increase in M_v seen at 2 weeks in the inner region for AI, ARI, and AHI may be due to shortening of the inflammatory phase. Also, the increase could be due to extra inflammatory cells included in the rabbit mononuclear cell fraction. For N_v, I and ARI were best in the inner region, while all treatments were good in the outer region. Overall, the injection alone (I) was the best treatment in the inner region for increasing fibroblasts while decreasing both macrophages and neutrophils, particularly at 2 weeks. In the outer region at 2 weeks, all of the injected treatments decreased inflammatory cells while increasing fibroblasts compared to C.

In conclusion, the injection of EPCs were generally successful at improving healing in full-thickness wounds by increasing ER and HR compared to the control, particularly at the 2-week time period. Additionally, the combination of injections with EPC-seeded scaffolds further improved healing by decreasing CR and the CR/ER ratio. When compared to data from a previous study, the injections were most influential during the 2-week period while local treatment alone improved healing more in the 3-week period. While the EPC treatments did lead to some increases in vascularization, the improvements in healing may also have been due to the secretion of growth factors and recruitment of cells by the EPCs. While the improvements in healing may have been due to the rabbit EPCs, it may also have been due in part to other cell types in the PBMC fraction. Thus, in future studies, it may be worth separating the rabbit cells through negative selection to remove inflammatory cells.

It is also important to note that no increased inflammatory response or adverse reactions were seen from using human cells in the rabbits or from using the rabbit PBMC fraction which contained inflammatory cells. This finding suggests that, clinically, using donor EPCs rather than autologous cells

could be advantageous for many groups of patients with poor quality or quantity of EPCs (such as those with cardiovascular disease, diabetes, and increasing age) and could improve healing without adverse reactions.

Future studies should also look at the effects of EPCs at earlier time periods, such as 1 week, as well as before the completion of healing at 3 weeks. Additionally, future studies should look at optimizing the matrix used for EPC seeding, possibly through the addition of growth factors or cytokines or the use of other matrix materials. More measurements should be made to further substantiate that removal and re-injection of EPCs increases the concentration in circulation, as well as to confirm the homing ability of the circulating EPCs. Improvements could also be made by increasing the number of EPCs used during treatment in order to have more cells available for vasculogenesis. This EPC expansion could be accomplished (1) *in vivo* using drugs or growth factors involved in EPC mobilization, such as GM-CSF, VEGF, angiopoietin-1, or statins;[6] (2) *in vitro* by expanding isolated cell populations in culture;[7] or (3) using gene therapy to overexpress growth factors involved in EPC mobilization.[24] While additional studies are needed to incorporate these improvements, systemic EPC treatment, alone or combined with local EPC treatment, looks promising as a treatment for improving healing of full-thickness chronic wounds.

ACKNOWLEDGMENTS

This material is based upon work supported under a National Science Foundation Graduate Research Fellowship and by the Centers for Disease Control through National Center for Injury Prevention and Control.

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FOOTNOTES

AI	treatment with albumin + injection
AHI	treatment with albumin + human EPC + injection
ARI	treatment with albumin + rabbit PBMC + injection
BV_v	volume fraction blood vessels
C	control (no treatment)
CR	contraction rate
EPC	endothelial progenitor cell
ER	epithelialization rate
F_v	volume fraction fibroblasts
HR	healing rate
I	treatment with injection
M_v	volume fraction macrophages
N_v	volume fraction neutrophils
PBMC	peripheral blood mononuclear cell
TF	tissue fill percentage

FIGURE LEGENDS

FIGURE 1. Image of a hematoxylin and eosin-stained histology slide. Vertical lines mark the original wound edges as determined by location of hair follicles. ER was determined by measuring the average length of the new epithelial layer between the lines divided by the time period. CR was determined by measuring the width of the wound between the lines compared to the original wound width divided by the time period. HR was the combination of epithelialization and contraction. TF was the amount of new tissue filling the wound divided by the amount required to completely fill the wound.

Table 1. Healing rate parameter measurements at 2 weeks

	Control	Injection	Albumin + Injection	Albumin + Rabbit PBMC + Injection	Albumin + Human EPC + Injection
ER (mm/wk)	1.18 ± 0.34	1.75 ± 0.92	1.51 ± 0.67	1.29 ± 0.13	1.45 ± 0.41
CR (mm/wk)	2.03 ± 1.26	4.39 ± 3.07	2.31 ± 2.00	1.22 ± 2.41	2.68 ± 1.79
HR (mm/wk)	3.20 ± 0.99	6.14 ± 3.24	3.83 ± 2.17	2.51 ± 2.36	4.13 ± 1.90
CR/ER Ratio	2.08 ± 1.75	2.55 ± 2.05	1.84 ± 2.07	1.02 ± 2.07	1.90 ± 1.16
TF (%)	86.39 ± 2.66	80.94 ± 4.78	83.18 ± 6.79	87.87 ± 4.58	84.64 ± 5.52

Data shown are mean ± standard deviation.

Table 2. Healing rate parameter measurements at 3 weeks.

	Control	Injection	Albumin + Injection	Albumin + Rabbit PBMC + Injection	Albumin + Human EPC + Injection
ER (mm/wk)	1.39 ± 0.31	1.38 ± 0.59	1.31 ± 0.56	1.37 ± 0.59	1.37 ± 0.54
CR (mm/wk)	3.68 ± 1.42	4.31 ± 1.50	2.66 ± 1.43	3.16 ± 1.08	3.49 ± 0.86
HR (mm/wk)	5.08 ± 1.65	5.69 ± 1.58	4.06 ± 1.85	4.53 ± 1.50	4.86 ± 0.76
CR/ER Ratio	2.61 ± 0.67	3.56 ± 1.82	1.91 ± 0.67	2.53 ± 0.76	2.97 ± 1.44
TF (%)	89.39 ± 7.15	91.91 ± 6.91	86.46 ± 2.50	89.80 ± 3.69	90.32 ± 3.06

Data shown are mean ± standard deviation.

Table 3. Volume fraction measurements at 2 weeks.

	Control	Injection	Albumin + Injection	Albumin + Rabbit PBMC + Injection	Albumin + Human EPC + Injection
BV_v - inner	4.30 ± 0.66	4.61 ± 0.87	4.30 ± 1.12	3.94 ± 1.41	5.58 ± 0.92
BV_v - outer	3.70 ± 0.84	4.24 ± 1.37	4.00 ± 1.04	3.27 ± 1.24	3.64 ± 2.11
F_v - inner	23.33 ± 8.86	25.58 ± 8.49	22.30 ± 6.60	24.67 ± 6.86	25.82 ± 3.80
F_v - outer	15.58 ± 4.34	20.06 ± 8.42	17.76 ± 5.30	18.85 ± 5.46	17.58 ± 4.93
M_v - inner	1.29 ± 1.46	0.85 ± 0.50	1.70 ± 0.55	1.33 ± 0.27	1.70 ± 0.66
M_v - outer	1.76 ± 0.54	1.52 ± 0.96	1.33 ± 0.46	1.15 ± 0.33	1.21 ± 0.43
N_v - inner	3.45 ± 1.26	3.45 ± 1.57	5.27 ± 1.94	3.55 ± 1.03	5.09 ± 1.27
N_v - outer	5.76 ± 1.05	5.15 ± 3.96	4.24 ± 1.60	3.82 ± 1.77	4.24 ± 0.61

Data shown are mean ± standard deviation.

Table 4. Volume fraction measurements at 3 weeks.

	Control	Injection	Albumin + Injection	Albumin + Rabbit PBMC + Injection	Albumin + Human EPC + Injection
BV_v - inner	3.70 ± 1.71	3.52 ± 1.17	3.39 ± 1.89	3.39 ± 1.18	3.64 ± 1.36
BV_v - outer	4.12 ± 0.95	3.85 ± 1.00	3.79 ± 1.54	3.64 ± 1.01	3.76 ± 1.61
F_v - inner	52.00 ± 4.60	51.61 ± 5.24	54.91 ± 2.59	49.94 ± 1.54	52.24 ± 7.81
F_v - outer	48.18 ± 6.27	49.00 ± 6.39	47.58 ± 5.08	45.39 ± 5.58	47.33 ± 5.31
M_v - inner	0.67 ± 0.25	0.73 ± 0.51	0.67 ± 0.40	0.91 ± 0.21	0.67 ± 0.33
M_v - outer	0.73 ± 0.35	0.58 ± 0.17	0.61 ± 0.30	0.73 ± 0.41	0.73 ± 0.46
N_v - inner	1.21 ± 0.37	1.12 ± 0.40	1.15 ± 0.69	1.39 ± 0.79	1.15 ± 0.45
N_v - outer	1.39 ± 0.46	1.00 ± 0.33	1.09 ± 0.46	1.27 ± 0.78	1.15 ± 0.50

Data shown are mean ± standard deviation.

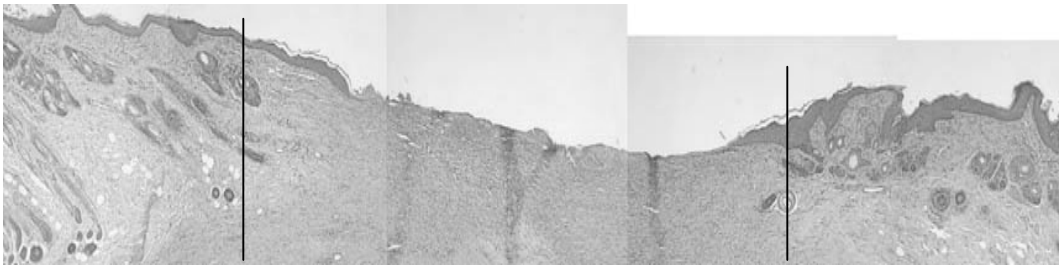

FIGURE 1