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Table of Contents

Cover..1

SF 298..2

Table of Contents..3

Introduction..4

Body...4

Key Research Accomplishments..12

Reportable Outcomes..12

Conclusions...12

References...13

Appendices...11

INTRODUCTION

To develop new therapeutic means to treat androgen-independent prostate cancer, we hypothesized that the apoptotic pathway of prostate cancer cells can be manipulated for therapeutic purposes. In support of our hypothesis, we presented preliminary data showing that a molecule of the apopotic pathway, caspase 7, induced therapeutic apoptosis after adenoviral-mediated overexpression. This was observed *in vitro* in LNCaP and LNCaP-Bcl-2 cells, and *in vivo* in the prostate of normal mice, which were inoculated with a virus overexpressing caspase-7 under the control of the powerful viral promoter RSV (AvC7).

That adenoviral mediated caspase-7 overexpression has the ability to induce therapeutic apoptosis in these two cell lines has now been reported by our group in two papers published in Cancer Research (1, 2). Based on this preliminary data, in aim 1 we proposed to investigate whether adenovirus AvC7 has the ability to modify the natural history of prostate cancer in the TRAMP mouse model, which was developed in the laboratory of Dr. Greenberg at Baylor College of Medicine (3).

First generation adenoviral constructs have some potential shortcomings, for instance they are significantly immunogenic, and their ability to maintain expression of the therapeutic gene of interest may be limited to a maximum of 2-3 weeks before they are neutralized by the immune system of the host. In view of this, first generation adenoviruses can only be directly inoculated in a cancerous lesion, and cannot be used systemically to reach metastatic deposits. For this reasons we have proposed in aim 2 to construct a gutless helper-dependent (HD) second-generation adenovirus. As these constructs are completely devoid of proteins of viral origin, they are only minimally immunogenic (4), are not recognized by the immune system, and have the ability to produce the gene of interest up to > 2 years after their initial inoculation (5, 6). Our original argument was that such constructs would be more amenable to treat not only primary neoplastic formations, but also metastatic deposits, and that this could be achieved by possibly inoculating the animal with multiple injections given over a long period of time.

In the third specific aim of our application we proposed to study if an HD vector carrying the RSV-Caspase-7 cassette has the ability to shrink sc xenografts of prostate cancer cell lines, following direct inoculation in the tumor, or systemic injection through the tail vein.

BODY

Task 1: To determine if an adenoviral construct containing a caspase-7 cDNA driven by the constitutively active RSV promoter changes the natural history of prostate cancer in the TRAMP model after direct intraprostatic inoculation.

Task 1 was largely developed during year 1. Task one was organized in four steps. In steps 1, 2 and 3 we wanted to identify the ideal dose and number of orthotopic inoculations of adenovirus AvC7 (in which the cDNA for caspase-7 is driven by the constitutively active viral promoter RSV), which induce maximal prostatic apoptosis and minimal systemic toxicity in TRAMP mice. In step 4 we wanted to compare AvC7 with a control virus AvGFP (in which the GFP cDNA is driven by the RSV promoter). We wanted to understand if treatment with AvC7, according to the modalities established in steps 1, 2 and 3, changes the natural history of prostate cancer in the TRAMP model compared to the control virus AvGFP. In steps 1 and 2 (performed last year) we identified that the ideal dose of adenovirus AvC7 causing maximal prostatic apoptosis

and minimal systemic toxicity was 10^9 pfu. In step 3 this dose of 10^9 pfu was orthotopically inoculated to the prostates of TRAMP mice once or twice, to understand if multiple inoculations were more effective than a single inoculation. As explained in the first progress report, we had to compromise on the number of intraprostatic inoculations, as mice started to die if more than three orthotopical administrations were given. We were therefore forced to compare two groups of TRAMP mice, group A which received one inoculation of AvC7 (10^9 pfu), and group B, which received two inoculations instead of the planned 4. Both groups were sacrificed one week after the last inoculation of therapeutic virus, and were analyzed for: a. wet weight of the prostates, b. scoring of TUNEL + and – prostate cells to quantitate apoptosis, c. macro and microscopic necroscopy to rule out systemic side effects, d. DNA was extracted from every organ to rule out extraprostatic invasion of virus by PCR using primers derived from viral sequences adjacent to the cloning site. No difference in any of these parameters (data not shown) was detected between the two groups, so we decided to use one single inoculation of virus for step 4. This result was in antagonism with our expectation that more than one administration should have been more effective than one. We think that this did not occur because we were unable to administer group B with four orthotopic inoculations of virus, as we had initially planned (see Table IIB of original application).

We then proceeded with step 4. Here we wanted to determine if the natural history of prostate cancer in TRAMP mice was changed after inoculation of AvC7 according to the modality established in step 1, 2 and 3. 30 TRAMP animals were treated with one orthotopic administration of AvGFP (group B) or AvC7 (Group A), given at the dose of 10^9 pfu's at age 12 weeks.

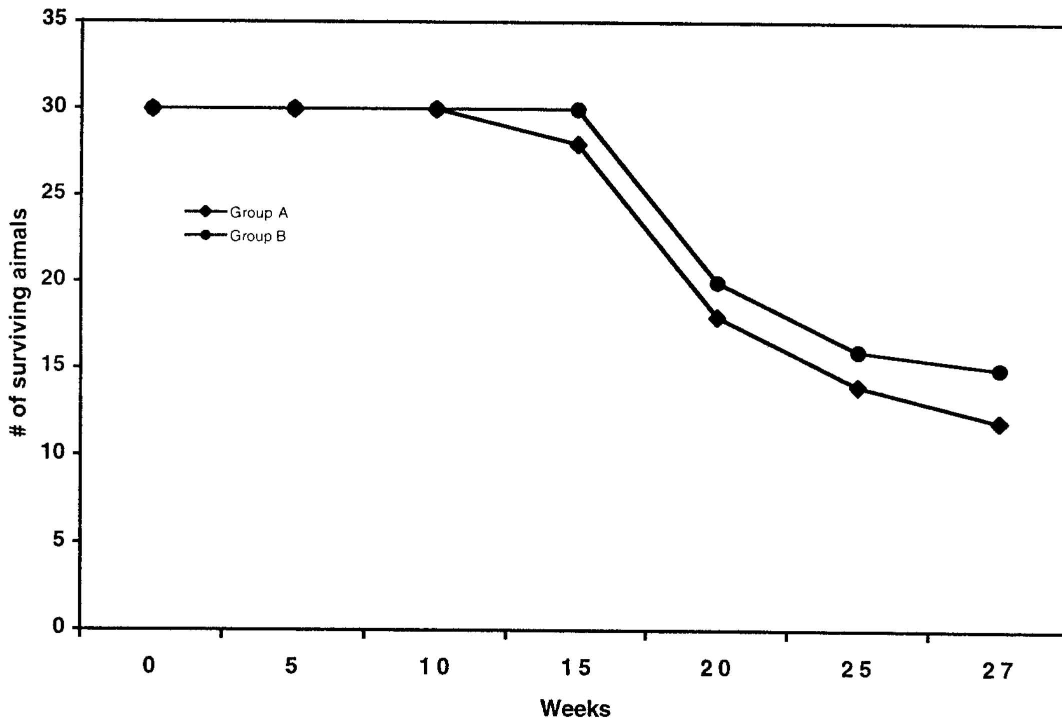

Fig 1: Survival curve of animals receiving 10^9 pfu's of AvC7 (A) or AvGFP (B).

As described in Table 2C of the original application, the experiment was organized in this way: "*Animals will receive the treatment at 12 week of age, the time in*

which primary disease has developed with a penetrance of 100%. The end point will be survival or prevention of metastatic disease after 27 weeks of age, when according to the natural history prostate cancer is 100% metastatic or has led TRAMP mice to death. Animals will be sacrificed at 27 week. A necroscopy will be performed, and the number of metastases in the various organs will be counted. The prostate of the animals will be harvested and analyzed histologically to verify absence of cancer, or to measure the size of the remaining lesion with computer-assisted image analysis". As shown in figure 1, no differences in the survival curve of animals treated with AvC7 or with AvGFP was identified. Table I shows the number of cancers and metastases that were macroscopically identified.

TABLE I

	Number of cancers	Weight of prostates	Number of metastases
Group A:	25	150 ± 45 g	39
Group B:	27	179 ± 57 g	44

No statistically significant difference was found. We have not yet done the histological analysis of tumors as we outlined in the original proposal.

Discussion of data presented for task 1; Overall we were surprised with the results produced by this experiment. The preliminary work was very promising in showing that AvC7 is a powerful weapon to induce apoptosis *in vivo* and *in vitro*. The results presented in table II did not show any appreciable change in the natural history of CaP in the TRAMP model after treatment with AvC7. We think that to see an effect we should have administered the virus AvC7 at least four times, as we intended to do in our original proposal. Unfortunately mice did not tolerate so many invasive surgeries, and we had to use a lower # of treatments. It is likely that one (or two) inoculations of the virus were not powerful enough to achieve a significant effect.

Task 2: To develop helper-dependent (HD) adenoviruses that drive caspase-7 (over)-expression under the control of the 6 Kb PSA promoter uniquely to human prostatic epithelium.

As described in the initial progress report, we have decided to use the ARR_2PB promoter instead of the 6 kb PSA promoter to direct expression of the gene of interest in prostatic epithelium. The paper describing the power of this promoter was published by our group in the Journal of National Cancer Institute last year (7). In view of the negative result of task 1, and of the result of task 3 in which we can see an effect of overexpressed caspase-7 in sc LNCaP tumors only if AvC7 is given at the same time with adenovirus Av-ARR2PB-Bax (which overexpresses Bax under the cpontrol of the DHT-dependent promoter ARR2PB, see below), for the purpose of task 2 we decided to prepare three gutless therapeutic adenoviruses. The names of the two therapeutic adenoviruses are:

$AvHDARR_2Pb$-C7 in which caspase-7 is under the control of the ARR_2PB promoter, and $AvHDARR_2PB$-Bax, in which Bax is under the control of the same promoter. The third construct is the control HD adenovirus AvHDARR2PB-GFP. We have done all the cloning necessary for the production of these viruses. The resulting plasmids have been cut with Pme to liberate a fragment containing the construct of interest (for instance ARR_2PB-Bax-poly-A), and the left and right Ad5 inverted terminal repeats, the packaging signal y, and a stuffing fragment consisting of the human hypoxanthine guanine phosphoribosyltransferase [hprtb] gene. This fragment was transfected in 293 Cre4 cells (containing Cre recombinase), which were then infected with a mutant Helper Adenovirus containing a packaging signal for adenoviruses that is flanked by lox sites. Upon infection of this Helper Adenovirus, cre recombinase excides the packaging signal and this induces formation of an adenovirus out of the Pme-I fragment described above. Viral plaques are removed from the plate, and a laborious process of purification is initiated. We have obtained a large number of these plaques, where we can amplify segments of DNA containing our therapeutic genes, however when we used these in assays of LNCaP infection and then we induce the ARR_2PB promoter by adding DHT to the medium, we have never seen induction of the therapeutic protein of interest. This is not unheard of, since it is always necessary to go through many purification processes to obtain an effective HD virus. Since we have gone through the process of purification of several plaques, and many of these plaques are PCR positive for the presence of the sequences of interest, we are confident that in the next few weeks we will obtain active HD viruses. The main use of these adenoviruses will be to complete the last item of task 3 (see below), in an attempt to induce obliteration of LNCaP tumors growing sc by inoculating iv the various HD viruses (alone or in combination). We also want to add that we have two experienced collaborators working with us on this task, one is Dr. BaBie Tang from the University of Texas in Houston. The other one is Dr. Sam Zhou from the Baylor Gene Therapy Center. They both are expert vectorologists, and they are very actively trying to overcome the problems of purification we had so far.

Task 3: To determine if HD constructs driven by the 6 Kb PSA promoter have the ability to induce therapeutic apoptosis of subcutaneous tumors consisting of PSA + LNCaP cells after direct or systemic administration.

As described above, we had problems in generating the HD viruses, and therefore this aim has been only partially completed, however we have generated at least one important andpromising result.

In experiment A of task 3 we proposed the following :" *Prove the fact that AvC7 induces therapeutic apoptosis of LNCaP and PC-3 tumors growing in nude mice. These experiments will be organized using nude mice inoculated with 1x 10^5 LNCaP or PC-3 cells (dispersed in Matrigel) on day 0. Treatment will start 30-40 days post-inoculation, when the tumors are 0.5 mm. There will be 20 mice with LNCaP tumors (group A) and 20 mice with PC-3 tumors (group B). Ten animals from group A or B will receive AvC7 or AvGFP using the modalities established after performing the experiments discussed in Table II A and B. The end point will be to demonstrate induction of apoptosis and obliteration of LNCaP or PC-3 tumors in a caspase-7 dependent way. This will be determined using the procedures discussed in Table II A and B*". We carried on the experiments for 4 weeks, using one administration of AvC7 or AvGFP every week directly inside the PC-3 or LNCaP tumor. The results with the two cell lines were very similar. Fig. 2 shows the outcome of the experiment performed with LNCaP cells. It is clear that AvC7 has an effect in preventing growth of the sc tumors, and this is

demonstrated by the significant difference in size between the two groups at the end of four treatments. However, AvC7 does not seem to be powerful enough to induce a clear decrease in tumor size, and this is demonstrated by the fact that there is not a significant difference in tumor size in the group receiving AvC7 at the beginning and at the end of the treatment.

We are carrying on a parallel investigation in which we are examining the potential role of proapoptotic Bcl-2 family members as therapeutic agents in prostate cancer. We were using the pro-apoptotic family member Bad, which when given alone had an effect very similar to that of Caspase-7, and prevented growth of the tumor, but was not powerful enough to decrease tumor size (Fig. 3). When Bad was given in association with the powerful pro-apoptotic Bcl-2 family member Bax, an additive effect was noticed compared to when the two therapeutic genes were given individually. In these experiments, tumors receiving Bax + Bad responded significantly better than tumors treated with each of the two genes individually (Fig 3), and this was associated with complete obliteration of two of the six tumors receiving combination therapy. Based on this we have hypothesized that maybe Caspase-7 is more effective when used in combination with an upstream gene of the apoptotic pathway. We reasoned that maybe simultaneous overexpression of a known inducer of apoptosis such as Bax and of Caspase-7 may elicit an amplification of the apoptotic response. Bax works at the beginning of the intrinsic pathway by incapacitating the mitochondria, while caspase-7 becomes active at the end of the same process, thus their association may prove very effective to achieve therapeutic apoptosis in cancer. To prove this, we performed an experiment similar to that shown in figure 3, but AvC7 instead of AvARR$_2$PB-Bad was used (Fig. 4). The results confirmed our hypothesis, and showed that under this experimental conditions AvC7 worked very well by amplifying the apoptotic response of the tumors to Bax. The association of Bax + Caspase-7 is very effective, as three of the six tumors of this experiment were completely obliterated at the end of the treatment (50%). They did not relapse after 8 weeks of follow up without therapy. Our working hypothesis (which we are trying to confirm in cell lines) is that Bax works at the beginning of the intrinsic apoptotic pathway by facilitating cytochrome c release to the cytosol, while the overwhelming amount of caspase-7 which is present in these cells, amplifies activation of the caspase pathway.

Discussion of the data presented for task 3: These data have demonstrated that caspase-7 does not have a major therapeutic role when given individually to sc LNCaP or PC-3 tumors. However they have shown that caspase-7 is an outstanding therapeutic partner of Bax, and that the two should be used simultaneously to achieve amplification of their therapeutic effects.

Missing experiments of task 3: We have critically reviewed the remaining experiments of task 3. These are items 2, 3 and 4. We have decided that given the amount of information we have generated with this project and with the parallel project supported by the Veterans Administration entitled "Bax-induced apoptosis in prostate cancer", items 2 and 3 are redundant at this time. In item 2 and 3 we wanted to use a HD virus with a prostate specific promoter driving caspase 7 to obliterate sc LNCaP tumors after direct intratumoral inoculation. We think items 2 and 3 are redundant in view of the data already produced in relation to item 1. We think that there is no difference between obliterating tumors with first generation adenoviruses compared to HD adenoviruses. Based on this we think that in the remaining of the funding period we should focus on the accomplishment of item 4 of task 3, where we propose to obliterate sc tumors growing in nude mice after *iv* inoculations of HD viruses. The strategy to follow will be the same outlined in Table IV experiment C, with the difference that HDAv-ARR$_2$PBC7 will be

used in combination with HDAv-ARR$_2$PB-Bax. We think that the results we have accomplished to date allow the conclusion that direct inoculation of a combination of viruses (Bad + Bax or Bax + Caspase-7) is effective in obliterating the sc tumor of interest in up to 50% of the cases. The main shortcoming of these experiments is that we have not yet developed a technology to reach tumors after systemic inoculation. We hope that we will be able to overcome this shortcoming by the successful performance of item 4 as explained above.

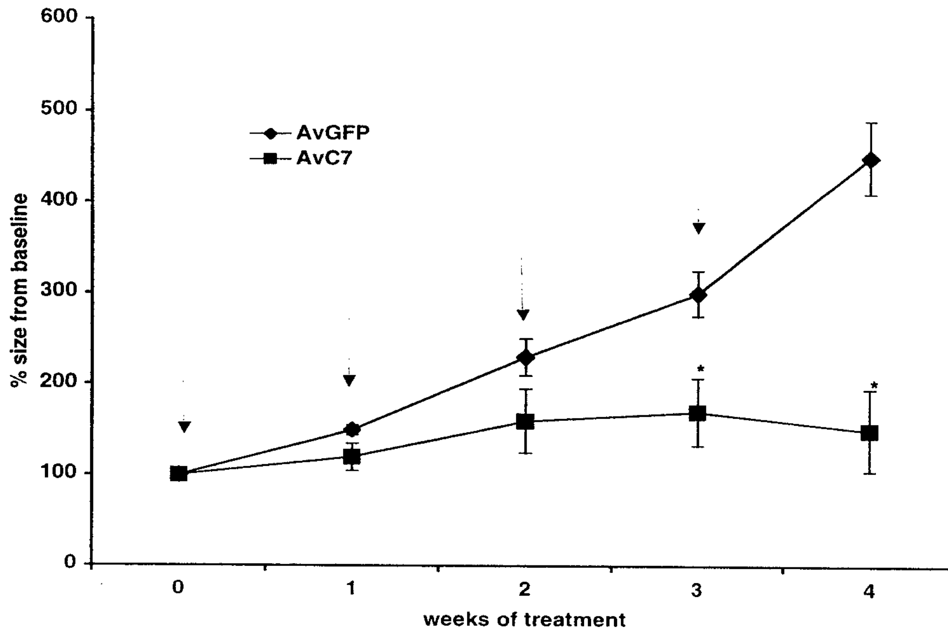

Fig. 2: Animals were injected with LNCaP dispersed in Matrigel (5 x 10^5). After approximately 20 days, when tumors had reached a diameter of 0.5 mm, the experiment was started by inoculating 10^9 pfu of adenovirus AvC7 (n = 6 tumors), or AvGFP (n= 6 tumors). The arrows indicate the time of viral administration. * indicate statistically significant difference between the two groups ($p < 0.05$).

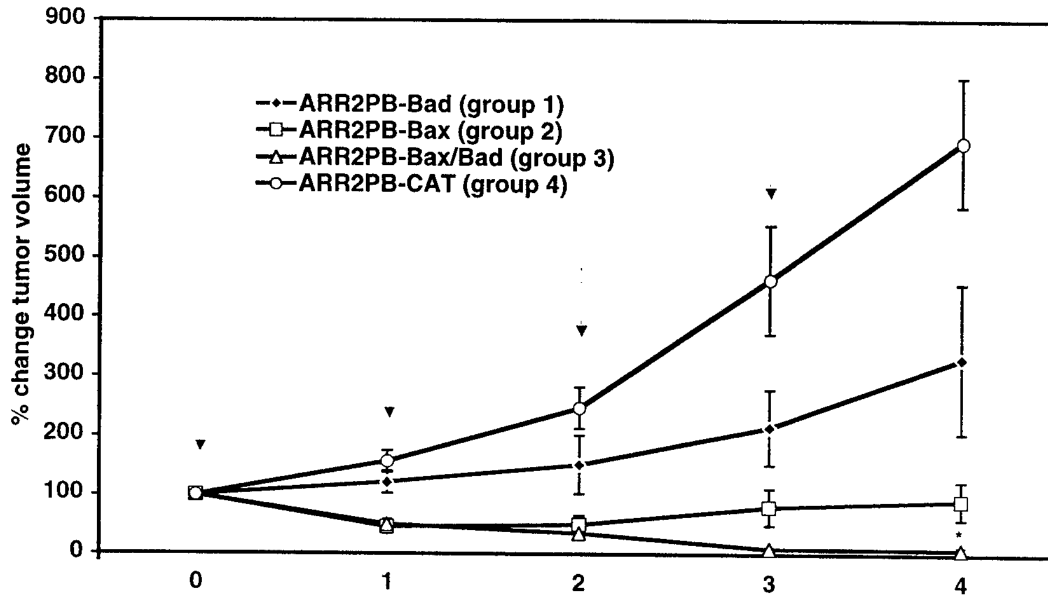

Fig. 3: LNCaP tumors respond differently to inoculation of different apoptotic adenoviruses. LNCaP tumors were produced by sc inoculation of 5×10^5 LNCaP cells dispersed in RPMI 1640 medium and 20% Matrigel. After approximately 30 days, when tumor volume was approximately 30 mm^3, treatment was begun using $AvARR_2PB$-Bad (group 1), $AvARR_2PB$-Bax (group 2), the combination $AvARR_2PB$-Bad and $AvARR_2PB$-Bax (group 3), and $AvARR_2PB$-CAT (control) (group 4) at the concentration of 1×10^9 pfu (each administration of virus is indicated by an arrow). Each group consisted of 6 tumors. Data are compared to their initial size set as 100%. Extensive statistical analysis of this experiment was performed, but for the purpose of this progress report we simply want to mention that a statistically significant difference between groups 2 and 3 was achieved after 4 weeks of the experiment, and between groups 1 and 3 after 2 weeks of treatment.

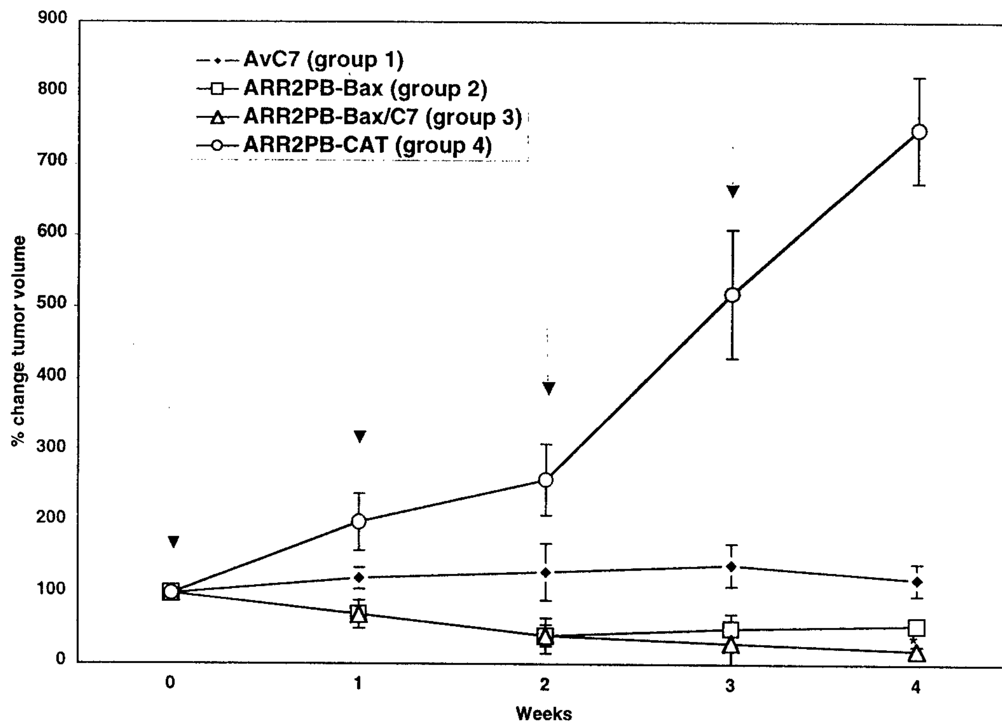

Fig. 4: LNCaP tumors were produced by sc inoculation of 5×10^5 LNCaP cells dispersed in RPMI 1640 medium and 20% Matrigel. After approximately 30 days, when tumor volume was approximately 30 mm^3, treatment was begun using AvC7 (group 1), AvARR$_2$PB-Bax (group 2), the combination AvC7 and AvARR$_2$PB-Bax (group 3), and AvARR$_2$PB-CAT (control) (group 4) at the concentration of 1×10^9 pfu (each administration of virus is indicated by an arrow). Each group consisted of 6 tumors. Data are compared to their initial size set as 100%. Extensive statistical analysis of this experiment was performed, but for the purpose of this progress report we simply want to mention that a statistically significant difference between groups 2 and 3 was achieved after 4 weeks of the experiment, and between group 1 and 3 after 2 weeks from the beginning of the treatment.

KEY RESEARCH ACCOMPLISHMENTS

The key research accomplishment is that the combination caspase-7 and Bax is very effective in inducing a significant effect in reducing tumor size, and in 50% of the cases in completing obliterating the LNCaP tumor. Although this is a significant achievement, we would like to confirm this experiment also with other cell lines, less likely to undergo apoptosis than LNCaP cells.

REPORTABLE OUCOME.

We have already sent the manuscript in which we report the positive effect of using poly instead of mono-gene therapy with pro apoptotic Bcl-2 family members. This manuscript was sent to "Human Gene Therapy", and it is pasted as an appendix. This was a very important manuscript, because it encouraged the hypothesis that caspase-7 could work in partnership with Bax to amplify its apoptotic effect. We have still a few experiment before we can publish the information that Caspase + Bax given together are very effective in obliterating LNCaP tumors.

We have presented some of the data described here at the Cancer Gene Therapy Meeting held in san Diego in December 2001: GENE THERAPY FOR THE INDUCTION OF THERAPEUTIC APOPTOSIS IN PROSTATE CANCER USING APOPTOTIC MOLECULES AND PROSTATE-SPECIFIC PROMOTERS. B. Nan, Y. Zhang, F. Andriani, J. Yu, B. Fang, S. Kasper, R. Matusik, L. Denner, M. Marcelli and at the Endocirne Society meeting, held in San Francisco in June 2002: GENE THERAPY FOR THE INDUCTION OF THERAPEUTIC APOPTOSIS IN PROSTATE CANCER USING APOPTOTIC MOLECULES AND PROSTATE-SPECIFIC PROMOTERS, by Ye Zhang, Jiang Yu and Marco Marcelli.

CONCLUSIONS

Overall this has been another productive year. The paper pasted in the appendix has been submitted, and another paper will shortly thereafter follow, after we have been able to complete a few additional experiments. Lack of an effect on the natural history of prostate cancer in TRAMP mice is probably non-reportable. We say this because we think that we have identified the cause for this negative result in our inability to treat the mice with more than one or two orthotopic inoculations of therapeutic virus. We hope that we will be able to obtain a therapeutic result with these experimental models of prostate cancer by systemic inoculation once the HD viruses will be ready.

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APPENDIX (privileged Information)

Mono- and poly-gene therapy for the treatment of experimental prostate cancers by use of the apoptotic genes Bax and Bad driven by the prostate-specific promoter ARR_2PB.

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Running Title: Poly- vs. mono-gene therapy of prostate cancer.

ABSTRACT

Aims: We have shown that adenoviral-mediated manipulation of apoptotic genes such as Bax could be a therapeutic option for prostate cancer. Unfortunately, the response of experimental prostate tumors to one single therapeutic gene of the apoptotic pathway is short-lived, and most of these tumors relapse after a short period of time. In this investigation we present data generated with adenovirus AvARR$_2$PB-Bad, in which the apoptotic gene Bad was placed under the control of the dihydrotestosterone (DHT)-inducible third generation probasin-derived promoter ARR$_2$PB. This therapeutic virus was given alone or in combination with other therapeutic viruses to a variety of *in vitro* and *in vivo* experimental models of prostate cancer. **Results**: Upon infection with AvARR$_2$PB-Bad, DHT-induced Bad overexpression occurred specifically in androgen receptor positive [AR(+)] cells of prostatic derivation. The apoptotic effect of AvARR$_2$PB-Bad (group 1) was compared to that of AvARR$_2$PB-Bax (group 2) (which overexpresses the apoptotic protein Bax), of the combination AvARR$_2$PB-Bad + AvARR$_2$PB-Bax (group 3) or of the control virus AvARR2PB-CAT (group 4) in the cell line LNCaP. In addition to identifying the modality of apoptosis induction by overexpressed Bad, the results suggested that group 3 contained more apoptotic cells than any other group. In additional studies, AR(+) androgen dependent LNCaP cells or AR(+) and androgen independent C4-2 cells were injected subcutaneously in nude mice. Four groups of six LNCaP or C4-2 tumors were treated with the same combinations of viruses of groups 1, 2, 3 and 4 discussed above. Treatment resulted in decreased tumor size in groups 1, 2 and 3 compared to group 4. There was a better response in group 3 compared to 2, and in 2 compared to 1. A better response in group 3 was confirmed during a eight week follow up period, in which no treatment was administered. Two LNCaP and C4-2 tumors of group 3 disappeared at the end of the treatment and did not recur after a eight

weeks follow up period. **Conclusions**: The data suggest that poly-gene therapy with apoptotic molecules is more effective in experimental models of androgen-dependent or -independent prostate cancer than mono-gene therapy.

OVERVIEW SUMMARY

We investigated the therapeutic efficacy of adenovirus $AvARR_2PB$-Bad (consisting of the apoptotic gene Bad driven by the androgen receptor-inducible promoter ARR_2PB) in prostate cancer cell lines growing *in vivo* and *in vitro*. We compared its ability to induce therapeutic apoptosis to that of $AvARR_2PB$-Bax (overexpressing the apoptotic gene Bax) or of the combination $AvARR_2PB$-Bad + Bax. Beside reporting the modalities of Bad overexpression-induced apoptosis in the cell lines, we also show both *in vitro* and *in vivo* that overexpression of the combination Bax + Bad is consistently more apoptotic than Bax alone, and, even more so, Bad alone. The results support the hypothesis that poly-gene therapy with apoptotic genes is more effective than mono-gene therapy for the induction of therapeutic apoptosis of experimental prostate cancers.

INTRODUCTION

The widespread use of prostate specific antigen (PSA) has significantly increased our ability to correctly identify patients affected by prostate cancer (CaP). This powerful diagnostic tool has changed the epidemiology of CaP, as an increasing number of patients are now diagnosed with organ-confined disease (Hankey, et al., 1999), and overall death rates are falling in many industrialized countries due to early diagnosis (Oliver, et al., 2001). Despite these encouraging statistics, prostate cancer is still the most frequently diagnosed visceral cancer in American men, and there will be an estimated 189,000 new cases and 30,200 deaths from it in 2002 (Jemal, et al., 2002). Thus, there is an urgent need to identify novel effective curative treatments.

Most patients with organ-confined disease undergo radical prostatectomy or irradiation, while those with non-organ-confined disease undergo palliative treatments consisting of surgical or medical castration or chemotherapy. As radical prostatectomy is associated with significant morbidity, and some cancers detected using PSA screening will not necessarily progress to clinical significance, it is questioned whether indiscriminate use of this aggressive procedure is warranted in all patients with preclinical disease (Harlan, et al., 2001, Yan, et al., 2000). In addition, currently available treatments for metastatic disease are inadequate and mostly palliative. Thus, novel treatments would be particularly beneficial for some patients affected by preclinical organ-confined disease, or and for those with metastatic CaP.

The community of cancer researchers has recently observed the development of two new successful cancer drugs directed against specific disease-causing abnormalities; Herceptin®, a monoclonal antibody against the Her-2/neu receptor tyrosine kinase, and STI-571, a small molecule inhibitor of the Bcr–Abl, c-kit and platelet derived growth factor receptor (PDGFR) tyrosine kinases. The clinical value of these drugs is now

firmly established with the success obtained in patients affected by Her-2/neu positive breast cancer (Slamon, et al., 2001), Bcr-Abl positive chronic myeloid leukemia (Druker, et al., 2001), and c-kit positive gastrointestinal stromal tumors (van Oosterom, et al., 2001). Development of cancer drugs directed against disease-specific mechanisms requires a sophisticated knowledge of the basic biology of the cancer of interest, and ideally presence of an established etiology, such as the Philadelphia translocation, which generates the fusion protein Bcr-Abl in 95% of patients with chronic myeloid leukemia (Sawyers, 1999). To create new therapeutic targets for malignancies such as prostate cancer where the etiology is unknown, we have previously demonstrated that manipulation of molecules regulating the intrinsic apoptotic pathway can be successfully utilized to induce therapeutic programmed cell death (PCD) in experimental models of prostate cancer. After dissecting the molecular mechanisms associated with apoptosis induced by HMGCoA and protein kinase inhibitors in prostate cancer cell lines (Li, et al., 2001, Marcelli, et al., 1998, Marcelli, et al., 1999, Marcelli, et al., 2000) and in a cell line derived from benign prostatic hyperplasia (BPH) stroma (Marcelli, et al., 2000), we identified the mitochondria as an attractive therapeutic target to induce therapeutic apoptosis in CaP cell lines. Using adenoviral technology to overexpress the powerful mitochondrial incapacitator Bax, we demonstrated that upon overexpression this molecule induces therapeutic apoptosis in all *in vitro* and *in vivo* experimental models of CaP tested (Li, et al., 2001). To minimize the possibility that overexpression of Bax may be associated with unwanted apoptosis in sites of accidental Bax accumulation, we have more recently developed a new adenoviral construct, in which Bax is driven by the modified probasin promoter ARR_2PB (Zhang, et al., 2000), which confers specific dihydrotestosterone (DHT)-induced expression only in androgen receptor positive (AR+) cells derived from prostatic epithelium (Andriani, et al., 2001).

Although this construct is effective in decreasing tumor size when it is delivered on a weekly base (Andriani, et al., 2001), in unpublished work we have noticed that tumors grow back to their original size around 6 to 8 weeks after the delivery of the virus was suspended. We have therefore hypothesized that treatment for a prolonged period of time, or with a regimen of multi-gene therapy may be more effective in inducing a complete eradication of these experimental tumors. Here we describe our experiments with adenovirus ARR_2PB-Bad, an additional weapon to target the apoptotic pathway, which was delivered *in vitro* and *in vivo* alone or in addition to ARR_2PB-Bax in experimental models of CaP. In addition to identifying the modality of apoptosis induction by overexpressed Bad, the results of this investigation suggest that poly-gene therapy (i.e using simultaneously the Bad and Bax overexpression systems) is more effective than mono-gene therapy (in which the overexpression systems for Bad and Bax were used individually) for the treatment of androgen-dependent or androgen-independent experimental models of CaP.

MATERIALS AND METHODS

Materials

Fetal bovine serum, tissue culture media and antibiotics were from Life Technologies, Inc. (Frederick, MD). Chemicals were from Sigma (St.Louis, MO) unless stated otherwise. Antibodies and other reagents for apoptosis assays have been described previously (Li, et al., 2001, Li, et al., 2001, Marcelli, et al., 1998, Marcelli, et al., 1999, Marcelli, et al., 2000, Marcelli, et al., 2000). The antibody to detect cleaved caspase-3 was from Cell Signaling Technology (Beverly, MA). Antibodies for Ser^{112}, Ser^{136}, and Ser^{155} Bad were # 9291, 9295 and 9297 from Cell Signaling (Beverly, MA), and for total Bad was # 943 from Santa Cruz Biotechnology (Santa Cruz, CA)

Cells Lines

Prostate cancer derived LNCaP (maintained in RPMI1640, 10% FBS and 1% P&S) (Horoszewicz, et al., 1980), PC-3 (maintained in F12 + 10% FBS + 1% P&S), and C4-2 (Thalmann, et al., 1994) (maintained in 80% DMEM low glucose and 20% Kaighn's F12 with 5% heat inactivated FBS supplemented with Insulin [5 μg/ml, Sigma], T3 [13.65 pg/ml,Sigma], apotransferrin [5 μg/ml, Sigma], d-Biotin [0.244 μg/ml, Sigma], adenine [25μg/ml, Sigma] and 1% P&S), have been described previously (Andriani, et al., 2001). C4-2 is a derivative of LNCaP. This cell line has acquired the ability to grow in a castrated host, and therefore represent a widely accepted model of androgen-independent and AR (+) CaP cell line (Thalmann, et al., 1994). Low passage 293 cells (maintained in IMEM zinc option with 10% HI-FBS, 2mM L-glutamine and 1% P&S) were purchased from Microbix Biosystem (Toronto, Canada), and utilized to produce adenovirus Av-ARR_2PB-Bad.

Construction of recombinant adenovirus ARR_2PB-Bad

Plasmid pCDNA-6-Bad was contructed using a Bad cDNA obtained by PCR from a LNCaP cDNA library. The following primers were used:

Bad-S: CA**AGATCT**CATCTTGTCCTCACAGCCCAGAGC

Bad-AS: CA**TCTAGA**TCACTGGGAGGGGGCGGAGCTTCCCCT

The product of the amplification was cut with the two custom restriction endonucleases Xho-I and Xba-I (in bold-face), and subcloned in a pCDNA-6 plasmid (Invitrogen, Carlsbad, CA) cut with the same enzymes. Sequence analysis was performed to ensure that no artifacts were added to the Bad sequence by the amplification process. Adenoviral particles containing the human Bad cDNA linked to the ARR_2PB promoter were prepared according to the technology previously reported (Marcelli, et al., 1999). Briefly, a full-length Bad cDNA was subcloned downstream of the ARR_2PB promoter in the background of the Bluescript plasmid (KS+) (Stratagene, San Diego CA). The ARR_2PB-Bad cassette was then cut with the restriction endonucleases EcoR-V and Sma-I and subcloned in the shuttle plasmid pXCJL-1-poly A+ (Marcelli, et al., 1999) cut with EcoR-V to obtain the construct pXCJL-1-ARR_2PB-Bad-poly-A. pXCJL-1-poly-A (a gift of Dr. BaBie Teng, UT Medical School, Houston TX) has a pBR322 backbone and contains the human adenovirus type 5 (Ad5) 59-inverted terminal repeat, the Ad5 origin of replication, the Ad5 encapsidation signal, the E1a enhancer, multiple cloning sites, a polyadenylation signal at the 3' of the polylinker, and Ad5 sequence from nucleotide positions 3328 to 6246 serving as homologous recombination fragment. The recombinant adenovirus Av-ARR_2PB-Bad was prepared by cotransfecting the pXCJL-1-ARR_2PB-Bad-poly-A with pJM17 (McGrory, et al., 1988), which contains a full-length adenoviral genome, into low passage 293 cells seeded onto a 60-mm culture dish the day before transfection at a density of $2x10^6$ cells/dish by the calcium phosphate coprecipitation method (Graham, et al., 1977). Two weeks after transfection, recombinant adenoviral plaques were picked, propagated, and screened for ARR_2PB-Bad sequences by polymerase chain reaction. Adenoviral particles (named Av-ARR_2PB-Bad)

that contained ARR_2PB-Bad were purified by a large-scale purification method described elsewhere (Marcelli, et al., 1999).

Other Adenoviral constructs

Adenovirus Av-ARR_2PBCAT[1], containing the CAT reporter gene under the control of ARR_2PB, was used as a control for Av-ARR_2PB-Bax. Adenovirus Av-ARR_2PB-Bax was previously described (Andriani, et al., 2001), and was used in combination with Av-ARR_2PB-Bad or alone in the *in vitro* and *in vivo* experiments described below.

Experimental protocols

Pilot experiments with a virus expressing the GFP (green fluorescent protein) cDNA under the CMV promoter (adenovirus AvCMV-GFP) determined that the optimal multiplicity of infections (MOI) for LNCaP and C4-2 cells was 100:1, and for PC-3 cells 1000:1 (data not shown). Optimal MOI is defined as the MOI resulting in infection of > 90% of the cells as assessed by the presence of green fluorescence 24 hours post-infection with MOI's of AvCMV-GFP ranging from 1:1 to 10,000:1.

Two days before infection, 1×10^5 cells were seeded in each well of a six well plate. On the day of the infection, one of the six wells was trypsinized and the cells counted. This information was used to infect each cell line at the desired MOI. Infections were carried out using various combinations of Av-ARR_2PB-Bad, Av-ARR_2PB-Bax or Av-ARR_2PB-CAT given alone or in combination in a 5% CO_2 incubator at 37 °C for 1 hour using 500 μl of infection medium (the same medium used for each cell line + 2% FBS and 1% P&S) on a rocker. One hour post-infection, cells were treated with dihydrotestosterone (DHT) at the concentration of 2 nM, or with vehicle alone. Cells were harvested at various time-points after infection. In the initial experiment cell

[1] Kasper S. et. al.: unpublished

lysates were used for the immunodetection of Bad at 0, 24, 48 and 72 hours post-infection by Western analysis, as described previously (Andriani, et al., 2001). β-Actin was included in each experiment to demonstrate equivalent amount of loading in each lane. When precise quantitation of DHT-dependent Bad induction was required, densitometric analysis was performed to correct expression of Bad with that of β-actin, which was immunodetected in the same sample. Densitometry was done by importing images to a Power Macintosh G4 personal computer using the Chemi Doc™ Documentation System, and the Quantity One quantitation software (both from BioRad, Hercules CA). Arbitrary densitometric units of Bad were then corrected for the densitometric units of β-actin.

Each experiment was performed in regular FBS. Use of regular FBS did not have consequences on AR trascritpional activation, as the concentrations of testosterone or dihydrotestosterone in FBS determined by radioimmunoassay were subsaturating [17 ng/dl (59 pM), and 3 pg/ml (0.01 pM) for T and DHT, respectively], and unable to induce significant ARR_2PB activity under the experimental conditions used throughout these studies.

Testosterone and Dihydrotetosterone Assay

One-hundred μl of fetal calf serum were diluted with double distilled water to 1 ml and approximately 5000 cpm of ^{3}H-testosterone (specific activity: 122.8 Ci/mmole) (New England Nuclear, Boston MA), or ^{3}H-dihydrotestosterone (specific activity 110 Ci/mmole) (Amersham, Piscataway NJ) were added. The sample was extracted twice with 3 ml of fresh diethylether (Mallinckrodt Baker, Paris KY), which was then evaporated to dryness with filtered air. The extract was dissolved in 0.5 ml of isooctane and applied to a celite column (ICN pharmaceuticals, Costa Mesa CA). After washing with 5 ml of isooctane, DHT was eluted with 4 ml of 3% ethylacetate in isooctane, while

T was eluted with 3.5 ml of 15% ethylacetate in isooctane. The eluates were dried, and the DHT and T residues were reconstituted in the 0 calibrator of the DHT (Diagnostic System Laboratory, Webster TX) or T RIA Kit (Diagnostic Product Corporation, Los Angeles CA) and assayed by radioimmuno assay. Aliquots were counted to determine the recoveries of DHT and T. The sensitivity of DHT was 10 pg/ml and that of T was 5 ng/dl.

Characterization of Bad overexpression

Bad undergoes post-translational changes in the form of phosphorylation at serines 112 (Zha, et al., 1996), 136 (Zha, et al., 1996) and 155 (Datta, et al., 2000). When phosphorylated, Bad is anchored to 14-3-3 proteins in the cytosol and kept inactive (Zha, et al., 1996). In response to apoptotic stimulations, Bad is dephosphorylated and binds the anti-apoptotic molecules $Bcl\text{-}_2$ and $Bcl\text{-}x_L$ on the mitochondrial surface (Datta, et al., 2000, Wang, et al., 1999). We studied Ser^{112}, Ser^{136}, and Ser^{155} Bad, and total Bad under various experimental conditions from total cell lysates, or from lysates containing mitochondrial or cytosolic fractions. The subfractionation technology used to obtain the various subcellular compartments has been previously reported (Li, et al., 2001, Marcelli, et al., 1999, Marcelli, et al., 2000). Most of these experiments were performed under control conditions (i.e. after infection with $AvARR_2PB$-Bad without addition of DHT) and after stimulation with DHT for 24 hrs.

Immunoprecipitation of Bad from uninfected LNCaP cells

LNCaP cells were grown in the presence or absence of 2 nM DHT for 30 minutes, 1, 6 and 22 hours. Harvested cells were washed with cold PBS and sonicated for 20 minutes in 500 μl lysis buffer [PBS + 1% NP40 + 20 μl of protease inhibitor cocktail (Complete^{TM}, Boehringer Mannheim)]. Ten μl of protein-G beads (Pierce, Rockford IL) were added to the samples, and rocked at 4 °C for 10 minutes, followed by 10 minutes of

microcentrifugation at 14,000 rpm to preclear the lysate of non-specific background. After addition of the primary antibody (Goat anti-(total)Bad, Santa Cruz Biotechnology, Santa Cruz CA), samples (1 μl/100 μg) were rocked overnight at 4 °C. The following morning protein G beads were added and the samples were rocked at 4 °C for 4 hrs to precipitate protein-beads complexes. After microcentrifuging the samples for 3 min at 14,000 rpm, the supernatant was removed, and the pellet containing the beads was washed with cold PBS twice. Pellets were resuspended in PBS + 1% NP40, diluted in loading buffer (0.45 g SDS, 1.875 ml TRIS [1M, pH 7.4], 1.5 ml glycerol, 0.75 ml βME, 2 mg Bromo-phenol Blue) boiled and sized in SDS-PAGE. Proteins were then transferred to nitrocellulose for the immunodetection of total Bad, and Ser^{112}, Ser^{136}, and Ser^{155} Bad by Western analysis.

Characterization of the intrinsic apoptotic pathway

Two days post-infection with Av-ARR_2PB-Bad (in the presence or absence of 2 nM DHT), cells were harvested and analyzed for Bad overexpression, cleaved caspase-3 expression, cytochrome c subcelluar localization, DNA fragmentation factor (DFF) cleavage, DEVDase activity and TdT-mediated-dUTP-X nick end labeling (TUNEL) (Marcelli, et al., 1998, Marcelli, et al., 1999, Marcelli, et al., 2000, Marcelli, et al., 2000).

***In vivo* experiments**

Animal studies were performed after approval by the center of Comparative Medicine of Baylor College of Medicine. 5 x 10^5 LNCaP cells dispersed in RPMI 1640 and 20% Matrigel were xenografted subcutaneously in twelve intact male nu/nu mice (Harlan Sprague Dawley, Indianapolis IN) (2 tumors in each mice, for a total of 24 tumors). In parallel experiments, C4-2 cells were inoculated subcutaneously in the same number of animals. An average of twentyfive days later, when tumors averaged a size of 30 mm^3, treatment was started using weekly intratumoral injection of 1.9 x 10^9 pfu of

virus (a total of four injections in four weeks). Each tumor was subdivided in four imaginary quadrants, and the total dose was delivered in four aliquots, one for each of the four quadrants. Six tumors were injected with ARR_2PB-Bad (group 1), six with ARR_2PB-Bax (group 2), six with a combination of ARR_2PB-Bad + ARR_2PB-Bax (group 3) (group 3 received half dose of each of the two virus, so the total dose of virus administered was the same as in groups 1, 2, and 4), and six with Av-ARR_2PB-CAT [the control group (group 4)]. Tumors were measured weekly using a caliper, and the volume calculated with the equation $m_1^2 \times m_2 \times 0.5236$ (where m_1 and m_2 are the smallest and largest diameters) (Janik, et al., 1975). One week after the last inoculation of virus, animals where sacrificed, the tumor excised and weighed. Although safety of the treatments was not a primary end point of this study, all animals underwent a macroscopic necroscopy. No unexpected deaths occurred in any of the animals before the time of sacrifice. Animals with complete tumor obliteration were kept alive for additional eight weeks without further therapy, to control for tumor recurrence. Intact males were used because the endogenous testosterone produced by these animals is necessary to sustain growth of xenografted LNCaP cells, and to drive ARR_2PB activity once constructs Av-ARR_2PB-Bax, Av-ARR_2PB-Bad or Av-ARR_2PB-CAT were inoculated inside the tumor. No exogenous androgens were used to carry on these experiments.

Statistical analysis

Following infection with various combinations of viruses, LNCAP cells were harvested, spun to a slide and subjected to TUNEL staining (Marcelli, et al., 1998). Five hundred cells were scored for each treatment (50 cells in 10 different random fields of the slide) for TUNEL positivity. Numbers of TUNEL positive cells in the various groups were compared using two tailed t-test for independent groups.

For the purpose of statistical analysis of the *in vivo* experiments, the weight of two tumors in the same animal was averaged, and statistical analysis of three treated mice vs. three control mice was performed. Size of the tumors in the two groups during the five weeks of the study were compared using two-tailed t-test for independent groups. Statistical significance was set at a p of < 0.05.

RESULTS

Av-ARR$_2$PB-Bad drives Bad overexpression in a DHT-dependent way

MOI's of Av-ARR$_2$PB-Bad known to infect approximately 100% of AR(+) LnCAP (100:1) and AR(-) PC-3 (1000:1) prostate cancer cell lines were utilized. Cells were grown under control or experimental conditions (i. e. with 10% FBS without or with 2 nM DHT, respectively) for 24, 48 and 72 hours. Immunoblot analysis demonstated DHT-induced overexpression of Bad (Fig. 1) in LNCaP cells. Bad expression peaked at 24 hours, and remained the same for the remainder of the experiment. As expected, no induction of Bad expression was detected in AR(-) PC-3 cells (not shown).

Detection of Phospho-Bad isoforms in LNCaP cells

We initially looked at total and phospho Bad expression using Av-ARR$_2$PB-Bad-infected LNCaP cells grown for 24 hours under control conditions, or after stimulation with 2 nM DHT. In agreement with the experiments discussed above, total Bad expression was stimulated approximately 250-fold by DHT (Fig. 2, lines 1 and 2). While under control conditions no phosphorylated form of Bad was identified, all three phosphorylated forms of Bad were correctly detected after stimulation with DHT [compare Fig. 2, lanes 3, 5 and 7 (control) with lanes 2, 4 and 6 (+ 2 nM DHT)]. This result could be consequent to increased expression of the phosphorylated forms of Bad paralleling DHT-induced total Bad overexpression. In alternative DHT could induce Bad phosphorylation, and facilitate immunodetection of the various forms of phospho-Bad regardless of total Bad overexpression. To sort this out, uninfected LNCaP cells were grown with or without DHT for 30 minutes, 1, 6 and 22 hours, and then subjected to immunoprecipitation using an antibody for total Bad. Immunoblot analysis of the immunoprecipitated material using phospho-antibodies correctly identified all three forms of phosphorylated Bad, and no DHT-dependent induction was detected (Fig 3). Thus, lack of phospho-Bad detection in whole cell lysates was due to inadequacy of the

antibodies to identify proteins that most likely were minimally expressed under control conditions.

Activation of the intrinsic apoptotic pathway by Bad overexpression

LNCaP cells were infected with AvARR$_2$PB-Bad (group 1), AvARR$_2$PB-Bax (group 2), AvARR$_2$PB-Bad + Bax (group 3) and AvARR$_2$PB-CAT (group 4) at a MOI of 100:1, treated in the presence or absence of 2 nM DHT, and subjected to TUNEL analysis. After 48 hours of DHT treatment 18, 40, 52 and 4% of LNCaP cells were apoptotic in groups 1, 2, 3 and 4, respectively. In contrast, only 5, 3, 5,and 6% of the cells grown under control conditions were TUNEL positive at the same time points ($p=0.009$, 0.0002, 0.0001 and non statistically significant between control vs. + 2 nM DHT in groups 1, 2, 3 and 4, respectively) (Fig. 4). The largest % of cells undergoing apoptosis after stimulation with DHT was in group 3, followed by group 2, 1 and 4. These differences were statistically significant (group 2 vs. 1 $p = 0.0007$, group 3 vs. 2 $p = 0.006$, group 3 vs. 1 $p = 0.0001$, groups 3, 2 and 1 vs. 4 $p<0.0001$).

In order to assess engagement of the apoptotic pathway in response to Bad overexpression, we next evaluated the subcellular location of Bad and cytochrome c in response to DHT. In the experiment shown in Fig 5 immunoreactive Bad was recognized by a total Bad antibody. Under control conditions (total) Bad and cytochrome c were recovered uniquely from the cytosolic and mitocondrial fractions, respectively (Fig. 5, lanes 1 and 2, respectively). Following infection with Av-ARR$_2$PB-Bad and treatment with DHT, a significant induction of (total) Bad expression was obtained, and a progressive shift of this molecule from the cytosol to a more evenly distributed cytosolic and mitochondrial location was seen (compare lanes 3 and 4 with 9 and 10). We also studied the intracellular location of the Ser^{112}, Ser^{136}, and Ser^{155} Bad phospho-isoforms in the same cell lysates, and found that phosphorylated Bad was present only in the cytosol

(Fig. 6). Thus, only unphosphorylated Bad (recognized by the total Bad antibody) migrated from the cytosol to the mitochondria.

Cytochrome c immunoreactivity shifted from the mitochondria to the cytosol following Bad translocation to the mitochondria (compare Fig. 5, lanes 1 and 2 with lanes 9 and 10). The passage of cytochrome c to the cytosol was followed by activation of caspase-3 [indicated by cleavage of pro-caspase-3 (Fig. 7A)], induction of DEVDase activity (Fig. 8), and cleavage of the death substrate DFF (DNA fragmentation factor (Liu, et al., 1997)) (Fig. 7B). Finally, we compared induction of DEVDase activity after 48 hours of stimulation with 2 nM DHT in group 1, 2, 3 and 4 (Fig. 8). We found that DHT treatment significantly stimulated DEVDase activity in groups 1, 2 and 3 compared to 4. At 48 hours DEVDase activity was significantly higher in groups 2 and 3 compared to 1 (group 2 vs. 1 $p = 0.0009$, group 3 vs. 1 $p = 0.0005$, group 2 vs. 3 NS). All groups treated with a therapeutic gene (groups 1, 2 and 3) had significantly higher DEVDase activity compared to the control group (group 4) (groups 1, 2, 3 vs. 4: $p < 0.0001$).

***In vivo* experiments**

The experiments discussed above showed that DHT-mediated overexpression of Bad was followed by apoptosis of LNCaP cells, and that this was due to induction of the intrinsic pathway. Overexpressed Bad was consistently less apoptogenic than Bax, and the combined infection with both viruses was the most effective way to induce programmed cell death. We then tested the efficacy of these reagents, given alone or in combination, using two *in vivo* experimental models of prostate cancer consisting of LNCaP or C4-2 cells inoculated subcutaneously in nude mice.

Fig. 9 shows changes in LNCaP tumor volume during a month of treatment (four inoculations, one inoculation every week) with 1×10^9 pfu's of AvARR$_2$PB-Bad (group 1), AvARR$_2$PB-Bax (group 2), a combination of AvARR$_2$PB-Bax and -Bad (group 3) or

$AvARR_2PB$-CAT (the control group, group 4). A statistically significant response was seen in groups 2 and 3 compared to 1 and 4 after one week of treatment and throughout the four weeks of the experiment (p value after one week of treatment: group 2 vs. 1 $p = 0.049$, group 2 vs. 4 $p = 0.002$, group 3 vs. 1 $p = 0.05$, group 3 vs. 4 $p = 0.002$). A statistically significant difference between groups 3 and 2 ($p = 0.045$) was reached at the end of the four weeks of treatment. Treatment with $AvARR_2PB$-Bad slowed tumor growth compared to control, but statistical significance was not achieved despite an obvious trend. By the end of the treatment, no tumor had disappeared from group 1 or 4, while two tumors were macroscopically invisible in groups 2 and 3. At the end of this initial phase of treatment, tumor size of groups 2 and 3 was measured for additional eight weeks, during which no treatment was administered. Interestingly, tumor size of group 3 remained significantly smaller than group 2. In addition, the two macroscopically invisible had relapsed in group 2 but not in group 3 by the end of these eight weeks of additional follow up (not shown).

Fig. 10 shows changes in C4-2 cells tumor volume during a month of treatment (one intratumoral administration/week) with 1×10^9 pfu's of $AvARR_2PB$-Bad (group 1), $AvARR_2PB$-Bax (group 2) a combination of $AvARR_2PB$-Bax and -Bad (group 3) or $AvARR_2PB$-CAT (group 4). The general trend observed was similar to that seen in LNCaP cells, i. e. control-treated cells continued to grow throughout the treatment, while all other groups showed a reduction in their original size, and group 2 and 3 shrunk more than group 1. The only difference found compared to the LNCaP experiment was that a statistically significant difference ($p=0.05$) was observed between group 1 and 4 beginning from week 1. By the end of the treatment two tumors had disappeared in groups 2 and 3, and after eight weeks of follow up with no treatment they had recurred

only in group 2 (data not shown). Tumor size of group 3 remained significantly smaller than group 2 during this period of follow up (not shown).

DISCUSSION

Adenovirus AvARR$_2$PB-Bad was developed for therapeutic purposes, with the intention to overexpress in a DHT-dependent way the pro-apoptotic protein Bad in AR (+) cell lines derived from prostatic adenocarcinoma. Following overexpression, while the phosphorylated forms of Bad remained anchored to the cytosol, unphosphorylated Bad migrated from the cytosol to the mitochondrial fraction, where it presumably heterodimerized with the anti-apoptotic proteins Bcl-2 and Bcl-x_L. This was followed by cytochrome c release from the mitochondria to the cytosol, activation of the caspase pathway and apoptotic death of the cell.

We then compared the ability of AvARR$_2$PB-Bad to induce DHT-dependent apoptosis of AR (+) CaP cells with that of the viral construct AvARR$_2$PB-Bax, which overexpresses the apoptotic protein Bax. For the *in vitro* experiments, we initially used the standard AR (+) CaP cell line LNCaP. While combination of the two adenoviruses (AvARR$_2$PB-Bax + Bad) was the most effective way to induce therapeutic apoptosis of LNCaP cells in group 3, monotherapy with AvARR$_2$PB-Bax (group 2) was more effective than with AvARR$_2$PB-Bad (group 1). Essentially similar results were obtained with a spectrum of other AR (+) CaP cell lines such as LAPC-4 (Klein, et al., 1997), C4-2 (Thalmann, et al., 1994) and PC-3 cells stably transfected with an AR cDNA (Andriani, et al., 2001) (data not shown). The Bad and Bax overexpressing systems were then used in two *in vivo* models of prostate cancer consisting of AR (+) and androgen dependent LNCaP cells, and AR (+) but androgen-independent C4-2 cells growing subcutaneously in nude mice. While AvARR$_2$PB-Bad achieved some success in slowing down tumor growth in both LNCaP and C4-2 cells, the AvARR$_2$PB-Bax overexpressing system was significantly more effective, and association of the two treatments was even more successful in decreasing the size of LNCaP and C4-2 tumors. Two LNCaP and two C4-2

tumors disappeared after four treatments both in groups 2 and 3. While none of these tumors recurred during 8 weeks of follow up in group 3, all of them reappeared in group 2 during the same period of time.

Several interesting observations were made while conducting this investigation. First, we confirmed in LNCaP cells the contribution of unphosphorylated Bad to cell death. It has been established that in a normal cell Bad is kept inactive in the cytosol through phosphorylation (Zha, et al., 1996). In the presence of apoptosis, Bad becomes dephosphorylated and migrates to the mitochondrial membrane where it heterodimerizes with the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-x_L (Zong, et al., 2001). Using the $AvARR_2PB$-Bad system and DHT, Bad was overexpressed to such high levels that its total and phosphorylated forms could be detected without the need of immunoprecipitation. During a 72 hours overexpression experiment we noticed that the three phoshorylated forms of Bad remained anchored to the cytosol, while increasing amounts of unphosphorylated Bad redistributed to the mitochondrial fraction. Our interpretation of these results is that upon treatment with DHT, Bad was overexpressed to a point that it overwhelmed the endogenous machineries responsible for its phosphorylation/inactivation. Accordingly, a portion of the protein was phosphorylated and remained anchored to the cytosol (Fig. 6), while increasing amounts were left unphosphorylated, moved to the mitochondrial surface (Fig. 5) where they presumably heterodimerized with the antiapoptotic Bcl-2 family members Bcl-2 and Bcl-x_L, and subsequently contributed to mitochondrial release of cytochrome c (Fig. 7), and to apoptosis through activation of the caspase pathway (Fig 7 and 8). In our hands, adenoviral-mediated overexpression of *wt* Bad equalized the effect of the Bad protein with mutated phosphorylation sites of Virdee et al (Virdee, et al., 2000), which acted as a

constitutive death-inducing molecule due to its inability to remain anchored to the cytosol.

The second significant finding of these studies is that AvARR$_2$PB-Bad was the least apoptotic of the three therapeutic combinations tested (AvARR$_2$PB-Bad, AvARR$_2$PB-Bax or AvARR$_2$PB-Bad + AvARR$_2$PB-Bax). This is explainable based on the different roles played Bad and Bax in the apoptotic pathway (Cheng, et al., 2001). These two molecules belong to the pro-apoptotic Bcl-2 family, which can be subdivided into multidomain members such as Bax and Bak [which posses the BH1, -2 and –3 (Bcl-2 Homology domain) domains], and BH-3 domain-only members, such as Bad, Bid and Bim, which display sequence homology only within this amphipathic α-helical segment which works as the critical death domain (Adams, et al., 1998). Additional important members of the Bcl-2 family are the anti-apoptotic molecules Bcl-2 and Bcl-x_L, which display conservation of four Bcl-2 homology domains (BH1-4). A paper by Cheng et al (Cheng, et al., 2001) has clarified the consequences of the heterodimerization occurring on the mitochondrial surface between the BH3 domain-only and anti apoptotic Bcl-2 family members, which takes place after the cell receives an apoptotic stimulation. According to their observations, the heterodimerization between BH-3 domain-only molecules and Bcl-2 or (Bcl-x_L) results in sequestration and inhibition of the formers. This inhibition can however be abrogated in the presence of an increased BH-3 domain-only /Bcl-2-like molecules ratio, which allows activation of the multidomain pro-apoptotic proteins Bax and Bak (Cheng, et al., 2001). In contrast to Bad, whose apoptotic activity depends on mediating the death signal to downstream molecules, active Bax is by itself a powerful effector of apoptosis by inducing the ultrastructural changes of the mitochondrial membrane which leads to cytochrome c (and other apoptotic molecules) efflux to the cytosol (Gross, et al., 1998). In previous work we showed that Bax

overexpression is sufficient to activate apoptosis without requirements of upstream signals, such as active BH-3 domain-only molecules (Andriani, et al., 2001, Li, et al., 2001), while in the current investigation we show that Bad overexpression is less apoptotic than Bax, or the combination Bax plus Bad. In all likelihood, the reason for these differences is that completion of the apoptotic program in a cell overexpressing Bad has to go through activation of the downstream endogenous multidomain pro-apoptotic Bcl-2 family members, of which presumably there is a limited amount. In contrast, direct overexpression of Bax does not require any rate limiting endogenous molecule for the apoptotic program to be completed. Cells infected with a combination of the Bad and Bax systems underwent apoptosis in an even more effective way. A plausible explanation for this is that they expressed large amounts of an active upstream mediator, Bad, and of the downstream executioner, Bax, and that simultaneous presence of two active molecules of the same pathway resulted in an additive apoptotic effect on the cell.

A further observation of this study is that a more robust response was noticed in tumors receiving the Bax and Bad overexpression systems compared to those receiving Bax alone, and more so, Bad alone. In support of a superior efficacy of the combination system, we noticed that two LNCaP and C4-2 tumors did not relapse two months after completion of the treatment in group 3, and that tumor size in group 3 was significantly smaller than in group 2 during this follow up period. Although this result could have been predicted from the *in vitro* experiments, we felt it was important to translate data obtained from an *in vitro* system into results derived from two sophisticated *in vivo* models of androgen-dependent and androgen independent prostate cancer.

In addition to the data discussed above we also performed a macroscopic necroscopy of all animals, and did not find any visible abnormality. Further, no early or unexpected deaths occurred in any of the four LNCaP or C4-2 groups. Thus, one could

argue that treatment with the various combinations of adenoviruses attempted in this study is relatively devoid of major systemic side effects under the experimental conditions and the lengths of time tested.

Although the data presented in this paper are encouraging and support the use of a combination of apoptotic genes for the treatment of prostate cancer, there are still a number of important challenges, such as the development of systems able to target metastatic disease. The experimental models presented in this paper mimic the primary lesions of the human disease, which are reachable by trans-rectal ultra-sound to deliver therapeutic genes (Herman, et al., 1999). Unfortunately, an even more challenging therapeutic problem for urologists is metastatic prostate cancer, and to date a reliable technology to reach metastatic lesions with gene therapy has not been developed.

The main lesson that can be derived from the studies presented in this paper is that "poly"-gene therapy appears to be more effective than "mono"-gene therapy to achieve reduction of experimental prostate cancers. This is reminiscent of the fact that chemotherapy, whether for microbial or neoplastic diseases, has evolved into poly-chemotherapy because combinations of drugs are more effective against resistant bacterial infections or cancers than a single agent. By analogy, it may very well happen that poly-gene therapy will eventually become a treatment of choice for resistant cancers, and that in addition to therapeutic genes from the apoptotic pathway one may be able to use genes targeting other vital functions of the cells, including known molecular defects present in specific types of cancer, pathways regulating the cell cycle, or rate limiting molecules necessary for the utilization of nutrients by the cancer.

Testing these hypotheses in these and other experimental models of CaP, as well as developing reagents to target metastatic disease are tasks currently pursued in our laboratory.

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LEGENDS TO FIGURES

Fig. 1: DHT-mediated induction of $AvARR_2PB$-Bad. Cells were infected at a multiplicity of infection (MOI) of 100:1 using adenovirus $AvARR_2PB$-Bad and treated for the indicated amount of time in the presence or absence of DHT (2 nM). Cells were were harvested and subjected to Western analysis for Bad and β-actin. β-actin was included to demonstrate equivalent amount of loading in each lane.

Fig. 2: Figure 2: Identification of phosphorylated Bad isomers after DHT-mediated Bad overexpression. Lane 1 and 2 show that induction of Av-ARR_2PB-Bad infected cells with DHT is associated with dramatic overexpression of total Bad [compare lane 1 (without DHT) and 2 (with DHT)]. Under control conditions (i. e. in the absence of DHT) none of the phosphorylated Bad isoforms is visible (lanes 3, 5 and 7). However, after induction with DHT all three phosphorylated forms of Bad are detectable.

Fig. 3: DHT does not induce phosphorylation of Bad in LNCaP cells. Cells were cultured for the indicated amount of time in the presence or absence of 2 nM DHT. Cells were then subjected to immunoprecipitation with an antibody recognizing total Bad. Immunoprecipitates were then used for Western analysis using antibodies recognizing the three phosphorylated forms of Bad. Lane 9 represents total Bad from untreated LNCaP cells.

Fig. 4: Overexpression of Bax and Bad is more apoptotic than overexpression of either protein alone in LNCaP cells. LNCaP cells were infected with $AvARR_2PB$-Bad (group 1), $AvARR_2PB$-Bax (group 2), a combination of the two viruses (group 3), or

AvARR2PB-CAT (group 4) at MOI's of 100:1 in the presence or absence (control) of 2 nM DHT for 48 hours. Cells were harvested and subjected to TUNEL staining. 500 cells from 10 random fields were scored for the presence of TUNEL positivity. Results are expressed as mean ± SD of at least 3 different experiments.

Fig. 5: Progressive shift of total Bad from a cytosolic location (lanes 1 and 3) to an equal distribution in the cytosol and mitochondria (lanes 5, 6, 9 and 10), and concomitant translocation of cytochrome c from the mitochondria (lane 2 and 4) to the cytosol (lanes 5 and 9), as a function of DHT-induced Bad overexpression. Cells were harvested before (lanes 1 and 2) and after (lanes 3-10) infection with AvARR$_2$PB-Bad at multiciplity of infection 100:1, and treated in the presence (lanes 5, 6, 9 and 10) or absence (lanes 1, 2, 3, 4, 7 and 8) of 2 nM DHT for the indicated amount of time. Aliquots containing mitochondrial or cytosolic fractions were obtained as described under material and methods and subjected to immunoblot analysis for total Bad, cytochrome c and β-actin. β-actin was included to demonstrate equivalent amount of loading in each lane.

Fig. 6: The three phospho-isoforms of Bad remain in the cytosol during DHT mediated Bad overexpression. Cell lysates corresponding to lanes 1, 2, 7, 8, 9, and 10 of the experiment shown in Figure 6 were utilized in a Western analysis using antibodies recognizing ^{112}Ser, ^{136}Ser and ^{155}Ser Bad. Cells were harvested before (lanes 1 and 2) and after (lanes 3, 4, 5 and 6) infection with AvARR$_2$PB-Bad at multiplicity of infection of 100:1, and stimulated in the presence (lanes 5 and 6) or in the absence (lanes 1, 2, 3 and 4) of 2 nM DHT for 72 hours. Immunoreactivity for ^{112}Ser, ^{136}Ser and ^{155}Ser Bad was identified only in the cytosolic fraction of cells stimulated with DHT for 72 hours (lane 5). β-actin was inserted to control for equal amount of loading.

Fig 7: Immunoblot analysis showing activation of procaspase-3 (A) and cleavage of the death substrate DFF (DNA fragmentation factor) (B) upon DHT mediated Bad overexpression. Cells were harvested after infection with $AvARR_2PB$-Bad at multiciplity of infection 100:1, and treated in the presence (lanes 2, 4, and 6) or absence (lanes 1. 3 and 5) of 2 nM DHT for the indicated amount of time. The apoptotic fragments of caspase 3 and DFF are observed only in cells treated with DHT.

Fig 8: Fig 8: Induction of DEVDase activity in LNCaP cells infected with apoptotic adenoviruses. Cells were infected with $AvARR_2PB$-Bad (group 1), $AvARR_2PB$-Bax (group 2), a combination of $AvARR_2PB$-Bad and Bax (group 3) , or $AvARR_2PB$-CAT (group 4) at a multiplicity of infection (MOI) of 100:1. Cells were then grown under control conditions or in the presence of 2 nM DHT for 48 hours, harvested and subjected to DEVDase assay. Induction of DVDase activity was calculated as a ratio of the activity detected in cells treated with DHT-containing medium vs. control medium. Each experiment was repeated in triplicate.

Fig. 9: LNCaP tumors respond differently to inoculation of different apoptotic adenoviruses. LNCaP tumors were produced by *sc* inoculation of 5×10^5 LNCaP cells dispersed in RPMI 1640 medium and 20% Matrigel. After approximately 30 days, when tumor volume was approximately 30 mm^3, treatment was begun using $AvARR_2PB$-Bad (group 1), $AvARR_2PB$-Bax (group 2), the combination $AvARR_2PB$-Bad and $AvARR_2PB$-Bax (group 3), and $AvARR_2PB$-CAT (control) (group 4) at the concentration of 1×10^9 pfu (each administration of virus is indicated by an arrow). Each

group consisted of 6 tumors. Data are compared to their initial size set as 100%. For statistical analysis of differences see text.

Fig 10: Different responses of C4-2 tumors to treatment with different apoptotic adenoviruses. C4-2 tumors were produced by *sc* inoculation of 5×10^5 C4-2 cells dispersed in RPMI 1640 medium and 20% Matrigel. After 30 days, when tumor volume was approximately 30 mm^3, treatment was begun using $AvARR_2PB$-Bad (group 1), $AvARR_2PB$-Bax (group 2), the combination $AvARR_2PB$-Bad and $AvARR_2PB$-Bax (group 3) or $AvARR_2PB$-CAT (group 4) at the concentration of 1×10^9 pfu for a total of four administrations (indicated by the arrows). Each group consisted of 6 tumors. Data are compared to their initial size set as 100%. A statistically significant difference in size was seen group 2 or 3 compared to 1 or 4 beginning from week 1, (groups 3 vs. 4 $p=0.01$, groups 2 vs. 4 $p=0.006$, groups 3 vs. 1 $p=0.01$, groups 2 vs. 1 $p=0.019$). A statistically significant difference was found between groups 1 and 4 beginning at 1 week ($p=0.05$). group 3 was consistently smaller than 2 and a statistically significant difference was obtained after 4 weeks ($p= 0.045$).