Adenovirus-mediated Bax Overexpression for the Induction of Therapeutic Apoptosis in Prostate Cancer

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ABSTRACT

Using adenoviral technology, we overexpressed the proapoptotic molecules pro-caspase-3, pro-caspase-7, and Bax to induce therapeutic apoptosis of prostate cancer cell lines growing in vitro and in vivo. Because overexpressed pro-caspase-3 did not undergo autocatalytic activation in any of the five prostate cancer cell lines evaluated, this strategy was unable to engage any component of the apoptotic pathway. Overexpressed pro-caspase-7 was proteolytically cleaved in LNCap and LnCaP-Bcl-2 cells but not in PC-3, DU-145, or TsnPr(1) cells. Cleavage was associated with engagement of many components of the apoptotic pathway, including DEVpase activity, cleavage of intracellular caspase targets such as the DNA fragmentation factor and the proapoptotic Bcl, release of cytochrome c from the mitochondria to the cytoplasm, and terminal deoxynucleotidyl transferase-mediated nick end labeling. No apoptosis was observed in the cells where caspase-7 did not undergo autocatalytic activation. Searching for an approach that would more reliably induce therapeutic apoptosis of prostate cancer cell lines, we used a binary adenoviral system to overexpress the proapoptotic molecule Bax. Bax was dramatically overexpressed and caused apoptosis of every cell line infected by engaging the mitochondrial pathway, including proteolytic cleavage and catalytic activation of the caspases, cleavage of caspase substrates, release of cytochrome c from the mitochondria, and DNA fragmentation. Furthermore, three injections of the Bax overexpression system into PC-3 cell tumors in nude mice in vivo caused a 25% regression in tumor size corresponding to a 90% reduction relative to continued tumor growth in animals that received injections with the control binary system expressing Lac-Z. These experiments show that adenovirus-mediated Bax overexpression is capable of inducing therapeutic programmed cell death in vitro and in vivo by activating the mitochondrial pathway of apoptosis. On the basis of these studies, we conclude that manipulation of Bax expression is an attractive new gene therapy approach for the treatment of prostate cancer.

INTRODUCTION

With 31,900 deaths estimated for the year 2000, prostate cancer is the second most frequent cancer-related cause of death among American men (1). Because of the widespread use of prostate-specific antigen screening of asymptomatic men, prostate cancer diagnoses and early intervention have dramatically increased in recent years. At presentation, prostate cancer can be an organ-confined or metastatic disease. Organ-confined disease is usually treated with radical prostatectomy or radiation therapy (2). Although these modalities of treatment are effective in many patients, there is significant morbidity and mortality associated with radical prostatectomy, and it is unclear from the outset how many of these cancers will progress to a clinically significant entity. Metastatic disease is mostly treated with a combination of hormonal manipulations (3). Unfortunately, most of these patients will relapse 12–18 months after treatment with a disease that is resistant to further hormonal or cytotoxic treatments (4). Thus, the currently available modalities of treatment are unsatisfactory. In the case of organ-confined disease, which may or may not progress to a clinical entity, the treatment of choice is an aggressive surgical procedure with potential morbidity and mortality, whereas in the case of metastatic disease, no curative treatment is available. Thus, the development of alternative therapies for prostate cancer is of critical importance.

In situ gene therapy represents an attractive alternative for the treatment of prostate cancer for several reasons. Primary prostate cancer is accessible by ultrasounds, and therapeutic genes can be directly inoculated into the neoplastic lesion. The prostate is relatively dispensable after the reproductive years so that, in contrast to cancers arising from organs regulating vital functions, specific promoters are unnecessary. In addition, disease progression or regression can be monitored using serial prostate-specific antigen measurements. Because prostate cancer is a relatively slow-growing disease, it is likely that treatment would include several inoculations of possibly more than one therapeutic gene. Another potential advantage of adenovirus-mediated gene therapy for prostate cancer is that intraprostatic gene delivery is associated with minimal side effects in patients treated to date (5) and minimal extraprostatic transduction of the therapeutic gene in experimental animals (6, 7). Thus, implementation of gene therapy for the primary lesion of prostate cancer is, at least in theory, a possible alternative to current treatment modalities. Nevertheless, reliable technology to reach metastatic lesions has not yet been developed.

Using a number of apoptotic substances, including lovastatin (8), sodium phenyl acetate, and staurosporine (9, 10), we have identified at least two steps of the apoptotic pathway whose activation is necessary to achieve apoptosis of prostate cancer cell lines: (a) activation of the caspase pathway (9); and (b) release of apoptotic molecules such as cytochrome c from the mitochondria to the cytosol (10). On the basis of these observations, we hypothesized that manipulation of these two steps of the apoptotic pathway by gene therapy may result in induction of therapeutic apoptosis of prostate cancer cells. Here we show that adenovirus-mediated overexpression of Bax induces therapeutic programmed cell death by triggering the mitochondrial pathway of apoptosis in a variety of prostate cancer cells growing in vivo and in vitro.

MATERIALS AND METHODS

Materials. Fetal bovine serum and tissue culture media were purchased from Life Technologies, Inc. (Frederick, MD). Antibodies for caspase-3 and caspase-7 were from Transduction Laboratories (Lexington, KY). The anti-cytochrome c, anti-Bax, and anti-caspase-9 antibodies were from PharMingen.
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(San Diego, CA). The anti-DFF4 (11) and Bid (12) antibodies were gifts of Dr. Wang (University of Texas, Southwestern Medical School, Dallas, TX). The *In Situ* Cell Death Detection kit was from Boehringer Mannheim (Indianapolis, IN). The fluorogenic substrate Ac-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin was from Enzyme System (Dublin, CA). The enhanced chemiluminescence detection kit was from Amersham Corp. (Arlington Heights, IL). TUNEL-positive cells were scored using a fluorescent microscope (Olympus IX70; Olympus America, Melville, NY). Images were recorded with a digital camera SPOT (Diagnostic Instruments, Sterling Heights, MI).

**Cell Lines.** LNCaP (13), LNCaP-Bcl-2 (14), PC-3 (15), DU-145 (16), and TspPr(1) (17) cells have been described previously (10). LNCaP, LNCaP-Bcl-2, and TspPr(1) cells were grown in RPMI 1640 supplemented with 10% FBS and 1% P&S (and 400 μg/ml G418 in LNCaP-Bcl-2). PC-3 cells were grown in F12 supplemented with 10% FBS and 1% P&S. DU-145 cells were grown in DMEM + 10% FBS and 1% P&S.

**Adenoviral Constructs and Infection Protocol.** First-generation adenoviruses driven by the constitutively active Rous sarcoma virus promoter and containing the CDNAs of caspase-3 and caspase-7 (AvC3 and AvC7) have been described previously (9, 18). The binary system for the overexpression of Bax consists of two adenoviruses (Ad/PKG/GV16 and Ad/GT-Bax) that have also been described previously (19). A previously described AvLac-Z virus (9) containing the Lac-Z cDNA was used as a control for AvC3 and AvC7. A binary system overexpressing Lac-Z (Ad/PKG/GV16 + Ad/GT-Lac-Z; Ref. 20) was used as a control in the experiments involving the binary system overexpressing Bax.

Two days before each experiment 1 × 10^4 cells were seeded in a six-well plate. On the day of the infection, one of the six wells was trypsinized, and the cells were counted. This information was used to infect each cell line at the desired MOIs. Infections were in 5% CO₂ incubators at 37°C for 1 h using 500 μl of medium. Following infection, the cell line was used for each cell line + 2% FBS and 1% P&S on a rocker. Pilot experiments with AvLac-Z determined the optimal MOI for each cell line. In these experiments (data not shown), all cell lines were infected with the control virus AvLac-Z, Bid was uncleaved and cytochrome c overexpression of pro-caspase-3 beginning 24 h and peaking at 96 h after infection. Pro-caspase-7 overexpression in LNCaP (Fig. 1) and other cell lines (not shown) was time dependent. Despite substantial pro-caspase-3 overexpression (Fig. 2), we were unable to detect DFF cleavage (Fig. 2), DEVDase activity (not shown), TUNEL (not shown), or cleavage of apoptotic substrates such as DFF (Fig. 2) in any of the cell lines tested. Thus, adenovirus-mediated pro-caspase-3 overexpression was not followed by auto-activation of the overexpressed protein and did not induce therapeutic apoptosis of prostate cancer cell lines.

**RESULTS**

**Adenoviral-mediated Overexpression of Pro-Caspase-3.** AvC3 infection of five prostate cancer cell lines resulted in significant overexpression of pro-caspase-3 beginning 24 h and peaking at 96 h after infection. Pro-caspase-3 overexpression in LNCaP (Fig. 1) and other cell lines (not shown) was time dependent. Despite substantial pro-caspase-3 overexpression (Fig. 2), we were unable to detect DFF cleavage (not shown), TUNEL (not shown), or cleavage of apoptotic substrates such as DFF (Fig. 2) in any of the cell lines tested. Thus, adenovirus-mediated pro-caspase-3 overexpression was not followed by auto-activation of the overexpressed protein and did not induce therapeutic apoptosis of prostate cancer cell lines.

**Adenovirus-mediated Overexpression of Pro-Caspase-7.** AvC7 infection of five prostate cancer cell lines resulted in significant overexpression of pro-caspase-7 beginning 24 h and peaking at 96 h after infection. Pro-caspase-7 overexpression in LNCaP (Fig. 1) and other cell lines (not shown) was time dependent. Despite substantial pro-caspase-7 overexpression (Fig. 2), we were unable to detect DFF cleavage (not shown), TUNEL (not shown), or cleavage of apoptotic substrates such as DFF (Fig. 2) in any of the cell lines tested. Thus, adenovirus-mediated pro-caspase-7 overexpression was not followed by auto-activation of the overexpressed protein and did not induce therapeutic apoptosis of prostate cancer cell lines.

**Adenovirus-mediated Overexpression of Pro-Caspase-7.** AvC7 infection of five prostate cancer cell lines resulted in significant overexpression of pro-caspase-7 beginning 24 h and peaking at 96 h after infection. Pro-caspase-7 overexpression in LNCaP (Fig. 1) and other cell lines (not shown) was time dependent. Despite substantial pro-caspase-7 overexpression (Fig. 2), we were unable to detect DFF cleavage (not shown), TUNEL (not shown), or cleavage of apoptotic substrates such as DFF (Fig. 2) in any of the cell lines tested. Thus, adenovirus-mediated pro-caspase-7 overexpression was not followed by auto-activation of the overexpressed protein and did not induce therapeutic apoptosis of prostate cancer cell lines.
experiments; determined as described previously (8–10). The results represent the means of three
infected at MOIs of 100:1 on day 0. DEVDase activity and TUNEL positivity were then
of TUNEL-positive cells in five prostate cancer cell lines infected with AvC7. Cells were
infected with Ad/Lac-Z, AvC3, respectively. However, cleavage of DFF (a marker of apoptosis) was present only
in LNCaP and LNCaP-Bcl-2 cells infected with AvC7.

Thus, it is likely that they represent either NH2-terminal proteolytic
epitope of which is between amino acids 4 and 125 of pro-caspase-7.

Thus, we concluded from these initial data that pro-caspase-3 or pro-caspase-7 overexpression induces therapeutic apoptosis only in a minority of prostate cancer cell lines and, thus, were not viable strategies for the treatment of prostate cancer.

Overexpression of Bax Induces Apoptosis of Prostate Cancer Cell Lines. Because we were unable to engage the apoptotic appara-
ratus by overexpressing the distal caspases, we turned our attention to
more proximal molecules. Specifically, we looked into the reported
ability of pro-apoptotic Bcl-2 family members, such as Bax, to trigger
apoptosis by functionally inactivating the mitochondria and forcing
the release of cytochrome c (22). Bax was overexpressed with a
system using two adenoviruses (19). The first (Ad/PKG/GV16) pro-
duces a powerful transcription factor, the GAL4-VP16 fusion protein
under the control of the constitutively active PGK promoter. The
second (Ad/GT-Bax) promotes Bax under the control of a GAL/
TATA minipromoter. Thus, the constituatively produced GAL4-VP16
binds the GAL/TATA minipromoter and drives transcription of the
Bax cDNA. Using this system, Bax was dramatically overexpressed in
each of the cell lines used within 24 h (as illustrated for PC-3 cells in
Fig. 5A). No Bax overexpression was present in cells infected with
the Ad/PKG/GV16 + Ad/GT-Lac-Z binary system (the control for the
Bax overexpression system; Fig. 5A). Within 24 h after infection, Bax
overexpression was followed by redistribution of cytochrome c to
the cytoplasm (Fig. 5, G and H). This, in turn, was followed by activation
of caspase-9, caspase-3, and caspase-7, induction of DEVDase activ-
ity, cleavage of the apoptotic substrate DFF, induction of TUNEL,
and DNA laddering. Fig. 5 shows this sequence of events in PC-3 cells,
compared with cells treated with the control binary system Ad/PKG/
GV16 + Ad/GT-Lac-Z. A similar sequence of events was also ob-
served in the other cell lines, with the only exception of DU-145 cells,
where no activation of caspase-7 was observed. Nevertheless, in all
cell lines Bax overexpression resulted in induction of DEVDase activity and TUNEL (Fig. 6).

In Vivo Experiments. Having demonstrated that the binary system
overexpressed Bax and induced apoptosis in every prostate cancer cell

D). Fig. 2 shows that adenovirus-mediated pro-caspase-7 overexpres-
sion was associated with the presence of smaller bands when the
various cell lines were analyzed by Western analysis using the anti-
caspase-7 antibody. These bands were recognized by an antibody, the
etope of which is between amino acids 4 and 125 of pro-caspase-7.
Thus, it is likely that they represent either NH2-terminal proteolytic
products of the full-length protein that are recognized by the NH2-
terminal antibody. In alternative, they may represent truncated pro-
teins initiated at the level of one of the internal methionines localized
before residue 125. In either case, the smaller bands cannot be the
active subunits of active caspase-7, because these are of COOH-
terminal derivation and would not be recognized by the antibody.

The molecular mechanism underlying the inability of overexpress-
ated pro-caspase-3 (in all five cell lines) and pro-caspase-7 (in
three of five cell lines) to undergo activation and induce apoptosis are
still uncertain (see “Discussion”). Nevertheless, in the mitochondrial fraction of LNCaP
and LNCaP-Bcl-2 cells infected with AvC7.

Fig. 2. Immunoblot analysis of pro-caspase-3, pro-caspase-7, and DFF in five prostate
cancer cell lines. Cells were infected at MOIs of 100:1 using adenoviruses AvLac-Z, AvC3,
or AvC7. After 72 h, cells were harvested. Five μg of cell lysate were subjected to Western
analysis using antibodies for caspase-3, caspase-7, or DFF. Overexpression of pro-
caspase-3 or pro-caspase-7 was detected in each cell line after infection with AvC3 or
AvC7, respectively. However, cleavage of DFF (a marker of apoptosis) was present only
in LNCaP and LNCaP-Bcl-2 cells infected with AvC7.

Fig. 3. DEVDase activity (expressed as fold-induction versus baseline) and percentage
of TUNEL-positive cells in five prostate cancer cell lines infected with AvC7. Cells were
infected at MOIs of 100:1 on day 0. DEVDase activity and TUNEL positivity were then
determined as described previously (8–10). The results represent the means of three
experiments; bars, SD.

Fig. 4. Cleavage of Bid and cytosolic redistribution of cytochrome c (Cyt c) is
detectable only in the cell lines where pro-caspase-7 underwent autocatalytic activation
(i.e., LNCaP and LNCaP-Bcl-2). LNCap, LNCaP-Bcl-2, DU-145, and PC-3 cells were
infected with AvC7 (A) or AvLac-Z (B) at MOIs of 100:1. After 72 h, cells were
harvested, and 5 μg of lysates were analyzed by Western analysis using an antibody for
Bid. A portion of the cell lysate of LNCaP-Bcl-2 (C) or PC-3 (D) was subfractionated to
obtain cytosolic (Lanes C) and mitochondrial (Lanes M) fractions. Five μg of each
subfraction were used to perform Western analysis using an antibody for cytochrome c.
Cytochrome c redistributed in the cytosol also in LNCap cells (not shown) and remained
in the mitochondrial fraction in DU-145 cells (not shown).
**DISCUSSION**

This investigation was performed to identify molecules of the apoptotic pathway that can be used as therapeutic targets to induce apoptosis of prostate cancer cells in *vitro* and *in vivo*. We reported previously that a necessary step of the apoptotic pathway for prostate cancer cells to acquire the typical morphological and biochemical phenotype of PCD consists in the activation of the caspase pathway (9). Thus, we initially attempted to activate the caspase pathway by overexpressing pro-caspase-3 or pro-caspase-7 using Rous sarcoma virus promoter-driven adenoviruses. This approach was based on the observations that plasmid-induced ectopic overexpression of several caspases is followed by apoptotic death of the target cell (24–27). We show here that the rate-limiting step for the induction of apoptosis of the various cell lines was the ability of the overexpressed pro-caspase to undergo autocatalytic activation. When pro-caspase-7 was cleaved in LNCaP and LNCaP-Bcl-2 cells, all components of the apoptotic apparatus were engaged. When pro-caspase-7 was not activated in the other three lines, no other apoptotic molecules were affected. In addition, pro-caspase-3 was not cleaved in any of the five cell lines, and none of these became apoptotic. These surprising results were in contrast with the ability of overexpressed Bax to induce apoptosis in all cell lines and in prostate cancer tumors in *vivo*.

The molecular mechanisms for the inability of overexpressed pro-caspase-3 to undergo activation are unclear. A previous report demonstrated that ectopic overexpression of pro-caspase-3 is followed by apoptosis of the host cells only if one uses a chimeric caspase-3 molecule in which the order of the various subunits is rearranged (27). Thus, it is conceivable that the native pro-caspase-3 molecule is not a good substrate for autoactivation after overexpression. It is also possible that overexpressed pro-caspase-3 is sequestered into a compartment that precludes autoactivation by, for example, acidification-dependent release of the safety catch.6

The ability of pro-caspase-7 to undergo cleavage in some cell lines but not others suggests a cell-specific mechanism that confers protection from apoptosis. Antia apoptotic molecules may prevent caspase-7 activation. For example, we showed by Western analysis that the caspase inhibitor XIAP (28) is expressed to a higher degree in DU-145 cells compared with LNCaP cells (not shown). Thus, it is possible that XIAP, other members of the IAP family (29), or the differential expression of IAP inhibitors such as Smac/Diablo (30, 31) may play a role in preventing activation of overexpressed caspases in prostate cancer cell lines. Interestingly, in LNCaP and LNCaP-Bcl-2 cells, active caspase-7 activated upstream, proximal components of the mitochondrial pathway, by, for example, cleaving the prosapotic Bcl-2 family member BID. In addition, caspase-7 induced apoptosis through direct cleavage of downstream, distal apoptotic substrates such as DFF.

Bax overexpression caused cytochrome c release, activation of the caspase pathway, and apoptosis of every prostate cancer cell line. It is possible that cytoplasmic release of the mitochondrial protein Smac (30, 31) contributed to the apoptotic effect of overexpressed Bax. These observations imply that manipulation of Bax expression has broad application to the induction of therapeutic apoptosis. Bax overexpression resulted in apoptotic death also of cell lines such as PC-3 and DU-145, which are resistant to some forms of chemically induced apoptosis (10). Furthermore, Bax overexpression had the ability to bypass the antiapoptotic effect of Bcl-2, which is stably overexpressed in LNCaP-Bcl-2 cells (14), and Bcl-XL, which is naturally overexpressed in PC-3 cells.5 Furthermore, Bax overexpression induced massive apoptosis in DU-145 cells, where the Bax protein is not expressed because of a nonsense mutation of the gene (32).

The apparent general ability of Bax overexpression to engage the apoptotic machinery and induce cell death in *vitro* suggested that Bax overexpression may be effective in *vivo* in models of prostate cancer. Indeed, three inoculations of the binary system overexpressing Bax caused regression of s.c. tumors of 4-fold reduction in tumor volume compared with the continued growth of tumors injected with the control virus. Because Bax overexpression induced apoptosis in PC-3 cells, which are particularly resistant to apoptosis (10, 33, 34), these results support the likely success of this strategy in treating all of the genotypically and phenotypically diverse cell types in both organ-confined and metastatic prostate cancer. Additional support of
the idea that Bax can act as a global inducer of therapeutic apoptosis comes also from the experience of other investigators, who have used different cell lines and experimental models with results similar to ours (19, 20, 35). Furthermore, it is likely that treatment optimization may cause complete tumor regression. For example, the dose chosen in the current experiments was dictated by technical considerations such that we used the largest volume that could be inoculated in these relatively small tumors without causing a major spread to either the surrounding tissues or the skin of the animals.

Limitations of currently available treatments have driven interest to develop new experimental therapies for prostate cancer. Attempts have been made to transduce the prostate with various therapeutic genes, including HSV-TK (5, 36–39), p53 (40, 41), p16 (41, 42), p21 (41, 43), IL-12 (44), and C-CAM (45) using a variety of vectors and prostate specific (7, 46) or constitutively active viral promoters. Most of the gene therapy approaches for prostate cancer that have been reported consist in the inoculation of adenoviruses containing the HSV-TK construct (47). The use of genes such as Bax represents an evolution from HSV-TK, because the former is cytotoxic without requiring exposure to substances like gancyclovir. On the basis of the results presented in this report, we think that the main issue with the use of apoptotic genes is not whether they will cause cell death but rather how to optimally target them to specific tissues and tumors. Although we did not identify any side effects attributable to the extravasation of the Bax virus to tissue other than the s.c. tumors, it is predictable that death genes such as Bax will induce a suicidal response in every tissue in which they are concentrated. This is especially true if they are driven, as in our studies, by a constitutively active promoter. Previous literature suggesting that treatment of patients with the intraprostatic delivery of adenoviruses containing HSV-TK is not associated with significant side effects (5, 39). Similarly, intraprostatic inoculation of adenoviruses in dogs is not associated with significant extraprostatic spread of the virus of interest (6, 7). Nevertheless, we think that prostate-specific promoters should be developed to target Bax overexpression uniquely to prostatic epithelium. Thus, one of the key challenges for this field is to identify prostate specific promoters that restrict expression of the therapeutic gene to prostatic epithelium. Promoters specific to prostate epithelial cells have been used successfully to create transgenic models of prostate cancer (48–50) and to direct gene expression to prostate cancer cells (7, 46, 51). The questions that remain to be asked is whether these promoters are powerful enough to sufficiently overexpress the protein of interest to obtain an apoptotic effect. In conclusion, although Bax overexpression is a powerful way to induce apoptosis in experimental models of prostate cancer, further work is necessary before this approach can be used for the treatment of human disease.

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