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ABSTRACT

Diphtheria toxin (DT) is a potent inhibitor of protein synthesis. As little as a single molecule of DT can result in cell-cycle independent cell death. This profound potency has led to difficulties in the development of DT as a suicide gene in cancer gene therapy, because toxicity appears to be related primarily to the fidelity of basal gene expression and the yield of viral titer. We evaluated the feasibility of prostate-specific DT gene therapy by cloning the catalytic domain (A chain) of DT under the control of the prostate-specific antigen (PSA) promoter, the PSA promoter and enhancer, or the cytomegalovirus promoter. The data on expression of DT from the plasmid constructs demonstrate that the basal level of DT gene expression determines the toxicity. To better test the potential therapeutic efficacy of DT suicide gene therapy, we first developed a DT-resistant adenoviral packaging line (293DTR). This allowed us to manufacture a relatively high titer adenoviral vector encoding the DT-A gene under the control of the PSA promoter and enhancer (Ad5PSE-DT-A) as well as an attenuated DT-A virus (Ad5PSE-tox176). In vitro studies showed that our viral constructs preferentially kill PSA-positive prostate cancer cells in the presence of exogenous androgen (R1881). In vivo studies showed that the nu/nu mice with PSA-positive cancer cell LNCaP xenograft treated with wild-type DT-A virus had a rapid regression of tumors and survived over a year without tumor progression, whereas the attenuated DT-A virus restricted tumor growth for only 1 month. The same constructs had no significant effect on the non-PSA-secreting cell line DU-145. These encouraging results suggest that DT-A viral gene transfer may ultimately have a therapeutic role in the treatment of advanced human prostate cancer.

INTRODUCTION

Despite escalating efforts to identify antiprostate cancer agents, advanced prostate cancer is still a universally progressive and fatal disease. This unusual resistance to conventional chemotherapeutic agents has led to the exploration of a variety of novel therapeutic strategies, including suicide gene therapy (1). This strategy relies on the delivery to cancer cells of genes that are either directly toxic or that produce toxic metabolites when administered with a prodrug. Examples of such a strategy include herpes simplex thymidine kinase, which can phosphorylate ganciclovir (an acyclic nucleoside analogue of 2'-deoxyguanosine), into the toxic monophosphate form. Other examples include cytosine deaminase and purine nucleoside phosphorylase. Because these suicide genes poison DNA synthesis by promoting abortive DNA chain elongation, they are uniquely effective at targeting rapidly dividing cells (e.g., leukemias, lymphomas, certain childhood malignancies, and germ cell tumors). Unfortunately, because <3% of prostate cancer cells are actively dividing at any given moment, this strategy is conceptually less appealing for our application (2). Thus, we have sought to develop a suicide gene therapeutic agent that is active against quiescent cells (i.e., cell cycle independent), yet potent and regulated.

DT is a potent cellular toxin that poisons protein synthesis by catalyzing ADP-ribosylation of elongation factor 2 (3) and kills cells primarily by an apoptosis-mediated pathway (4). In certain situations (e.g., mutant p53 expression) the cells may die by necrosis rather than apoptosis; however, the pharmacokinetics is similar regardless of the pathway of cell death (5). DT is composed of three functional domains located in two subunits, the A chain and B chain, which are joined by a disulfide bond (6). The A chain of DT has the catalytic domain, whereas the B chain comprises the receptor binding and translocation domains. It has been estimated that a single molecule of DT is capable of killing a cell (7); therefore, strategies must be used that limit its delivery to or expression in specific target cells. These strategies have included delivering expression constructs directly to diseased cells by liposomal gene transfer under the control of a regulatory element or tissue-specific promoter (8–11), or delivering the toxin A-chain molecules by virtue of fusion to cloned antibody fragments (12) or peptide ligands for cell-specific receptor-mediated endocytosis (13–15). Previous attempts at limiting toxicity of DT-A by use of a tissue-specific promoter have led to variable results. For example, Maxwell et al. (16) used a truncated form of the metallothionein promoter to demonstrate that basal expression of this promoter, even in the absence of heavy metals, resulted in substantial inhibition of protein synthesis. This inhibition could be augmented by the addition of an immunoglobulin enhancer element but only minimally by cadmium. The authors were never able to demonstrate true specific cytotoxicity but rather only a preferential cell susceptibility to DT-A-mediated cell death, presumably as a result of basal expression of this highly toxic gene. As a result, subsequent efforts by this group and others concentrated on introducing an attenuated mutant of DT-A (17) or on tightly regulating gene expression using prokaryotic control elements (18, 19). In both cases, although preferential cell killing could be demonstrated, complete abolition of nonspecific cell killing was not achieved. As a follow-up study, Keyvani et al. (20) used the same tet repressor-based system as they had reported previously with the wild-type DT-A gene with an attenuated DT-A mutant and were still unable to demonstrate complete abolition of background expression and subsequent cell death. These results stand in contrast to the efforts of Kunitomi et al. (11) and Murayama et al. (21), who demonstrated that selective inhibition of hepatocellular carcinoma cell lines can be achieved by α-fetoprotein promoter control of DT-A in a cationic liposomal gene transfer system (11, 21). Although differences among these studies may reside in the technical aspects in which DT-A activity is assessed, differing basal level activities of the various promoters used may also play a role.

3 The abbreviations used are: DT, diphtheria toxin; PSA, prostate-specific antigen; PSE, prostate-specific enhancer; CMV, cytomegalovirus; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MOI, multiplicity of infection.
Therefore, we initiated our studies first with conventional plasmid expression analysis of DT-A controlled by various prostate-specific promoters in liposomally mediated transient transfection assays. These experiments suggested that the PSA promoter and enhancer did not confer absolute specificity for DT-A toxicity; however, the poor gene transfer efficiency and indirect nature of the assessment of toxicity led us to question whether these results were valid or simply an artifact of the method of analysis. Therefore, we extended our analysis by developing an adenoviral expression system for DT under the control of the PSA promoter and enhancer. By using a high-efficiency gene transfer methodology coupled with direct measurement of cytotoxicity, we have been able to establish the feasibility of DT-mediated suicide gene therapy. Our viral construct does exhibit preferential toxicity to PSA-producing cells, although higher doses of the virus can affect non-PSA-producing cell lines. Nonetheless, to our knowledge, this is the first demonstration of an adenoviral gene transfer of DT. This development allows us to finally assess cytotoxicity directly in a population of cells. Such methods will allow us the tools to develop subsequent gene therapy treatments for a variety of applications. Furthermore, the DT-expressing adenovirus showed a strong inhibition of tumor growth in a PSA-producing prostate cancer cell LNCaP tumor xenograft model while not affecting the non-PSA-producing cell line DU-145 tumor xenograft model.

MATERIALS AND METHODS

Cells and Cell Culture. LNCaP, DU145, and TSU are human prostate cancer cell lines. DLD is a human colon cancer cell line. All were obtained from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented with 10% FBS and 50 units each penicillin and streptomycin per ml in an atmosphere containing 5% CO2. DMEM was used to similarly maintain 293 cells (Quantum Biotech) with the same supplements.

Plasmid Construction. pbK-CMV (Stratagene) is the basis for the plasmid vectors used in this study. Plasmids CN65 and CN33 (22) were provided by D. Henderson (Calydon, Inc., Menlo Park, CA). The CMV promoter was removed from pbK-CMV and replaced by the PSA promoter (−541 to +12), which was amplified from the plasmid CN33, to create pbK-PSA. The PSA enhancer and promoter (−5322 to −3722 and −541 to +12) were amplified by PCR from the plasmid CN65 and cloned into the pbK backbone to create pbK-PSE/PSA. The DT-A catalytic fragment (196 amino acids at NH2 terminus, which was amplified from the plasmid CN65 and cloned into the pBK backbone to create pbK-PSE/PSA) was inserted into the pBK-PSE/PSA-DT-A. The fragment from pBK-PSA-CMV (Stratagene) was cotransfected with the DT-A expressing plasmids with DT on the DT-resistant cell line and its parental 293 cells. Two pools of DT-resistant packaging cell lines were generated by cotransfecting 293 cells with plasmids pCMV-β-galactosidase and pBK-PSA-DT-A. The fragment from pBK-PSA-DT-A containing the DT-A coding sequence was cloned into pbK-PSE/PSA to make the plasmid pbK-PSE/PSA-DT-A.

Cotransfections to Determine the Effect of Promoter on DT Expression and Cell Type Specificity. LNCaP Cells were plated 1–2 days before the transfection in antibiotic-free RPMI 1640 with 10% FBS. Reporter plasmid pbK-CMV-luc (100 ng; firefly luciferase gene under control of the constitutive CMV promoter) was cotransfected with the DT-A-expressing plasmids with different promoters (varied between 0 and 150 ng/well), without promoter, or the pbK-CMV plasmid without DT-A transgene using Lipofectamine Plus Reagent (Life Technologies, Inc.). Cells were added to RPMI 1640 with dextran-charcoal-stripped serum (10% final concentration) containing R1881 (final concentration 5 nM) at 4 h after transfection. The Luciferase assays were performed at 48 h after transfection.

Viruses. CN702 is adenovirus type 5 attenuated by a deletion of E3 region. Ad5PSE-DT-A or Ad5PSE-Tox176 virus, which is E3 region deleted and the E1 region replaced by the DT-A or attenuated-toxin tox 176 gene under the control of the PSA enhancer and promoter replacing, was made by cotransfecting 293 cells with plasmids CP98 and pBH910 (Microbix Biosystems Inc.). The cloned tox 176 coding sequence had a G-to-A transition at nucleotide 383 as the only difference from the wild-type sequence. This resulted in replacement of the glycine at position 128 by aspartic acid in the amino acid sequence (17). CP98 was constructed by inserting the enhancer region of the PSA gene from −5322 to −3739 and a promoter region from −532 to +11 from CN65 (22) into the HindIII site of pE1sp1A (Microbix Biosystems, Inc.). The DT-A or attenuated-toxin tox 176 gene was inserted into the Xbal and BamH1 sites of pE1sp1A downstream of the PSE. Ad5-PSE-null virus was created similarly and contains the enhancer/promoter of PSA but lacks DT-A gene. Ad5LacZ and Ad5TK were adeno viral constructs expressing bacterial β-galactosidase gene or herpes virus thymidine kinase gene driven by CMV promoter in E1A region (Somatix, Inc.).

PCR Analysis of DT-A Insert and E1A in Adenovirus Constructs. PCR analysis was undertaken on the wild-type toxin virus Ad5PSE-DT-A, attenuated-toxin virus Ad5PSE-Tox176, and control virus Ad5PSE-null used in this study. The following primer pairs were designed to determine the presence or absence of DT and AdenoE1A sequences:

AdenoE1A 5’-CATGCCAACGTTCTCCTATAGC-3’ and 5’-GAGACATATTATCTGCCACGAGG-3’

DT-A 5’-AAACTTCCCCGTCGACATT-3’ and 5’-AGGTATACAAAGCCAAAATCGTG-3’

The expected sizes of the resulting fragments from AdenoE1A and DT-A are 540 bp and 270 bp, respectively. A 25 µl aliquot of each type of viruses was heated at 100°C for 5 min then cooled to 4°C, spun at 14,000 rpm for 5 min to pellet debris, and the supernatant was transferred to a fresh tube. A 10 µl aliquot of the cleared supernatant was used for each PCR reaction. PCR reactions (50 µl) were performed with 0.5 units Taq polymerase (Life Technologies, Inc., Rockville, MD) in the manufacturer’s buffer, 1.5 mM MgCl2, 200 µM of each deoxyribonucleotide triphosphate, and 1 µM of each primer. Initial denaturation at 98°C for 1 min, then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min were performed, with an additional 5 min extension at 72°C during the last cycle. Products were visualized after electrophoresis of 10 µl of each reaction through a 1% agarose gel.

Cytotoxicity of Virus Constructs. The cytotoxicity of Ad5-PSE/PSA-DT-A, Ad5-PSE-null, and CN702 viruses for various cell lines was assessed by MTT assay (Trevigen). Approximately 10,000 cells (TSU, LNCaP, DU145, or DLD) per well were plated in 100 µl of RPMI 1640 with 10% FBS in Falcon 96-well collagen coated plates. The next day the cells were infected with a MOI of 0, 2, 5, 12.5, or 30 for the dose response experiment. The wells were measured at 4 days after infection.

Establishment of DT-resistant Packaging Cell Lines. Using various concentrations of Lipofectamine, 293 cells in six-well plates were transfected with pHED-7 DNA (2 µg/well; kind gift of T. Uchida), which contained the gene for the DT-resistant EF-2 from Chinese hamster ovary cells (24). Three days after transfection, cells were trypsinized, and each well was expanded to 2–100 mm2 dishes with different dilutions (containing 1 of 10 and 9 of 10 of the original cells, respectively) and selected by the addition of 1.7 × 10−6 M DT holoenzyme. After 6 weeks of selection, two dishes were positive for growth, containing 15 and 8 colonies. The colonies in each dish were pooled and called 293 DTP#1 and 293 DTP#2, respectively. A third plate had only 1 colony, which was harvested and called 293 DTRC#1.

Resistance of DT Virus Packaging Cells to the Toxic DT Holoenzyme. MTT assays (Trevigen) were performed to determine the cytotoxic effect of DT on the DT-resistant cell line and its parental 293 cells. Two pools of DT-resistant cells, 293 DTP#1 and DTP#2, 1 clone 293 DTRC#1, and their parental 293 cells were plated at 25,000 cells/well in 96-well plates. The following day the cell lines were assayed in quadruplicate at each concentration of DT in DMEM with 10% FBS. Three days after DT addition, MTT assays were performed as follows: 10 µl of MTT reagent was added to the 100 µl of medium in the wells. Plates were incubated at 37°C for 4 h, then 100 µl of Detergent reagent was added to each well. The plates were incubated at room temperature overnight and read at 570 nm the next day.

Determining the Packaging Efficiency of the DT-resistant Cell Line and Its Parental 293 Cells. We plated 800,000 cells (293, or 293 DTPR1#2 or DTRC#1) per well in 12-well plates in duplicate. The following day the cells were infected with either vAd5LacZ or vAd5TK (MOI = 10). After infection (40 h) the medium was removed, and the cells were subjected to three freeze-thaw cycles to release cell-associated virus. The lysate was combined with the reserved medium. Dilutions (10−2 to 10−5) of virus from the various cell lines were added to 293 monolayers in six-well dishes and allowed to absorb overnight. The following day each well was overlaid with 0.8% LMP agarose (Life Technologies, Inc.) in DMEM (Dul-
beco’s Modified Eagles Medium, Life Technologies, Inc.) with 10% FBS and Pen/Strep (50 units of each/ml). Plaques were counted after 10 days of growth.

**Mouse Tumor Xenograft Analysis.** Approximately $2 \times 10^6$ LNCaP or DU145 cells in 0.2 ml of 50% Matrigel-50% PBS were injected s.c. into one side flank of athymic male nude mice (Taconic Farms, Germantown, NY) at 4–6 weeks of age. Tumors started to develop by 4–6 weeks. When tumors grew to average volume 300 mm$^3$, 1 $\times 10^9$ viral particles in 0.3 ml of PBS was administered intratumorally through multiple needle passes. Tumor size was measured at indicated times postinjection of recombinant viruses, as described previously (25).

**RESULTS**

**Transient Transfection Analysis of DT-A Expression by Using Various Promoters.** To test the specificity and toxicity of expression of DT-A by a promoter known to be active in prostate cells, transient cotransfection experiments were performed with plasmids expressing DT-A from the constitutive CMV promoter (CMV-DT-A), the PSA minimal promoter (PSA-DT-A), or the PSA promoter with the upstream enhancer (PSE-DT-A) in the LNCaP prostate cancer cell line. The cotransfected reporter plasmid (pBK-CMV-luc) contained the firefly luciferase gene driven by the CMV promoter. DT activity was inferred from the reduction of luciferase activity with increasing DT plasmid concentration by a modification of the strategy originally described by Maxwell et al. (16). Our approach differed only in that we used the luciferase reporter gene activity as the indirect measure of DT toxicity rather than that of the chloramphenical acetyl transferase gene. DT expression was found to be proportional to the basal strength of the promoter (Fig. 1A) so that the minimal PSA promoter had the lowest activity, the PSA promoter and enhancer had an intermediate activity, and the CMV promoter had the highest degree of activity. Previous studies had shown that expression from the PSA promoter with enhancer is highly inducible by androgen (22). To determine whether DT-A toxicity could be enhanced in our constructs by the addition of androgen, we performed the cotransfection experiment in the absence or presence of 5 nM R1881 (a synthetic, stable, and highly potent androgen). Only minimal induction of DT-A activity was noted among all of three promoters tested in the presence of R1881 (Fig. 2A), despite the presence of functional androgen receptor in the LNCaP cell line. To demonstrate that the LNCaP cell line was in fact responsive to enhanced transcriptional induction by R1881, we replaced the DT-A insert with the luciferase gene under control of the same promoter, which may overcome the inhibitory effect of DT on protein synthesis. This resulted in the expected high induction of the
construct with PSA promoter and enhancer by R1881 (Fig. 2B). The result suggests that the induced DT-A activity has exceeded the threshold level required in complete inhibition of host protein synthesis. The conclusion from this system is consistent with the previous observation with the inducible metallothionein system that toxicity was directly related to basal gene transcription rather than maximal induction.

Next, we wanted to determine whether the expression of DT-A from PSA or PSE would be tissue and cell specific. Two non-PSA-producing prostate cancer cell lines, DU145 and TSU, as well as the colon cancer cell line DLD, were cotransfected with the DT plasmids and the pBK-CMV-luc reporter plasmid. The results obtained for each of these cell lines were comparable with those seen in the LNCap cell line (Fig. 1, B–D) in that the minimal PSA promoter had modest activity in all of the lines tested, the PSA promoter with enhancer had an intermediate activity, and the positive control of CMV-DT-A had maximal activity. Of note, the nonprostate DLD line did seem to require relatively higher concentrations of the PSE-DT-A plasmid before inhibition of the coreporter luciferase was achieved. However, the differences were minor and difficult to interpret because of the indirect nature of this toxicity assessment.

Generation and Characterization of DT-resistant Packaging 293 Cell Lines. Previous attempts to make DT-A-expressing adenovirus were thwarted by the sensitivity of the 293 packaging cell line to the basal expression of the toxin gene. Therefore, as an initial step we generated DT-resistant 293 packaging cells by stably transfecting a DT-resistant genomic gene EF-2 mutant gene (pHED-7), which was isolated from Chinese hamster ovary cells (24, 26), and by selecting resistance to the DT holoenzyme. Two pools of resistant cells (293 DTRP#1 and 293 DTRP#2) and one clone (293 DTRC#1) were isolated, and the EF-2 mutation was proved by PCR amplification of the gene and digestion with Mbo II, which demonstrated the unique point mutation (data not shown). Furthermore, these resistant cells have been characterized for their resistance to DT holoenzyme and their ability to package nontoxic viruses. In comparison to the parental 293 cells, which were sensitive to $10^{-11}$ M DT holoenzyme, both pools of DT-resistant cells and the DT-resistant clone were resistant to $10^{-7}$ M DT, the highest dose tested (Fig. 4). To obtain high titer DT virus, it was necessary to determine the ability of the DT-resistant 293 cells to package nontoxic viruses, because this property could have been altered by transfection, DT selection, or extensive passaging of the cells that was required to produce the resistance. Two nontoxic viruses, Ad5TK encoding the herpes virus thymidine kinase gene and Ad5lacZ encoding the bacterial β-galactosidase gene, were independently used to infect each cell line at a MOI of 10. After infection (40 h), viruses were harvested from each cell line, and the amount of virus produced was assessed by plaque assay on wild-type 293 cells. None of the 293-resistant cell lines differed significantly from the wild-type 293 cells in their ability to package infectious virus (Fig. 5). Because the results obtained with 293DTRP#2 were marginally better in packaging the Ad5lacZ virus, that cell line was used for producing all of the subsequent Ad5PSE-DT virus stocks. With this packaging cell line, titers of Ad5PSE-DT-A reached as high as $3 \times 10^9$/ml (data not shown). The difference between total number of Ad5PSE-DT-A virus particles prepared from 293DTRP#2 versus wild-type 293 cells is 87-fold based on quantitative PCR analysis using DT-A-specific primers.

Replication-incompetent adenoviruses have several hundred nucleotides of homology to the 293 genome, in the proximal E1 region, because 293 cells are transformed with adenovirus. Thus, recombination leading to replication-competent adenovirus is a common problem in the generation of replication-incompetent constructs. Because of the profound cytotoxicity of DT, we expected to encounter a high
fresh frequency of wild-type version of replication-competent adenovirus under such severe selection pressure. Using our DT-resistant packaged 293 cells, we consistently found that a very faint band corresponding to an E1A-specific band (540 bp) was amplified from the Ad5PSE-DT-A virus preparations, whereas the E3-deleted Ad5 virus CN702 showed intensive band for the E1a gene, and Ad5PSE-null lacked this band (Fig. 3B). Because these preparations were purified by two rounds of CsCl centrifugation, this faint band likely represents a low frequency of reversion to wild-type rather than a contaminating genomic DNA in the virus preparation. Moreover, we continued to be able to detect this faint band despite multiple rounds of plaque purification, additionally supporting that this was likely a low frequency reversion from toxic selective pressure.

Viral Cytotoxicity in PSA-producing and Non-PSA-producing Cell Lines. To characterize the cytotoxicity of virally delivered DT, a dose-response analysis of DT activity was undertaken for all three of the viruses in PSA-producing cell line LNCaP and non-PSA-producing prostate cancer cell line DU145 day 4 after infection. The survival of the androgen-sensitive prostate cancer cell LNCaP was much lower than that of the androgen-insensitive cell DU145 at a very low MOI (2–5 pfu/cell) of wild-type DT-A virus (Ad5PSE-DT-A; Fig. 6), but both cell lines were insensitive to the Ad5PSE-null infection. The result indicated that DT-A gene expression by the viral construct was specific to PSA-producing cells in potency and cytotoxicity. The observation from infection of attenuated DT-A virus suggests that Tox176 gene expression was less toxic at a MOI <10. However, the cytotoxicity did converge at the highest MOIs. Such high levels of viral infection are beyond what is feasible for delivery in situ to tumors or clinically in commercial viral preparations, and, thus, this degree of specificity may already be reasonable for subsequent use.

Inhibition of Tumor Xenograft Growth. Because the potent cytotoxicity of recombinant viruses was achieved in an in vitro assay, additional studies of the therapeutic effect were conducted in tumor xenograft models. Both PSA-producing prostate cancer cells (LNCaP) and non-PSA-producing prostate cancer cell (DU145) xenografts were established. After a single intratumoral injection of either Ad5PSE-DT-A or Ad5PSE-Tox176, or Ad5PSE-Null, the male nude mice with LNCaP tumor xenografts treated with Ad5PSE-DT-A (∗ = 5) showed a halt of tumor growth within 12 days and after that were visually free of tumors (Fig. 7A). The mice with LNCaP tumor xenografts were treated with...
xenograft treated with attenuated virus Ad5PSE-Tox176 (n = 5) showed a similar inhibition of tumor growth; however, the tumors then showed subsequent progression after a month (Fig. 7B), whereas those animals treated with control virus Ad5PSE-Null (n = 5) showed no inhibition of tumor growth. Interestingly, the inhibitory effect was not observed in DU145 tumor xenograft model treated with either Ad5PSE-DT-A (n = 5) or Ad5PSE-Tox176 (n = 5; Fig. 7, C and D), whereas the Ad5PSE-DT-A appears to have a relative high background toxicity in vivo experiments. The in vivo experiments suggest tumor cell selectivity of the DT-A viral killing, i.e., the cytotoxicity of Ad5PSE-DT-A viruses is restricted to PSA-producing cells only to a greater degree than seen in the in vitro experiments.

Another important observation is that four of five mice with LNCaP tumor xenograft treated with Ad5PSE-DT-A survived more than a year without tumor progression, but this was not the case with Ad5PSE-Tox176 treatment, where the survival rate started dropping at day 138 (Fig. 8A). The survival rate in the DU145 tumor xenograft mice started dropping at day 67, 83, or 91 when treated with Ad5PSE-null, Ad5PSE-tox176, or Ad5PSE-DT-A (Fig. 8B). Therefore, Kaplan-Meier analysis revealed a substantial reduction of mortality in the LNCaP tumor xenograft mice treated with wild-type toxin virus Ad5PSE-DT-A compared with attenuated-toxin virus Ad5PSE-Tox176 and control virus Ad5PSE-Null (Fig. 8A), and no difference in mortality in the DU145 tumor xenograft mice (Fig. 8B).

DISCUSSION

Previous liposomal-gene transfer studies of DT-A expression have shown that the toxin is so potent that cell death occurs before enough DT-A can accumulate for immunological detection. Thus, expression was detected by cotransfection of the DT-A plasmid with another constitutively expressed reporter gene (16, 17). The indirect assessment of cell death coupled with a low efficiency of gene transfer and variability in the accuracy of initial cell counts raises the possibility of artifact in these assays. Alternative uses of the DT-A gene, for instance in the generation of transgenic animals with ablation of specific cell lineage, have demonstrated the utility of this strategy with both the wild-type DT-A gene (27–29) as well as attenuated mutants of the gene (30), which was necessary to limit nonspecific cell lineage ablation from low level expression of the promoters used in those studies. Such findings, therefore, have suggested that an appropriately selective promoter might be a feasible method of controlling DT-A expression for applications in human cancer gene therapy. Therefore, we sought first to determine whether our tissue-specific promoter system demonstrated specificity or lack of specificity in cell death when driving the DT-A gene, and then we compared the results with a direct measure of cellular viability on a population of cells infected with a DT-encoded adenovirus, controlled by the same tissue-specific promoter. By generating a DT-resistant packaging cell line, we were able to propagate high titer of recombinant adenoviruses encoding the A chain of DT. This is the first demonstration of such an adenoviral construct, and its high gene transfer efficiency has enabled us to directly assess the specificity and potency of this approach in prostate and nonprostate cancer cells. Our results indicate that the cotransfection of indirect reporter gene method for assessment of specificity is useful but is unable to fully discriminate the subtleties of cell-specific regulation. Higher efficiency gene transfer systems such as monoclonal antibody facilitated liposomal gene transfer (31) or fusogenic liposomal macromolecule transfer (32) result in cell-specific death.

Our results extend these observations and demonstrate that adenoviral-mediated gene transfer of the DT-A gene under the control of the PSA promoter and enhancer confers excellent potency and acceptable specificity in prostate-specific cell death. In vitro studies, the androgen-responsive and PSA-positive cell line LNCaP displayed a preferential sensitivity to the Ad5PSE-DT-A, which is consistent with the results of treatment of the LNCaP tumor xenograft. Whereas the non-PSA-producing prostate cancer cell line DU145 showed an intermediate sensitivity to the Ad5PSE-DT-A, the growth of DU145 tumor xenograft was not inhibited by treatment with this virus. The colon cancer cell line DLD displayed the least sensitivity to our prostate-specific DT-A virus in vitro study, which required a longer time period for toxicity and fairly high doses of the DT-A virus before resulting in cell death (data not shown).

Additional improvements in the specificity of cell death are likely to be achieved by decreasing basal gene expression and using tightly regulated promoters; however, using attenuated mutants of DT such as tox176 does not appear to be feasible as it lessens the sustained antitumor efficacy substantially. Because the nu/nu mice with LNCaP tumor xenografts treated with Ad5PSE-DT-A survived more than a year without tumor progression compared with those treated with Ad5PSE-Null and Ad5PSE-tox176 it will be of interest to additionally study the mechanism of inhibition of tumor growth by DT-A.

We demonstrate the first successful generation of a DT-expressing recombinant adenovirus. Generation of a DT-resistant packaging cell line has allowed us to obtain a high titer of this recombinant virus. We find that such a vector demonstrates a potent elimination of established tumors in a nude mouse model with remarkable selectivity, and the cure of these established tumors was sustained for > 1 year. Our attenuated DT recombinant adenovirus (i.e., Ad5PSE-Tox176) retained a high degree of potency against PSA-producing cell lines, with diminished toxicity in non-PSA-producing lines in vitro studies; however, this effect was sustained in our xenograft model, and the tumors recurred and progressed after 1 month. Our data demonstrate that selective cytoreduction appears to be enhanced in our xenograft models compared with the in vitro tissue culture experiments, which may reflect an artifactual toxicity bias in the tissue culture experi-

![Fig. 8. Kaplan-Meier analysis of in vivo studies (software Statgraphics Plus 5). The LNCaP and DU145 tumor xenografts were established by s.c. injection of ~2 × 10⁶ LNCaP or DU145 prostate cancer cells in 50% Matrigel. Three different recombinant adenoviruses were intratumorally administered when tumors grew to average volume 300 mm³. Tumors were measured as indicated times postinjection of recombinant viruses. The arrow indicates the date of injection with the adenoviral vectors.](https://cancerres.aacrjournals.org/content/56/11/2581/F8.large.jpg)
ments. These results appear very promising as a potential novel suicide gene therapy for the treatment of advanced prostate cancer.

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Ying Li, John McCadden, Fernando Ferrer, et al.


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