Cancer Research

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Cancer Res 1997;57:2559-2563.

[CANCER RESEARCH 57, 2559–2563, July 1, 1997]

Advances in Brief

Prostate Attenuated Replication Competent Adenovirus (ARCA) CN706: A Selective Cytotoxic for Prostate-specific Antigen-positive Prostate Cancer Cells[1]

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Abstract

Prostate-specific antigen (PSA) is a widely used marker for the diagnosis and management of prostate cancer. Minimal enhancer/promoter constructs derived from the 5′ flank of the human *PSA* gene (prostate-specific enhancer) were inserted into adenovirus type 5 DNA so as to drive the *E1A* gene, thereby creating a prostate-specific enhancer-containing virus, CN706. *E1A* was expressed at high levels in CN706-infected human PSA-producing LNCaP cells but not in CN706-infected DU145 cells, which are human prostate cells that do not express PSA. The titer of CN706 was significantly higher in LNCaP cells compared to several human cell lines that do not produce PSA (HBL100, PANC-1, MCF-7, DU145, and OVCAR3). Furthermore, in LNCaP cells, the yield of CN706 was dependent on exogenous androgen (R1881). CN706 destroyed large LNCaP tumors (1×10^9 cells) and abolished PSA production in *nu/nu* mouse xenograft models with a single intratumoral injection.

Introduction

PSA[4] is the most widely used serum marker for the diagnosis and management of any form of cancer. It is produced in PCA cells and prostate ductal epithelia (which represents less than 5% of the cells of the prostate); it is also produced in much smaller amounts in the periurethral glands and, very rarely, in tumors of the skin, salivary, and breast (1–3). With advances in PSA screening followed by tissue biopsy, newly diagnosed PCA represents 43% of all diagnoses of nonskin cancer in men. Despite these advances in PCA diagnosis and management, effective systemic treatment remains elusive; metastatic PCA is the second leading cause of cancer death of men in the United States (2). There is no curative treatment for PCA once the disease has progressed beyond the organ (4, 5). Human gene therapy using tissue-specific expression of cytotoxic genes may be a rational treatment strategy. The regulatory regions of the *PSA* gene are a reasonable choice for such an approach. Recently, we described an androgen-responsive and tissue-specific PSE located upstream of the PSA promoter, which, when fused to the promoter in a minimal enhancer/promoter unit, causes PSA to be expressed at almost wild-type levels (6). Because the prostate is an accessory organ, removal or ablation of the entire gland has no serious health repercussions (7–10).

Most current methods of gene therapy use viral vectors because the efficiency of gene transfer with viruses is superior to that achieved by nonviral systems (11–13). We chose an adenoviral vector. We reasoned that placing its *E1A* gene under the control of the PSE would create a virus, the replication of which would be restricted primarily to PSA-producing cells within the prostate and PSA-expressing PCA cells. Here, we describe the construction of such an ARCA and demonstrate its selective cytotoxicity toward PSA-expressing PCA cells *in vitro* and *in vivo*.

Materials and Methods

Cells and Cell Culture. The following cell lines, all obtained from the American Type Culture Collection were used: LNCaP, a human PCA cell line derived from a cervical lymph node metastasis that produces PSA and a mutated but functional androgen receptor (14, 15); HBL100, a human lung cell line; MCF-7, a human breast cancer cell line; PANC-1, a human pancreatic cancer cell line; DU145, a human PCA cell line that lacks the androgen receptor and does not produce PSA, as determined by reverse transcriptase-PCR; and OVCAR3, a human ovarian cancer cell line. The human embryonic kidney cell line, 293, which expresses the adenovirus E1A and E1B gene products, was obtained from Microbix Biosystems, Inc. (Toronto, Canada; Ref. 16). Cells were maintained in DMEM, with the exception of LNCaP cells (maintained in RPMI 1640) and suspension culture 293 cells (maintained in Joklik's MEM). Cultures were supplemented with 10% FCS, with the exception of suspension culture 293 cells, which were supplemented with 10% horse serum. All media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin and maintained at 37°C in 5% CO_2.

Construction and Purification of CN706 and CN702. pXC.1 and BHG10 (17, 18) were purchased from Microbix Biosystems. pXC.1 contains human adenovirus 5 (Ad5) sequences from base pairs 22 to 5790 (17). BHG10 contains Ad5 sequences with two deletions: an E1 deletion of base pairs 188-1339 and an E3 deletion of base pairs 28,133–30,818. BHG10 DNA is noninfectious, whereas cotransfection of pXC.1 and BHG10 creates infectious virus by homologous recombination (18). A unique *Age*I restriction site in the promoter of adenovirus E1A at Ad5 nucleotide 522 was created in pXC.1. The first set of PCR primers, 15.131a (5′-TCGTCTTCAAGAATTCTCA) and 15.133d (5′-TTTCAGTCACCGGTGTCGGA), produced a 927-bp PCR fragment from the unique *Eco*RI site in the pBR322 backbone of pXC.1 to the unique *Age*I site at Ad5 nucleotide 552. A second set of PCR primers, 15.133c (5′-TCCGACACCGGTGACTGAAA) and 15.133b (5′-GCATTCTCTAGACACAGGTG), produced a 787-bp fragment from the *Age*I site to the *Xba*I site at Ad5 nucleotide 1339. Combining equal amounts of the 927-bp product with the 787-bp product, a second PCR was performed with the two outside primers, to yield a product of 1714 bp that could be cut with *Age*I into the two smaller fragments. The 1714-bp fragment was cleaved with *Eco*RI and *Xba*I and cloned into similarly cleaved pXC.1 to yield CN95.

CN65 contains the enhancer and promoter of the human *PSA* gene, consisting of the enhancer at base pairs −5322 to −3738 fused to the PSA promoter at base pairs −541 to +12, separated by 76 bp of a multiple cloning site from BSKSII+ (Stratagene; Ref. 6). The 2213-bp PSE with *Age*I ends was prepared by PCR of CN65 (6) with primers 15.176a (5′-CATTAACCGGTACCTCTAGAAAATCTAGC), which introduces an *Age*I site at the 5′-end of the

Received 4/7/97; accepted 5/22/97.

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[1] R. R., H. Y. L., and J. W. S. have no financial interest in Calydon, Inc., and were supported by NIH Specialized Programs of Research Excellence in Prostate Cancer Grant CA-58230 and the CapCure Foundation.

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[4] The abbreviations used are: PSA, prostate-specific antigen; PCA, prostate cancer; PSE, prostate-specific enhancer; ARCA, attenuated replication-competent adenovirus; Ad5, human adenovirus 5; pfu, plaque-forming unit(s); i.t., intratumoral.

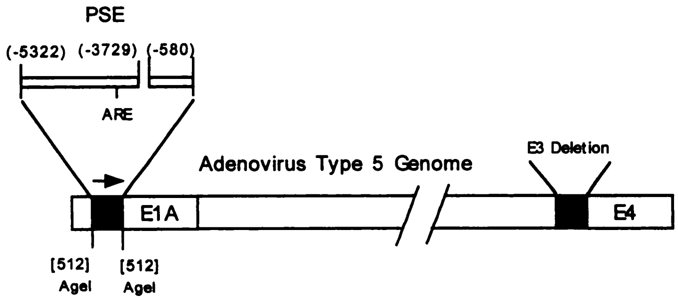

Fig. 1. Structure of CN706. A composite enhancer/promoter element from the human *PSA* gene was inserted in the Ad5 genome at nucleotide 512 as shown. As a control, CN702, identical to CN706 but lacking the composite PSA enhancer/promoter, was constructed. Both CN706 and CN702 contain the E3 deletion derived from the BHG10 plasmid, as shown.

PSE, and primer 15.176b (5′-CATTAACCGGTAAGCTTGGGGCTGGGG), which introduces an *Age*I site at the 3′-end of the PSE. The PCR product was cleaved with *Age*I and cloned into *Age*I-cleaved CN95 to yield CN96.

Recombinant virus was prepared by homologous recombination (19). Ten μg of CN96 were mixed with 20 μg of BHG10, precipitated with $CaCl_2$, and used to transfect 293 cells (16) as described (20). Plaques were picked after 15 days, replaqued, and grown on 293 cells to yield virus. A single plaque was designated CN706. Similarly, a single plaque prepared by homologous recombination of pXC.1 and BHG10 was designated CN702. Both CN706 and CN702 contain identical 2685-bp deletions in the Ad5 E3 region (nt 28133 to 30818) derived from BHG10. However, whereas CN702 contains a wild-type E1 region, CN706 contains the PSE inserted at Ad5 nucleotide 512 driving the *E1A* gene. CN702's growth was identical to that of wild type. Ad5LacZ, a replication-defective Ad5 devoid of E1A and E1B but containing the cytomegalovirus immediate early promoter driving the β-galactosidase *LacZ* gene, was a kind gift of L. Cohen (Somatix Therapy Corporation).

Virus was prepared by infecting 30 15-cm plates of 293 cells at a multiplicity of infection of 10 pfu and harvesting the detached cells after 48 h. The virus remains associated with the cell. Virus purification was performed at 4°C. Cells were collected by centrifugation at 3000 rpm for 10 min in a Sorvall RC-2B centrifuge. The cells were twice resuspended in 25 ml of cold PBS (Ca^{2+}- and Mg^{2+}-free) and collected by centrifugation. The supernatant was carefully removed, and the cell pellet was resuspended in 20 ml of cold 0.1 M Tris-HCl (pH 8.0). The cells were lysed by adding 10% sodium deoxycholate to an overall concentration of 0.5% and kept on ice for 30 min. The suspension was treated for 1 min with a Tissue-Tearer at full speed to reduce viscosity. The suspension was layered onto a cold CsCl block gradient of equal parts 1.45 g/ml and 1.20 g/ml CsCl in 10 mM Tris-HCl (pH 8.0) and centrifuged for 2.5 h at 30,000 rpm at 4°C in a Beckman Ti70 rotor. The virus band was removed, diluted, rebanded in a preformed CsCl gradient by ultracentrifugation for 16 h, and dialyzed into cold PBS (Ca^{2+}- and Mg^{2+}-free) containing 10% glycerol. Virus was sterilized through a syringe-mounted sterile 0.22-μm filter and stored at −80°C.

Western Blot of Ad5 E1A Expression. LNCaP and DU145 cells were infected at multiplicities of 10 and 20 pfu, respectively. Untreated and CN702- and CN706-infected cells were prepared in parallel. Cell pellets were extracted after 5 days with 500 μl of cell lysis buffer [0.1 M Tris-HCl (pH 7.4), 0.5% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol] and heated at 100°C for 10 min. Lysates were centrifuged at 14,000 rpm for 20 min, and the supernatants were removed. Total protein was estimated by dye binding (Bio-Rad). Protein (25 μg) was loaded onto Laemmli gels. Gels were run overnight at 8 ω, transferred to nitrocellulose paper (Schleicher & Schuell; Trans-Blot, Bio-Rad), probed with a rabbit polyclonal antibody against E1A protein (Santa Cruz Biotechnology), and developed (enhanced chemiluminescence kit; Amersham). Prestained molecular weight markers (Rainbow Markers; Bio-Rad) were run in the same gels for comparison of molecular weight and estimation of transfer efficiency. Films were developed after incubation for 1 min with the horseradish peroxidase substrate provided in the Amersham enhanced chemiluminescence kit.

LNCaP Tumors in Nude Mice. Tumors were induced in 6–7-week-old BALB/c *nu/nu* male mice by s.c. injection of 1×10^6 LNCaP cells in 0.5 ml of 50% Matrigel (21)-50% PBS. Tumors were allowed to grow to approximately 1 cm in diameter (4 weeks) prior to initiation of experiments. For i.t. injection, 0.1 ml of virus suspension in PBS (Ca^{2+}- and Mg^{2+}-free)-10% glycerol was injected into tumors using a 25-gauge needle.

Tumors were measured at the indicated times postinjection in their longest dimension and at 90° to their longest dimension. Tumor volumes were calculated using the formula: (length $\times$ width^2)/2 (22). Tumor volumes were normalized to 100% on day 0. The relative percent tumor volume was calculated for each mouse. The relative value for each group of mice were averaged, and the SEs were calculated. Blood samples for measurement of serum PSA levels were obtained by tail vein incision (23). Serum PSA levels were determined using the Tandem-E PSA ELISA kit (Hybritech, San Diego, CA). PSA values were calculated as for tumor volumes.

Results

Structure of CN706. Ad5 containing PSE, so as to drive the E1A gene, CN706 (Fig. 1), was constructed from derivatives of pXC.1 (17), CN96, and BHG10 (18). pXC.1 contains wild-type Ad5 sequences from Ad5 nucleotides 22 to 5788 in pBR322; BHG10 contains Ad5 sequence at nucleotide 1339 to the right terminus of the Ad5 genome, with an E3 deletion of 2685 bp from nucleotides 28,133 to 30,818; and CN96 contains a PSE of 2213 bp in pXC.1 in the left-to-right orientation of adenovirus, so as to drive the Ad5 *E1A* gene inserted into a unique *Age*I site at Ad5 nucleotide 552. The structure shown in Fig. 1 was confirmed by PCR, restriction enzyme analysis, and DNA sequencing of the insert and joints of the insert with the Ad5 sequence.

Western Blot of E1A Expression. Ad5 E1A protein expression in LNCaP and DU145 cells following infection with CN706 and CN702 was determined by Western blot analysis. E1A protein was not de-

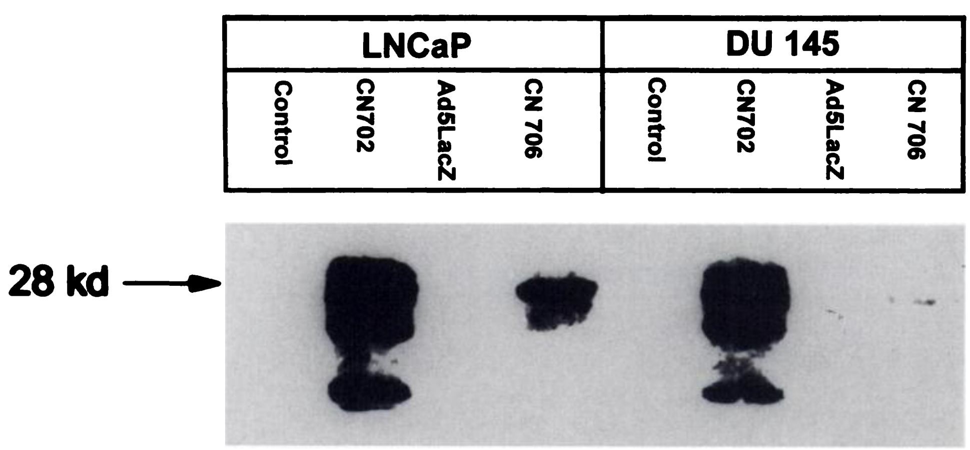

Fig. 2. Expression of E1A by CN706. LNCaP or DU145 cells were infected with CN702 (wild-type Ad5 E1 region with E3 deletion), Ad5LacZ, or CN706; mock-infected cells were included as a control. Cells were harvested by lysis in SDS-PAGE sample buffer, electrophoresed, and transferred to nitrocellulose. The immobilized proteins were then probed with a polyclonal antiserum to Ad2 E1A proteins and bound antibody detected by chemiluminesence as described in "Materials and Methods." *Arrow*, location of the M_r 28,000 protein marker.

tected in either cell line prior to infection or in cells infected with Ad5LacZ. CN702 expressed E1A at significant levels in both LNCaP and DU-145 cells, as did CN706 in LNCaP cells (Fig. 2). In CN706-infected DU15 cells, however, E1A expression was reduced by 99%. Northern blot analysis of CN706-infected LNCaP cells did not detect E1A mRNA from the endogenous viral enhancer/promoter, which was displaced 1681 bp upstream (data not shown). Thus, the PSE in CN706 provided E1A expression selectively in PSA-producing cells (LNCaP) but not in non-PSA-producing cells (DU145).

Ability of CN702 and CN76 to Multiply in Several Cell Types. Differential titers have been used to compare growth of mutant viruses in different cell lines (24–30). The differential titer of CN702 and CN706 in various human cell lines is shown in Table 1. The absolute number of plaques was normalized to 5.0×10^5, and relative titers were calculated. The results show that CN702 and CN706 grew equally well in LNCaP cells. However, in all other cell lines, CN702 gave a higher titer than CN706; the titer of CN706 was reduced 3000:1 in HBL100 (human lung) cells, 20:1 in MCF-7 (human breast carcinoma) cells, 20:1 in PANC-1 (human pancreatic carcinoma) cells, 2000:1 in DU145 (human non-PSA producing prostate carcinoma) cells, and 60:1 in OVCAR3 (human ovarian carcinoma) cells. These differential titer data may reflect a potential therapeutic index for ARCA in different tissue types *in vivo*. Thus, depending on cell type, the therapeutic ratio of CN706 varies from a low of 20:1 to a high of 3000:1. Therapeutic ratios of many conventional cytotoxic drugs range from 1.5:1 to a high of 6:1 (31).

The activity of the PSE has been shown to be inducible by androgen (6). An androgen-induced increase in CN706 titer as compared to CN702 titer was shown in LNCaP cells. LNCaP cells were infected with a constant amount of CN702 or CN706, and cultures were overlayed with agar containing increasing concentrations of the non-metabolizable synthetic androgen R1881. CN702 titer was not effected by androgen, whereas 1 nM and 10 nM R1881 induced additional 5- and 7-fold increases, respectively, in CN706 titer compared to no treatment with R1881 (data not shown).

Treatment of Prostate Tumors with CN706. LNCaP tumors were injected i.t. with 5×10^8 pfu CN706 on day 0 (Fig. 3*A*). Tumors were measured at the indicated times. There was a slight increase in tumor volume during the first 2 weeks after i.t. injection, followed by a rapid decrease. After 6 weeks, 5 of 10 mice were visually free of tumor.

These experiments show tumor cell selectivity and tumor cell killing, but, whereas wild-type Ad5 can infect and occasionally transform mouse cells, Ad5 cannot propagate significantly in mouse cells. Thus, tissue selectivity of CN706 must be shown indirectly in mouse xenografts. Indeed, as expected, CN702 can also eliminate human LNCaP xenografts in nude mice (data not shown). DU145 is a PCA cell line that does not produce PSA or androgen receptor. In contrast, all, or nearly all, human PCAs produce PSA *in situ* (1, 7, 32–34). However, PSA expression is lost within hours in primary cultures of PCA cells.[5] Tumors of DU145 cells were induced in nude mice and challenged with buffer, CN702, and CN706 (Fig. 3*B*; $n = 5$ for each group of mice). The results show CN702 inhibits growth of DU145 tumors, whereas CN706 has no effect on tumor growth. Thus, the prostate-specific CN706 virus shows selectivity for cells producing PSA *in vivo*.

Blood samples were harvested from the mice shown in Fig. 3*A* at the same time as tumor volumes and serum PSA levels were measured (Fig. 4). PSA levels also increased slightly, as did tumor volume, after infection but then fell rapidly. The fall in PSA levels preceded the

Table 1 *Titer of CN702 and CN706 in human cell lines*

	Virus	
Cell line	CN702	CN706
293	5.0×10^5	5.0×10^5
LNCaP	2.0×10^4	1.5×10^4
HBL100	5.0×10^5	5.0×10^2
MCF-7	2.0×10^3	1.0×10^2
PANC-1	2.0×10^3	1.0×10^2
DU145	2.0×10^5	1.0×10^2
OVCAR3	6.0×10^4	1.0×10^3

Cells were plated and infected as described in "Materials and Methods." Infected cells were overlayed with nutrient agarose and plaques counted at 5 days. Titers were normalized to 5×10^5 pfu/ml in 293 cells.

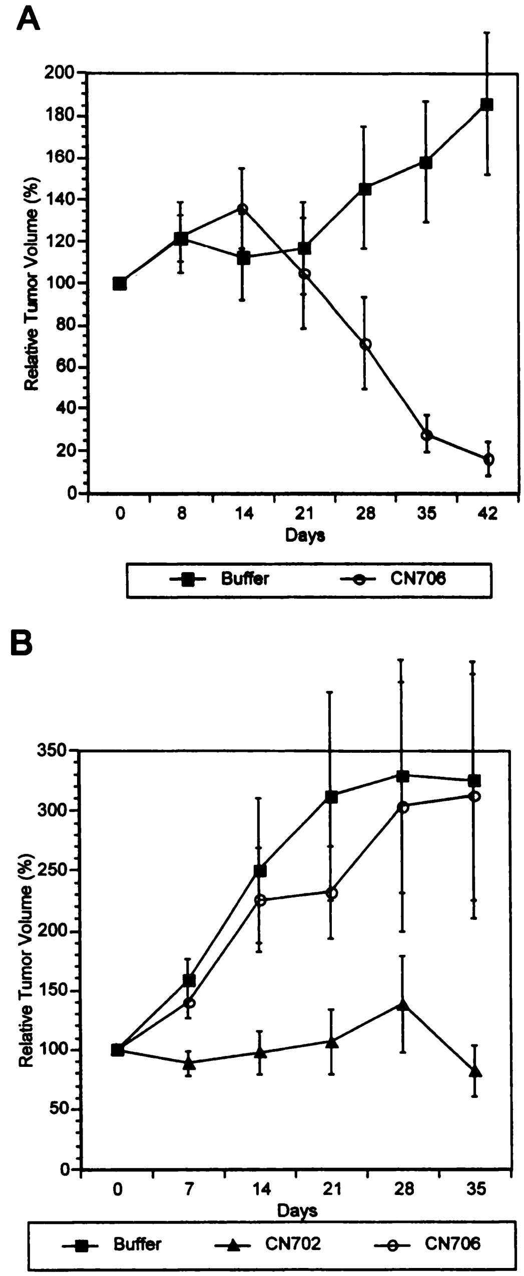

Fig. 3. Treatment of tumor xenografts with recombinant viruses. Tumor xenografts were grown s.c. in BALB/c *nu/nu* male mice to approximately 1 cm in diameter. Tumors were treated with recombinant viruses by i.t. injection on day 0, and measurements were taken weekly. *A*, LNCaP tumors were treated with PBS-10% glycerol (buffer, $n = 5$) or CN706 in buffer ($n = 10$). Average tumor volumes were normalized to 100% on day 0. *B*, DU145 tumors were treated with buffer ($n = 5$), CN702 ($n = 5$), or CN706 ($n = 5$), and weekly tumor measurements were taken as in *A*.

[5] D. Peehl, personal communication.

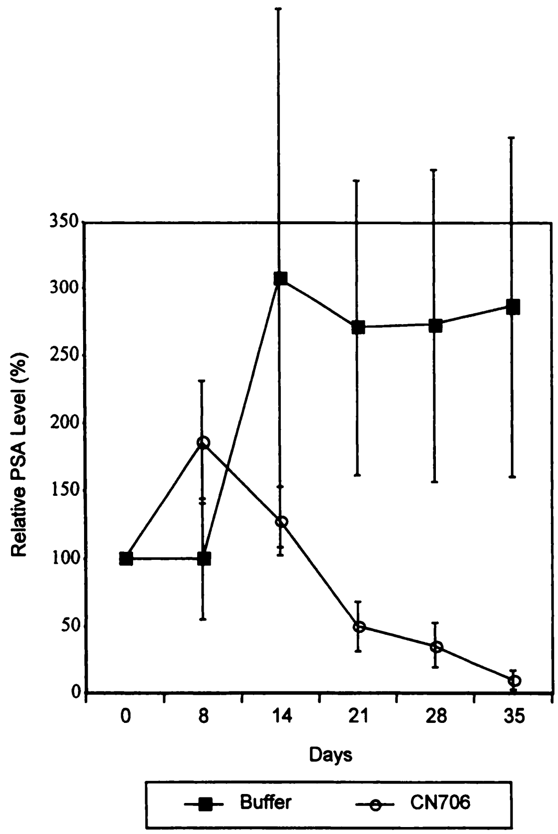

Fig. 4. Serum PSA Levels of mouse LNCaP xenografts treated with CN706. Weekly blood samples were taken from the mice in the experiment shown in Fig. 3*A* at the time of the tumor measurements. Serum from the samples was used to measure PSA levels using a PSA ELISA as described in "Materials and Methods."

reduction in tumor volume by 1 week. Although these simultaneous small increases in tumor volume and PSA levels were within experimental error, the delay in the decrease in tumor size compared to the decrease in PSA levels may reflect the time required for readsorption and clearance of dead cells. Late recurrence of tumors has not been detected though 70 days. All CN706-treated animals were free of serum PSA by 6 weeks after infection. Control tumors injected with buffer, Ad5LacZ, or UV-irradiated CN706 continued to grow.

Discussion

In vivo therapy with replication-deficient adenoviruses involves a balance between achieving a useful therapeutic end point before the patient clears the virus (35) and the immune system prohibits further virus use (36). Even changing adenovirus serotypes can only be expected to achieve 25–30% of the original treatment efficacy (37). Thus, an effective adenoviral therapeutic will need as high a therapeutic potential as possible, from as low an immunogenic dose as possible. Repeat doses are encumbered as the host defense immune system is activated (38–40).

An ARCA designed to attack a specific therapeutic target may address some of these concerns. By replicating preferentially in target cells, the therapeutic will deliver a therapeutic dose at the intended site of action, with a small input immunogenic dose, and require a minimum number of therapeutic doses. By producing viral antigens specifically at the site of the desired therapeutic action, the patient's immune system may be elicited to enhance the target cell killing of the cytolytic replicating adenovirus (36).

Recently, Bischoff *et al.* (12) constructed an adenovirus with a mutated *E1B* gene that replicates in and selectively destroys cancer cells lacking or containing mutated p53. Such a virus could conceivably be a PCA antineoplastic. However, only 8–20% of human PCAs carry mutations in p53 (41, 42), whereas more than 95% of PCAs are PSA-positive (32, 43).

Vectors like CN706 have several advantages for clinical evaluation: they can be engineered for specific cell type targeting; they amplify dose and PCA cell killing by replicating; they express viral antigens that may elicit immune cell killing of desired target cells; and only one dose may be required to eliminate tumors. Human safety has already been established for replication-competent adenoviruses; in 1956, Smith *et al.* demonstrated responses in tumors of 26 of 40 patients with cervical cancer injected with wild-type adenovirus (44). Studies of optimum dose and route of administration of CN706 are ongoing. Human clinical testing of CN706 and vectors like it will permit evaluation of the selective toxicity and therapeutic potential of attenuated adenovirus *in vivo* cytoreductive therapy for PCA.

Acknowledgments

We thank Drs. Don Coffey, William G. Simon, and W. K. Joklik for critically reading this manuscript.

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