Application of a Tumor Suppressor (C-CAM1)-expressing Recombinant Adenovirus in Androgen-independent Human Prostate Cancer Therapy: A Preclinical Study

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ABSTRACT

Recently, we demonstrated that an androgen-regulated cell adhesion molecule, C-CAM, acts as a tumor suppressor in prostate cancer development. In this study, we further explored the possibility of applying C-CAM as a potential agent for developing prostate cancer gene therapy using an adenoviral delivery system. We found that prostate cancer cells, in general, were sensitive to adenoviral infection. In vitro characterization indicated that C-CAM1 protein was detected only in C-CAM1 adenovirus-infected cells but not in antisense control virus-infected cells, and the levels of expression showed dose dependency. Because of the stability of the protein, C-CAM expression in virus-infected cells appeared to be a long-lasting event, indicating that C-CAM may be superior to many other known tumor suppressors that have a short protein half-life. Most importantly, the delivery of a single dose of C-CAM adenovirus was able to repress the growth of PC-3-induced tumors in nude mice for at least 3 weeks. Taken together, these data indicate that C-CAM is a potential candidate for human prostate cancer therapy.

INTRODUCTION

Prostate carcinoma, a disease of epithelial origin, has become the leading cancer among U.S. men. Almost 50% of patients diagnosed with prostate cancer have already developed metastatic lesions at initial clinical presentation, and an additional 20% of patients who initially have local disease will eventually develop metastasis. The standard regimen for metastatic prostate cancer treatment is by androgen ablation. Despite the initial responsiveness to this treatment, tumor cells inevitably relapse to an AI state. The mortality associated with this cancer is mostly due to recurrent AI disease for which effective therapeutic regimens are lacking.

CAMs are known to play important roles in regulating normal cell growth and differentiation, and changes in their expression have been suggested to have effects on tumorigenesis (1, 2). We recently demonstrated that the expression of an epithelium-specific CAM, C-CAM, in prostate could be regulated by androgen (3). The pattern of expression correlated with prostatic epithelial differentiation. From clinical investigation, we also observed that C-CAM levels were decreased in a preneoplastic lesion, i.e., prostatic intraepithelial neoplasia, and were diminished in all the different grades of prostate cancer examined (4). These observations indicate that C-CAM may be involved early in prostate cancer development. To further demonstrate the role of C-CAM in prostate cancer, we transfected a C-CAM1 (a C-CAM isoform) expression vector into a tumorigenic prostate cancer cell line (PC-3). The C-CAM-expressing cells exhibited a reduced growth rate in vitro and a decreased tumor take rate and tumor growth in vivo (5).

MATERIALS AND METHODS

Construction and Large-scale Production of the C-CAM1 Recombinant Adenovirus. To construct a recombinant adenovirus containing the C-CAM1 gene, as shown in Fig. 1, C-CAM1 cDNA was cloned into the EcoRI site of the multiple cloning sites of the pBSK vector (Stratagene, La Jolla, CA). Either a sense or antisense strand of C-CAM1 cDNA was directionally cloned into the HindIII and NotI sites of a shuttle vector (pAdE1CMV/pA) so that its expression was under the control of a CMV promoter, one of the very strong promoters (7). The recombinant adenovirus was generated from cotransfection of the shuttle vector (30 μg) with the C-CAM1 cDNA insert and a pM17 vector (20 μg) carrying both adenoviral genome (Ad5), and pBR322 sequences were cotransfected into 293 cells using DOTAP reagent (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer’s protocol. The presence of the pBR322 insert in pM17 renders the entire adenoviral genome larger than its packing capacity, which can prevent the appearance of wild-type virus from pM17. In this study, two replication-deficient recombinant viruses, one containing the sense orientation of C-CAM1 cDNA (i.e., AdCAM902) and the other containing the antisense orientation of C-CAM1 cDNA (i.e., AdCAM101), were generated from homologous recombination in a packaging cell line (293 cells). Each clone of viruses was isolated from a single plaque after reinfecting 293 cells (6). Subsequently, a large amount of viral stocks was produced from 293 cells and purified by Inland Laboratories, Inc. (Austin, TX). The average titers of viral stocks were about 1 × 1011 virions/ml as determined by A260 assay. However, using a plaque assay, we noticed that the titer of AdCAM101 was 1 × 1011 plaque-forming units/ml, whereas the titer of AdCAM902 was 1 × 1010 plaque-forming units/ml.
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Determination of Viral Infectivity of PC-3 Cells by Fluorescent-activated Cell Scanning Analysis. PC-3 cells (5 × 10^3 cells) were plated on a 65-mm plate for 24 h, then different concentrations of viruses were added and incubated at 37°C for 24 h. Cells were trypsinized into a single-cell suspension and incubated with either preimmune serum or anti-C-CAM polyclonal antibody (Ab669) and FITC-conjugated secondary antibodies as described by Cheung et al. (9). The percentage of positive cells was determined by a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA) as described previously (5).

Determination of C-CAM1 Expression by C-CAM1 Recombinant Adenovirus using Northern and Western Analyses. To determine the levels of C-CAM1 expression in viral-infected cells, we performed both Northern and Western assays as described previously (5). For the dose-dependent experiments, PC-3 cells (5 × 10^4 cells) were infected with either AdCAM101 or AdCAM902 at various ratios of virus:cells (0:1, 1:1, 5:1, and 20:1) for 24 h. Cells were harvested for the determination of C-CAM1 mRNA and protein levels. For the time course experiments, the same amount of PC-3 cells was infected with either AdCAM101 or AdCAM902 at a 5:1 virus:cell ratio, and medium was changed every 3 days. At the indicated time, cells were harvested for the determination of C-CAM1 mRNA and protein levels.

Measurement of PC-3 Tumor Growth by in Vivo Administration of Recombinant Adenovirus. To determine the efficacy of C-CAM1 adenovirus on the growth of PC-3 tumors, we injected 1 × 10^6 cells/site at four sites in the flanks of 8- to 10-week-old male nude mice s.c. Once the tumor became palpable, 50 μl of either recombinant adenovirus or buffer control (PBS+10% glycerol) were injected near the tumor area (i.e., s.c. above the tumors). The change in tumor volume was measured weekly and calculated using a formula described previously (5).

RESULTS

Structural Analysis of C-CAM1 Adenovirus. To ensure the completion of homologous recombination and the presence of C-CAM in these recombinant adenoviruses, we performed PCR using different sets of primers as described in “Materials and Methods.” As shown in Fig. 2, using a C-CAM-specific primer set (i.e., primer set A) from the COOH-terminal portion (from amino acid 413 to amino acid 519) of C-CAM (10), we were able to amplify a 0.3-kb transcript from both AdCAM101 and AdCAM902 DNA, indicating that both adenoviruses contained the C-CAM sequences. Also, using primer set B for the flanking sequences from the human CMV promoter and SV40 polyadenylation site, a 2.1-kb full-length C-CAM cDNA transcript was detected in both viruses. These data indicated that the C-CAM insert was located between CMV promoter and SV40 polyadenylation site. Moreover, using an adenovirus-specific primer (i.e., primer set C), an expected 0.86-kb PCR transcript appeared from both viruses, indicating that both viruses were generated from homologous recombination. Taken together, these data indicate that these two viruses contain the expected gene structure.

Fig. 1. C-CAM1 recombinant adenovirus construction. A full-length C-CAM1 cDNA (2.1 kb) was first cloned into the EcoRI site of the pBSK vector with two possible orientations (sense and antisense). Subsequently, either strand of C-CAM1 insert was directionally cloned into the HindIII (5') and NotI (3') sites of pAdE1CMV/pA under the control of a CMV promoter. The pJM17 vector contains both viral genome and pBR322 orientations (sense and antisense). Subsequently, either strand of C-CAM1 insert was (2.1 kb) was first cloned into the EcoRl site of the pBSK vector with two possible

primer set B (Ref. 8); or primer set C (Ref. 8), and 1.0 unit of Taq DNA polymerase (Promega, Madison, WI). The reactions were carried out for 30 cycles, and each cycle was performed at 94°C for 0.5 min, at 56°C for 0.5 min, and 72°C for 1 min.

Fig. 2. Determination of the genome structure of C-CAM1 adenovirus by PCR. A, DNAs prepared from either AdCAM101 (Lanes 1, 2, and 5) or AdCAM902 (Lanes 3, 4, and 6) were subjected to PCR with primer set A (Lanes 1 and 3), primer set B (Lanes 2 and 4), and primer set C (Lanes 5 and 6). B, The AdCAM genome of 36 kb is divided into 100 map units (1 μm = 0.36 kb). The C-CAM expression cassette replaced the E1 region (1.3-9.2 μm) of the Ad5 genome. To confirm the structure of AdCAM, three different sets of primers were used. Primer set A is designed to amplify a 300-bp transcript from the COOH terminus of C-CAM1 cDNA; primer set B, including the forward primer from the first intron downstream of human CMV major IE gene promoter and reverse primer from SV40 early polyadenylation signal, is designed to amplify 2.1-kb of the cDNA insert. Primer set C, with two primers localized at 13 and 13.4 μm, is designed to amplify the Ad5 genome-specific sequences (~0.86-kb PCR product). μm, map unit.

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In Vitro Characterization of C-CAM1 Adenovirus. To characterize the infectivity of adenovirus to prostatic cancer cells, PC-3 cells (5 × 10^5 cells) were plated in a 65-mm dish overnight prior to infection; then various ratios of viral particles/cell were added to cells and incubated at 37°C for 1, 2, and 4 days. The efficiency of viral infection was determined by fluorescent-activated cell scanning based on positive membrane staining of C-CAM1. As shown in Fig. 3, the background fluorescence was set at 20 FITC units based on the scanning of PC-3 cells infected with buffer control (Fig. 3, A, E, and I). PC-3 cells infected with AdCAM101 (1000:1 virions:cell; B, F, and J), AdCAM902 (100:1 virions:cell; C, G, and K; 1000:1 virions:cell; D, H, and L), or PBS as a control (A, E, and I) for 1 day (A, B, C, and D), 2 days (E, F, G, and H), or 4 days (I, J, K, and L). At the indicated times, the percentage of infected cells was determined using immunofluorescence staining by fluorescent-activated cell scanning analysis. The dual-parameter histogram represents relative cell size (abscissa) measured from forward light scatters (FSC) and log of C-CAM1 expression levels (ordinate) measured from the green fluorescence intensity emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 20 FITC units, which was the background level from control cells as well as the levels from AdCAM101-infected cells stained with antibodies. Fractions of C-CAM-positive cells: 2% (A); 1% (B); 48% (C); 93% (D); 4% (E); 62% (G); 93% (H); 2% (I); 77% (K); 95% (L).

![Fig. 3. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM101 (1000:1 virions:cell; B, F, and J), AdCAM902 (100:1 virions:cell; C, G, and K; 1000:1 virions:cell; D, H, and L), or PBS as a control (A, E, and I) for 1 day (A, B, C, and D), 2 days (E, F, G, and H), or 4 days (I, J, K, and L). At the indicated times, the percentage of infected cells was determined using immunofluorescence staining by fluorescent-activated cell scanning analysis. The dual-parameter histogram represents relative cell size (abscissa) measured from forward light scatters (FSC) and log of C-CAM1 expression levels (ordinate) measured from the green fluorescence intensity emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 20 FITC units, which was the background level from control cells as well as the levels from AdCAM101-infected cells stained with antibodies. Fractions of C-CAM-positive cells: 2% (A); 1% (B); 48% (C); 93% (D); 4% (E); 62% (G); 93% (H); 2% (I); 77% (K); 95% (L).](image-url)
study, during the first few weeks of viral injection, we observed a
small degree of tumor inhibition by antisense (AdCAM101) adeno-

virus treatment, suggesting that the virus itself may cause some toxic
effect on cells since the titer of AdCAM101 was 10-fold higher than

that of AdCAM902. However, from the AdCAM101-treated group, the growth of PC-3-induced tumors eventually resumed, and the size of
tumors are similar with that of control (Fig. 6).

Since C-CAM1 adenovirus can suppress the growth of small tu-
mors, we further examined the efficacy of AdCAM902 treatment on
larger tumors, ranging from 40 to 75 mm3, which represent more
heterogenous tumors. Based on previous results, we estimated that the
effective viral dosage needed to increase at least 3-fold to inhibit the
growth of larger tumors. However, it is difficult to deliver such a large
volume (i.e., 150 μl) to tumors at once; therefore, the viruses were
equally divided into three dosages and delivered every week for 3
consecutive weeks. In this study, we observed that AdCAM902 (Fig.
7C), but not control (Fig. 7A) and AdCAM101 (Fig. 7B), was still
able to suppress the growth of PC-3 tumors for at least 5 weeks,
starting from the first viral injection. However, in some cases, Ad-
CAM902 failed to suppress the tumor growth if the tumor size
reached more than 100 mm3 (data not shown); this may be due to the
poor penetration of the viruses. These data suggest that a more
frequent treatment schedule may be needed.

**DISCUSSION**

With advances in molecular genetics and biology, it has become
obvious that altered expression of normal genes leads to initiation of
cancer cells. The conventional therapy for malignancy, such as chem-
otherapy and radiation, has focused on mass cell killing without
specific targeting and often causes damaging side effects. The new
direction in cancer therapy is to deliver a normal gene to replace or
correct the mutated gene and thereby alter the malignant phenotype of
transformed cells. In order to deliver a gene into somatic cells with
high efficiency, two major genetically engineered vector systems,
retrovirus and adenovirus, have been well developed. Several prop-
erties of the retrovirus have limited its use in prostate cancer treatment
(12): (a) infection by retrovirus depends on host cell division. In
human prostate cancer, very few mitotic cells can be found in tumor
lesions (13); (b) the integration of retrovirus into the host genome may
cause adverse effects on target cells because malignant cells are high
in genetic instability; (c) retrovirus infection is often limited by a certain
host range; (d) retrovirus has been associated with many malignancies in

Fig. 6. The tumor-suppressing effect of C-CAM1 adenovirus on PC-3 tumors. Fourteen

tumors were treated in each group. A single dose of 50 μl of either control (PBS+10%
glycerol) or viral solution (5 × 10^{11} AdCAM101 or AdCAM902 virions/ml in PBS+10%
glycerol) was injected s.c. above the tumor mass 20 days after inoculation with PC-3 cells.

Tumor volume was recorded every week and tabulated as the mean; bars, SD. Arrow, viral
injection. ▲, control; ○, AdCAM101; •, AdCAM902.

Fig. 4. C-CAM1 mRNA expression in PC-3 cells infected with C-CAM adenovirus.

Total RNAs were prepared from PC-3 cells infected with AdCAM101 or AdCAM902
adenovirus and analyzed by Northern blot using a random-primer-labeled C-CAM cDNA
probe. A, dose-dependence of C-CAM1 expression. PC-3 cells were infected with either
AdCAM101 or AdCAM902 at various ratios of virion:cells (0:1, 1:1, 5:1, and 20:1) for
24 h. B, time course of C-CAM1 expression. PC-3 cells were infected with either
AdCAM101 or AdCAM902 at a 5:1 virion:cell ratio for various lengths of time as
indicated. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal
control. C, mock infection control; A, AdCAM101 (antisense) infection; S, AdCAM902
(sense) infection.

Fig. 5. C-CAM1 protein expression in PC-3 cells infected with C-CAM1 adenovirus.

Cell lysates were prepared from PC-3 cells infected with C-CAM1 adenovirus and
analyzed by Western immunoblot using polyclonal antibodies against C-CAM (Ab669).

A, dose-dependence of C-CAM1 expression. PC-3 cells were infected with either
AdCAM101 or AdCAM902 at various ratios of virion:cells (0:1, 1:1, 5:1, and 20:1) for
24 h. B, time course of C-CAM1 expression. PC-3 cells were infected with either
AdCAM101 or AdCAM902 at a 5:1 virion:cell ratio. The medium was changed every 3 days. At the
indicated times, cells were collected for Western blot analysis. C, mock infection control;
S, AdCAM902 (sense) infection.
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both mammals and vertebrates; and (e) the titer of retrovirus, in general, is 100- to 1000-fold lower than that of adenovirus.

Knowledge of the genetic organization of adenovirus (~36 kb), a linear and double-stranded DNA virus, allows substitution of a large piece of adenoviral DNA with foreign sequences up to 7 kb (14). In contrast to retrovirus, the infection of adenoviral DNA into host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells, regardless of their cell cycle stage. Thus far, adenoviral infection appears to be linked only to mild disease, such as acute respiratory disease in humans. Based on these unique features of the adenovirus system, it is worthwhile to explore adenovirus-based gene therapy in prostate cancer treatment. In the current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector (Fig. 1). Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure (Fig. 2).

The natural history of prostate cancer is intrigued by the unique metastatic pattern (15). Prostate cancer appears to spread selectively to the bone where AI prostate cancer cells often relapse (16, 17). Therefore, the bone appears to be a critical target for developing effective therapeutic strategies for recurrent AI prostate cancer. PC-3 cells, originally developed from patients with osseous metastasis, have been characterized as an AI and highly tumorigenic prostate cancer cell line when inoculated in athymic animals. And, the tumor incidence by inoculating PC-3 in vivo ranges from 67 to 94% (5, 18). These tumors became palpable within 3 weeks, indicating that PC-3 line is a fast-growing tumor and a suitable tumor model for screening potential therapeutic agents.

Data from this study (Figs. 4A and 5A) and others (19) show that prostate cancer cells, in general, are sensitive to adenoviral infection. Because adenovirus can maintain its infectivity for a long period of time extracellularly (Fig. 3), we suggest that the optimal dosage of virus should be determined from in vitro infectivity experiments, which may avoid the excessive in vivo administration of adenovirus. On the other hand, we observed a prolonged expression pattern of C-CAM mRNA as well as protein in viral-infected cells (Figs. 4B and 5B), suggesting that C-CAM may be a stable molecule with a long half-life as compared to other tumor suppressors, e.g., p53, which have a short half-life (20). Recent studies (21, 22) also indicated that the frequent administration of p53 adenovirus is required for achieving the therapeutic efficacy. Therefore, this long-lasting expression of C-CAM may help to reduce the frequency of in vivo viral administration, which may elicit an adverse immunological response. In addition, it is known that C-CAM is present in many different cell types under normal physiological conditions (23); it is likely that overexpression of C-CAM protein in normal cells would not impose any toxicity. Therefore, C-CAM may be a potential agent for prostate cancer therapy.

In vivo gene therapy results (Figs. 6 and 7) confirmed our previous data (5) that C-CAM1 can specifically suppress the proliferation of AI prostate cancer cells. In the first experiment, knowing that 50 μl of either control (PBS+10% glycerol; A) or viral solution [5 × 10^11 AdCAM101 (B) or AdCAM902 (C) virions/ml in PBS+10% glycerol] was injected once a week for 3 consecutive weeks when tumor volume was larger than 40 mm^3. Tumor volume was recorded every week. Arrow, viral injection.
mechanisms such as a bystander effect may contribute to this dramatic tumor suppression. Bystander effect, often seen in gene therapy using suicide gene (24–27), may be caused by unknown paracrine factor(s) produced by infected cells that elicit a toxic effect on surrounding uninfected cells. This possibility needs to be further investigated. On the other hand, we noticed that adenovirus may not inhibit the larger tumors efficiently, which may be a result of limited penetration of virus. Therefore, a more frequent treatment schedule may be required in order to inhibit the growth of tumors effectively. To achieve better therapeutic efficacy, it is conceivable that C-CAM1 gene therapy needs to be combined with other regimens.

As gene therapy is a new approach for cancer therapy, many steps are in experimental stages (21, 22). Based on these compelling results, we conclude that the C-CAM1 adenovirus is able to inhibit prostate cancer growth and achieve an effect similar to our previous results (5). In addition, no apparent pathological symptoms have been observed in these animals (data not shown), suggesting that C-CAM1 recombinant adenovirus did not cause significant toxicity in the hosts. Moreover, use of adenovirus has an advantage in that it has high infectivity, and adenovirus did not cause significant toxicity in the hosts. Moreover, these animals (data not shown), suggesting that C-CAM1 recombinant adenovirus is able to inhibit prostate cancer growth and achieve an effect similar to our previous results (5). In addition, no apparent pathological symptoms have been observed in these animals (data not shown), suggesting that C-CAM1 recombinant adenovirus did not cause significant toxicity in the hosts. Moreover, use of adenovirus has an advantage in that it has high infectivity, and adenovirus did not cause significant toxicity in the hosts. Moreover, these animals (data not shown), suggesting that C-CAM1 recombinant adenovirus is able to inhibit prostate cancer growth and achieve an effect similar to our previous results (5).

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