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 viral-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-CAM (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).

Consistently, transfection of an antisense C-CAM1 vector into a nontumorigenic prostatic epithelial cell line, NbE, resulted in tumor formation in nude mice. The sublines derived from the NbE-induced tumors showed reduced levels of C-CAM compared to control cells (5). These observations suggest that C-CAM1 acts as a tumor suppressor in prostate cancer.

In this study, we further tested whether C-CAM1 could be a potential agent for prostate cancer therapy. Since adenovirus appears to be an efficient delivery system (6), we constructed a recombinant adenovirus carrying the C-CAM1 gene. The recombinant adenovirus AdCAM902 and its antisense construct, AdCAM101, which served as a control virus, were evaluated for their tumor-suppressing efficacy in vivo. In vitro characterization indicated that AdCAM902 mediated high-level expression of C-CAM1 protein in PC-3 cells. Furthermore, in vivo results showed that a single dose of C-CAM1 adenovirus administered into PC-3-induced tumors was able to suppress the tumor growth for at least 3 weeks. From an in vitro time course study, we observed that C-CAM1 expression in viral-infected PC-3 cells is a long-lasting event. Because of the high stability of C-CAM1 protein, the C-CAM1 adenovirus may be superior to other tumor suppressor adenoviral vectors. Therefore, we believe that C-CAM1 adenovirus should be further explored for developing new prostate cancer therapy.

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 eDNA 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 pBRS322 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 reinfesting 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 × 10^11 virions/ml as determined by A260 assay. However, using a plaque assay, we noticed that the titer of AdCAM101 was 1 × 10^11 plaque-forming units/ml, whereas the titer of AdCAM902 was 1 × 10^10 plaque-forming units/ml.

Analysis of the Structure of the Recombinant Adenovirus by PCR. Adenoviral DNA was prepared as described previously (8). The PCR was performed in a 50-μl reaction mixture containing 4 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 200 μM of each deoxyribonucleotide triphosphate, 10 mM Tris-HCl (pH 9.0), 2 μM of the different primer sets [primer set A, (5'-GA-TCCAACACAAGGAAATTCC-3' and 5'-TCATCTTTTTTGTACTAC-3')]
Determination of Viral Infectivity of PC-3 Cells by Fluorescent-activated Cell Scanning Analysis. PC-3 cells (5 × 10⁵ 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 confirm the integrity 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 × 10⁵ cells) were infected with either AdCAM101 or AdCAM902 at various ratios of virus:cell (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 × 10⁶ 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 flanking sequences from the human CMV promoter and reverse primer from SV40 early polyadenylation signal, is designed to amplify 2.1-kb of the cDNA insert. 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 11 and 13.4 µm, is designed to amplify the AdS genome-specific sequences (~0.86-kb PCR product). μm, map unit.

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 11 and 13.4 μm, is designed to amplify the AdS genome-specific sequences (~0.86-kb PCR product). μm, map unit.
In Vitro Characterization of C-CAM1 Adenovirus. To characterize the infectivity of adenovirus to prostatic cancer cells, PC-3 cells (5 x 10⁵ 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 (Fig. 3, B, F, and J) gave the same fluorescence profile as for the buffer control, indicating that cells infected with AdCAM101 did not express C-CAM protein. On the other hand, C-CAM was expressed by PC-3 cells infected with AdCAM902 (Fig. 3, C, D, G, H, K, and L). When we determined the percentage of positive cells after AdCAM902 infection for 1 day, we found that 48% of cells expressed C-CAM1 when the cells were infected at a 100:1 virion:cell ratio (Fig. 3C), and 93% of cells expressed C-CAM when infected at a 1000:1 ratio (Fig. 3D), which is comparable to another report using β-galactosidase adenovirus (11). These data indicate that C-CAM1 expression in AdCAM902-infected PC-3 cells is dose dependent. On the other hand, we observed that maximal infection (~93%) plateaued within 24 h at the saturated viral concentration (Fig. 3, D, H, and L). However, under the subsaturated viral concentration, the infectivity rate increased from 48% at day 1 to 77% at day 4 in a time-dependent manner (Fig. 3, C, G, and K), suggesting that this recombinant adenovirus can maintain its infectivity at least 4 days.

When PC-3 cells were infected with sense recombinant adenovirus (i.e., AdCAM902), both Northern and Western blot analyses showed that C-CAM1 expression could be detected 24 h after infection and that the level of C-CAM1 expression depended on the number of viruses used for infection (Figs. 4A and 5A). A much weaker signal was detected from PC-3 cells infected with AdCAM101, using a C-CAM1 cDNA probe (Fig. 4), suggesting the presence of the antisense C-CAM1 message. Since antisense mRNA, in general, has a short half-life, this weak signal is expected. However, AdCAM101 showed no C-CAM1 protein expression (Fig. 5A). These results indicate that the sense recombinant adenovirus (AdCAM902) is capable of expressing C-CAM1. From a time course study, a continuous elevation of C-CAM1 mRNA was detected in viral-infected PC-3 cells 14 days after infection (Fig. 4B). Similarly, the C-CAM1 proteins were still detectable 20 days after infection (Fig. 5B). Since the viral genome was not detected 10 days after infection (data not shown), these data suggest that the prolonged C-CAM1 expression in sense recombinant virus-infected cells may be due to the high stability of C-CAM1 protein. This long-lasting expression of C-CAM1 may also provide an advantage for C-CAM1 as a potential agent for prostate cancer therapy.

Tumor-suppressing Effect of C-CAM1 Recombinant Adenovirus. Our previous results (5) indicated that increased C-CAM1 expression in PC-3 cells can reduce the in vitro growth of this cell line. Based on the in vitro time course of C-CAM expression in virus-infected cells (Fig. 5B), we tested the therapeutic efficacy of C-CAM1 on preexisting PC-3 tumors by injecting (50 μl) either sense or antisense C-CAM1 adenovirus or buffer control into the area near the tumor mass. The growth of tumors was then monitored weekly. As shown in Fig. 6, the growth of PC-3-induced tumors was suppressed by C-CAM1 adenovirus treatment for at least a 3-week period. At week 6, we observed that tumors regained the growth activity. The second dose (50 μl) of virus delivered at week 7 was able to continue the tumor suppressing for at least a 2-week period (Fig. 6). In the same
study, during the first few weeks of viral injection, we observed a small degree of tumor inhibition by antisense (AdCAM101) adenovirus 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 tumors, 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, AdCAM902 failed to suppress the growth of tumors eventually. The 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 chemotherapy 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 properties 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
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 (D) 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 mm³. Tumor volume was recorded every week. Arrow, viral injection.

Fig. 7. Determination of the treatment schedule of C-CAM1 adenovirus for PC-3 tumors. Five tumors (each indicated by a different symbol) were treated in each group. A dose of 50 μl of either control (PBS+10% glycerol; A) or viral solution [5 × 10^11 AdCAM101 (D) 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 mm³. 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
it has less potential risk compared to retrovirus because it does not
integrate into the host chromosome. Therefore, we believe C-CAM1
adenovirus should be further explored for its clinical applications.

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