Suppression of Human Bladder Cancer Growth by Increased Expression of C-CAM1 Gene in an Orthotopic Model

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Abstract

Recently, we demonstrated that an immunoglobulin-like cell adhesion molecule, C-CAM, acts as a tumor suppressor in prostate cancer. It is known that C-CAM is expressed in many epithelial cell types. In this study, we tested the possibility that C-CAM may also suppress bladder cancer progression. We used an orthotopic tumor model, which provides a relevant organ condition for examining the interaction between primary tumor cells and their microenvironment; this interaction has a critical impact on the behavior of carcinoma. We constructed a recombinant adenovirus expressing C-CAM1 (an isoform of C-CAM) and infected the 253J B-V cell line, a tumorigenic human bladder carcinoma subline. In vitro, C-CAM1 protein was detected in C-CAM1 adenovirus-infected cells but not in antisense control virus-infected cells, and the levels of expression showed dose dependency. When these cells were injected orthotopically in nude mice, we found that the increased expression of C-CAM1 in the 253J B-V cells repressed the growth of 253J B-V-induced tumors. Taken together, these data indicate that C-CAM1 is a potent tumor suppressor in human bladder cancer.

Introduction

Cancer of the urinary bladder is increasing in incidence; it has become the fifth most common cancer among U.S. men and the eighth most common cancer among U.S. women (1). Although in most patients, disease originates from the transitional epithelia, cancer cells exhibit diverse biological potential. It is believed that such heterogeneity is controlled by a cascade of molecular changes. Accumulating molecular genetic evidence has also indicated that losses of negative regulators (i.e., tumor suppressors) and/or increases in positive regulators (i.e., oncogenes) in normal cells result in cellular cancers. Recently, we demonstrated that an epithelial-specific CAM, C-CAM, may be associated with an early event in prostate carcinogenesis (2) and that increased expression of C-CAM1 (an isoform of C-CAM) in a tumorigenic prostate cancer cell line (PC-3) results in a reduced growth rate in vitro and decreased tumor incidence and tumor growth in vivo (3). Furthermore, we showed that C-CAM1 could be a potent candidate for prostate cancer gene therapy (4). C-CAM is also known to be expressed in a variety of epithelia, including transitional epithelium (5). Therefore, in this study, we tested whether C-CAM1 could also suppress human bladder cancer progression.

Interaction between stromal and epithelial components in a para-

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4 The abbreviations used are CAM, cell adhesion molecule; m.o.i., multiplicity of infection.

The normal physiological function of an organ (6, 7). In addition, recent data show that the stromal component from tumor-surrounding microenvironments may also play a crucial role not only in changing the biological behavior of tumor cells but also in determining the metastatic pattern of tumor cells (8–10). Very often, this interaction appears to be organ specific (11). Therefore, to examine the effect of any agents on tumor behavior, it is crucial to consider the potential influence from the surrounding microenvironment. In this study, we decided to use an orthotopic bladder model (12) using hosts of both genders to assess the tumor suppressive effect of C-CAM1 on human bladder cancer progression.

Materials and Methods

Determination of Sensitivity of 253J B-V Cells by C-CAM1 Adenovirus Using Fluorescent-activated Cell-scanning Analysis. In this study, two replication-deficient recombinant viruses, one containing the sense orientation of C-CAM1 cDNA (AdCAM902) and the other containing the antisense orientation of C-CAM1 cDNA (AdCAM101), were generated as described previously (4). To produce a large amount of viral stocks, the recombinant viruses were harvested from the 293 cells after 36 h of infection and subjected to two cycles of CsCl gradient ultracentrifugation (13). After dialysis overnight, the stock of viruses was aliquoted and stored at −80°C until use. The average titers of viral stocks were determined using the plaque assay in triplicate, and viral concentrations were measured by absorbance at 260 nm. The titer of the virus used in this study is equivalent to 1 log of the viral concentration, that is, 1 m.o.i. = 10:1 (virus:cells).

To determine the sensitivity of 253J B-V cells, 5 × 10⁵ cells were plated on a 65-mm plate for 24 h, and then cells were infected with different concentrations of viruses (m.o.i., ranging from 1 to 100) at 37°C for another 24 h. The infected cells were trypsinized into a single-cell suspension and incubated with anti-C-CAM polyclonal antibody (Ab669) and a secondary antibody labeled with FITC conjugate, as described previously (3). The percentage of positive cells was defined as the fluorescence intensity higher than 100 FITC units, obtained from the staining of uninfected cells using a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA).

Measurement of in Vitro Tumorigenicity of 253J B-V Cells by C-CAM1 Adenovirus. To obtain the optimal dose of adenoviral infection, the levels of C-CAM1 expression in virus-infected cells were determined using both Northern and Western assays (3). 253J B-V cells (5 × 10⁵ 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, and then infected cells were equally divided: one part for the isolation of total cellular RNA and the other for the extraction of membrane protein. In time course experiments, the same amount of 253J B-V cells was infected with either AdCAM101 or AdCAM902 at a 5:1 virus:cell ratio, and the medium was changed every 3 days; at 24 h after infection, cells were harvested and equally divided for total cellular RNA purification and membrane protein extraction.

Assessment of the in Vivo Tumorigenicity of 253J B-V Cells. The orthotopic bladder model was established by intrabladder inoculation of a low-tumorigenic human bladder carcinoma cell line, 253J; subsequently, a tumorigenic subline, 253J B-V, was derived from the primary tumor as described previously (11). In this study, 253J B-V cells were infected with either AdCAM902 or AdCAM101 18 h before injection into mice; then cells...
Fig. 1. Efficiency of infection by adenovirus in human bladder cancer cells as determined by flow cytometry. 253J B-V cells were infected with either the control buffer (PBS and 10% glycerol; A), AdCAM101 (B–D), or AdCAM902 (E–H) with different ratios of viruses. After 24 h, the percentage of infected cells was determined using immunofluorescence staining by fluorescent-activated cell scanning analysis, as described previously (4). Positive staining was defined as staining intensity greater than 100 FITC units, which was the background level for control cells as well as the level for AdCAM101-infected cells stained with antibodies. A, m.o.i. = 0; B and F, m.o.i. = 0.5; C and G, m.o.i. = 5; D and H, m.o.i. = 10; E, m.o.i. = 0.1. Fractions of C-CAM-positive cells: A, 1%; B, 1%; C, 1%; D, 1%; E, 1%; F, 5%; G, 42%; H, 63%. FL1, FITC unit; FSC, forward light scatters unit for relative cell size.

Results and Discussion

Sensitivity of 253J B-V Cells to C-CAM1 Adenoviral Infection. For delivering recombinant DNA into somatic cells with high efficiency, the adenoviral vector is a suitable system for epithelial cells because of its high titer and superb infectivity with a wide spectrum of target cells. In this study, we first characterized the recombinant adenoviral infectivity in a human bladder cancer cell line (253J B-V). As shown in Fig. 1, the sensitivity of 253J B-V cells to viral infection was determined by the numbers of C-CAM1-positive cells detected with fluorescent-activated cell scanning. The background fluorescence was set at 100 FITC units based on the scanning of 253J B-V cells incubated with control buffer (PBS and 10% glycerol; Fig. 1A). The fluorescence profiles of 253J B-V cells infected with AdCAM101 with various increments of m.o.i. (Fig. 1, B–D) were identical to that of the buffer control, indicating that cells infected with AdCAM101 cannot express any C-CAM protein. In contrast, the infectivity of AdCAM902 on 253J B-V cells (Fig. 1, E–H) exhibited dose dependence. At a low m.o.i., such as 10, 63% of cells were C-CAM1 positive, indicating that 253J B-V cells are very sensitive to adenoviral infection.

In Vitro C-CAM1 Expression in Adenovirus-infected Cells. When 253J B-V cells were infected with either antisense (AdCAM101) or sense (AdCAM902) recombinant adenovirus, C-CAM1 expression could be detected 24 h after infection, and the elevated levels of C-CAM1 mRNA expression correlated with viral concentration (Fig. 2A). In the Northern analysis, we also noticed that a much weaker signal was detected in 253J B-V cells infected with AdCAM101 (Fig. 2, A and B), suggesting that the antisense C-CAM1 message may have a short half-life. These results were consistent with those of our recent study using human prostate cancer cells (4). However, Western blot analysis revealed that only the sense adenovirus (AdCAM902) was able to translate an authentic C-CAM1 protein (i.e., 105 kilodaltons) in infected cells (Fig. 2, C and D).

In the time course experiment (Fig. 2, B and D), we found that significantly elevated levels of C-CAM1, dictated by a strong cyto...
Table 1  
Suppressive effect of C-CAM expression on the establishment of human bladder cancer in different gender hosts

<table>
<thead>
<tr>
<th>Host</th>
<th>Treatment</th>
<th>m.o.i.</th>
<th>n/a</th>
<th>Bladder weight</th>
<th>Bladder size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Mock infection</td>
<td>0</td>
<td>12</td>
<td>260 ± 40</td>
<td>266 ± 39</td>
</tr>
<tr>
<td>Male</td>
<td>AdCAM101</td>
<td>20</td>
<td>5</td>
<td>260 ± 30</td>
<td>298 ± 64</td>
</tr>
<tr>
<td>Male</td>
<td>AdCAM101</td>
<td>50</td>
<td>8</td>
<td>100 ± 20</td>
<td>87 ± 19</td>
</tr>
<tr>
<td>Male</td>
<td>AdCAM902</td>
<td>5</td>
<td>10</td>
<td>40 ± 10</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>Male</td>
<td>AdCAM902</td>
<td>20</td>
<td>5</td>
<td>32 ± 2</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Female</td>
<td>Mock infection</td>
<td>0</td>
<td>9</td>
<td>270 ± 50</td>
<td>206 ± 44</td>
</tr>
<tr>
<td>Female</td>
<td>AdCAM101</td>
<td>20</td>
<td>10</td>
<td>270 ± 60</td>
<td>229 ± 59</td>
</tr>
<tr>
<td>Female</td>
<td>AdCAM902</td>
<td>20</td>
<td>11</td>
<td>21 ± 1</td>
<td>14 ± 1</td>
</tr>
</tbody>
</table>

a Total numbers of animals used in three different experiments.
b Bladder weight was measured on an analytical balance, and bladder size was determined using the formula length × width × height × 0.5326 (11); length, weight, and height were measured with a caliper after dissecting the bladders from animals.

Mean ± SE.

megalovirus promoter, peaked 7 days after infection; then its levels gradually diminished. Nevertheless, C-CAM1 was consistently expressed in viral-infected 253J B-V cells through 21 days after infection, which may be due to the high stability of the C-CAM1 molecule. Similar results were also observed in human prostate cells infected with C-CAM1 adenoviruses (4). This prolonged expression of C-CAM1 may provide an advantage for C-CAM1 as a potential agent for bladder cancer therapy.

Tumor-suppressing Effect of C-CAM1 in an Orthotopic Bladder Model. It is known that the progression of any tumor cells is influenced by both the genetic composition of the tumor cells and the surrounding microenvironment. Genetic alterations in tumor cells certainly have a great impact on tumor behavior. However, microenvironment factors such as autocrine, paracrine, and endocrine factors...
contributed by either neighboring normal cells, the stromal component, or the circulation often can modulate phenotypic changes of tumor cells. An in vivo orthotopic tumor model can simulate the progression of human tumor cells. In a recent study (12), we established an orthotopic bladder tumor model by injecting low-tumorigenic human bladder cancer cells (253J) into the bladder walls of athymic nude mice. Subsequently, we were able to obtain a highly tumorigenic subline, 253J B-V, from the primary tumor.

In this study, prior to injection, cells were infected with either the C-CAM1 sense adenovirus (AdCAM902) or the control virus (AdCAM101); then the growth of tumors was determined 4 weeks after injection. As shown in Table 1 and Fig. 3G, the sizes of 253J B-V-induced tumors in male and female hosts without any viral insulin were about the same. However, in the presence of AdCAM101 adenovirus at the low m.o.i. of 20, both the weight and size of 253J B-V-induced tumors are about the same as those of tumors treated with control buffer, suggesting that the control virus failed to inhibit the growth of 253J B-V cells. In addition, we observed a small degree of tumor inhibition by AdCAM101 adenovirus treatment at a high m.o.i. of 50, suggesting that too much virus may cause some toxic effect on cells. To determine whether those enlarged bladders are tumors, H&E staining data indicated that those enlarged bladders indeed were filled with carcinoma cells (Fig. 3, A-D).

In the presence of the C-CAM1 sense adenovirus (AdCAM902), we observed a very significant suppression of the growth of 253J B-V-induced tumors. Basically, the weights and sizes of bladders determined from the AdCAM902-treated group were identical to those of normal mouse bladders, and H&E staining confirmed these results (Fig. 3, E–G). These data (Table 1) also suggest that bladder cells are very sensitive to adenoviral infection, because the tumor-suppressing activity already reached a maximal effect at the low m.o.i. of 5.

Consistently, in both the male and female hosts, we observed similar degrees of tumor inhibition by AdCAM902 adenovirus treatment (Table 1), indicating that the tumor-suppressing effect of C-CAM1 is active in human bladder cancer cells regardless of the different surrounding environments and hormonal conditions contributed by hosts from different genders.

**Physiological Role of C-CAM in Bladder Epithelial Cells.** Cell-cell interaction and communication are required for both morphogenesis and ontogenesis of multicellular organisms. CAMs are known to play an essential role in these processes. Overwhelming data indicate that the spatiotemporal expression of C-CAM, an epithelial-specific CAM, correlates with the differentiation of epithelia in many organs (2, 5, 14, 15). In our recent study (2, 16), the subcellular localization of C-CAM in the epithelium may vary during the different developing stages. For example, C-CAM spreads over the whole cell surface in the poorly differentiated basal epithelium of the prostate gland; in contrast, C-CAM localizes on the apical surface of the well-differentiated luminal epithelium of the prostate. These data suggest that C-CAM may be critical in the cell-cell interaction of basal cells, and it may be important in the organization of polarized luminal cells. Therefore, any alteration in C-CAM function may result in the hyperplastic growth of normal cells that precedes neoplastic transformation.

Positive staining for C-CAM is also detected in the transitional epithelia of the urinary bladder and ureter (14). Furthermore, Hunter et al. (17) showed that C-CAM was localized at sites of cell-cell contact of NBT II cells, a rat bladder carcinoma cell line, and the interaction between C-CAM molecules can direct the reorganization of actin filaments in the adhesion complex. In the presence of a differentiating agent such as Ultroser G, the altered C-CAM expression parallels the morphological conversion in NBT II cells, which resembles the epithelial-to-mesenchymal transition of early embryogenesis (17). Taken together, these data suggest that C-CAM may play a homeostatic role in maintaining the physiological function of the transitional epithelium.

**Potential Application of C-CAM in Bladder Cancer Progression.** Neoplastic transformation is considered a “dedifferentiation” process that may recapitulate the early development of the embryonic stage. Multiple genetic or epigenetic alterations have to take place in a cascade before the malignant phenotype appears. In addition to C-CAM, Fearon et al. (18) showed that DCC, a member of the immunoglobulin gene superfamly with a structure similar to that of C-CAM, is often deleted in colon carcinoma. These data indicate that loss of cell communication signifies an early event leading to neoplastic transformation. It is likely that reestablishment of the cell communication between malignant cells will restore their pathological status to the normal differentiation pathway. Data from this study and previous reports (3, 4) demonstrate that increased expression of C-CAM correlated with enhanced cell adhesion in C-CAM-transformed cells can diminish both tumor growth and tumorigenicity in vitro and in vivo. Based on these results, we believe that C-CAM may act as a key mediator in cell communication and that C-CAM has the potential to develop as an agent for human bladder cancer therapy.

Unlike the case with many other tumor suppressor genes, the down-regulation of C-CAM expression does not result from gene deletion. Data from Rosenberg et al. (19) and Neumaier et al. (20) indicate that hypermethylation of cytidine is associated with the regulatory sequences of the C-CAM gene, which may result in the inactivation of C-CAM gene expression. Interestingly, another potential tumor suppressor, glutathione S-transferase, has been shown to have a similar pattern of hypermethylation occurring in prostatic cancer tissues (21). Taken together, these data suggest that mechanisms other than DNA mutation or deletion leading to altered RNA transcriptional regulation may also contribute to the loss of tumor suppressor gene expression in malignant transformation. Therefore, the exploration of this class of tumor suppressor genes should provide an alternative means of cancer therapy. Further understanding of the regulatory mechanism of the C-CAM gene may offer an additional strategy for altering the malignant phenotype of cancer.

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**References**


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