Loss of Adenoviral Receptor Expression in Human Bladder Cancer Cells: A Potential Impact on the Efficacy of Gene Therapy

Yingming Li, Rey-Chen Pong, Jeffrey M. Bergelson, M. Craig Hall, Arthur I. Sagalowsky, Ching-Ping Tseng, Zhi Wang, and Jer-Tsong Hsieh

Abstract

There is great interest in the development of gene therapeutic strategies for the treatment of benign and malignant diseases. Recombinant adenovirus has a wide spectrum of tissue specificity and is an efficient vector delivery system. Successful gene delivery, however, requires viral entry into the target cells via specific receptor-mediated uptake. Recently, a cDNA clone (the coxsackie and adenovirus receptor [CAR]) encoding a 46-kDa protein was identified as the receptor for group C adenovirus (e.g., adenovirus type 2 and 5). Currently, little is known regarding the expression of adenoviral receptor in normal tissue and cancer. In this paper, we have documented a significant difference in viral receptor levels that may be due to transcriptional regulation of the CAR gene in several human bladder cancer cell lines. The differences in viral receptor levels in these cells correlated with their sensitivity to viral infection. Transfection of receptor-negative cell line with CAR cDNA led to increased virus binding and increased susceptibility to adenovirus-mediated gene delivery. Our results demonstrate that the expression of adenoviral receptor is variable among human bladder cancer cells. This variability may have a significant impact on the outcome of adenovirus-based gene therapy.

Introduction

Genetic alteration is one of the major causes of malignant transformation and cancer progression. It is conceivable that the malignant phenotype can be altered by replacing absent critical functional genes in target cells. Gene therapy is an innovative way to achieve this goal. The replication-deficient adenovirus derived from adenovirus type 5 (1), in contrast to other vectors available for gene therapy, is highly infectious and capable of transferring genes into nondividing cells. This vector system appears to be especially suitable for malignancies characterized by a low mitotic index. Adenoviral entry into target cells is the rate-limiting step of gene delivery. The initial binding of adenovirus to the cell surface has been shown to be a receptor-mediated process (2, 3). The adenoviral fiber protein is responsible for attachment of the virus to the cellular receptor. The fiber protein can be divided into three domains: head, spike, and base. It is known that the head domain of the fiber contains the receptor attachment site. Some evidence (1, 4, 5) suggests that two distinct receptors interact with group C (adenovirus type 2 and 5) and group B (adenovirus type 3). Recently, two different groups reported that CAR (4) is a common receptor for adenovirus type 2 and 5 (6–8). The expression pattern of CAR in either normal tissue or cancer cells has not been defined.

In our laboratory, we are evaluating the efficacy of gene therapy for urogenital cancer using replication-deficient adenovirus. Recently, we observed several human bladder cancer cell lines that appeared to be resistant to viral infection. Therefore, we decided to determine the levels of CAR in those cell lines. We found that the level of CAR correlated with viral infectivity. Increased viral sensitivity could be restored in a resistant line after transfecting a functional CAR cDNA vector. We believe that these findings have significant biological and therapeutic implications.

Materials and Methods

All of the human bladder cancer cell lines except for SWBC1 and WH (9) used in this study were obtained from American Type Culture Collection (Manassas, VA), and all of the cell lines were grown in T medium (10) containing 5% FBS. The oligonucleotides were synthesized by Life Technologies, Inc. (Gaithersburg, MD). A mammalian expression vector, pCDNA3.1/V5/His-TOPO, was purchased from Invitrogen (Carlsbad, CA).

Primary Culture of Human Bladder Cancer Cells. The primary human bladder cancer cell (SWBC1) was derived from a cancer patient diagnosed with invasive transitional carcinoma (T2N0M0) after radical cystoprostatectomy. The tumor specimen was dissected into 3–5-mm³ pieces and planted on a 60-mm dish with T medium containing 5% FBS. After cells grew out from the explants, we carefully removed contaminated fibroblasts by trypsinization and continued to pass the cells. Biochemical analysis using both cytokeratin and vimentin antibodies confirmed that this cell is of epithelial origin. Currently, these cells have been cultivated in vitro for 8 months with more than 40 passages.

Recombinant Virus Construction and Purification. The replication-deficient recombinant virus, AdCMV-β-gal, was generated as described previously (11). Another replication-deficient recombinant virus, dl312, obtained from Dr. Shenk (12), was labeled with [3H]thymidine as described previously (13). To produce a large amount of viral stock, the recombinant viruses were harvested from the cell pellet after 36 h of infection and subjected to two cycles of CsCl gradient ultracentrifugation (1). After dialysis overnight, the stock of viruses was aliquoted and stored at −80°C until use. The titers of viral stocks were determined using the plaque assay in triplicate and viral concentrations were measured by A260 nm.

Detection of Virus-mediated Gene Delivery, Virus Binding, and Immunostaining. To determine the viral sensitivity of human bladder cancer cells, 5 × 10³ cells were infected with different concentrations of viruses at 37°C in a 5% CO₂-humidified incubator. We determined the virus-mediated gene delivery with two different approaches. β-gal staining and activity assay. At the indicated time, the infected cells were washed with PBS, fixed, and stained for β-gal activity (14). The adenovirus-infected cells were counted microscopically by the number of the positive β-gal-positive cells. In the second approach, the β-gal activity was determined as follows: infected cells were counted in a 260-nm ultraviolet (UV) assay.

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4 The abbreviations used are: m.o.i., multiplicity of infection; CAR, coxsackie and adenovirus receptor; β-gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription PCR; CMV, cytomegalovirus; Ad, adenovirus.
trypsinized and washed once with PBS, and then the protein concentration of each sample was determined by the Bradford dye-binding procedure (Bio-Rad, Hercules, CA). β-Gal activity (15) was measured in a 200-μl cell lysate and normalized to the protein concentration of each sample.

For virus binding assays, [3H]thymidine-labeled dl312 (∼10^6 plaque forming units) was incubated with 1 ml of cells ranging from 5 × 10^3 to 1 × 10^7 at 4°C for 1 h, and then the cell suspension was loaded onto 3 ml of PBS containing 2% BSA and 10% sucrose and centrifuged in a swinging bucket rotor at 2000 rpm for 5 min (13). The cell pellet was lysed in 100 μl of 0.3 N NaOH solution and subjected to liquid scintillation counting to determine the amount of virus bound.

Cytometric analysis of viral receptor by monoclonal antibody (RmcB [16]).

**Table 1 In situ β-gal activity among different human bladder cancer lines infected with β-gal adenovirus**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>m.o.i.</th>
<th>24 h (%)</th>
<th>48 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT4</td>
<td>1</td>
<td>37</td>
<td>86</td>
</tr>
<tr>
<td>253J</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>UMUC3</td>
<td>1</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>SWBC1</td>
<td>1</td>
<td>27</td>
<td>80</td>
</tr>
<tr>
<td>WH</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TCC</td>
<td>1</td>
<td>12</td>
<td>34</td>
</tr>
<tr>
<td>T24</td>
<td>1</td>
<td>85</td>
<td>100</td>
</tr>
</tbody>
</table>

a No. of blue cells (%)

The results were obtained from two separated experiments. Numbers of blue cells were average counts of cells obtained by at least 100 cells by three investigators. The SD is less than 10%.

membrane fluorescence staining was performed on a single-cell suspension and FITC-conjugated secondary antibodies as described previously (17). Fluorescence-activated cell scanning was performed with a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA) delivering 50 mW at 488 nm with an Enterprise air-cooled laser. Analysis was performed using LYSYS II software (Becton Dickinson, Mountain View, CA).

Cloning of CAR cDNA by RT-PCR and Construction of an Expression Vector. CAR cDNA was isolated by RT-PCR with total cellular RNA isolated from both 253J and RT4 cell lines. Two sets of primer were synthesized: CAR1, 5′-AATTTCCAGGACGAGAG-3′; CAR2, 5′-TCCAGACTACT-CAGAAGAG-3′; and CAR4, 5′-CTCTTCAATGAGTTAC-3′; CAR3, 5′-GGCTTCCAGGAGATGTTAC-3′; and CAR4, 5′-GAACCGGAGGGCCAGATGAGAC-3′. For amplifying the 5′ end of CAR cDNA (nucleotides 2–684), first strand cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies, Inc.) with random primers, and then one-fifth of the cDNA was subjected to PCR (40 cycles of 92°C [15 s], 55°C [30 s], and 65°C [3 min]) using primers CAR1 and CAR2. The final PCR products were cloned into pcDNA3.1/V5/His/TOPO vector and, to avoid any PCR-induced mutations, sequenced using the Thermosequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech, Chicago, IL). Among these clones, we identified clone pCAR12s with the sense orientation to the CMV promoter of pcDNA3.1/V5/His/TOPO. For amplifying the 3′ end of CAR cDNA (nt 479–1192), first strand cDNA was synthesized using Superscript II reverse transcriptase with primer CAR4, and then one-fifth of the cDNA was subjected to PCR (40 cycles of 92°C [15 s], 55°C [30 s], and 72°C [2 min]) using primers CAR3 and CAR4. The final PCR products were cloned into PCR 2.1 vector (Invitrogen, Carlsbad CA) and sequenced. Full-length CAR cDNA was assembled in an expression vector (pTOPOCAR) by ligating a 573-bp fragment from the 3′ end of CAR cDNA (digested with pflMI and NotI) with pCAR12s digested with pflMI and NotI.

Measurement of CAR mRNA and Protein Using Quantitative RT-PCR and Northern Analysis. For quantitative RT-PCR, 2 μg of total cellular RNA from each cell line was reverse transcribed into first strand cDNA as described...
Previously, one-fifth of the cDNA was subjected to a 100-μl PCR (30 cycles of 92°C [15 s], 55°C [30 s], and 72°C [2 min]) using both the CAR primer set (i.e., CAR3 and CAR4; 1 ng/μl each) and the GAPDH primer set (5’-TCGTTGAGGACCTACACGTACC-3’ and 5’-TCCACACCCTGGTGTGATA-3’; 0.5 ng/μl). The final PCR products (10 μl) were electrophoresed in a 2% NuSieve agarose gel (3:1, FMC Bioproducts, Rockland, ME) and quantified with BioMax 1D image analysis software (Eastman Kodak, Rochester, NY). The relative level of CAR mRNA from each sample was normalized to GAPDH transcript from the same reaction. To determine the levels of CAR expression among human bladder cancer lines, we performed Northern blot analysis as described previously (17).

**Southern Blot Analysis of CAR Gene in Human Bladder Cancer Cells.** For Southern analysis, high molecular weight DNA was purified by the procedure of Davis et al. (18). Twenty μg of DNA were digested with restriction endonucleases overnight at 37°C and then subjected to Southern blot analysis as described previously (17) with a full-length CAR cDNA probe.

DNA Transfection into Human Bladder Cancer Cells. T24 cells (2 × 10^5 per p-35 plate) were transfected with 2 μg of pcDNA3.1/V5/HisTOPO or pTOPOCAR using LipofectAMINE transfection reagent. Forty-eight h after transfection, cells were split and were selected for neomycin resistant clones with 600 μg/ml G-418. Resistant colonies were either pooled or cloned by ring isolation after 2 weeks of selection.

**Results and Discussion**

**The Sensitivity of Human Bladder Cells to Adenoviral Infection.** Previously, we demonstrated that recombinant adeno virus appears to be an effective gene therapeutic vector to deliver exogenous DNA into a human bladder cancer cell line (253J) in an orthotopic animal model (19). However, when we extended our investigation to examine the effect of several different recombinant viruses on many other cancer cell lines, we observed a variable level of protein expression among different human cancer lines (20). In particular, no detectable levels of exogenous protein were found in a transitional carcinoma line (T24). Then led us to hypothesize that virus may fail to infect this cell line.

To test this hypothesis, we examined the infectivity of human bladder cancer cells by a recombinant adenovirus, AdCMV-β-gal. As shown in Fig. 1A, the positive blue cells were very visible in the RT4 cells infected with virus at m.o.i. 1, whereas no blue cells were seen in cells treated with the buffer control. The infectivity also increased with longer viral incubation (Table 1). In contrast to both 253J and RT4 cells, WH, TCC, and T24 cells showed either few or no blue cells even 48 h after infection; these results correlated with the previous results obtained using different kinds of adenoviruses (20). Similarly, in the presence of the same amount of AdCMV-β-gal, the β-gal activity per cell in RT4 cells was at least 50-fold higher than that in T24 cells 48 h after infection (data not shown). Both RT4 and 253J cells bound significantly more radiolabeled virus than T24 cells did (Fig. 1B). As summarized in Fig. 1C and Table 1, human bladder cancer lines exhibited a wide spectrum of sensitivity to virus attachment and virus-mediated gene delivery. RT4 and 253J bound the most virus and were the most sensitive to the β-gal viral infection. TCC and T24 bound the least virus and were resistant to viral infection. It is known that the entry of adenovirus is mediated by the presence of a specific receptor on the target cells (1–5). Therefore, these data suggested that human bladder cancer cells may possess different levels of receptor for adenovirus and that receptor expression may correlate with sensitivity to adenoviral infection.

**The Loss of Viral Receptor in the Resistant Human Bladder Cancer Line.** Data from recent studies indicate that CAR is a common receptor for both coxsackievirus and group C adenovirus (6, 7). Therefore, we decided to determine whether the presence of CAR mRNA in these human cancer lines correlated with the dramatically different β-gal activity detected. We first performed quantitative RT-PCRs (Fig. 2A) using RNA extracted from these human cancer cells, and we observed a significant difference (approximately 20-fold) in the intensity of CAR PCR product (i.e., 714 bp) between T24 and RT4 cells with no difference in the intensity of the GAPDH (i.e., 452 bp) control. CAR mRNA levels paralleled the viral sensitivity in human bladder cancer lines tested (Fig. 2A). The CAR PCR product was cloned into a PCR vector and sequenced. Sequences of this PCR product were completely identical to those of CAR cDNA previously reported (6–8). Using CAR cDNA as a probe (Fig. 3C), we detected at least four different sizes (i.e., 2.4, 4.8, 6.4, and 8.0 kb) of RNA transcript in human cancer cell lines as well as other cells (7, 8), suggesting that there may be a gene family or different splicing variants associated with the adenoviral receptor gene. CAR mRNA (Fig. 3C) levels were significantly higher in RT4 cells than in T24 cell line.

Such dramatic differences in the CAR mRNA levels detected in T24 and RT4 prompted us to further examine the status of the CAR gene in both cells. We used Southern blot analysis to determine whether gene amplification or DNA rearrangement of the CAR gene can be detected in human bladder cancer lines. As shown in Fig. 2B, the data indicated that the overall hybridization intensity of CAR gene in both RT4 and T24 cells is similar to each other, indicating that gene amplification does not account for the elevated levels of CAR mRNA in RT4 cells. In addition, the restriction enzyme patterns of the CAR transcript in both RT4 and T24 cells were identical, revealed by five different enzymes (such as BamHI, EcoRI, HindIII, PstI, and Mspl) suggested that no large gene alteration in the CAR gene can account
for the low levels of CAR mRNA in T24 cells. In four other human bladder cancer cell lines tested, their restriction enzyme patterns of the CAR gene were identical. Taken together, these data suggested that neither DNA amplification nor DNA arrangement of the CAR gene can be accounted for such dramatic difference in CAR mRNA levels in these human bladder cancer cells. Therefore, transcriptional regulation of the CAR gene may be critical for modulating CAR levels in each cell line. More detailed analyses of the CAR gene in T24 cells are warranted.

**Increased Viral Infectivity after CAR cDNA Transfection.** To test whether the CAR protein is responsible for adenoviral infection in human bladder cancer cells, we constructed a mammalian CAR expression vector and transfected it into T24 cells. After G-418 selection, three independent clones (N2, N3, and N4) and vector-transfected clones (T6, T8, and T10) were chosen based on their different DNA integration pattern (Fig. 3A). Data from the quantitative RT-PCR (Fig. 3B) indicated that the levels of CAR mRNA expression among those transfected sublines were N2 > N4 > N3. The control sublines were completely negative. Northern blot analysis indicated that these three sublines expressed a single CAR mRNA band with a predicted size of 1.1 kb (only transcribed from the open reading frame of CAR cDNA) that was identical to the 2.4 kb of CAR mRNA detected in RT4 cells (Fig. 3C). Furthermore, results obtained from virus binding and β-gal activity showed that the N2 subline, consistent with its higher levels of CAR mRNA expression, was the most sensitive cell to adenoviral infection (Table 2). We also noticed that the β-gal activity 24 h after viral infection in N4 cells is relatively high, but the receptor binding, determined by 1 h binding assay, to this
subline as similar to that in parental T24 cells. This may be due to the fact that the kinetics for virus binding in N4 cells is much slower than the other two sublines.

Because CAR is a membrane-associated protein, fluorescence-activated cell scanning using the monoclonal antibody RmcB was used to determine the presence of CAR protein on cell membrane. As shown in Fig. 3D, RT4 cells exhibited a dramatic shift in the fluorescence intensity with RmcB antibody (thick line) staining compared to control antibody (thin line) staining, indicating that a high levels of CAR protein expression was detected in RT4 cells. In contrast to RT4 cells, parental T24 cells did not have any shift between RmcB and control antibodies, indicating that T24 cells did not have any detectable CAR protein. However, the fluorescence intensity in the N2 cells with RmcB antibody staining had a moderate increase compared to control antibody staining (Fig. 3D), indicating that N2 cells did express the CAR protein on their cell membrane. In addition, the profile of CAR staining exhibited a wide spectrum of intensity on their cell membrane, suggesting that the levels of CAR protein expressed in N2 cells were very heterogeneous (Fig. 3D). Similar results were seen with β-gal staining (data not shown). Although, the total CAR mRNA levels in N2 cells were equivalent to that in RT4 cells (Fig. 3C), the majority of N2 positive cells expressed low levels of CAR protein on their cell membrane (Fig. 3D). With further cloning of N2 cells, we did not obtain any single clone with the enriched positive cells. We have found, on the basis of selecting more than 30 clones from two separate transfection studies, that most clones had only 10–20% positive cells (data not shown). Therefore, it is possible that the CAR protein levels in T24 cells may be modulated posttranscriptionally.

The Biological Significance of Viral Receptor in Human Bladder Cancer Lines. Gene therapy is an innovative approach to the treatment of malignant and benign disorders characterized by genetic alterations that may serve as therapeutic targets. Several practical and theoretical considerations (21) make recombinant adenovirus an attractive vector for cancer gene therapy. One advantage is that the adenoviral receptor CAR is ubiquitous and is detected in a variety of organs (7). In this study, we observed a wide spectrum of adenoviral sensitivity in several human bladder cancer lines (Table 1). Although those cell lines originate from the transitional epithelium, each line exhibits a diversity of phenotypes and growth potential. For example, the RT4 cell line is derived from well-differentiated transitional cell papilloma, and the T24 cell line is a rapidly growing transitional carcinoma with a mutated ras oncogene. Obviously, more detailed studies will be required to understand whether CAR levels correlate with the grade of cancer cells. If similar results can be observed clinically, these data will certainly have an impact on the efficacy of adenovirus-based gene therapy.

Adenoviruses are nonenveloped DNA viruses that can infect target cells by binding to cellular receptors. More than 40 human adenovirus serotypes have been identified based on nucleic acid homology, oncogenic potential, and their protein component. The receptor for adenovirus type 5, which is frequently used as a viral vector for gene therapy, has been cloned (6, 7). Sequence analysis indicates that this adenoviral receptor CAR cDNA encodes a typical immunoglobulin-like membrane protein with two immunoglobulin domains that may interact with adenovirus fiber protein. In addition to the extracellular domain, CAR cDNA contains a 22-amino acid transmembrane domain and a 107-amino acid intracellular domain that has a putative tyrosine phosphorylation site. Based on the CAR protein structure, it is likely that CAR not only may function as the receptor for adenovirus but also may have other physiological functions, such as a cell adhesion molecule.

Our Northern blot analysis indicated that there are four CAR mRNA transcripts detected in RT4 cells using a full-length CAR cDNA. Biologically, these transcripts could represent various isoforms of CAR that are likely to have different viral binding affinities or other distinct physiological function. Based on our RT-PCR results using both CAR3 and CAR4 primers, including both transmembrane and intracellular domains, we only observed one single PCR product from all of the human bladder cancer lines tested. However, with both CAR1 and CAR2 primers, including extracellular domain, we did observe several larger size transcripts from RT-PCRs. Thus far, we have sequenced at least 20 different clones, and none of them appeared to be CAR homologues (data not shown). Other studies using RmcB antibody also indicated that a single protein of 46 kDa was detected by both Western blot and immunoprecipitation (6, 7). Taken together, these results suggest that the higher molecular weight RNA transcripts may be splicing intermediates of CAR mRNA.

Although neither CAR mRNA nor protein levels are detectable in T24 cells, the genomic structure of the CAR gene in T24 cells and several other human bladder cancer lines is identical. This suggests that DNA rearrangement or mutation of the CAR gene does not account for the dramatic difference between RT4 and T24 cells. Therefore, transcriptional regulation of the CAR gene appears to be an important aspect of modulating CAR gene activity. Obviously, understanding the gene regulation of CAR leading to the induction of endogenous CAR gene activity could be a new strategy for increasing the efficiency of gene delivery.

Results from this study demonstrate that the expression of adenoviral receptor is heterogeneous among human bladder cancer cells. This may have significant implications concerning the design and efficacy of gene therapy trials for human bladder cancer. Our data suggest that determining the receptor status of given patient’s tumor prior to adenovirus-based gene therapy may be important.

Acknowledgments

We thank Dr. Victor Lin for providing the primer set for GAPDH and Dr. Leland Chung for providing the WH cell line.

References

3. Freimuth, P. A human cell line selected for resistance to adenovirus infection has reduced levels of the virus receptor. J. Virol., 70: 4081–4085, 1996.

Table 2 The adeno viral sensitivity among several CAR cDNA-transfected T24 sublines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Virus binding (cpm)$^b$</th>
<th>β-Gal activity (A$_{650}$ uncorrected)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24</td>
<td>92 ± 24 (1)$^b$</td>
<td>1.96 ± 0.11 (1)$^b$</td>
</tr>
<tr>
<td>N2</td>
<td>823 ± 51 (9)</td>
<td>24.91 ± 2.82 (13)</td>
</tr>
<tr>
<td>N3</td>
<td>240 ± 30 (3)</td>
<td>11.95 ± 2.00 (6)</td>
</tr>
<tr>
<td>N4</td>
<td>94 ± 10 (1)</td>
<td>15.01 ± 2.49 (8)</td>
</tr>
<tr>
<td>N6</td>
<td>0 (0)</td>
<td>0.55 ± 0.15 (6)</td>
</tr>
<tr>
<td>T8</td>
<td>0 (0)</td>
<td>0.52 ± 0.24 (0)</td>
</tr>
<tr>
<td>T10</td>
<td>106 ± 20 (1)</td>
<td>0.59 ± 0.28 (0)</td>
</tr>
</tbody>
</table>

$^a$ Each value was determined in triplicate from two separate experiments.

$^b$ Numbers in parentheses are fold induction was normalized to parental T24 cells.
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