Variability of Adenovirus Receptor Density Influences Gene Transfer Efficiency and Therapeutic Response in Head and Neck Cancer

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ABSTRACT

Despite encouraging preclinical studies in many tumor types including head and neck squamous cell carcinoma (HNSCC), initial clinical trials with adenovirus-mediated gene therapy have been disappointing. Although the adenovirus is a “highly efficient vector,” it is still limited by the extent of effective in vivo transduction. In our studies with multiple human HNSCC cell lines, we have noted a variation in both in vitro and in vivo responses to the same recombinant adenovirus therapeutic construct. We hypothesize that adenovirus receptor density among tumor cell populations, even of the same histology, greatly influences transduction efficiency and therapeutic results of a variety of adenovirus-based gene therapy strategies. To investigate this hypothesis, the numbers of adenovirus receptors on three well-characterized HNSCC cell lines were determined. Marker and cytokine gene transfer efficiencies as well as therapeutic outcomes after adenovirus-mediated tumor suppressor gene and suicide gene therapies were evaluated and correlated with receptor status.

A 5-fold variation in adenovirus receptor density was identified among the HNSCC cell lines (P < 0.002, t test). This variation directly correlated with adenovirus type 5 (Ad5)-mediated green fluorescent protein marker gene and Ad5-interleukin 2 cytokine gene transfer efficiency and resulting protein expression in each individual cell line. The receptor density also directly correlated with therapeutic response after Ad5-thymidine kinase or Ad5-p16 gene transfer in each HNSCC line. The role of the adenovirus receptor in gene transfer efficiency was further supported by recombinant Ad5 fiber knob blocking experiments. The marker gene transfer was increasingly blocked by the same concentration of Ad5 recombinant fiber knob in relation to decreasing levels of adenovirus receptor in the HNSCC lines.

An Ad5 recombinant construct that carries the shared coxsackie and adenovirus receptor (CAR) was created and used to up-regulate receptors on each cell line. Ad5-CAR infection significantly increased Ad5-β-Gal gene transfer efficiency and expression (P = 0.0003, Mann-Whitney test). This increased marker gene expression remained consistent with the established pattern of gene transfer efficiency among the HNSCC cell lines.

These data confirm the importance of the adenovirus receptor on individual tumor cell lines with respect to investigating novel adenovirus-mediated gene therapy strategies. This work further supports consideration of assaying adenovirus receptor status, even in tumors of the same histology from patients enrolled in gene therapy clinical trials. Adenovirus receptor status may prove valuable for selecting or stratifying patients as well as assessing outcomes among patients within adenovirus-based cancer gene therapy trials.

INTRODUCTION

Human HNSCC 1 is not uncommon and represents 6% of all new cancers in the United States or ~50,000 cases each year (1–4). In 1998, HNSCC caused approximately 12,500 deaths in the United States, and the 5-year survival rate remains at 50% overall, despite ongoing advances in surgery, radiation, and chemotherapy strategies (1–4). For advanced stages III and IV disease, 2-year survival drops to 30%. HNSCC is, therefore, an ideal target for the development of new and more effective therapeutic modalities.

Gene therapy has shown promise in preclinical studies as a novel molecular treatment for malignant tumors including HNSCC. Multiple gene transfer strategies that involve the introduction of foreign genes that directly kill tumor cells, restore a defective tumor-suppressor gene, and induce apoptosis, or that enhance immune responses are presently under investigation for HNSCC (4–8). Among the available well-studied cloned genes, herpes simplex virus tk, p53, p16 INK4A, and IL-2 have been reported in preclinical investigations and selected clinical trials against HNSCC (5–7, 10–13).

The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; tk, thymidine kinase; IL, interleukin; AD5, adenovirus 5; CAR, coxsackie and adenovirus receptor; FBS, fetal bovine serum; PS, penicillin-streptomycin; β-Gal, β-galactosidase; CMV, cytomegalovirus; GFP, green fluorescent protein; MOI, multiplicity of infection; GCV, ganciclovir; MFI, mean fluorescence intensity.

Received 6/23/99; revised 9/20/99; accepted 9/22/99.

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1 Supported in part by NIH/National Institute of Dental and Craniofacial Research Grant R29 DE11 772-03 (BWO).
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3 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; tk, thymidine kinase; IL, interleukin; AD5, adenovirus 5; CAR, coxsackie and adenovirus receptor; FBS, fetal bovine serum; PS, penicillin-streptomycin; β-Gal, β-galactosidase; CMV, cytomegalovirus; GFP, green fluorescent protein; MOI, multiplicity of infection; GCV, ganciclovir; MFI, mean fluorescence intensity.
The effective administration of therapeutic genes to target tumor cells requires an efficient delivery vehicle. The replication-defective adenoviral vector possesses several important advantages among the available vehicles for directly delivering foreign genes into mammalian cells. The most cited advantages are the high efficiency of gene transfer as compared to other presently available systems, the ability to infect nondividing cells, and the established general safety in preclinical and early clinical investigations. The recombinant adenovirus also remains as a nonreplicating extrachromosomal entity and therefore has little chance to activate a dormant oncogene or interrupt a tumor suppressor gene. To date, there has been no evidence of insertional mutagenesis in connection with the adenovirus-mediated gene delivery.

The adenovirus is a nonenveloped, icosahedral, double-stranded DNA virus. At present, 47 human adenovirus serotypes have been distinguished on the basis of their resistance to neutralization by antisera to other known adenovirus serotypes. The Ad5-based vector has been the predominate vehicle for adenovirus gene therapy strategies.

It has been shown that Ad5 enters the host cell by a receptor-mediated endocytosis mechanism, and its attachment relies on its fiber protein (14, 15). The distal, COOH-terminal domain of the fiber protein terminates in a knob that directly binds to the recently identified shared CAR (15, 16). It has been also demonstrated that several different adenovirus serotypes including Ad2, Ad5, and Ad12 share the same receptor (15, 17–18). The identified and isolated CAR plays an important role in Ad5 gene transfer.

We have observed significant variations in transduction efficiency and therapeutic outcome after adenovirus-mediated gene transfer among separate HNSCC cell lines that have the same or similar histology characteristics. This variation in gene transfer efficiency imposes limitations on the application of adenovirus-mediated gene therapy. In this work, we establish a correlation between adenovirus receptor density and limitations in gene transfer efficiency, subsequent protein expression, and therapeutic outcomes after adenovirus-mediated gene transfer.

**MATERIALS AND METHODS**

**Cell Lines.** The human HNSCC cell lines were generated and maintained at Johns Hopkins University Department of Otolaryngology-Head and Neck Surgery (JHU012 and JHU022) or acquired from the American Type Culture Collection (FaDu). All three cell lines were maintained in RPMI 1640 with 10% FBS, and 1% PS. These cell lines have been genetically characterized (Table 1; Refs. 13 and 19).

**Table 1 Characterization of HNSCC cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue origin</th>
<th>p16</th>
<th>p14ARF</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>JHU012</td>
<td>Oral cavity</td>
<td>Meth&lt;sup&gt;a&lt;/sup&gt;</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>JHU022</td>
<td>Larynx</td>
<td>WT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>FaDu</td>
<td>Pharynx</td>
<td>Pt. mut&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Pt. mut</td>
<td>WT</td>
</tr>
</tbody>
</table>

<sup>a</sup> Promoter hypermethylation.

<sup>b</sup> Sequence is wild-type (WT) with low but detectable endogenous protein by Western blotting.

<sup>c</sup> Point mutation.

**Construction of Recombinant Adenovirus Ad5-tk, Ad5-IL-2, Ad5-p16, Ad5-β-Gal, and DL 312.** The original recombinant Ad5-tk and Ad5-IL-2 constructs were generously provided by Drs. Savio L.C. Woo and Shu-Shia Chen (presently at Mount Sinai Medical Center, New York, NY), and the generation and purification have been published previously (20, 21). The replication-defective virus (DL 312), kind gift of Dr. Shenk (Princeton University), was used as a control vector. These original stocks were further amplified and purified by cesium chloride ultracentrifugation using established methods in our laboratory (21). The viral titer (pfu/ml) of purified stocks was determined by plaque assays on 293 cells.

The recombinant Ad5-p16 was constructed in our laboratory with the p16<sup>INK4A</sup> cDNA under the transcriptional control of the CMV immediate-early gene promoter as described previously (10, 13). The recombinant Ad5-β-Gal (gift from Dr. Frank Graham, McMaster University, Hamilton, Ontario, Canada) was amplified and purified using the same methods as aforementioned.

**Construction of Recombinant Adenovirus Ad5-CAR and Ad5-GFP.** A new AdEasy system has been developed recently for simplifying the generation of recombinant adenovirus (22). This new technique was used in generating recombinant adenovirus Ad5-CAR and Ad5-GFP in the present study. In short, a plasmid containing the CAR gene (generously provided by Dr. Jeffrey Bergelson, The Children’s Hospital of Philadelphia, Philadelphia, PA) was first cloned into a pAdTrack-CMV shuttle vector (generously provided by Drs. Tong-Chuan He and Bert Vogelstein, The Johns Hopkins Oncology Center, Baltimore, MD) including the gene encoding enhanced GFP under the transcriptional control of an independent CMV immediate-early gene promoter. The resultant plasmid was linearized by digesting with restriction endonuclease PmeI and subsequently cotransformed into Escherichia coli. B35183 cells with an adenoviral backbone plasmid, pAdEasy-1 (obtained from Drs. Tong-Chuan He and Bert Vogelstein, The Johns Hopkins Oncology Center). The linearized recombinant plasmid was then transfected into 293 cells, and recombinant adenovirus Ad5-CAR was generated in 8 days. A clonal recombinant virus containing Ad5-CAR and without wild-type adenovirus contamination was confirmed by PCR analysis. The recombinant Ad5-CAR was amplified and purified using the same methods as aforementioned. The viral titer (pfu/ml) of purified Ad5-CAR stocks was determined by plaque assays on 293 cells using standard methods (21). The recombinant Ad5-GFP (gift from Dr. Tong-Chuan He and Bert Vogelstein, The Johns Hopkins Oncology Center) was amplified and purified using the same methods as Ad5-CAR.

**Monoclonal Antibody RmcB.** The monoclonal antibody RmcB that recognizes CAR protein was used in the present study. Monolayers of each HNSCC cell line, positive receptor control (HeLa), and murine negative control (SCCVII) cell lines were trypsinized and washed twice in wash buffer (PBS with 0.1% sodium azide). Cells were then resuspended in round-bottomed tubes (Falcon 2054) in triplicate at a concentration of 5 × 10<sup>5</sup> cells. The cells were mixed with RmcB (kindly provided by Dr. Jeffrey Bergelson, the Children’s Hospital of Philadelphia) to a concentration of 10<sup>7</sup> cells/ml of RmcB and
incubated for 24 h. Each of three Ad5-\(\text{tk}\) triplicate sets at a dilution of 1 \(\times\) 10\(^5\) were solubilized by the addition of 100 \(\mu\)l of wash buffer. FACScan analysis was performed on a Becton Dickinson FACScan, and data from each cell line were analyzed with CellQuest software. Statistical analysis (\(t\) test) was performed using Statmost software for Windows.

**In Vitro Ad5-IL-2.** Each of three HNSCC cell lines was plated in T-25 tissue culture flasks (Corning) in two triplicate sets at a dilution of 1 \(\times\) 10\(^6\) cells in each flask in RPMI 1640 with 10% FBS and 1% PS at 24 h prior to adenovirus infection. Cells were either treated with Ad5-\(\text{IL-2}\) at MOI of 10 or mock-treated with Ad5-empty (Ad5 vector alone). The treated cells were then incubated for 24 h. After 24 h of culturing, the medium was collected, and the IL-2 expression was then measured using a commercially available ELISA kit of IL-2 immunoassay (R & D Systems). The intensity of the color change was proportional to the amount of IL-2 bound in the cell supernatants and was read by an ELISA plate reader (Bio-Tek Industries) at 450/570 nm. A \(t\) test analysis was performed on data from all tested cell lines (Statmost for Windows).

**In Vitro Ad5-tk.** Each of three HNSCC cell lines was seeded into T-75 tissue culture flasks (Corning) at a dilution of 6 \(\times\) 10\(^6\) cells in each flask in RPMI 1640 with 10% FBS and 1% PS at 12 h prior to adenovirus infection. Cells were treated either with Ad5-\(\text{tk}\) or with PBS. The treated cells were then incubated for 24 h. Each of three Ad5-\(\text{tk}\) treated cell lines was then trypsinized and plated into 96-well plates (Nunc) in two triplicate sets at a dilution of 1 \(\times\) 10\(^5\) cells in each well in RPMI 1640 with 10% FBS and 1% PS with or without GCV at 80 \(\mu\)g/ml. At 72 h, the medium was removed, and the cells were assayed by the method according to Mosman (21). In short, the medium was aspirated, and fresh medium with 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide substrate (ICN) was added to each well and incubated for 4 h at 37\(^\circ\)C, 5% CO\(_2\), in a humidified incubator. The cells were solubilized by the addition of 100 \(\mu\)l of an HCl isopropanol solution and analyzed on an ELISA plate reader (Bio-Tek Industries) at 570/630 nm.

**Tumor Cell Growth Rates.** Cells from each HNSCC cell line were seeded into six-well plates at a dilution of 4 \(\times\) 10\(^5\) cells/well in RPMI 1640 containing 10% FBS and 1% PS. At 24-h time points over 6 consecutive days, the cells were trypsinized and counted one well/day with a hemocytometer.

**In Vitro Ad5-\(p\text{16}\).** Each of three HNSCC cell lines was seeded into T-75 tissue culture flasks (Corning) at a dilution of 6 \(\times\) 10\(^6\) cells in each flask in RPMI 1640 with 10% FBS and 1% PS at 12 h prior to adenovirus infection. Cells were treated either with Ad5-\(p\text{16}\) at MOI of 5 or with PBS. The treated cells were then incubated for 24 h. Each of three treated cell lines was then trypsinized and plated into six-well plates (Falcon) in triplicate at a dilution of 1 \(\times\) 10\(^5\) cells in each well in RPMI 1640 with 10% FBS and 1% PS. At 24-h time points over 6 consecutive days, the cells were trypsinized and counted with a hemocytometer, respectively.

**In Vitro Ad5-CAR and Ad5-\(\beta\text{-Gal}.** Each of three HNSCC cell lines was plated into 6-well plates in three triplicate sets at a concentration of 1 \(\times\) 10\(^6\) cells in each well in RPMI 1640 with 10% FBS and 1% PS at 12 h prior to adenovirus infection. Cells were treated either with Ad5-CAR or with DL 312 at MOI of 10 or with PBS. After 8 h of incubation, the treated cells were infected with Ad5-\(\beta\text{-Gal}\) at MOI of 10. The cells were then incubated overnight. The next day, the medium was removed, and the cells were fixed with 1.5% glutaraldehyde (Sigma) in PBS and stained with 5-bromo-4-chloro-3-indolyl-\(\beta\text{-d-galactopyranoside as described in our previous publication (5). Cells were counted in four random fields/well, and the results were expressed as positive \(\beta\text{-Gal}\)-stained cells. Mann-Whitney statistical analysis was performed using Statmost for Windows.

**Recombinant Ad5 Knob and Ad5-GFP.** The recombinant Ad5 knob, which inhibits Ad5 infectivity by binding Ad5 receptor, was obtained from Dr. David Curiel (Gene Therapy Program, University of Alabama at Birmingham, Birmingham, AL) and used in the present study. Each of three HNSCC cell lines was plated into six-well plates (Falcon) in two triplicate sets at a concentration of 1 \(\times\) 10\(^6\) cells in each well in RPMI 1640 with 10% FBS and 1% PS for 24 h. The cells were washed once in PBS and mixed either with Ad5 knob at 2 \(\mu\)g/ml per well or with PBS. After 20 min of incubating at room temperature, all of the cells were incubated with Ad5-\(\text{GFP}\) for 10 h and incubated in the incubator. Twenty-four h after incubation, the cells were washed once in PBS and then trypsinized. The cells were resuspended in round-bottomed tubes (Falcon 2054) in 0.5 ml of PBS. FACScan analysis was performed on a Becton Dickinson FACScan using CellQuest software.

**RESULTS**

**Adenovirus Receptor Density in HNSCC Cell Lines.** The adenovirus receptor density was determined in FaDu, JHU012, and JHU022 cell lines using the monoclonal antibody RmC8 that recognizes and binds the CAR protein (15, 24). Fig. 1 depicts the MFI for each cell line that is directly proportional to the number of adenovirus receptors. The MFI was 208.66 for FaDu, 494.32 for JHU012, and 983.74 for JHU022. The JHU022 cell line showed a significantly higher density of adenovirus receptors compared with both JHU012 and FaDu (\(P <\)...
The difference between JHU012 and FaDu was also statistically significant ($P < 0.03$).

**Transfection Efficiency of Ad5-GFP Reporter Gene and Ad5-IL-2 Among Different HNSCC Lines.** *GFP* reporter gene incorporated into the Ad5 vector allows direct tracking of adenovirus infection. Fig. 2 shows the result of intracellular expression of a *GFP* reporter gene in each of the HNSCC cell lines. The different MFIs illustrate the level of GFP expression after Ad5-GFP infection. The cell line having the highest concentration of adenovirus receptors, JHU022, also demonstrated the highest transfection efficiency. The low receptor density FaDu tumor cell line had the lowest gene transfer efficiency. There were significant statistical differences among all three HNSCC cell lines as shown: $P < 0.0004$ (JHU022 versus FaDu); $P < 0.0007$ (JHU012 versus FaDu); and $P < 0.0053$ (JHU022 versus JHU012). Marker gene transfer efficiency, therefore, correlates with the level of adenovirus receptor on each tumor cell line.

Adenovirus-mediated transfer of the cytokine IL-2 has been investigated as a gene therapy strategy to generate tumor-specific immunity mediated by CD4+ and CD8+ T-lymphocytes as well as natural killer cell activity. The following experiment was performed to assess whether the potentially therapeutic level of cytokine expression varies according to receptor density among the HNSCC lines. IL-2 protein from IL-2-transfected cells of each of three HNSCC cell lines was detected using an IL-2 ELISA kit. The quantity of IL-2 expression in Ad5-IL-2 transfected FaDu, JHU012, and JHU022 cells are shown in Fig. 3. IL-2 expression was not detected in any of Ad5-empty vector (control)-treated cell lines (data not shown). Differences in IL-2 expression were found among the tested cell lines of FaDu (4,082.5 pg/ml), JHU012 (57,477.5 pg/ml), and JHU022 (121,216.7 pg/ml). Statistical significance was determined using a $t$ test analysis as shown: $P < 0.0078$ (FaDu versus JHU022); $P < 0.011$ (FaDu versus JHU012); and $P < 0.005$ (JHU022 versus JHU012).

**Receptor Density Influences Therapeutic Outcomes after Ad5-tk and Ad5-p16 Treatment in Vivo.** The strategy of Ad5-tk gene transfer and GCV administration has proven effective in directly killing tumor cells including HNSCC (5–7, 9–12). Ad5-tk gene transfer was performed in each of the HNSCC cell lines at the same MOI. Fig. 4 shows different tumor cell killing among the HNSCC cell lines infected with Ad5-tk and treated with GCV. The FaDu cell line was the most resistant to the cytotoxic effects of Ad5-tk because 50% of the tumor cells survived compared with 25.4% cell survival for JHU012 and 13.7% for JHU022. These findings demonstrate differences in tumor cell susceptibility to Ad5-tk and GCV that

![Fig. 2](image1.png) Expression of GFP in FaDu, JHU012, and JHU022 cell lines after transfection with Ad5-GFP at MOI 10 (performed in triplicate). FACS profiles from each cell line are shown with three-dimensional views. A significant difference in *GFP* marker gene expression was detected among each cell line and concurred with the variable pattern of adenovirus receptor numbers.

![Fig. 3](image2.png) IL-2 expression in FaDu, JHU012, and JHU022 cell lines. Cells were transfected with Ad5-IL-2 at MOI 10 for 24 h. The level of IL-2 in supernatants of each cell line was determined using ELISA analysis. The significant difference in IL-2 expression among each cell line was consistent with the variation in adenovirus receptor density. Bars, SD.

![Fig. 4](image3.png) Sensitivity of FaDu, JHU012, and JHU022 cell lines to the cytotoxic effects of Ad5-tk. Cell survival rate was assessed 72 h after transfection at MOI 10. Significantly different cytotoxic effects were observed among the cell lines. FaDu was the least sensitive cell line, and JHU022 was the most sensitive. These data are consistent with the variable pattern of adenovirus receptor density.

![Fig. 5](image4.png) Growth curves for three untreated human HNSCC cell lines in *vitro*. Mean cell numbers were assessed at days 1, 2, 3, and 4 (performed in triplicate). FaDu demonstrated a significantly higher growth rate by day 4 as compared with JHU012 and JHU022. Bars, SD.
directly correlate with the number of adenovirus receptors on each HNSCC cell line.

To determine whether variable tumor cell growth rates are the reason for the differences in the responses to Ad5-\(tk\) and GCV, growth rates were assessed for each HNSCC line. The cell growth curves of FaDu, JHU022, and JHU012 lines are depicted in Fig. 5. FaDu demonstrated a significantly faster growth rate by day 3, whereas JHU012 and JHU022 were essentially the same. We would have predicted that the fastest growing FaDu cell line should be more sensitive to Ad5-\(tk\) gene therapy because the therapeutic results (direct tumor cell killing) require active cell division. Therefore, other cellular differences besides growth rate are governing the sensitivity to Ad5-\(tk\) and GCV.

It has been shown that \(p16^{INK4A}\) is a tumor suppressor gene, and expression of the introduced \(p16^{INK4A}\) gene in tumor cells inhibits the cell growth by inducing G1–S cell cycle arrest (9, 13). A significant antitumor effect of Ad5-\(p16\) against HNSCC both in vitro and in vivo has been demonstrated previously (13). The therapeutic effects of Ad5-\(p16\) gene transfer were determined for FaDu, JHU012, and JHU022 tumor cell lines. Fig. 6 illustrates cellular growth curves of each HNSCC cell line up to 6 days after a single Ad5-\(p16\) treatment. Growth rates were significantly inhibited in JHU022 and JHU012 cell lines after Ad5-\(p16\) treatment by 80 and 70% on day 6, respectively, whereas FaDu demonstrated a very poor response to Ad5-\(p16\), as evidenced by 31% inhibition rate. The response to Ad5-\(p16\) treatment in all three cell lines, which are wild-type for retinoblastoma (Rb), indicated a functional consequence of the wild-type Rb status of these cell lines. The poor therapeutic result to Ad5-\(p16\) in the FaDu cell line further illustrated the importance of Ad5 receptor.

**Ad5 Receptor (CAR) Blocking Supports the Correlation of Receptor Number to Actual Gene Transfer Efficiency.** It has been shown that Ad5 attachment to host cells is mediated by CAR that is recognized by the knob of the fiber protein of adenoviral capsid (15). By binding Ad5 receptor on host cells, the recombinant Ad5 knob inhibits adenovirus attachment. The capability of recombinant Ad5 knob to block Ad5-GFP gene transfer was evaluated among the HNSCC cell lines. Fig. 7 shows the results of GFP expression in all three HNSCC cell lines with or without recombinant Ad5 knob pretreatment. The recombinant Ad5 knob significantly reduced Ad5-GFP gene transfer and GFP protein expression in FaDu, JHU012, and JHU022 cell lines. At a concentration of 2 \(\mu\)g/ml, the knob inhibited 75, 59, and 47% of the GFP expression in FaDu, JHU012, and JHU022 cell lines, respectively. As expected, the cell line with the lowest adenovirus receptor density (FaDu) was the most susceptible cell line to Ad5 knob block because of a relatively high concentration of the knob.

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**Fig. 6** Growth curves depicting the effect of Ad5-p16 treatment on FaDu, JHU012, and JHU022 cell lines versus PBS controls in vitro. Mean cell numbers were determined on days 1 through 6. Each cell line showed either a decrease in growth or an apparent growth arrest after Ad5-p16 treatment versus controls. However, a significant variation of growth inhibition was identified among each cell line that followed the pattern of adenovirus receptor density. Bars, SD.
Up-Regulating Adenovirus Receptor (CAR) Significantly Enhances the Efficiency of Ad5-mediated Gene Transfer in Each HNSCC Line in Correlation with the Level of Baseline Receptor Density. To determine whether marker gene expression in each HNSCC cell line could be increased through up-regulating CAR expression, an Ad5 recombinant construct carrying the CAR gene was created. Cotransfection with Ad5-CAR and Ad5-β-Gal was performed in FaDu, JHU002, and JHU012 cell lines. Fig. 8 shows the results of β-Gal reporter gene expression with cotransfected Ad5-CAR or DL 312 or PBS in each cell line. As would be expected, all of CAR-transfected groups revealed a significantly higher expression of β-Gal reporter gene as compared with either DL 312 or PBS groups ($P = 0.0003$). There were no statistical differences between DL 312 and PBS-treated groups when looking at the same cell line. A 7-, 6-, and 5-fold increase in susceptibility to Ad5-mediated reporter gene transfer was found in Ad5-CAR (MOI 10) treated FaDu, JHU012, and JHU022 cell lines. The FaDu cell line, having the lowest receptor density, demonstrated the greatest percentage increase in marker gene expression after Ad5-CAR treatment. Comparisons of the different expressions of β-Gal among Ad5-CAR-transfected cell lines showed significant statistical differences between FaDu and JHU022 ($P = 0.0003$), FaDu and JHU012 ($P = 0.0003$), and JHU022 and JHU012 ($P = 0.0003$).

DISCUSSION

Limited in vivo transduction efficiency remains a major issue for effective clinical application of adenovirus-mediated gene therapy (25). Because viral binding is a critical step for initiating gene uptake and subsequent expression, we hypothesized that variations in receptor status would greatly influence gene therapy outcomes.

Using the monoclonal antibody RmcB that recognizes the CAR protein, we identified significant variations in adenovirus receptor density among three HNSCC cell lines. To determine the influence of this receptor variation on gene uptake and expression, each HNSCC cell line was transfected with the same MOI of Ad5-GFP. The GFP expression was significantly different among three HNSCC cell lines and varied according to the level of adenovirus receptor, therefore suggesting a correlation between receptor number and gene transfer efficiency. This pattern also translates to the level of cytokine expression after infection with Ad5-IL-2 that has been reported previously in murine head and neck tumor models (10, 26). The demonstration that IL-2 expression varies according to receptor density among different HNSCC cell lines is an important finding because we have reported recently that the in vivo level of IL-2 expression within the local tumor environment may govern immune-mediated antitumor responses (27). The adenovirus receptor variations in human HNSCC cells may therefore limit the clinical application of Ad5-IL-2 alone or in combination therapy.

Given these findings, it was necessary to investigate whether receptor density would influence the outcomes of gene therapy strategies that have a direct effect on tumor cells. In experiments with both Ad5-5k and Ad5-p16 constructs, direct cytotoxic and tumor suppressor effects occurred in each of the cell lines; however, they varied significantly according to receptor number in the identical pattern as seen with the initial marker and cytokine gene transfer experiments. Growth rates were evaluated for each cell line, and they did not appear to explain the variations in therapeutic responses.

It is well known that the promoter initiates transcription of introduced exogenous DNA in a hosted cell and plays an important role in gene product expression. The difference in susceptibility in different HNSCC cell lines to Ad5-mediated gene transfer strategies could be attributable to promoter differences. However, the same pattern of transfection efficiency in FaDu, JHU022, and JHU012 cell lines was found in both the CMV-driven system (Ad5-GFP and Ad5-p16) and RSV-driven system (Ad5-5k, Ad5-IL-2, and Ad5-β-Gal). Therefore, it is unlikely that promoter activity explains the variations in susceptibility that were identified.

The importance of adenovirus receptor density was further supported by the recombinant Ad5 fiber knob and Ad5-CAR experiments. Ad5 knob was effective in blocking 47–75% of adenovirus infection at a concentration of 2 μg/ml. The highest percentage of blockage was seen in FaDu, a finding that had been expected based on the relative high concentration of knob to receptor number for this cell line. The experiments with Ad5-CAR were valuable for two rea-
rons: (a) the general importance of receptor number on gene transfer and expression was further demonstrated by the finding that Ad5-CAR treatment dramatically increased transduction efficiency; and (b) because Ad5-CAR infection increased gene expression in all of the HNSCC cell lines, it may prove useful in a combination gene therapy strategy to augment therapeutic responses.

The nonlinearity of both Ad5 knob blocking and the variance in GFP and IL-2 expression between the three cell lines suggest that the cell surface receptor (fiber-CAR interaction) does play an important role but is not all inclusive in governing adenovirus-mediated gene transfer. The data support that other factors, such as cell surface integrins and coreceptors, may also play important roles in adenovirus binding and entry (28). Evaluation of these other mechanisms will be the basis for future experimentation.

Differences in adenovirus receptor number may provide explanations for identifiable variations in antitumor responses and clinical outcomes among patients. More importantly, adenovirus receptor status could prove valuable as a means of selecting or stratifying patients who have the greatest chance of success in adenovirus-mediated gene therapy. Use of the Ad5-CAR construct provides a new tool that may help overcome low levels of in vivo transduction efficiency and broaden the clinical application of adenovirus vectors.

ACKNOWLEDGMENTS

We thank Bin Duan for technical assistance with this project.

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