

Selective Gene Delivery to Head and Neck Cancer Cells via an Integrin Targeted Adenoviral Vector¹

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ABSTRACT

In vivo cancer gene therapy approaches for squamous cell carcinoma of the head and neck (SCCHN) based on adenoviral vector-mediated gene delivery have been limited by the suboptimal efficacy of gene transfer to tumor cells. We hypothesized that this issue was due to deficiency of the primary adenoviral receptor, the coxsackie-adenovirus receptor (CAR), on the tumor targets. Studies of CAR levels on SCCHN cell lines confirmed that their relative refractoriness to the adenoviral vector was based on this deficiency. To circumvent this deficiency, we applied an adenoviral vector targeted to a tumor cell marker characteristic of SCCHN. In this regard, integrins of the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ class are frequently overexpressed in SCCHN. Furthermore, these integrins recognize the RGD peptide motif. On this basis, we applied an adenoviral vector genetically modified to contain such a peptide within the HI loop of the fiber protein as a means to alter viral tropism. Studies confirmed that the CAR-independent gene delivery achieved via this strategy allowed enhanced gene transfer efficiencies to SCCHN tumor cells. Importantly, this strategy could achieve preferential augmentation of gene transfer in tumor cells compared with normal cells. The ability to achieve enhanced and specific gene transfer to tumor cells via adenoviral vectors has important implications for gene therapy strategies for SCCHN and for other neoplasms in general.

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INTRODUCTION

A number of considerations have suggested that gene therapy represents a rational approach for SCCHN³ (1, 2). Among these is the fact that conventional therapeutic modalities of surgery coupled with radiotherapy and chemotherapy have not led to meaningful improvements in the overall survival rates for this disorder (3–6). It is thus apparent that novel therapeutic modalities are required for this disease. In this regard, specific SCCHN disease features have suggested that it represents an appropriate target for the evaluation of gene therapeutics. Of special note, the surface accessibility of the tumor potentially allows for direct gene delivery for implementation of a variety of cancer gene therapy approaches that are based on *in vivo* tumor transduction.

To this end, a number of distinct cancer gene therapy approaches have been developed for SCCHN that are predicated upon direct gene delivery to tumor cells *in situ* (1, 2). For achievement of this gene delivery, recombinant adenoviral vectors have been used, based on their superior *in vivo* efficacy characteristics (7–10). In this regard, a number of distinct approaches have been tried in both animal models and human clinical trials. These approaches have included delivery of the *p53* tumor suppressor gene or toxin-encoding genes, such as herpes simplex virus thymidine kinase and cytosine deaminase (11–14). Thus, the basic paradigm of these gene therapy interventions assumes *in vivo* transduction of adequate efficiency to realize the potential therapeutic benefit of the delivered transgenes.

Whereas adenoviral vectors are understood to exhibit superior levels of *in vivo* gene transfer compared with available alternative vector systems, their present level of efficiency may nonetheless be suboptimal for SCCHN gene therapy applications (15). In several reported human clinical trials based on *in vivo* gene delivery, relative resistance of target tissues to adenoviral infection has been noted (16). Deficiency of the primary Ad receptor, CAR, has been understood to be the biological basis of this phenomenon (17, 18). There is a significant disparity between the adenoviral vector efficacies observed *in vitro* using cell lines and the tumor transduction rates achievable in the context of *in vivo* gene delivery schemas (19). In addition, it has been observed in human clinical trials that dose-rated vector toxicities may limit the overall therapeutic index achievable with adenoviral vectors (20, 21). Strategies that increase the gene transfer efficacy of adenoviral vectors may allow the use of

³ The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; CAR, coxsackie-adenovirus receptor; FBS, fetal bovine serum; DMEM/F-12, DMEM/Ham's F-12; mAb, monoclonal antibody; pfu, plaque-forming unit; m.o.i., multiplicity of infection; Ad, adenovirus; RLU, relative light unit.

lower vector doses and improve the overall therapeutic potential of gene therapy approaches.

We have endeavored to modify the tropism of adenoviral vectors to achieve cell-specific targeting to tumor cells as a means of improving the overall feasibility of these cancer gene therapy strategies (22). Retargeting approaches based on immunological modification of the virus have achieved cell-specific gene delivery via routing the virus to nonnative cellular entry pathways (18, 23, 24). In addition, we have achieved Ad retargeting using genetic strategies to alter tropism. These latter methods are based on genetic alterations of the virion capsid proteins as a means to modify the interaction of the vector with target cells (25, 26). In the current study, we demonstrate that SCCHN tumor cells exhibit relative resistance to Ad vectors based on the deficiency of the primary Ad receptor, CAR. Furthermore, we show that we can circumvent this resistance by using an adenoviral vector genetically modified to allow CAR-independent cellular targeting (27). Importantly, this retargeting was achieved via dysregulated cellular receptors characteristic of SCCHN, thereby providing a level of tumor cell-specific gene delivery. These studies provide the rationale to modify current SCCHN gene therapy paradigms by incorporating the next generation of adenoviral vectors that use modified gene delivery characteristics. Such vector modifications are also clearly relevant to a variety of other cancer gene therapy approaches.

MATERIALS AND METHODS

Cell Lines and Primary Human SCCHN Tumor Samples. Three human head and neck tumor cell lines and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). The cell lines studied were FaDu (pharyngeal squamous cell carcinoma), SCC-4 and SCC-25 (tongue squamous cell carcinoma), and HeLa. FaDu cells were grown in MEM supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY), 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate. SCC-4 and SCC-25 cells were grown in DMEM/F-12 at a 1:1 ratio by weight with 10% FBS, 2 mM glutamine, and 400 ng/ml hydrocortisone (Sigma, St. Louis, MO). HeLa cells were cultured in DMEM/F-12 with 10% FBS and 2 mM glutamine. 293 cells (28) obtained from Microbix (Toronto, Canada) were maintained in DMEM/F-12 supplemented with 10% FBS. All cells were cultured at 37°C in a 5% CO₂ atmosphere. Primary human SCCHN tumor samples obtained during surgery (The University of Alabama at Birmingham, Birmingham, AL) were transported to the laboratory and processed for experiments. Briefly, tumor or normal tissue was finely minced, distributed into approximately equal aliquots, weighed, and then overlaid with 100 µl of Opti-MEM (Life Technologies, Inc.). For all experiments, 10–20 mg samples of tissue were used.

Recombinant Adenoviral Vectors. A recombinant E1A/B-deleted Ad (29) expressing the luciferase reporter gene (AdCMVLuc) was provided Dr. R. D. Gerard (University of Leuven, Leuven, Belgium). A similar Ad that also expresses luciferase from the cytomegalovirus promoter but contains an integrin targeting peptide within the HI loop (Ad5lucRGD) was constructed as described previously (27). Recombinant Ads were propagated on the permissive 293 cell line, purified using

a cesium chloride gradient, and subsequently plaque titered on 293 cells using standard methods (30). Virus stocks were stored at –80°C until use.

Immunohistochemical Reagents. Mouse anti-CAR monoclonal antibody (RmCB) prepared as ascites fluid was obtained from Dr. R. L. Crowell (Hahnemann University, Philadelphia, PA; Ref. 31). The anti- $\alpha_v\beta_3$ mAb LM609, the anti- $\alpha_5\beta_5$ mAb P1F6, the anti- $\alpha_2\beta_1$ mAb BHA2.1, and the anti- $\alpha_3\beta_1$ mAb MAB1992 were purchased from Chemicon International Inc. (Temecula, CA). Control mouse IgG and FITC-conjugated F(ab')₂ fragments of antimouse IgG were purchased from Sigma.

Purification of Ad5 Knob Protein. The knob domain of Ad5 fiber was expressed in *Escherichia coli* with an N-terminal 6×His tag using the pQE30 expression vector (Qiagen, Hilden, Germany) as described previously (32). In brief, the knob domain plus the last repeat of the shaft domain of the fiber gene was cloned into *Bam*HI-*Hind*III-digested pQE30, resulting in plasmid pQE.KNOB5. Recombinant knob was expressed in *E. coli* M15(pREP4) cells harboring pQE.KNOB5 and purified on Ni-NTA agarose columns (Qiagen). The ability of the recombinant knob to block infection by Ad was verified as described previously (32).

Ad Infection Assays. To assess adenoviral vector infection, 10⁵ cells of each cell line were plated in triplicate into each well of 12-well plates in the presence of 1 ml of culture media. The cells were then incubated overnight to allow adherence. Prior to infection, the cells were incubated in 300 µl/well of the media containing 2% FBS, with or without knob protein at a final concentration of 20 µg/ml for 15 min. Infection complexes mixed in a final volume of 300 µl containing AdCMVLuc or Ad5lucRGD (10–250 pfu/cell) or AdCMVLuc/knob protein (20 µg/ml) or Ad5lucRGD/knob protein (20 µg/ml) were added to each well. The cells were incubated at 37°C in 5% CO₂ for 1 h, washed with PBS (pH 7.4), and then supplemented with 1 ml of complete media. Forty-eight h after the infection, the cells were rinsed with PBS and assayed for luciferase expression by enzyme assay or by *in situ* hybridization of luciferase mRNA. For all luciferase enzyme assays, the cells were lysed in 200 µl of lysis buffer (Promega, Madison, WI). Ten µl of each sample were subsequently mixed with 50 µl of Promega luciferase assay reagent according to the manufacturer's instructions, and duplicate determinations of triplicate samples were assayed in a Berthold luminometer. For primary tissues, the minced aliquots of tissue were incubated in 1 ml of Opti-MEM with or without 20 µg/ml knob protein for 30 min and then infected by AdCMVLuc or Ad5lucRGD (5 × 10⁷ pfu/10 mg tissue) for 1 h. After replacing the media (Opti-MEM containing antibiotics), the tissue was incubated for an additional 24 h. The tissue was then homogenized and centrifuged. The collected supernatant was then used for the luciferase assay and the measurement of protein concentration. All assays were performed in triplicate.

Indirect Flow Cytofluorometry. Cultured cells were washed with PBS and harvested with Versene (Life Technologies, Inc.) for 15 min. Detached cells were centrifuged and resuspended in PBS containing 1% BSA and 0.1% sodium azide (1% BSA/PBS) at a concentration of 10⁵ cells/ml. The cells were then incubated with primary antibodies for 1 h on ice. Subsequently, the cells were washed and incubated with FITC-

conjugated antimouse IgG for an additional 1 h. After washing with 1% BSA/PBS, the cells were analyzed by flow cytometry.

In Situ Hybridization of Luciferase mRNA. A protocol for the *in situ* hybridization technique is described in detail elsewhere (33, 34). Briefly, cells were plated into each well of 12-well plates in the presence of 1 ml of culture media. After the cells reached subconfluence, they were transduced by AdCMVLuc or Ad5lucRGD at 250 pfu/cell for 1 h. After an additional 48 h of incubation, the cells were rinsed with PBS and resuspended in Versene (Life Technologies, Inc.). After centrifugation, the cells were finally resuspended in diethyl pyrocarbonate-treated PBS at a concentration of 10^6 cells/ml. Cells in 100 μ l of each sample were attached to the glass slide by a cytospin. The cells were then rinsed with PBS and fixed for 1 h in 3% paraformaldehyde at room temperature. Fixed cells were treated with 0.2 M HCl to inhibit endogenous alkaline phosphatase activity, acetylated with 0.1 M triethanolamine and acetic anhydride to decrease background staining, and hybridized overnight at 50°C with 400 pg/ml/kb of relevant riboprobe in hybridization solution. The hybridization solution consisted of 50% formamide, $4\times$ SSC, $1\times$ Denhardt's solution (Sigma), 500 mg/ml heat-denatured herring sperm DNA, 250 mg/ml yeast tRNA, and 10% dextran sulfate. After hybridization, the cells were rinsed with $2\times$ SSC followed by sodium chloride-Tris-EDTA buffer and treated with RNase A (20 mg/ml in sodium chloride-Tris-EDTA) for 30 min at 37°C to remove excess nonhybridized probe. Next, a series of progressive stringency washes were performed with $2\times$ SSC, $1\times$ SSC, $0.5\times$ SSC, and 0.15 M Tris-NaCl (pH 7.5) with normal horse serum. The cells were then stained with alkaline phosphatase-conjugated anti-digoxigenin antibody at a concentration of 1:5000 for 1 h. The cells were washed with Tris-NaCl and transferred to a basic Tris buffer with $MgCl_2$ (pH 9.5). Finally, the slides were incubated with the enzyme substrate solution (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Boehringer Mannheim) overnight in dark, humid chambers at 4°C. The color reaction was stopped by rinsing the slides in Tris-EDTA buffer (pH 8.0).

RESULTS

Adenoviral Vector-mediated Gene Transfer to Human SCCHN Cell Lines. Based on their high *in vivo* efficiency, adenoviral vectors have been used in a variety of cancer gene therapy approaches (35). Nevertheless, dose-related toxicities and poor *in situ* transduction rates in human trials have suggested that adenoviral vectors, in their present form, may be suboptimal for this application (36). To address this issue, we evaluated the efficiency of adenoviral vectors for human SCCHN lines as a measure of their utility in this disease context. For these studies, we used a replication-defective adenoviral vector encoding the luciferase reporter gene, AdCMVLuc, as a means to quantitatively determine transduction levels. The viral vector was delivered to cells in culture at a fixed m.o.i. of 10 pfu/cell, and 48 h later, cells were evaluated for luciferase gene expression. In addition, parallel experiments were carried out in the presence of recombinant fiber knob protein. Recombinant knob protein has been shown to specifically inhibit CAR-dependent Ad infection (18, 27, 32). Knob protein blocks the interaction of the adenoviral vector with its target receptor, CAR

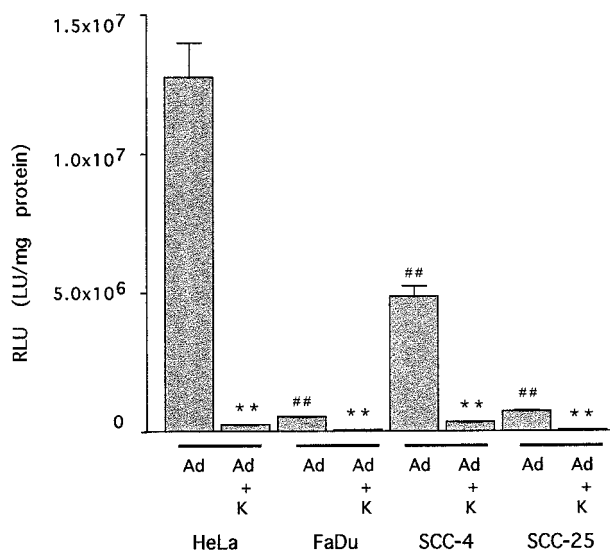


Fig. 1 Gene transfer to human cell lines via the luciferase-expressing replication-defective adenoviral vector AdCMVLuc. Human SCCHN cell lines FaDu, SCC-4, and SCC-25 and the positive control cervical carcinoma cell line HeLa were infected at a m.o.i. of 10 vector particles/cell and analyzed for the product of the luciferase gene after 48 h. Blocking experiments were also carried out with recombinant fiber knob (K). The protein concentration of the lysates was determined, and the RLU of luciferase/milligram of total cellular protein is shown graphically. The results are shown as the mean of multiple assays. The number of replicates (n) = 3; error bars, SE. *P*s were determined using the knob protein negative control (**). The knob protein negative control in HeLa cells (##).

(32), providing an index of the degree to which observed gene transfer is mediated through CAR pathways. The highly infectible human cell line HeLa was used as a positive control for comparing relative levels of infection by Ad.

In these studies, the control HeLa cell line demonstrated high levels of vector-mediated gene delivery, as anticipated (Fig. 1). However, the human SCCHN cell lines were significantly less susceptible to Ad-mediated infection than the control HeLa cells (Fig. 1). In this regard, the observed luciferase activity was 4.8×10^5 RLU/mg protein for FaDu and 6.9×10^5 RLU/mg protein for SCC-25. These reporter gene magnitudes were 4.0% and 5.7%, respectively, of the levels observed for HeLa. The SCCHN cell line SCC-4 exhibited a slightly higher degree of susceptibility, demonstrating luciferase levels that were 38% of those observed for HeLa. Parallel studies carried out with knob competition exhibited a >90% blockade in both the HeLa and SCCHN cell lines. Thus, the observed levels of transduction were accomplished via CAR-dependent pathways. These data indicated that SCCHN cells were significantly less susceptible than HeLa to adenoviral vector-mediated gene transfer. Furthermore, these data suggested that the major cellular factor involved in adenoviral vector infection of human SCCHN cell lines was the primary adenoviral receptor, CAR.

Expression of CAR and Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on Human SCCHN Cell Lines. We next investigated the biological basis of the relative resistance of human SCCHN cell

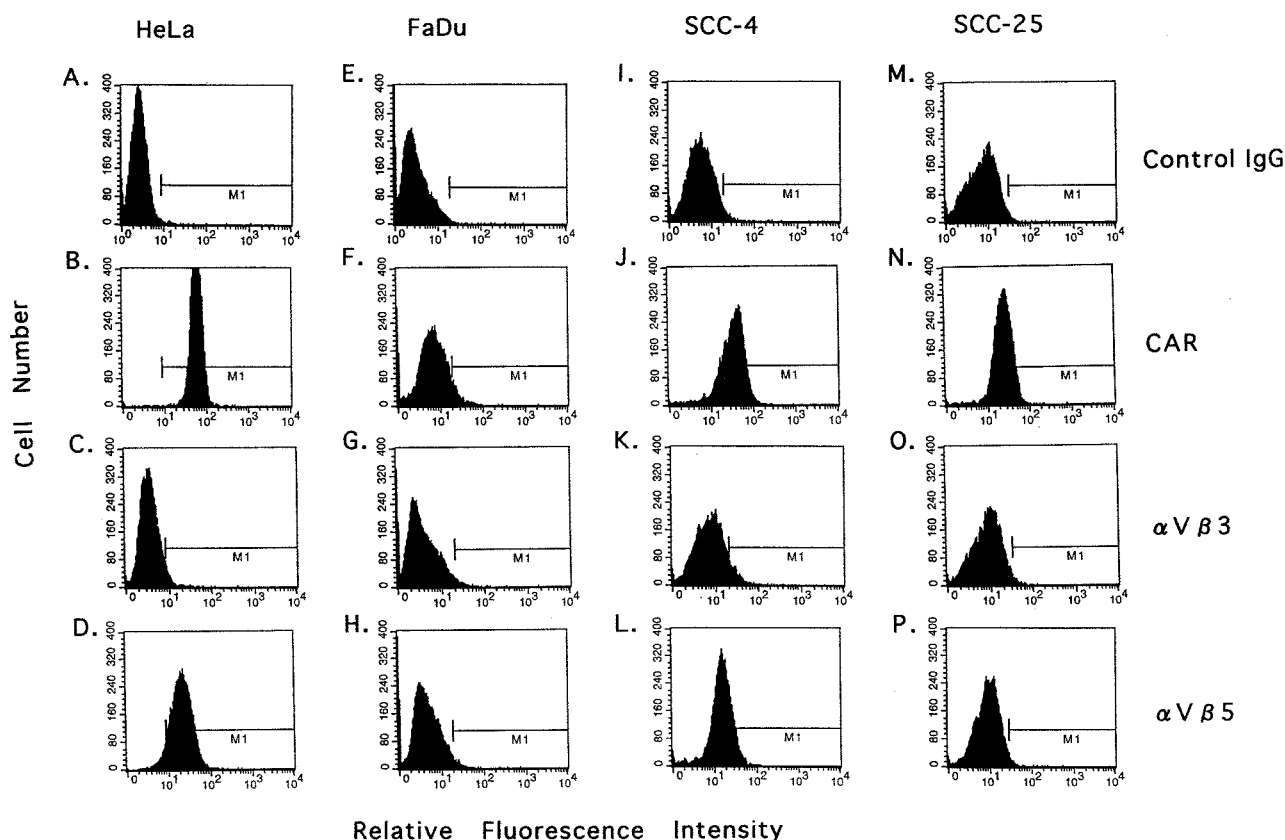


Fig. 2 Expression of CAR and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in SCCHN human tumor cell lines. Indirect flow cytometry is shown for the human SCCHN cell lines FaDu, SCC-4, and SCC-25 and for the positive control cervical carcinoma cell line HeLa.

lines to adenoviral vector-mediated gene transfer. In this regard, Ad accomplishes initial binding to target cells via the primary receptor, CAR (17). After the initial binding step, the particle achieves internalization via interaction with integrins of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ class (37–39). Relative resistance to adenoviral vectors has been noted to occur based on a deficiency of either or both of these cellular receptor classes (17, 18, 40). On this basis, we evaluated the relative levels of both receptor classes in the resistance of SCCHN cell lines as compared with levels in the highly permissive HeLa cell line. Direct flow cytometry was carried out using antibodies specific for CAR and the indicated integrins.

These studies demonstrated that the positive control HeLa cells expressed high levels of CAR (Fig. 2B). The positive ratio and mean fluorescent intensity of CAR on HeLa were 99.6% and 57.9, respectively. In contrast, the levels of CAR detected for the SCCHN cell lines were greatly reduced. In this regard, the mean fluorescent intensity of CAR in FaDu, SCC-4, and SCC-25 cells was 8.9, 36.2, and 25.3, respectively (Fig. 2, F, J, and N). In contrast, the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin levels did not differ from those of HeLa by a margin as substantial as that noted for CAR. On this basis, we concluded that the relative CAR deficiency in SCCHN cells provided the principal explanation for the relative resistance of these cells to adenoviral vector transduction.

Expression of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ Integrins on Human SCCHN Cell Lines. The paucity of the primary adenoviral receptor CAR on the SCCHN tumor targets mandated the consideration of alternative receptors to allow adenoviral vector infection of these cells. In this regard, a number of specific growth factor receptors and integrins have been shown to be specific markers of SCCHN. Two candidates in the latter category include the integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$. Of note, these integrins function as potential receptors with recognition of RGD peptide motifs. We have previously demonstrated the capacity of RGD peptide motifs genetically incorporated into the adenoviral capsid to alter vector tropism (27). On this basis, we rationalized that the presence of these candidate receptors on the human SCCHN cell lines might serve as targets for gene transfer in the present context. We thus used a similar flow cytometry analysis to determine the presence of the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins (Fig. 3). These key SCCHN markers were highly expressed in our study target cells and represent candidate tumor-specific receptors. The presence of these receptors provides the rationale for a retargeting strategy based upon using a RGD peptide motif for specific recognition of these tumor-specific integrins.

Gene Transfer to Human SCCHN Cell Lines via CAR-independent Pathways. The CAR deficiency that was observed on the SCCHN cells suggests a requirement to reroute

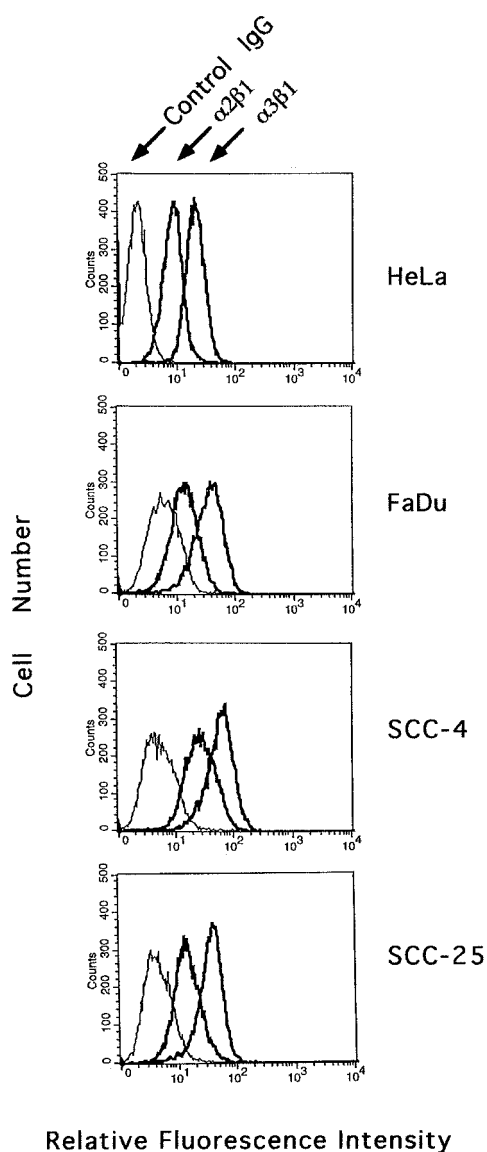


Fig. 3 Expression of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins in SCCHN human tumor cell lines. Indirect flow cytometry was carried out as described for Fig. 2, but mAbs directed against the cellular integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ were used.

the virus via non-CAR pathways to achieve efficient gene delivery. In this regard, we have developed methods to achieve tropism modification of adenoviral vectors to allow retargeting of the particle via alternative pathways (22). We have genetically engineered retargeting motifs into the adenoviral capsid to accomplish tropism alterations (25, 26). Specifically, the RGD-4C peptide was incorporated into the HI loop of the fiber knob. This modified virion can achieve primary interaction with and attachment to target cells via the affinity of the incorporated RGD-4C peptide for cellular integrins (27). Of note, our initial studies have shown that this schema allows the achievement of CAR-independent gene transfer to cells deficient in CAR expression. Furthermore, such a peptide targeting motif would be

predicted to be of utility for the dysregulated integrins that are characteristic of SCCHN. Thus, this vector may offer useful capacities to achieve enhanced and targeted gene delivery to this tumor cell type.

We thus used the genetically modified Ad encoding the luciferase gene, Ad5lucRGD, for transduction of the human SCCHN cell lines. Direct comparison was made in these studies with the nonmodified control virus AdCMVLuc. Of note, the reporter gene is driven by the cytomegalovirus promoter in both of these Ad vectors. The application of Ad5lucRGD to HeLa cells resulted in a 4-fold augmentation of gene transfer compared with the control virus AdCMVLuc (Fig. 4A). The addition of recombinant knob had no significant inhibitory effect on gene transfer via Ad5lucRGD, confirming that the augmented levels of transgene expression represented transduction that had occurred via non-CAR pathways. We next applied Ad5lucRGD to the human SCCHN cell lines. In these studies, a very dramatic augmentation in gene transfer was noted in these otherwise Ad-refractory cells. Specifically, the FaDu (Fig. 4B), SCC-4 (Fig. 4C), and SCC-25 (Fig. 4D) cells show 35-, 18-, and 77-fold enhancements in gene transfer, respectively. Significantly, knob competition had no effect on the gene transfer to these cells accomplished via the Ad5lucRGD vector. Thus, the achievement of CAR-independent gene transfer to SCCHN cells and potentiated a highly augmented level of gene transfer.

To confirm these studies, we next evaluated the actual transduction frequency achieved via this modified vector approach. For this analysis, we used *in situ* hybridization analysis to detect the mRNA transcript of the luciferase reporter gene. To this end, luciferase mRNA was hybridized with a digoxigenin-labeled riboprobe and detected by using the enzyme cytochemical technique. As a control, uninfected cells of the SCCHN cell line SCC-25 showed no positive signal (Fig. 5A). Due to the detection limits of the *in situ* hybridization assay, we chose to use a m.o.i. of 250 pfu/cell. The use of a lower m.o.i. resulted in undetectable staining in cells infected with Ad5CMVLuc. Infection of these cells with AdCMVLuc at a m.o.i. of 250 pfu/cell induced limited positive staining. In contrast, cells infected with Ad5lucRGD at the same m.o.i. showed an enhanced signal, indicating an infection frequency of >80%. The relative luciferase activities achieved by these vectors, 4.1×10^7 and 3.3×10^9 relative light units per mg of protein, respectively, were compatible with the results of the *in situ* hybridization. On the basis of these studies, it is thus apparent that Ad5RGDluc infects a significantly greater fraction of the target tumor cells. Thus, the expedient of CAR-independent gene transfer allows a dramatic augmentation in both gene transfer levels and the transduction frequency in human SCCHN cells. This latter parameter is the key factor that predicts the ultimate utility of any cancer gene therapy approach.

Gene Transfer to Primary Explants of Human SCCHN.

Whereas cell lines provide an index of the tissue-specific parameters relevant to gene transfer, the analogy to human tumors is imprecise. In this regard, vector efficiencies in primary material are frequently distinct from those obtained with cell lines (18). In addition, gene transfer frequencies in human cell lines/murine xenograft models frequently overestimate the infection rates ultimately obtainable in the context of human clinical gene

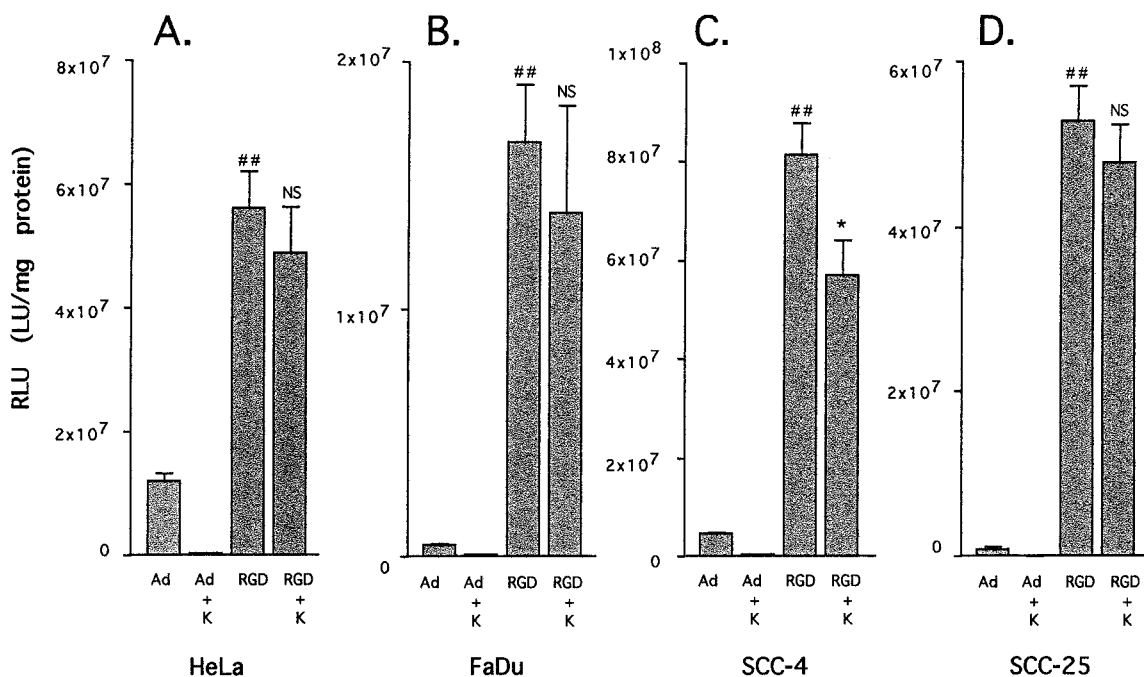


Fig. 4 Comparison of the relative gene transfer efficiencies of AdCMVLuc and Ad5lucRGD for HeLa (A) and the human SCCHN tumor cell lines FaDu (B), SCC-4 (C), and SCC-25 (D). Ad5lucRGD contains a RGD motif in the HI loop of the fiber protein for targeting to specific integrins. Analysis was carried out as described for Fig. 1, where $n = 3$. P s were determined using the knob protein negative control (**) and the knob protein negative control in HeLa cells (##). The difference is not statistically significant (NS) when compared with the knob protein negative control.

therapy trials (41, 42). On this basis, primary human material represents a key substrate for studies to establish the validity of developed vector approaches. In addition, the parallel analysis of targeted vectors in tumor and corresponding normal tissue provides insight as to the infection differential that may be accomplished in human clinical use. This differential may be the key factor that dictates the therapeutic index of a given gene therapy approach. We also explored our tropism-modified adenoviral vectors in the context of normal buccal mucosa, the normal tissue substrate relevant to SCCHN.

In these studies, primary tumor cells exhibited a relative resistance to the adenoviral vector AdCMVLuc as compared with the human SCCHN cell lines (Fig. 6). These findings validate the frequently noted disparity in primary tumor and cell line data and highlight the difficulty in achieving meaningful transduction rates to human tumors *in situ*. We next compared these findings with the Ad5lucRGD virus. Of note, Ad5lucRGD accomplished augmented gene transfer to this otherwise refractory tumor target. Specifically, augmentations of 2.4- and 5.8-fold were noted in two independent isolates of primary tumor. Competition studies with knob confirmed that the observed augmentations occurred via the achievement of CAR-independent gene transfer. Thus, for fresh primary tumor material, which represents the clinically relevant study substrate for vector analysis, we have shown that CAR-independent gene transfer allows significant augmentation of gene transfer to human SCCHN tumors. Of further note, no infection differential between AdCMVLuc and Ad5lucRGD could be noted in normal buccal mucosa (Fig. 6). This important finding predicts that this

CAR-independent approach will allow an improved tumor to normal gene transfer differential and thus a potentially improved therapeutic study.

DISCUSSION

Fundamental to the realization of therapeutic gain via gene therapy is the ability to deliver genes to target cells with a requisite level of efficiency. On this basis, a number of cancer gene therapy strategies have been developed embodying methods of *in vivo* gene transfer to tumor cells (35). SCCHN has been a particularly attractive candidate for these approaches, based on disease context factors. For these strategies for SCCHN, as well as for a variety of other *in vivo* cancer gene therapy approaches, limited *in situ* transduction has precluded any understanding of valid therapeutic potential. Adenoviral vectors used for *in vivo* gene delivery have shown limited efficacy in this stringent context (36). It is thus understood that any maneuvers to enhance the gene delivery efficacy of adenoviral vectors will have a high level of significance for realizing the therapeutic potential of these cancer gene therapy schemas.

To this end, we have explored the basis of SCCHN tumor refractoriness to adenoviral vectors. We have found that a relative paucity of the primary adenoviral receptor, CAR, is the major factor limiting gene transfer by this vector. Of note in this regard, primary SCCHN tumor cells exhibited an even more extreme resistance to adenoviral infection than their cell line counterparts. This phenomenon has been noted in other tumor contexts in which a deficiency of the adenoviral primary recep-

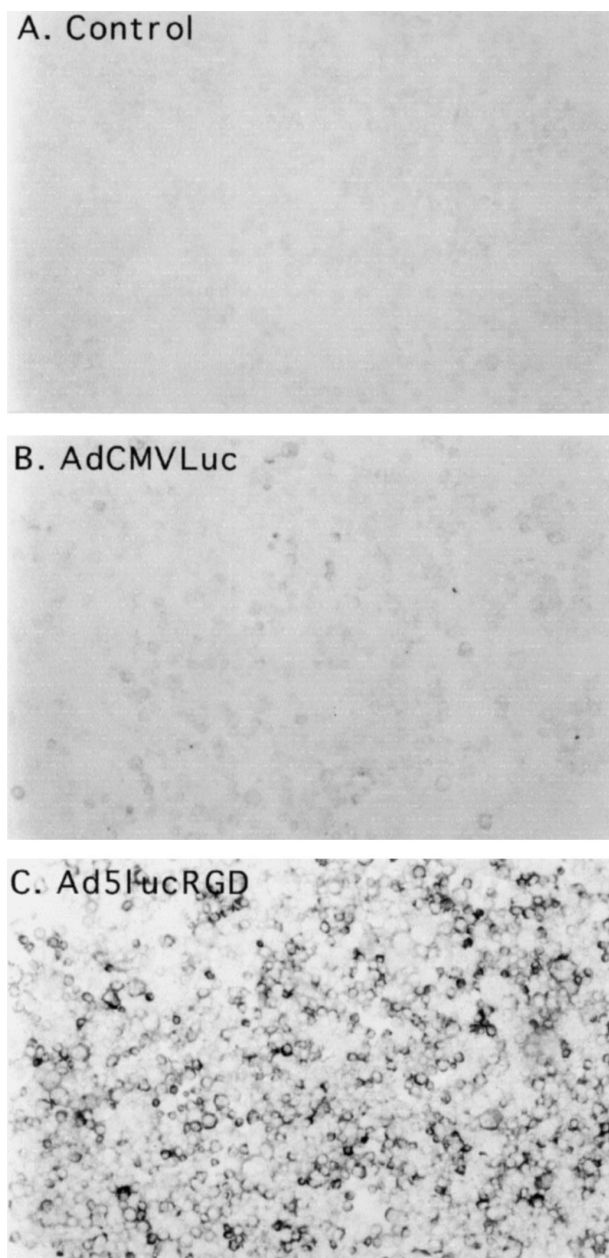


Fig. 5 Analysis of the relative gene transfer frequency of AdCMVLuc and Ad5LucRGD for SCCHN cell lines. Infection of SCC-25 cells was performed at a m.o.i. of 250 particles/cell. Forty-eight h after infection, cells were analyzed for the product of the reporter gene by *in situ* hybridization with a probe for luciferase mRNA.

tor limits vector efficacy (18). It is thus clear that the issue of CAR deficiency may present a more general problem that will represent a confounding factor in all cancer gene therapy strategies based on *in vivo* gene delivery.

As a means to address this issue, we attempted to achieve gene delivery to these refractory tumor cells via CAR-independent pathways. We used an adenoviral vector capable of achieving primary cell attachment via binding to dysregulated cellular integrins characteristic of SCCHN tumor cells. This was

achieved by genetic modifications of the virion to allow the incorporation of an integrin-binding peptide motif in the HI loop of the fiber knob. We demonstrated that this vector accomplished CAR-independent gene transfer to SCCHN cell lines and primary tumor explants. Importantly, such CAR-independent gene transfer allowed dramatic augmentations of the efficacy of transgene expression in these tumor cells. On this basis, methods that accomplish CAR-independent gene transfer may present a more general means to augment gene transfer efficiency in those contexts in which native CAR levels may be suboptimal for gene delivery processes. In addition, the achievement of gene transfer via a dysregulated receptor cellular receptor characteristic of the SCCHN neoplastic phenotype potentially provided a level of tumor cell specificity.

The disease context of SCCHN has allowed gene therapy approaches based on *in situ* delivery into the tumor (7–10). Whereas this scenario allows a means to achieve a level of cell specificity via the locoregional delivery approach, suboptimal tumor transduction has represented a major limitation. Thus, our vector schema described here was designed to allow the enhancement of gene delivery by addressing the CAR deficiency aspect of SCCHN tumor biology. In addition, the exploitation of a tumor-specific marker allows an enhanced tumor:normal infection differential. The dramatic augmentation of gene transfer noted with Ad5LucRGD for SCCHN tumor cells was not noted for normal buccal mucosa. Of note in this regard, integrin dysregulation has been described in the context of SCCHN (43). Whereas this has mainly been noted for integrins of the $\alpha_v\beta_3$ class in SCCHN, it is noteworthy that at least 12 of the described integrin heterodimers, including $\alpha_2\beta_1$ and $\alpha_3\beta_1$, recognize the RGD sequence contained in the HI loop of the modified adenoviral vector (44–47). Whereas we cannot rule out the possibility that other RGD-binding integrins are used by the integrin-targeted vectors used in this study, the expression patterns of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ in SCCHN cell lines and tumors make them likely candidate targets in the present study. In this study, we also demonstrated the expression of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins on SCCHN tumor cell lines. The differential augmentations of Ad5LucRGD for tumor tissue and normal tissue in this case may thus be understood to potentially allow a more favorable therapeutic index than is currently achievable via unmodified adenoviral vectors.

Studies by O'Malley *et al.* (8) have suggested that adenoviral vector doses required to achieve adequate tumor transduction may be associated with limiting toxicity in the context of SCCHN gene therapy. Indeed, vector dose appears to be a critical issue in relation to adenoviral vector-induced immunogenicity (35, 36). Clearly, maneuvers to reduce the viral dose may have a beneficial effect with respect to these parameters. Thus, our description of a viral vector with dramatically augmented efficiencies clearly has potential implications in a variety of genetic diseases for which adenoviral vector-based approaches have been proposed, such as cystic fibrosis and Duchene's muscular dystrophy (48). In these contexts as well, CAR deficiency is a major factor limiting vector utility. It may thus be critical to the overall usage of adenoviral vectors to devise CAR-independent gene schemas relevant to specific tissue contexts. Maneuvers such as those we describe here may thus allow the use of viral doses commensurate with achieving

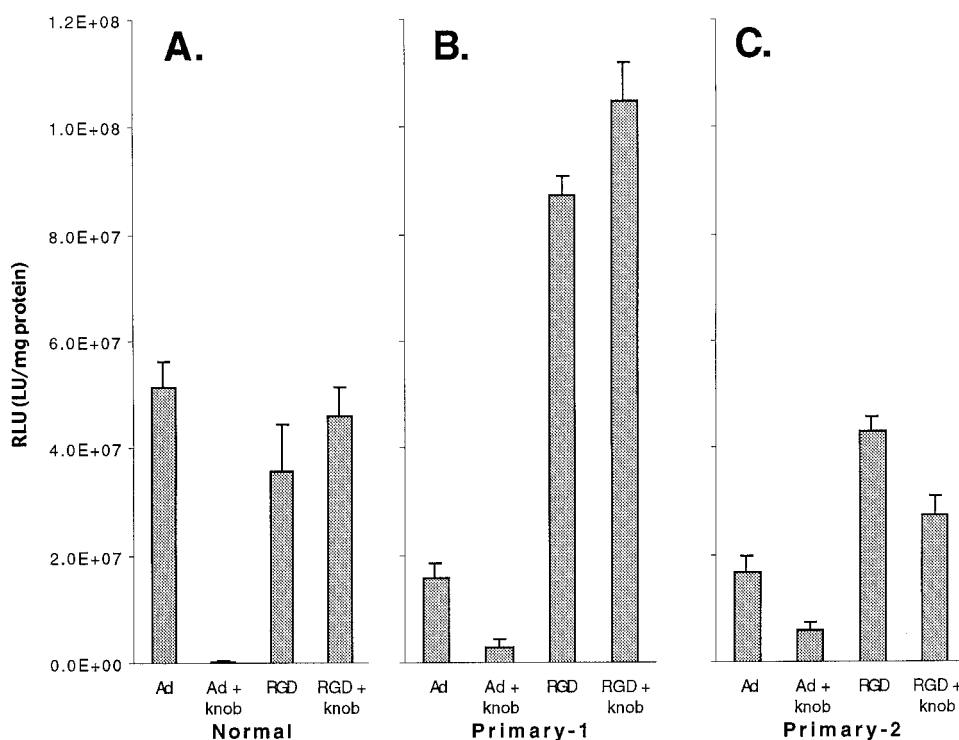


Fig. 6 Analysis of the differential gene transfer efficiency of AdCMVLuc (*Ad*) and Ad5lucRGD (*RGD*) for primary SCCHN tumor and normal buccal mucosa. Fresh tissue (10–20 mg) was prepared from patients and infected with the adenoviral vectors (10^6 vector particles/mg tissue). Twenty-four h later, cells were analyzed for the expression of the luciferase gene product. Results are the mean from duplicate assays. Error bars, SE.

meaningful therapeutic results in a variety of gene therapy approaches.

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