Retargeting to EGFR Enhances Adenovirus Infection Efficiency of Squamous Cell Carcinoma

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Background: Adenovirus-mediated gene therapy has been used for squamous cell carcinoma of the head and neck (SCCHN), but the in vivo efficacy has been limited by a lack of tissue specificity and low infection efficiency. We are interested in improving cancer gene therapy strategies using targeted adenovirus vectors.

Objective: To determine if the infection efficiency of adenovirus-mediated gene transfer to SCCHN cells could be enhanced by retargeting to the epidermal growth factor receptor (EGFR), which is known to be overexpressed in these tumors.

Design: Epidermal growth factor receptor retargeting in SCCHN cells was accomplished with a bispecific antibody that recognized the knob domain of adenovirus as well as EGFR. Using this retargeting schema, we compared the infection efficiency and specificity of unmodified and EGFR-retargeted adenovirus.

Results: Squamous cell carcinoma of the head and neck cell lines were shown to be infected by adenovirus with low efficiency, which is likely because of the low level of adenovirus receptor expressed in the SCCHN cells. Epidermal growth factor receptor retargeting markedly enhanced transduction in both SCCHN cell lines and primary tumor tissue, as indicated by the elevated levels of reporter gene expression. Furthermore, retargeting enhanced infection of tumor tissue compared with normal tissue from the same patient.

Conclusions: Epidermal growth factor receptor retargeting enhanced adenovirus infection of SCCHN cells and, in doing so, augments the potency of the vector. This modification makes the vector potentially more valuable in the clinical setting.


IN 1998, AN estimated 52 000 new cases of oral cavity cancers will be diagnosed in the United States, accounting for approximately 16 000 deaths.1 The majority of these oral cancers are squamous cell carcinoma of the head and neck (SCCHN), and 20% to 30% of the patients die from the disease within 5 years.2 The prognosis for advanced (stage IV) SCCHN disease is poor, and there is no effective salvage therapy for patients in whom conventional treatments fail. Of note, the standard treatments of radiation, chemotherapy, and surgery for SCCHN have failed to improve patient outcome for the last 3 decades, which argues for the development of new therapeutic modalities. In this regard, gene therapy represents one novel therapeutic modality that has been proposed for SCCHN.3-10 Gene therapy strategies for SCCHN include mutation compensation and molecular chemotherapy. Mutation compensations attempt to correct the specific genetic defects in cancer cells. For SCCHN, such approaches have included replacement of defective proteins important in cell cycle regulation, including p53, p16, and p21.3,11 Another mutation compensation strategy was designed to abrogate a dysregulated signal transduction pathway by downregulating expression of the epidermal growth factor receptor (EGFR).12 Molecular chemotherapy approaches applied to SCCHN have included delivery of the herpes simplex virus thymidine kinase gene and Escherichia coli cytosine deaminase, both of which direct local prodrug conversion to cytotoxic products.

Several inherent characteristics of SCCHN suggest that such gene therapy approaches may be uniquely feasible in this disease. In this regard, most SCCHN tumors are localized to the oral pharynx cavity, making the tumors accessible to direct intratumoral injection of the gene therapy vector. Intratumoral injection of the vector has the added benefit of

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METHODS

VIRUSES AND ANTIBODIES

An E1-, E3-deleted adenovirus vector expressing firefly luciferase from the cytomegalovirus (CMV) immediate early promoter, AdCMV-Luc,26 was obtained from Robert Gerard, PhD (University of Leuven, Leuven, Belgium). The adenovirus vector AdCMV-LacZ, which expresses E coli β-galactosidase (β-Gal), was originally obtained from Jay Kolls, MD (Louisiana State University, New Orleans). Viruses were propagated and plaque-titered on permissive cell line 293 and purified by double centrifugation on cesium chloride gradients, as described elsewhere.27 All virus preparations were dialyzed against phosphate-buffered saline (PBS), aliquoted, and stored at −80°C. Titers were determined using standard plaque assays.

Ascites fluid containing monoclonal antibody (mAb) RmcB, a neutralizing mAb that recognizes CAR, was received from Robert W. Finberg, MD (Dana-Farber Cancer Institute, Boston, Mass), and has been described previously.28 Murine mAb 425 to human EGFR was received from Zenon Steplewski, PhD (Wistar Institute, Philadelphia, Pa), and the neutralizing murine mAb 1D6.14 specific for the knob domain of adenovirus serotype 5 fiber protein has been previously described.18 The bispecific antibody Fab-425 was generated as described elsewhere.21 Monoclonal antibody 425 was chemically conjugated to the Fab fragment of mAb 1D6.14 using the bifunctional crosslinker N-succinimidyl 3-(2-pyridyldithio)propionate to a molar ratio of 1:1. The resulting conjugate was then purified by size exclusion chromatography.

TUMOR CELLS

The human SCCHN cell lines SCC-25, FaDu, and SCC-4 were purchased from the American Type Culture Collection, Rockville, Md. The FaDu and SCC-25 cells were cultured in Eagle minimum essential medium (MEM) with Earle balanced salt solution (BSS) containing 1% t-glutamine; nonessential amino acids, 1 mmol/L; and 10% fetal calf serum (FCS) (Gibco BRL, Life Technologies Inc, Rockville, Md). The SCC-4 cells were cultured in Ham F12/Dulbecco modified Eagle medium containing 1% t-glutamine; sodium pyruvate, 1 mmol/L; glucose, 10 mmol/L; hydrocortisone, 0.2 mg/mL; and 10% FCS. All cell lines were cultured at 37°C in 5% carbon dioxide atmosphere. Primary human SCCHN tumor samples obtained during surgery at the University Hospital of the University of Alabama at Birmingham were transported to the laboratory and processed for experimentation. Tumor and normal tissues were finely minced, distributed into approximately equal aliquots, weighed, and then overlaid with 100 µL of OptiMem (Gibco BRL, Life Technologies Inc). Normal tissue was defined as tissue that appeared clinically normal and was taken from the mucosa or underlying submucosa at a site that was at least 2 cm from the resection margin. For all experiments, 10 to 50 mg of tissue was used. For some experiments, a cell suspension was made by incubating the minced tumor tissue in Dispase protease solution (Worthington Biochemical Corp, Lakewood, NJ) and then filtering through glass wool. The cells were then grown in cell culture medium in a carbon dioxide incubator at 37°C. Twenty-four hours later, the cells were subjected to EGFR retargeting as described above. Permission to obtain excess surgical tissue was reviewed and approved by our Institutional Review Board for Human Experimentation.

FLOW CYTOMETRY

The cell monolayer was washed once with PBS (without magnesium and calcium) and then incubated with versene (Gibco BRL, Life Technologies Inc) at 37°C in a carbon dioxide incubator until cells were released from the plate surface. After centrifugation (400g for 5 minutes), the cell pellet was resuspended into PBS/bovine serum albumin (BSA)/azide (PBS containing 1% BSA and 0.1% sodium azide) at 1.2 × 10^6 cells per milliliter. Next, 1.2 × 10^7 cells were incubated with anti-CAR ascites (5.0 µL/mL), mAb 425 (2.0 µg/mL), or mouse IgG (2.0 µg/mL) for 1 hour at 4°C. Cells were then washed 3 times with PBS/BSA/azide and incubated with fluorescein isothiocyanate–conjugated antismouse IgG antibody (0.1 µg/mL; Sigma, St Louis, Mo) for 1 hour at 4°C. Cells were then washed 3 times with PBS/BSA/azide and incubated with flow cytometry at the Immunopathology Program flow cytometry facility of the University of Alabama at Birmingham. Positive CAR or EGFR expression was defined by a 1.5-fold or greater shift to the right in the mean fluorescence compared with the isotype antibody control.

ADENOVIRUS GENE TRANSFER

To assess native or EGFR-retargeted adenovirus infection efficiency, 5 × 10^4 cells were plated in 6-well plates and allowed to adhere overnight at 37°C. To demonstrate the specificity of infection, some cells were then blocked with either recombinant adenovirus type 5 (Ad5) knob protein (10-20 µg/mL) or mAb 425 (30 µg/mL) for 1 hour at 4°C. During the blocking period, 4 × 10^6 plaque-forming units (pfu) per milliliter of AdCMV–β-Gal were incubated with either PBS or Fab-425 in PBS (3.0 ng of Fab-425 per 4 × 10^6 pfu/mL) for 45 minutes at room temperature. The virus was then diluted with OptiMem, and the cells were infected at a multiplicity of infection (MOI) of 100 pfu per cell for 1 hour at 37°C. Cells were then washed with PBS, incubated in complete media, and assayed for β-Gal expression by X-Gal staining 24 hours after infection. Representative areas of the monolayer were photographed at ×20 magnification. To quantify native or EGFR-retargeted adenovirus gene transfer, 1 × 10^5 cells were plated in triplicate wells of 24-well plates and allowed to attach overnight at 37°C. During the blocking period, Fab-425 (described above and in the “Results” section) was incubated with either AdCMV–β-Gal or AdCMV-Luc for 45 minutes at room temperature. Following the blocking period, the cells were infected with native or EGFR-retargeted AdCMV-Luc at an MOI of 100 pfu per cell for 1 hour at 37°C, as described above. One hour later the cells were washed once with PBS and incubated for 36 hours in complete media. Cell lysates were assayed for luciferase expression 36 hours after infection in a Berthold luminometer using the Luciferase Assay System (Promega Corp, Madison, Wis), and the protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories Inc, Hercules, Calif) according to the manufacturer’s protocols.
increasing the specificity of the cells that are infected. Also, metastasis of the tumor is a late-stage occurrence, making early local-regional control of the tumor of primary importance. On the basis of these favorable characteristics, gene delivery strategies have been developed for SCCHN. To date, the majority of approaches for gene therapy of SCCHN have used adenovirus vectors. Adenovirus vectors are favored for their ability to transduce non-dividing cells with relatively high efficiency. In addition, high-titer preparations of adenovirus vectors can be produced with relative ease. Of note, phase 1 clinical trials employing adenovirus have been carried out for SCCHN. As observed for other tumor tissue types, a major concern associated with using adenovirus vectors in SCCHN is the limited tumor infection rates achieved in vivo. Thus, despite the fact that the adenovirus vector possesses the most favorable gene delivery characteristics, its utility for tumor infection in the SCCHN context has been disappointing.

To overcome the problems associated with in vivo gene delivery, retargeting of adenovirus has been endeavored as a means to improve its specificity and efficacy. Adenovirus serotypes 2 and 5 bind to the recently identified Cox-sackievirus-adenovirus receptor (CAR) via the knob domain of the fiber protein. Subsequently, a specific amino acid sequence motif (ie, Arg-Gly-Asp, or RGD) in the adenovirus penton base protein interacts with cellular αv integrins that facilitate internalization by receptor-mediated endocytosis. Retargeting allows adenovirus to bind to alternative cellular receptors, resulting in CAR-independent infection. One such method is based on immunological retargeting. This method uses an antifiber antibody or antibody fragment that is chemically conjugated to either a cell-specific ligand, receptor, or antireceptor antibody. It has been shown recently in cell culture that adenovirus can be targeted to specific cellular receptors, such as the folate receptor, basic fibroblast growth factor receptor, and EGFR. In cells that are poorly transduced by adenovirus because of their low levels of functional adenovirus fiber receptor, immunological retargeting may offer a means to increase the efficiency of gene transfer. Retargeting of adenovirus to EGFR was recently shown to enhance gene transfer in primary, low-passage glioma tumor cells, which suggests further clinical relevance for retargeting. Of note, EGFR is frequently overexpressed in SCCHN, making this a relevant receptor for targeting in SCCHN. In addition, EGFR has been suggested to be an early marker for carcinogenesis in SCCHN. On this basis, we evaluated the utility of an EGFR-retargeting approach for SCCHN. We demonstrate in the current study that such a retargeting approach has merit in the context of this disease by augmenting gene transfer to SCCHN tumor cells. These findings have important bearings on the clinical gene therapy approaches for SCCHN.

RESULTS

ADENOVIRUS GENE TRANSFER IN SCCHN AND HEla CELL LINES

To evaluate the utility of adenovirus vectors for gene transfer into SCCHN cells, we first investigated the infection efficiency of an unmodified recombinant adenovirus on a panel of SCCHN cell lines. For comparison, the highly transducible cervical cancer cell line HeLa was used. Three human SCCHN cell lines, SCC-23, FaDu, and SCC-4, were selected for the adenovirus infection assays. A quantitative analysis of gene transfer was performed by infecting cell lines with AdCMV-Luc, which expresses the luciferase reporter gene (Figure 1). Reporter gene expression in SCC-25, FaDu, and SCC-4 cells was 6.4-fold, 16.6-fold, and 10-fold less, respectively, than the luciferase gene expression in HeLa cells. These data suggest that SCCHN cells are relatively refractory to adenovirus infection, and, on this basis, we considered alternative strategies to achieve efficient gene transfer to these target cells using a modified adenovirus vector.

ANALYSIS OF TARGET RECEPTORS IN HUMAN SCCHN CELL LINES RELEVANT TO ADENOVIRUS INFECTION

The amount of gene transfer by adenovirus to the SCCHN cell lines in the previous experiment suggested that these cells express low levels of the native adenovirus receptor CAR. To ascertain the level of CAR expression in the 3 SCCHN cell lines, flow cytometry was performed using an anti-CAR antibody (Figure 2). In comparison to HeLa cells, in which greater than 90% of the cells expressed CAR, the percentage of SCCHN cells positive for CAR expression was 31% in SCC-25 cells, 17% in FaDu cells, and 9% in SCC-4 cells. These findings were consistent with the concept that a deficiency in the native adenovirus receptor results in cells being refractory to adenovirus infection. Thus, we considered alternative receptors known to be upregulated in SCCHN that could be exploited for targeting. Epidermal growth factor receptor has been reported to be frequently overexpressed on SCCHN tumors and expressed at low levels in the upper aerodigestive tract mucosa of normal epithelium in patients without SCCHN. Therefore, we evaluated SCCHN cell popula-
425 ablates endogenous tropism by inhibiting the bind-
ing of knob to CAR, and the anti-EGFR portion of Fab-425 redirects the viral particle to EGFR on the cell surface. Hereafter, EGFR retargeting refers to the infection of cells using an adenovirus that has been coated with Fab-425. For these experiments we used an MOI of 100 pfu per cell because the use of lower MOIs (ie, <10 pfu/cell) resulted in nondetectable levels of gene transfer using the unmodified adenovirus. In addition, Miller et al21 have shown experimentally that this virus-cell ratio is within the range for achieving an optimal EGFR retargeting index. Multiplicities of infection ranging from 20 to 200 pfu per cell gave similar EGFR retargeting indexes (data not shown). To demonstrate EGFR retargeting, SCCHN cells were infected with either native or EGFR-retargeted AdCMV-Luc. Twenty-four hours after the infection, cells were lysed and luciferase activity was measured. The protein concentration was determined for each cell extract, and the number of luciferase units was adjusted for the concentration of each cell extract. T-shaped bars indicate SEs.

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Figure 3. Quantification of epidermal growth factor receptor (EGFR) retargeting in squamous cell carcinoma of the head and neck cell lines. Retargeting was quantified using an adenovirus vector that expresses the luciferase reporter gene (AdCMV-Luc). FaDu (A) and SCC-4 (B) cell lines were incubated in the presence or absence of knob protein (10-20 µg/mL) or monoclonal antibody (mAb) 425 (50 µg/mL) for 1 hour at 4°C. Cells were then infected with native or EGFR-retargeted AdCMV-Luc at a multiplicity of infection of 100 plaque-forming units per cell. Twenty-four hours after infection, the cells were lysed and luciferase activity was measured. The protein concentration was determined for each cell extract, and the number of luciferase units was adjusted for the concentration of each cell extract. T-shaped bars indicate SEs.

Figure 2. Analysis of relevant receptors in cell lines. Coxsvackievirus-adenovirus receptor (CAR) and epidermal growth factor receptor (EGFR) levels were analyzed in HeLa and squamous cell carcinoma of the head and neck (FaDu, SCC-4, and SCC-25) cell lines using flow cytometry. Cells were harvested with versene and incubated with an isotype normal mouse IgG (M), anti-CAR ascites (C), or anti-EGFR (E) antibody. After washing, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody and subjected to flow cytometry analyses. Cell lines expressing CAR or EGFR were considered positive when a 1.5-fold or greater shift to the right in the mean fluorescence was observed compared with the isotype antibody control.
EGFR RETARGETING INCREASES THE FREQUENCY OF INFECTION OF SCCHN CELLS

The previous experiments demonstrated that adenovirus infection of SCCHN cells can be redirected to EGFR, resulting in enhanced reporter gene expression. The observed enhancement of gene transfer could have arisen by 2 mechanisms. An increased number of viruses could have infected each susceptible cell. Alternatively, more cells in the population may have been rendered susceptible to infection because of high levels of EGFR on the cell surface. To ascertain which mechanism accounted for the enhanced levels of reporter gene expression, EGFR retargeting was performed using an adenovirus that expresses the β-gal reporter gene. The SCCHN cells were infected with either native or EGFR-retargeted AdCMV–β-Gal at an MOI of 100 pfu per cell, stained for β-Gal expression, and visualized by microscopy. Figure 4 shows the results of EGFR retargeting in FaDu (A-F) and SCC-4 (G-L) cells. A low number of cells in both cell lines were infected by native adenovirus (A and G). In addition, native adenovirus infection was efficiently blocked when the cells were preincubated with recombinant knob protein (B and H); however, infection was not significantly blocked when the cells were preincubated with mAb 425 (C and I). Epidermal growth factor receptor retargeting resulted in increased numbers of cells that stained positive for β-Gal expression in the FaDu and SCC-4 cell lines (D and J). Preincubation of the cells with recombinant knob protein did not block EGFR-retargeted adenovirus infection (E and H), confirming that the EGFR-retargeted adenovirus was entering the cells through a pathway independent of CAR. Preincubation of the cells with mAb 425 significantly reduced EGFR-retargeted adenovirus infection (F and L), providing additional evidence that EGFR functioned as the receptor for the retargeted adenovirus. Taken together, these data indicate that all SCCHN cells in the 2 cell line populations tested were equally susceptible to EGFR retargeting. Furthermore, retargeting of the adenovirus to EGFR increased the number of SCCHN cells infected and accounted for the primary mechanism of enhanced gene transfer.

COMMENT

In this study we have demonstrated that retargeting of adenovirus provides a means to enhance gene transfer efficiency to SCCHN cells. An EGFR-retargeted entry pathway was found to be more efficient than the native adenovirus pathway in SCCHN cell lines and primary tumors. Redirecting the virus to the highly expressed EGFR molecule increased the overall number of cells infected. Retargeting was specific for EGFR, as demonstrated by the ability of an mAb directed against EGFR to block infection. Furthermore, the inability of the knob protein to block infection indicates that the retargeted adenovirus uses a mechanism independent of CAR. In 1 sample of matched tumor and normal tissue, the EGFR-retargeted adenovirus showed greater enhancement in tumor than in normal tissue. This differential infection efficiency by the retargeted adenovirus is likely caused by the elevated level of EGFR expression in SCCHN tumors compared with normal epithelial tissue. These findings support the hypothesis that targeting of receptors that are relevant to certain tumors is likely to increase the therapeutic index of the vector (ie, the differential effect between normal and tumor tissue).

Since adenovirus normally infects the epithelium of the upper aerodigestive tract, it was initially expected that adenovirus would efficiently transduce these cells. However, the findings in the current study demonstrated that adenovirus infected aerodigestive epithelial tumor cells with relatively low efficiency. Flow cytometry analyses demonstrated that the low infection efficiency is likely caused by low levels of CAR expression in SCCHN cell lines. These data indicate that relatively low levels of CAR

EGFR RETARGETING IN PRIMARY SCCHN TUMOR EXPLANTS

To determine whether the findings in cell lines extended to primary cells, EGFR retargeting was analyzed in primary SCCHN tumor explants that were derived from fresh tissue specimens. This was important to this study because there are frequently differences between vector efficiency in cell lines and primary cells. Furthermore, primary materials more accurately reflect the human clinical situation. Epidermal growth factor receptor retargeting enhanced adenovirus infection of an SCCHN primary explant (Figure 5, A) and a solid tumor (Figure 5, B) by approximately 9.5- and 11-fold, respectively. Thus, in primary cells, retargeting significantly enhances gene transfer. We also hypothesized that the difference of EGFR expression levels in normal vs tumor tissue would produce a favorable therapeutic index (ie, the differential ability of the vector to efficiently transduce tumor vs normal issue). Normal tissue was defined as tissue that appeared clinically normal and was taken from the mucosa or underlying submucosa at a site that was at least 2 cm from the resection margin. We tested the hypothesis that adenovirus would display differential infection efficiency of the 2 tissue types by employing EGFR retargeting in normal and tumor tissue from the same patient with SCCHN (Figure 5, C). Epidermal growth factor receptor retargeting enhanced adenovirus infection by 27-fold in tumor tissue, but only 8-fold in normal tissue. This experiment again demonstrates that EGFR retargeting enhances adenovirus gene delivery and suggests that tumor tissue is preferentially targeted over normal tissue. Such enhancement of tumor tissue infection compared with normal tissue suggests that immunological retargeting may increase the therapeutic index in vivo. Finally, the use of these primary tissue samples represents a substrate that is highly relevant to an in vivo human situation.
in SCCHN tumors would present an obstacle for the use of adenovirus as a gene therapy vector in human clinical trials. One means to overcome this obstacle is to increase the dose of adenovirus. In the report of 1 recent clinical trial, multiple doses of up to $10^{11}$ pfu ($3 \times 10^{12}$ pfu total) of adenovirus-p53 resulted in no dose-limiting toxic effects or severe adverse side effects; however, fever and edema were noted and gene transfer efficiency was low. Similarly, studies by Knowles et al also demonstrated inefficient adenovirus gene transfer to the nasal epithelium, with dose-limiting toxic effects occurring before efficient gene transfer was achieved.

**Figure 4.** Infection frequency in squamous cell carcinoma of the head and neck cell lines using epidermal growth factor receptor (EGFR)-retargeted adenovirus. Squamous cell carcinoma of the head and neck cell lines were infected with native (A-C and G-I) or EGFR-retargeted (D-F and J-L) AdCMV-β-Gal to demonstrate immunological retargeting. FaDu (A-F) and SCC-4 (G-L) cell lines were incubated without a blocking reagent (A, D, G, and J) or in the presence of knob protein (B, E, H, and K) or monoclonal antibody (mAb) 425 (C, F, I, and L) for 1 hour at 4°C. Cells were infected with native or EGFR-retargeted AdCMV-β-Gal at a multiplicity of infection of 100 plaque-forming units per cell. Twenty-four hours after infection, the cells were fixed in phosphate-buffered saline containing 2% formaldehyde and 0.05% glutaraldehyde, stained for β-galactosidase (β-Gal) expression, and photographed (original magnification ×20).
Evidence of gene transfer in clinical trials, even with high dose adenovirus, has been disappointing because of toxic effects. In summary, the results of these clinical trials mandate that mechanisms be developed for more efficient adenovirus gene delivery in vivo.

Immunological retargeting begins to address issues of tissue specificity and gene transfer efficiency that will be important in developing new vectors that are efficacious in the clinical setting. This study provides proof of the principle that a modification of the viral vector potentiates efficient gene transfer to SCCHN cells that are normally refractory to adenovirus. In addition, this study provides evidence to suggest that the low level of CAR expression contributes to poor gene transfer efficiency in vivo. Determining the expression levels of relevant receptors in SCCHN tumors and surrounding tissues may be an important factor when considering the use of adenovirus as a gene therapy vehicle. Additional studies will be directed at determining the expression levels of CAR and other target receptors in patient SCCHN tumor tissues. Also, we are currently investigating the utility of retargeting in a suicide gene transfer system using a retargeted adenovirus vector carrying the enzyme cytosine deaminase. To this end, future studies using the retargeted adenovirus vector combined with suicide gene delivery in an animal oral cancer model will be pursued to demonstrate that retargeting increases the therapeutic index in an in vivo setting. Finally, the findings in this study bring into question the utility of unmodified adenovirus in future SCCHN human clinical trials, since it is apparent that tropism-modified viruses are likely to improve gene delivery to certain types of tumors.

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