Targeting adenovirus to CD80 and CD86 receptors increases gene transfer efficiency to malignant glioma cells

ILYA V. ULASOV, PH.D.,1 ANGEL A. RIVERA, PH.D.;2 YU HAN, B.A.,1 DAVID T. CURIEL, M.D., PH.D.,2 ZENG B. ZHU, M.D.,3 AND MACEJ S. LESNIAK, M.D.1

1Division of Neurosurgery, The University of Chicago, Chicago, Illinois; and 2Division of Human Gene Therapy, Departments of Medicine, Pathology, Surgery, and The Gene Therapy Center, University of Alabama at Birmingham, Alabama

Object. Gene therapy protocols for malignant gliomas utilize adenoviral vectors that rely almost exclusively on the adenovirus serotype 5 (Ad5) backbone. The authors have previously shown that chimeric vectors that bind to the Ad3 receptor, or CD46, increase the transduction efficiency of malignant brain tumors. In light of the debate regarding the efficacy of CD46 compared with CD80/CD86 in binding Ad3 virions, the authors now examine the expression and transduction efficiency of Ad5/3 chimeras that bind via CD80/CD86.

Methods. The authors first analyzed CD80/CD86 expression in glioma cell lines. They then used three replication-defective vectors containing a luciferase reporter gene: Ad5/3 (containing the tail and shaft domain of Ad5 and the knob domain of Ad3); Ad3/5 (containing the tail of Ad5, shaft of Ad3, and knob of Ad5); and Ad3/3 (containing the tail of Ad5, shaft of Ad3, and knob of Ad3). These vectors were analyzed both in vitro and in vivo against malignant glioma cells. To examine further the effect of Ad5/3 fiber modification, the authors created an oncolytic vector, conditionally replicative Ad5/3 (CRAd5/3).

Results. The Ad5/3 vector showed a 10- to 100-fold enhanced transduction efficiency of malignant glioma compared with replication-defective wild-type adenovirus (reAd5) (p < 0.05). Moreover the use of Ad5/3 reduced transgene expression by more than 90% in normal human brain cells compared with reAd5. Finally, the use of CRAd5/3 inhibited tumor cell proliferation by 43% more than replication-competent wild-type virus in vitro (p < 0.05).

Conclusions. The results of this study demonstrate that the Ad5/3 vector offers superior transduction efficiency and low toxicity in the setting of brain tumors, and therefore represents a potential new approach to gene therapy for malignant gliomas. (DOI: 10.3171/JNS-07/09/0617)

Key Words • adenoviral vector • adenovirus serotype 3 • adenovirus serotype 5 • CD80/CD86 • chimeric vector • gene therapy • malignant glioma

HIGH-GRADE glioma is the most common primary malignant tumor of the adult central nervous system.22,27 Despite recent advances, the prognosis in patients with these lesions remains poor, and novel approaches to treatment are desperately needed to make an impact on this devastating disease. Efforts aimed at developing new therapies have focused on new treatment strategies that specifically target tumor cells while sparing normal cells. One such modality, gene therapy, has shown special promise in the spectrum of agents used against brain tumors.

Recombinant adenoviruses are an attractive vehicle for gene therapy, and most of the adenoviral vectors used against malignant brain tumors are derived from human Ad5. Although preliminary studies have shown that Ad5 vectors are more effective than retroviral vectors28 and recent preclinical and clinical studies have shown promising results,5,8,21,28,29,36,40 continued efforts to improve transduction efficiency and transgene expression in tumor cells, while simultaneously decreasing endogenous binding to normal brain cells, are likely to enhance the clinical success of glioma gene therapy in the future.

The efficiency of Ad5 vectors in the setting of malignant brain tumors is probably influenced by two important constraints. First, the expression of the primary receptor for Ad5, CAR, is highly variable on cancer cells. In fact, several studies have demonstrated poor transduction efficiency of malignant glioma with adenoviral vectors, a finding that was attributed to the quantitative deficiency of CAR.

Abbreviations used in this paper: AdWT = replication-competent wild-type adenovirus; Ad3 = adenovirus serotype 3; Ad5 = adenovirus serotype 5; CAR = Coxsackie–adenovirus receptor; CRAd5/3 = conditionally replicative Ad5/3; DMEM = Dulbecco modified Eagle’s medium; EDTA = ethylenediaminetetraacetic acid; FACS = fluorescence-activated cell sorting; PBS = fetal bovine serum; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GBM = glioblastoma multiforme; HEK = human embryonic kidney; NHA = normal human astrocyte; PBS = phosphate-buffered saline; reAd5 = replication-defective wild-type adenovirus; RLU = relative luciferase units; SD = standard deviation; VP = viral particle.
expression on brain tumor cells.\textsuperscript{3,13,18} Thus, the variability of adenovirus receptor density expressed by tumors may influence gene transfer efficiency and the ultimate therapeutic response observed in patients. Second, although tumor expression of the adenoviral receptor may be low, normal human tissues, including NHAs,\textsuperscript{31} show widespread distribution of CAR expression,\textsuperscript{9,10,19,30,44} thus precluding the ability to target tumor-specific cell types and causing dose-related vector toxicities that limit the overall therapeutic index achievable with adenoviral vectors.

To further enhance adenoviral gene transfer to malignant glioma cells and bypass the dependence on CAR expression, we decided to replace the receptor-binding fiber knob domain of Ad5 with the human Ad3 group B knob. The rationale for this approach was based on our finding of the Ad3 receptor (CD46) on glioma cells\textsuperscript{32} and on previously published reports of chimeric Ad5/3 vectors used against melanoma and ovarian carcinoma.\textsuperscript{2,16,37} However, despite the role of CD46 in mediating Ad3 infectivity, a growing body of evidence suggests that CD80/CD86 plays an equally important, if not complementary role, in Ad3 binding and internalization.\textsuperscript{35} To analyze further the role of CD80/CD86, we used sets of replication-defective and replication-competent adenoviral vectors containing different combinations of components of Ad5 and Ad3 fibers. We then analyzed CD80/CD86 expression in glioma cells and examined the transduction activities of our chimeric vectors against these tumors in vitro and in vivo since the potential role of such viruses in the treatment of malignant brain tumors has not been previously elucidated.

Materials and Methods

Cell Lines and Tissue Culture Conditions

The human glioma cell lines U87MG and U373MG were kindly provided by Dr. Shaun Sparacio (Department of Cell Biology, University of Alabama at Birmingham). The U118MG+ cells (transduced with the CAR gene) were kindly provided by Dr. J. T. Douglas (Division of Human Gene Therapy, University of Alabama). The No. 10 glioma cells were obtained from the Japanese Glioma Tumor Bank, and the HEK293 cells and HEK911 cells were obtained from American Type Culture Collection. Primary glioblastoma cells were obtained from patients undergoing tumor resection according to a protocol approved by the Institutional Review Board at the University of Chicago.

The U87MG, U373MG, No. 10, U118MG+, and primary tumor cells were maintained in DMEM/Ham F-12 medium (1:1, vol/vol) supplemented with 10% FBS in a humidified atmosphere containing 5% CO\textsubscript{2} at 37°C. The HEK293 and HEK911 cells were maintained in high-glucose DMEM with 10% heat-inactivated FBS. Normal human astrocytes (a gift from Dr. D. Benos, Department of Biophysics, University of Alabama) were cultured in DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin. These cells were originally obtained from Cambrex BioScience and represent a fetal astrocyte cell line that has not been retrovirally transduced.

Primary Human Tissue Samples

Human liver specimens were obtained from the hepatectomy remnants of patients after liver transplantation. Nonneoplastic human brain tissue samples were obtained from patients undergoing a temporal lobectomy for seizure focus resection. Sample tissue from the part of the brain that was normal and free of disease (as confirmed by an attending neuropathologist) was then used in our infectivity studies. The protocols we used were approved by the institutional review boards at the University of Alabama and the University of Chicago. To generate human liver and brain organ cultures, the tissue was serially dissected into 0.5-mm-thick slices using the Krumdieck tissue slicer (Alabama Research Development). Next, the tissue was cultured in six-well plates (1 slice/well) containing 2 ml of complete culture media (RPMI 1640, Media Facility, University of Alabama) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml insulin. The plates were incubated at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} for up to 48 hours. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2}. Three tissue slices were examined per group.

Flow Cytometry

Adherent glioma cells grown in a T-75 flask (Falcon) were detached by treatment with 1 mM ethylenediaminetetraacetic acid and washed three times with PBS. After washing, the cells were resuspended in PBS supplemented with 1% FBS wash buffer at a concentration of 10\textsuperscript{6} cells/ml. The cells were then incubated for 1 hour at 37°C with monoclonal antibodies specific for anti-human CAR (ab9891, Abcam), CD86 (557655, clone 2331, 1:50 final dilution; BD Pharmingen), or CD80 (557223, clone L307.4, 1:50 final dilution; BD Pharmingen). Subsequently, cells were washed three times with wash buffer and incubated with fluorescein isothiocyanate-labeled goat anti–mouse (554001) or phycoerythrin-labeled (550589) goat anti–mouse immunoglobulin G antibodies (both at a final dilution of 1:100; BD Pharmingen) for 45 minutes at 4°C. Tumor samples were stained with CD86 (557655, clone BU63, 1:50 final dilution; Chemicon International) or CD80 antibodies (217625, Calbiochem). The secondary antibodies were fluorescein isothiocyanate-labeled (554001). After incubation with secondary antibodies, cells were washed three times with wash buffer and subjected to flow cytometry. Flow cytometry was performed with a FACSCalibur (Becton Dickinson) and analyzed with FlowJo and CELLQuest software.

Construction of Recombinant, Replication-Defective, Fiber-Modified Adenovirus

Three replication-incompetent adenovirus vectors were used which contained a firefly luciferase transgenic cassette in place of the deleted E1 region. The Ad5 vector was generated as previously described.\textsuperscript{9} The Ad5/3 vector (containing chimeric fiber with the tail and shaft domain of Ad5 and the knob domain of Ad3) was constructed as previously described.\textsuperscript{8} The Ad3/5 vector (containing chimeric fiber with the tail of Ad5, shaft of Ad3, and knob of Ad5) was constructed using recombinant plasmid pNEB.PK F 3/5 in a scheme described by Dimitriev et al.\textsuperscript{1} The same vector (designated pNEB.PK F 3/3) was used for the cloning of short shaft and short knob domain from human Ad3 to generate Ad3/3 (containing chimeric fiber with the tail of Ad5, shaft of Ad3, and knob of Ad3). All vectors were rescued by transfecting HEK293 cells with the resultant adenovirus genome. The viruses were propagated on HEK293 cells and purified by two rounds of cesium chloride density centrifugation. The VP concentration was determined spectrophotometrically, using a conversion factor of 1.1 × 10\textsuperscript{11} VP per absorbance unit at 260 nm,\textsuperscript{31,34} and standard plaque assays on HEK293 cells were performed to determine the number of infectious particles.\textsuperscript{20}

Construction of Recombinant, Replication-Competent, Fiber-Modified Adenovirus

The conditionally replicative virus, CRAd5/3, was constructed as previously described.\textsuperscript{8} Both CRAd5/3 and AdWT were propagated in E1–transcomplementing HEK911 cells, purified with double cesium chloride density gradient ultracentrifugation, and dialyzed in PBS with 10% glycerol. Viral aliquots were stored at −80°C.

Determination of Transductional Efficiency

The Ad3/5, Ad5/3, Ad3/3, and reAd5 replication deficient adenoviruses were used to infect the U87MG, U118MG+, U373MG, No. 10 glioma, and NHA cells in a 24-well plate at 10 and 100 VP/cell. Cells were plated at a concentration of 5 × 10\textsuperscript{4} cells/ml and grown overnight at 37°C. The following day, media was removed from cell monolayers and the cells were infected with 10 and 100 VPs of each virus for 1 or 24 hours at 37°C. All viruses were removed from the
Chimeric adenovectors for malignant glioma

cells and the media was replaced with 1 ml DMEM containing 10% FBS. Forty-eight hours later, the cells were tested in a luciferase assay. The cells were washed with PBS. After a PBS rinse, 200 µl of lysis buffer was added to each well. Plates were incubated at −80°C for 20 min. The lysates were thawed and gene transfer luciferase expression was determined using the Promega Luciferase detection kit. Experiments were performed in triplicate, and luciferase activities are presented in RLUs.

Blocking Studies

Human anti-CD80 or anti-CD86 antibodies (557655 and 557223; Pharmingen) were incubated with 10⁴ cells (96-well plates) on ice at a concentration of 50 µg/ml. One hour later, the cells were washed twice with PBS, and reAd5, Ad5/3, Ad3/5, or Ad5/3 viruses (1000 multiplicity of infection) were added to the cells. After 1 hour of incubation at 37°C, the cells were washed several times with PBS, and 0.2 ml of growing medium was added to each well. Forty-eight hours later, the cells were assessed for luciferase activity. Results were determined as a percentage of inhibition of infection.

Viral Injection of Liver and Brain Slices

Liver and brain slices were prepared as described in Primary Human Tissue Samples. All viral infections were done at 500 VP/cell in complete culture medium supplemented with FBS. Cell numbers for tissue slices were estimated at 10⁴ cells/slice based on a 10-cell thick slice (approximately 250 µm) and a 8-mm slice diameter. Infections were allowed to proceed overnight, and on the subsequent day the media was removed and replaced with complete culture media. Slices were removed after 48 hours, and the media was subjected to luciferase assays.

Evaluation of In Vivo Transduction Efficiency

Human glioma xenografts were generated in the brains of three athymic nude mice. Briefly, 10⁶ U87MG glioma cells were inoculated intracranially into nu/nu BALB/c mice (Day 0). Ten days after tumor inoculation, mice were randomized into five groups, each to receive a replication-defective virus: Ad3/3, Ad5/3, Ad3/5, reAd5, or normal saline. A total of 10⁵ VP in a total volume of 5 µl of PBS was injected into each mouse. The animals were killed 48 hours later, and the tumors were removed and subjected to analysis. Specimens were divided into two equal portions: one was fixed in 4% paraformaldehyde, the other was processed for protein isolation and analysis. For quantification of tumor luciferase expression levels, tumors were disrupted in a lysis buffer (Promega). The resultant cell suspension was lysed by three freeze–thaw cycles and centrifuged for 5 minutes at 13,000 rpm. The supernatant was analyzed for luciferase activity with a luminometer. Activity was corrected for protein concentration, determined using the bovine serum albumin protein assay reagent (BIO-RAD Lab).

Immunohistochemical Assays

Tumor tissues were stained for hexon antigen using standard immunohistochemical techniques and a histostain kit (DAKO). Anti-human Ad5 hexon immunostaining was performed on sections from tumors in each group. Sections were washed in PBS, fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Each tumor sample was cut into tissue slices of 760 µm with an automatic oscillating tissue slicer (Electron Microscopy Sciences). After the fixation period, the sections were postfixed in ethanol and acetic acid for 10 minutes. After this, sections were counterstained with a luminometer. Activity was corrected for protein concentration, determined using the bovine serum albumin protein assay reagent (BIO-RAD Lab).

Evaluation of Cell Growth Inhibition with CRAd5/3

The U87MG cells were grown at 10⁶ cells/well in a 96-well plate. Once the cells achieved approximately 80% confluency, they were rinsed with PBS and infected with AdWT or CRAd5/3 at 1000 VP per cell in 2% growing medium. Control cells were mock infected. After 24 hours’ adsorption, the cells were washed with PBS, and 0.2 ml of fresh medium supplemented with 10% FBS was added to the wells. Forty-eight hours postinfection, 20 µl of 2% media containing 10 µM [3H]thymidine was added to the cells. Twenty-four hours later, the cells were harvested, and radioactivity was measured using a liquid scintillation counter (LS 6000TA; Beckman Instruments). Results are expressed as a percentage of proliferating cells (thymidine count in infected cells/thymidine count in mock-infected cells × 100).

Statistical Analysis

The luciferase activities measured in this study were analyzed using the Student t-test and commercially available software (SAS 8.2). A probability value less than 0.05 was considered statistically significant.

Results

Three replication-defective vectors containing a luciferase gene were created: Ad5/3 (containing the tail and shaft domain of Ad5 and the knob domain of Ad3); Ad3/5 (containing the tail of Ad5, shaft of Ad3, and knob domain of Ad5); and Ad3/3 (containing the tail of Ad5, shaft of Ad3, and knob domain of Ad3). We used reAd5 as a control. In addition, a conditionally replicative Ad5/3 chimera, CRAd5/3, was created and compared with a replication-competent wild-type virus, AdWT.

Glioma cells demonstrate low levels of CAR expression and high levels of adenovirus B1 group (CD80/CD86) receptor expression. To evaluate the level of primary Ad5 receptor, or CAR, in several glioma cell lines, we performed a FACS analysis for protein expression on the surface of tumor cells. The results indicate that, although NHAs overexpress CAR (Fig. 1A), the majority of glioma cell lines show poor CAR expression, with the exception of U118MG+ (Fig. 1B). These results are summarized in Table 1.

Next, we examined the levels of Ad3 cellular receptors, CD80 and CD86, on the same cell lines. As shown in Fig. 2A, U87MG, U373MG, U1185MG+, and No. 10 tumor cells significantly overexpressed CD80/CD86 compared with NHA cells, suggesting that the Ad3-knob-modified virus can infect these cells in a CD80/CD86

Quantitative Analysis for Detection of E4 Gene Expression

Total cellular DNA was extracted from one sample of glioblastoma tissue, GBM-T13, 6 hours postinfection using a DNA tissue Kit (Qiagen). The E4 gene was detected in DNA samples by using an oligonucleotide pair (forward primer 5’GGAGTGCGCCGA-GACAAC, and reverse primer 5’ACTAGTCCCCGCGTTCATC) and the probe (ORF6-TGGCGATGACACTACGACCAAACG-ATC). The oligonucleotides were designed using Primer Express 1.0 (Perkin-Elmer) and synthesized by Applied Biosystems. The real-time polymerase chain reaction specifics have been described.16,24 Negative control testing without templates was performed for each reaction series, and an internal control (human GAPDH) was used to normalize the copy number for the E4 genes. Results are presented as E4 copies per human GAPDH.
The expression of CD80/CD86 was further confirmed in several human samples of GBM, with the representative sample GBM-T13, illustrated in Fig. 2B. These results are summarized in Table 2.

Effect of Capsid Modifications

Capsid Modifications Increase Gene Transfer to Glioma Cell Lines. The relatively high degree of CD80 and CD86 expression in these tumor cell lines provided a rational re-targeting strategy for malignant gliomas. We therefore tested transgene expression using our retargeted vectors, and compared it with reAd5. As shown in Fig. 3, cell lines

![Image](https://example.com/image.png)

**TABLE 1**

*Expression of CAR on malignant glioma cells*

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% CAR Expression ± SD</th>
<th>p Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>82.9 ± 9.12</td>
<td>NA</td>
</tr>
<tr>
<td>U87MG</td>
<td>1.76 ± 0.38</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U118MG+</td>
<td>31.3 ± 2.96</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U373MG</td>
<td>4.87 ± 0.65</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>No.10</td>
<td>1.11 ± 0.21</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>NHA</td>
<td>36.2 ± 2.41</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GBM-T13</td>
<td>6.26 ± 0.54</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* NA = not applicable.
† Compared with HEK293 cells.

Fig. 1. A FACS analysis of CAR expression on NHAs and glioma cell lines. Flow cytometric analysis of NHAs (A) as well as passaged and primary glioma cells (GBM-T13) (B) for CAR expression. The red dots represent the isotype control, and the blue dots are target staining. Data were obtained from three different experiments performed in triplicate. Results are presented as plots from a single experiment.
U87MG, U118MG+, and No. 10 demonstrated a significantly higher level of luciferase activity for Ad5/3 compared with reAd5, especially at the end of the 24-hour period of infection (p < 0.05).

To further assess the specificity of the observed response, we first incubated U87MG cells with antibodies to CD80/CD86 and then investigated the effect of viral transduction of tumor cells. Mouse monoclonal antibodies to CD80/CD86 had a moderate effect on the binding of reAd5 vectors (36.6–39.1% inhibition), most likely secondary to the expression of CD46 on U87MG cells. However, anti-CD80/CD86 monoclonal antibody efficiently inhibited the binding of Ad5/3 (93.4–95.1% inhibition; p < 0.05), indicating that these antibodies blocked all specific binding sites.

Capsid Modifications Decrease Gene Transfer to NHAs and Normal Human Brain Cells. One of the most important considerations in designing an adenoviral vector is the specificity of the virus for tumor tissue with minimal infectivity of normal, healthy tissues. To examine the specificity of our viruses, we infected NHAs as well as nonneoplastic human brain cells with our retargeted vectors. As illustrated in Fig. 5A, reAd5 luciferase activity was significantly elevated in comparison to Ad3/5, Ad5/3, and Ad3/3 in NHAs. In fact, at a concentration of 10 VP/cell, we observed an 11.6- and 32.3-fold increase in luciferase activity using the reAd5 compared with the Ad3/5 and Ad5/3, respectively. These findings were even more pronounced at a concentration of 100 VP/cell, in which we observed a 20-, 30-, and 1100-fold increase in infectivity over Ad3/5, Ad5/3, and Ad3/3, respectively (p < 0.05). Most important, however, a significant decrease in infectivity was shown with Ad3/5, Ad5/3, and Ad3/3 viruses (p < 0.05). Of the three, the Ad3/3 virus demonstrated the lowest level of infectivity in the setting of normal astrocytes.

Because NHAs represent a cell line and may not accurately reflect the infectivity of our vectors, we infected nonneoplastic human brain tissue samples with our retargeted vectors and analyzed luciferase activity as a percentage of reAd5 activity (Fig. 5B). The results indicate that Ad5/3 showed less than 10% of infectivity compared with reAd5 (p < 0.05). Taken together, our data reveal that the reAd5 virus infected astrocytes more efficiently than Ad5/5, Ad3/5, or Ad3/3, regardless of the dominant knob receptor used.

**TABLE 2**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% CD80/86 Expression ± SD</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHA</td>
<td>35.8 ± 0.92</td>
<td>NA</td>
</tr>
<tr>
<td>U118MG+</td>
<td>65.8 ± 5.04</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U373MG</td>
<td>75.2 ± 3.18</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>No. 10</td>
<td>75.2 ± 7.13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GBM-T13</td>
<td>63.6 ± 1.53</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U87MG</td>
<td>62.1 ± 5.21</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Compared with NHAs.

Fig. 2. A FACS analysis of human adenovirus group B receptors on glioma cells. Passaged (A) or primary (B) glioma cells were incubated with anti-CD80 and anti-CD86 monoclonal antibodies, followed by detection with phycoerythrin- or fluorescein isothiocyanate–labeled secondary antibodies. Although three different primary glioma specimens were tested, only a representative sample, GBM-T13, is illustrated. Data were obtained from three different experiments performed in triplicate. Results are presented as plots from a single experiment.
In this setting, Ad5/3 offers an improved safety profile, which warrants further examination of this virus in vivo.

**Capsid Modifications Decrease Gene Transfer to Normal Human Liver Cells.** Next, given the affinity of wild-type adenoviruses for liver cells, we analyzed the infectivity of our retargeted vectors in normal hepatocytes (Fig. 6). The results indicate that both reAd5 and Ad5/3 bind to hepatocytes and cause elevated transgene expression. Comparison of the average total luciferase activity between reAd5- and Ad5/3-infected cells shows a twofold increase in Ad5/3 infectivity. At the same time, adenovirus vectors Ad3/5 and Ad3/3 show very low levels of liver transduction (25 and 30% of the wild-type infectivity level, respectively; \( p < 0.05 \)) compared with reAd5.

**Capsid Modifications Enhance Gene Transfer In Vivo.** To validate our results further, we performed in vivo studies using an intracranial xenograft model of human U87MG glioma. As shown in Fig. 7A and B, Ad5/3 demonstrated increased infectivity compared with reAd5. This data was further corroborated by transgene expression. As shown in Fig. 7C, the Ad5/3 virus showed a 10-fold increase in luciferase expression compared with the expression of luciferase by reAd5 (\( p < 0.05 \)).

**Capsid Modifications Increase the Oncolytic Potential of CRAd5/3 In Vitro.** Finally, to confirm that genetic capsid modifications enhance gene therapy of malignant brain tumors, we utilized a conditionally replicative vector with a 5/3 modification, CRAd5/3. As shown in Fig. 8A, CRAd5/3 enhanced the transduction efficiency in primary glioma...
cells by more than 10-fold, as shown by the number of E4 copies/human GAPDH (p < 0.05). Most important, treating U87MG glioma cells with CRAd5/3 in vitro inhibited tumor cell proliferation by 43% compared to treatment with reAd5 virus (p < 0.05).

Discussion

A key component to successful treatment using gene therapy lies in the design of the optimal gene delivery vehicle. Effective vehicles must produce a sufficient level of gene transfer to neoplastic cells while minimizing damage to healthy brain tissue. To achieve these goals, both viral and nonviral vectors have been explored. However, nonviral delivery systems, including liposomes and synthetic polymer vectors, often have only transient gene expression due to enzymatic degradation by the host endosome, lysosome, or endoplasmic reticulum. In contrast, through the course of evolution, viral vectors have adapted mechanisms to evade these cellular processes and are in fact able to take advantage of the host cell machinery to synthesize their own proteins. The most common viral vectors used for gene therapy include: retrovirus, herpes simplex virus, measles virus, reovirus, and adenovirus. Among these choices, the adenovirus has several features advantageous for gene delivery, including a high titer, efficient transduction, broad tropism, a large carrying capacity for transgenes, and mild pathogenicity. In addition, its viral genome has been well-characterized and fully sequenced, making the vector a convenient system for gene delivery. In light of these advantages, adenoviruses are the most commonly used system in clinical trials for glioma gene therapy.

Although adenoviral vectors have been used successfully in both preclinical and clinical studies of malignant glioma, increasing evidence suggests that the efficiency of these vectors may be limited by the poor expression of the Ad5 receptor—CAR—on brain tumor cells. Native Ad5 tropism can be selectively modified to circumvent CAR deficiency in cancer cells. Transductional targeting of adenovirus aims at enhancing transduction of the tumor cells by incorporating targeting moieties into the fiber knob region. In the present study, we demonstrated that chimeric viruses with an Ad3 knob domain inserted into an Ad5 fiber backbone utilize the group B receptor CD80/CD86 and show enhanced transduction of glioma compared with wild-type Ad5. Moreover, an oncolytic virus with the 5/3 modification shows increased transduction efficiency and glioma-cell killing compared with the unmodified Ad5 wild-type virus, further supporting the development of clinical vectors that do not depend on CAR for adenoviral entry and replication.

Although there is an ongoing debate as to the exact nature of the adenoviral group B receptor, recent evidence points to CD46 as well as to CD80/CD86. Specifically, although group B2 serotypes 11, 14, 16, 21, and 35 appear to utilize the CD46 receptor, Ad3 (group B1) appears to depend on CD80/CD86 for entry into the cell. Based on these data and our interest in Ad3, we first examined the expression of CD80/CD86 on malignant glioma cells and...
confirmed overexpression of these receptors on tumors compared with NHAs. Subsequently, we developed a set of chimeric adenoviral vectors that utilize different components of the Ad3 fiber within the Ad5 backbone fiber. Our in vitro and in vivo data demonstrate that an E1 replication-deficient adenovirus that contains an Ad5 shaft and an Ad3 knob domain provides a high level of transgene expression compared with wild-type Ad5 in malignant glioma cells.

Specifically, both Ad3/5 and Ad5/3 vectors showed superior levels of tumor transduction as evidenced by luciferase transgene expression. Although the results obtained with Ad5/3 were consistent with our hypothesis, the relatively high level of transgene expression associated with Ad3/5 was at first surprising given that Ad3/5 contains the Ad5 knob and therefore probably utilizes the Ad5 receptor (CAR). However, Takayama et al. \(^{37}\) have recently shown that chimeric vectors exhibit enhanced gene expression, probably because they utilize both primary receptors. Indeed, those authors generated a mosaic virus via incorporation of both Ad5 and Ad3 knobs into the same particle. This mosaic virus was able to use either receptor (CAR or the Ad3 receptor) for attachment to cells. Enhanced adenovirus infectivity with the mosaic virus was shown in a panel of cell lines, with receptor profiles ranging from CAR-dominant to Ad3 receptor–dominant. Similarly, Kawakami et al. \(^{17}\) examined the effects of tropism modification in a conditionally replicative Ad5/3 virus. Overall, the chimeric Ad5/3 virus was progressively more efficient at each step of the replication cycle compared with its Ad5 counterpart. The higher replication efficiency of the chimeric Ad5/3 vector translated into improved therapeutic efficacy in a murine in vivo tumor rejection model. Taken together, these findings suggest that the mosaic virus strategy may improve adenovirus-based gene therapy approaches by infectivity enhancement and tropism expansion.

Although the knob domain of fiber remains an important determinant of viral tropism, recent studies indicate that the length of the fiber shaft has equally important implications for viral infectivity and spread. Shayakhmetov and Lieber \(^{34}\) reported that efficient gene transfer to the target cells depends on the shaft length of the fiber protein. In their study, the authors constructed a series of Ad5 capsid–based vectors containing long or short fibers with knob domains derived from Ad5, Ad9, or Ad35 and tested them in adsorption, internalization, and transduction studies. For Ad5 or

**Fig. 7.** Immunohistochemical studies (A) and bar graphs (B and C) demonstrating in vivo transgene expression utilizing the chimeric vectors. The U87MG human glioma cells were used for evaluation of transduction efficiency in an intracranial xenograft model. One million U87MG cells were implanted intracranially in nu/nu Balb/c mice. Ten days later, the animals received intratumoral injections of the virus (total dose of 10\(^8\) VPs). Mice treated with replication-defective vectors (reAd5, Ad3/5, Ad5/3, or Ad3/3) were killed 48 hours later and the tumors were removed. Tumor tissue was then divided into two parts which were used for luciferase expression and immunohistochemical analysis. In part (A), the tumor sections were fixed, sectioned, and stained with H & E. In parallel, anti–human Ad5 hexon immunostaining (IHC) was performed on sections from tumors in each group. The data show significantly elevated expression of the viral hexon staining (black arrows) within the tumor mass. Original magnification × 40. Digital imaging was then performed to measure the percentage of staining cells/\(\mu\)m\(^2\) as shown in (B). Finally, the tumors were homogenized in luciferase lysis buffer. Luciferase activity was determined as RLU/mg of tissue (C). Each point represents the mean of three experiments ± SD. The Ad5/3 showed an increased level of luciferase expression as compared with reAd5 (*p < 0.05).
Chimeric adenovectors for malignant glioma

Ad9 knob–possessing vectors, a long-shafted fiber was critical for efficient adsorption, internalization, and transduction of CAR/αv integrin–expressing cells. In contrast, for the chimeric vectors possessing Ad35 knobs, which enter cells by a CAR/αv integrin–independent pathway, fiber-shaft length had no significant influence on the binding or infectivity of the tested cells. This study demonstrates that the fiber–CAR interaction is not the sole determinant for tropism of adenovirus vectors and that the length of the fiber shaft may influence transgene expression. This conclusion is further supported by our own experiments, in which we found that the transduction efficiency of Ad5/3 was greater than that of Ad3/3. Because the long shaft of Ad5 contains 22 repeat domains with six repeat domains in Ad3, the greater length of Ad5/3, as opposed to Ad3/3, probably increases receptor–ligand binding and interaction, thereby increasing the level of adenoviral infectivity.

As with any targeted vector therapy, the benefits of enhanced gene expression in tumor tissues must be counterbalanced with decreased systemic toxicity. Our results demonstrate that chimeric Ad3/3, Ad3/5, and Ad5/3 vectors exhibit reduced infectivity and transduction in NHAs compared with wild-type Ad5. Of note, two (Ad3/5 and Ad3/3) of the three retargeted vectors also showed a diminished level of liver infectivity. Only Ad5/3, which has been shown by other authors to cause diminished hepatotoxicity in mice, exhibited elevated infectivity and transgene expression in human liver tissue. However biodistribution and safety studies in mice are of limited value because the mouse analog of the B group adenovirus receptor is expressed only in murine testis. This finding should not preclude further development of Ad5/3 vectors. In fact, in cancers such as glioma, for which local rather than systemic administration of the vector would be the preferred route of therapy, the increased infectivity of tumor cells with diminished neurotoxicity is likely to outweigh any potential hepatotoxicity. In fact recent clinical studies using direct intracranial injection of replication-defective adenoviral vectors in humans and primates have failed to show any side effects related to systemic transgene expression.

The capacity to selectively target locally recurring tumor cells with diminished toxicity to the surrounding brain tissue offers a potentially improved therapeutic advantage over local administration of chemotherapy and/or radiotherapy in the setting of malignant glioma.

Conclusions

The design of chimeric vectors that do not depend on the expression of CAR for transduction efficiency offers a potential new approach for gene therapy of gliomas. Our results indicate that CD80/CD86 play an important role in Ad3-mediated infectivity and that an Ad5/3 vector offers superior transduction efficiency with low toxicity in the setting of brain tumors. This study provides further basis for the application of chimeric vectors for in vitro and in vivo gene transfer and for gaining an understanding of the factors that determine adenovirus tropism in the context of glioma therapy.

Acknowledgment

We would like to thank Dr. Andrew Khramtsov (Department of Pathology, University of Chicago) for his assistance with this research.

References


34. Short JJ, Pereboev AV, Kawakami Y, Vus C, Holterman MJ, Curiel DT: Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. Virology 322:349–359, 2004


41. Ulasov IV, Tyler MA, Zheng S, Han Y, Lesniak MS: CD46 rep-
Chimeric adenovectors for malignant glioma


Grant support: American Cancer Society, Illinois Division; The American College of Surgeons, and the National Institute of Neurological Disorders and Stroke, K08 NS046430-01A1 (M.S.L.).

Address reprint requests to: Maciej S. Lesniak, M.D., The University of Chicago, Section of Neurosurgery, 5841 South Maryland Avenue, MC 3026 Chicago, Illinois 60637. email: mlesniak@surgery.bsd.uchicago.edu.