

Enhanced transduction of malignant glioma with a double targeted Ad5/3-RGD fiber-modified adenovirus

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Abstract

Malignant brain tumors remain refractory to adenovirus type 5 (Ad5)–based gene therapy, mostly due to the lack of the primary Ad5 receptor, the coxsackie and adenovirus receptor, on brain tumor cells. To bypass the dependence on coxsackie and adenovirus receptor for adenoviral entry and infectivity, we used a novel, double targeted Ad5 backbone–based vector carrying a chimeric Ad5/3 fiber with integrin-binding RGD motif incorporated in its Ad3 knob domain. We then tested the new virus *in vitro* and *in vivo* in the setting of malignant glioma. Ad5/3-RGD showed a 10-fold increase in gene expression in passaged cell lines and up to 75-fold increase in primary tumors obtained from patients relative to the control. These results were further corroborated in our *in vivo* human glioma xenograft model, where the Ad5/3-RGD vector showed a 1,000-fold increase in infectivity as compared with the control. Taken together, our findings indicate that

Ad5/3-RGD may be a superior vector for applications in glioma gene therapy and therefore warrants further attention in the field of neuro-oncology. [Mol Cancer Ther 2006;5(9):2408–16]

Introduction

Malignant glioma, in particular glioblastoma multiforme, represents a devastating form of primary brain cancer. Despite aggressive therapy, which may include surgery, radiotherapy, and chemotherapy, the median survival continues to be measured in months rather than years (1, 2) and local recurrence leads to rapid progression of this fatal disease (3). Gene therapy is a novel strategy that can be used to treat glioblastoma multiforme. Because these tumors rarely metastasize outside of the central nervous system and recur in proximity to the original site, direct delivery of a highly therapeutic gene offers the potential to effectively target these tumors. Adenoviral vectors (Ad) are especially suitable in this treatment strategy and recent clinical trials have established the safety of such vectors in the central nervous system. For example, a recent phase I trial using ONYX-015, a mutated oncolytic adenovirus, showed that injection of up to 10¹⁰ plaque-forming units was well tolerated in patients with malignant glioma (4). Whereas further research will be needed to ascertain the therapeutic value of this virus, studies such as this clearly provide the scientific rationale for further development of targeted adenoviral gene therapies for glioblastoma multiforme.

The therapeutic efficacy of any adenovirus-based cancer gene therapy approach depends on the efficacy of vector-mediated tumor transduction. Of note, human trials carried out to date have shown relatively inefficient gene transfer to tumor achieved by Ad vectors employed in *in vivo* delivery schemas (5–10). This has been understood to result from a relative paucity of the primary adenovirus receptor, the coxsackie and adenovirus receptor (CAR), on brain tumor cells (11–15). Indeed, a relative paucity of CAR has been shown to limit Ad vector efficacy in a number of tumor contexts, possibly representing a fundamental practical barrier to realizing the full benefit of adenoviruses for cancer gene therapy applications. On this basis, it has been proposed that cellular transduction via “CAR-independent” pathways may be required to circumvent this key aspect of tumor biology (16, 17). Thus, it is clear that augmenting the gene transfer efficacy of Ad vectors via transductional modification of the fiber protein is essential to deriving their full benefit in the context of conceptually promising adenovirus-based gene therapy strategies.

To bypass the dependence on CAR for adenoviral entry and replication, a number of different approaches have

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been used in the past few years. For example, several groups have genetically modified the knob domain of fiber in an attempt to retarget Ad vectors. Genetic alterations include virions containing chimeric fiber proteins composed of the tail and shaft domains of adenovirus type 5 (Ad5) fiber and the knob domain of Ad3 (18, 19) or the exchange of fiber with alternative serotypes such as Ad11 and Ad35 (20–22). Indeed, we have previously shown that chimeric Ad5/3 vectors that target the Ad3 cellular receptor, either via CD80/86 or CD46, enhance the transduction of malignant brain tumors (23).⁶ A different approach includes the incorporation of COOH-terminal polylysine sequences (24) or an integrin-binding RGD motif at the COOH terminus of Ad5 fiber (25). Because gliomas express high levels of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (26–31), Ad vectors carrying the RGD modification have shown a significant increase in transduction of CAR-negative glioma cell lines (15, 32). However, whereas both Ad5/3- and RGD-modified Ad5 fibers have been successfully used in gene therapy applications, the effect of combining both types of genetic modifications in a single-fiber molecule has not previously been explored for malignant glioma and therefore represents a novel development in the field of neuro-oncology.

In this study, we hypothesized that a double-modified Ad5 vector, comprising Ad3 serotype chimerism and an RGD-modification in a single-fiber molecule, would show a superior transduction efficiency and gene delivery as compared with vectors with either modification alone. To test this hypothesis, we genetically incorporated an RGD motif into either the HI loop or the COOH terminus of the Ad3 knob domain of a chimeric fiber bearing the tail/shaft domain of Ad5 and the knob of Ad3. We then examined the transduction efficiency and gene delivery efficiency of this vector *in vitro* and *in vivo* in the context of malignant glioma. Our results show an enhanced transduction profile for the double targeted Ad5/3-RGD vector in the setting of malignant glioma and therefore warrant further preclinical as well as clinical investigation.

Materials and Methods

Cell Culture

The human malignant glioma cell lines U373MG, U118MG, and U87MG and the human kidney cell line HEK293 were purchased from the American Type Culture Collection (Manassas, VA). Kings and No.10 glioma cell lines were purchased from the Japanese Tumor Tissue Bank (Tokyo, Japan). Normal human astrocytes were purchased from Cambrex-Clonetics (East Rutherford, NJ). All cells were grown in DMEM with 4.5 g/L glucose and L-glutamine, supplemented with 10% fetal bovine serum (FBS; Mediatech, Herndon, VA), and incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Human primary brain tumor cells (T5, T7, T19, and T20) were obtained from patients undergoing surgery in accordance with a protocol approved by the Institutional Review Board at the University of Chicago. All specimens were confirmed as grade 4 gliomas by an attending pathologist. The tissue was minced and cultured in flasks containing 20% FBS-DMEM with 100 µg/mL ampicillin/streptomycin mixture in a humidified atmosphere of air containing 5% CO₂ at 37°C.

Viruses

Six replication-deficient Ad vectors (WT, WT-HI-RGD, WT-C-RGD, Ad5/3, Ad5/3-C-RGD, and Ad5/3-HI-RGD) containing a firefly luciferase transgenic cassette in place of the deleted E1 region were used in this study. AdWT was generated as previously described (33). The AdWT and Ad5/3 vectors (chimeric fiber with the tail and shaft domain of Ad5 and the knob domain of Ad3) containing an RGD motif in either the HI or the C-loop were constructed as previously described by our group (34, 35). All vectors were rescued by transfecting HEK293 cells with the resultant adenoviral genome. The viruses were propagated on HEK293 cells and purified by two rounds of cesium chloride density centrifugation. The viral particle (vp) concentration was determined spectrophotometrically using a conversion factor of 1.1×10^{12} vp per absorbance unit at 260 nm (36, 37), and standard plaque assays on HEK293 cells were done to determine the number of infectious particles (38).

Flow Cytometry

To analyze $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin expression, the cells were washed with PBS, detached by 0.05% trypsin solution for 3 to 5 minutes at room temperature, resuspended in 2% FBS-DMEM, and then collected by centrifugation. One million cells were used for incubation with 1 µg of mouse anti-human $\alpha_v\beta_3$ antibody (CBL 544; Chemicon Europe, Temecula, CA) and mouse anti-human $\alpha_v\beta_5$ monoclonal antibody (mAb1961z; Chemicon International, Temecula, CA) for 30 minutes at 4°C. As a secondary antibody, we used goat anti-mouse fluorescein-conjugated antibody (BD Biosciences PharMingen, Franklin Lakes, NJ). Incubation with the secondary antibody was done for 30 minutes at 4°C. The cells were then washed three times using $1 \times$ Dulbecco's PBS without calcium and magnesium after incubation with each corresponding antibody. Cell samples were resuspended in 1 mL of Dulbecco's PBS and then analyzed on a FACScalibur (Beckton-Dickinson, Erembodegem-Aalst, Belgium). HEK293 cells were evaluated for integrin expression as a positive control. A cytometric analysis of 10,000 events per sample was conducted using FlowJo software version 6.3 (Tree Star, Inc., Ashland, OR).

Gene Transfer Assay in Glioma Cells

Cultured human glioma cells were harvested and resuspended in 10% FBS-DMEM medium. After centrifugation, 5×10^4 cells were seeded in 24-well tissue culture plates. Twenty-four hours later, cells were infected in triplicates with replication-deficient viruses (WT, WT-HI-RGD, WT-C-RGD, Ad5/3, Ad5/3-C-RGD, and Ad5/3-HI-RGD) at 1,000 vp/cell for 1 hour at 37°C. Following the

⁶ I.V. Ulasov, A.A. Rivera, Y. Han, et al. Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to malignant glioma. *Neuro-Oncology* 2006; Submitted.

infection step, the 2% FBS-DMEM was replaced with 10% FBS-DMEM. Forty-eight hours postinfection, the cells were lysed in 200 μ L of Cell Lysis Reagent (Promega, Madison, WI) at -20°C for 20 minutes and then freeze-thawed once. Luciferase activity was evaluated by adding 20 μ L of luciferase substrate (Promega) to 50 μ L of lysed-cell solution and measured with a Modulus luminometer (Turner Biosystems, Sunnyvale, CA).

Antibody-Mediated Blocking Assay

Antibody-mediated blocking assays using U87MG human glioma cells and anti- $\alpha_v\beta_3$ or anti- $\alpha_v\beta_5$ integrin antibodies were done as previously described (39). Briefly, 1, 10, and 100 $\mu\text{g}/\text{mL}$ antibody solutions were prepared in 2% FBS-DMEM and incubated with 1×10^4 cells in 96-well plates for 2 hours on ice. After several washes with PBS, the viruses (1,000 vp/cell) were added to the antibody-blocked cells and incubated for 1 hour at 37°C . Medium with unbound viruses was then aspirated and fresh growth medium was added to each well. Forty-eight hours postinfection, a luciferase assay was done. In the control samples, cells were not treated with the antibodies and were only incubated with medium alone.

Virion Binding Assay

To assess cell binding ability of the viruses, 0.5×10^6 of U87MG, U118MG, and No.10 human glioma cells were seeded on six-well plates in 6 mL of F-12 medium per well and grown to 60% confluence. The medium was aspirated on the next day and the cells were infected at 1,000 vp/cell for 1 hour at 37°C in a humidified 5% CO_2 atmosphere (40). Cells were then washed thrice with $1 \times$ Dubelcco's PBS solution, detached from the wells with 1 mL/well of 0.05% of Trypsin-EDTA (Mediatech), and washed with 3 mL/well of 10% DMEM. Viral genomic DNA was isolated from the cells following a standard protocol from DNeasy Tissue Kit (Qiagen Sciences, Germantown, MD) and quantitative real-time PCR assay for *E4* gene was done (41). The sequences of specific primers used for *E4* were as follows: sense, 5'-GGAGTGGCGCCGAGACAAC3'; anti-sense, 5'-ACTACGTCCGGCGTTCCAT 3'. PCR amplification procedures were described by Taki et al. (42). The PCR was done with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific primers (TaqMan *GAPDH* control reagent; Applied Biosystems, Foster City, CA) to create an internal standard. Quantification using SYBR Green PCR Master Mix (Applied Biosystems) was done according to vendor recommendations. All data were presented as the ratio of *E4* copy number to the human *GAPDH* gene copy number.

Immunochemical Assessment of Viral Infectivity

To estimate efficiency of viral binding to target cells by immunohistochemical approach, 2×10^5 No.10 glioma cells were seeded and grown on polylysine-coated coverslips (Sigma, St. Louis, MO). Twenty-four hours later, the cells were washed and infected with a set of replication deficient viruses at a multiplicity of infection of 10 vp/cell. After 1 hour of viral adsorption, cells were washed with PBS and incubated in the growth medium. Forty-eight hours later, the cells were washed with PBS and fixed with 4%

paraformaldehyde for 30 minutes at room temperature. Immunohistochemical staining was done with primary rabbit polyclonal antiserum (1:100; Ab24240, Abcam, Cambridge, MA) raised against the major adenoviral structural hexon protein. The secondary antibody consisted of FITC-conjugated rabbit anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA). Cell nuclei were stained with nucleic acid stain 4,6-diamino-2-phenylindol (Sigma). Cell images were captured using a confocal laser scanning microscope (Leica TCS-SP, Leica, Wetzlar, Germany) and 63 \times objectives with dual laser excitation and equipped with an imaging software, Leica-TCS-NT version 1.6.551.

Animal Studies

BALB/c *nu/nu* mice, ages 4 to 5 weeks, were obtained from Taconic (Germantown, NY). All experimental studies were approved by The University of Chicago Institutional Animal Committee Board. Each animal was given a single s.c. injection of 1×10^7 U373MG tumor cells in a 100- μ L volume to establish a tumor into the right flank. Once tumors reached 0.7 cm in size, the animals were randomized into seven groups, where each group had five mice with comparable tumor sizes. Each mouse then received i.t. injections of 1×10^8 vp of one of the following viruses: WT, WT-C-RGD, WT-HI-RGD, Ad5/3, Ad5/3-C-RGD, Ad5/3-HI-RGD in RPMI medium, or RPMI medium alone as a control. Forty-eight hours later, the mice were euthanized and tumor xenografts were subjected to immunohistochemistry and quantitative PCR analysis of the viral *E4* gene copy number. At the time of euthanasia, tumors were excised and fixed in 10% formalin, embedded in paraffin, and then cut into 4-mm sections. Immunohistochemical analysis was done on tumor sections with goat anti-hexon antibodies (Virostat, Portland, ME) that recognize the hexon protein, and processed with histostain kit according to the instructions of the manufacturer (DAKO, Carpinteria, CA).

Quantitative PCR Analysis of Viral Genomes in Infected Tumors

Five-millimeter paraffin sections were washed with PBS and paraffin was melted with 1% xylene at room temperature. After spinning down at 4,000 rpm, the tumor tissue was processed for DNA isolation. DNA was isolated with DNeasy Tissue Kit (Qiagen Sciences) and subjected to the quantitative PCR with primers specific for adenoviral *E4* region as described above in the virion binding assay.

Statistical Analysis

The significant differences between groups were assessed by calculation of the Student *t* value. $P < 0.05$ was considered statistically significant.

Results

Assessment of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Expression in Malignant Glioma Cell Lines by Fluorescence-Activated Cell Sorting Analysis

To investigate the potential of RGD-mediated transduction of glioma, we evaluated the level of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin expression in the passaged glioma lines (Fig. 1A)

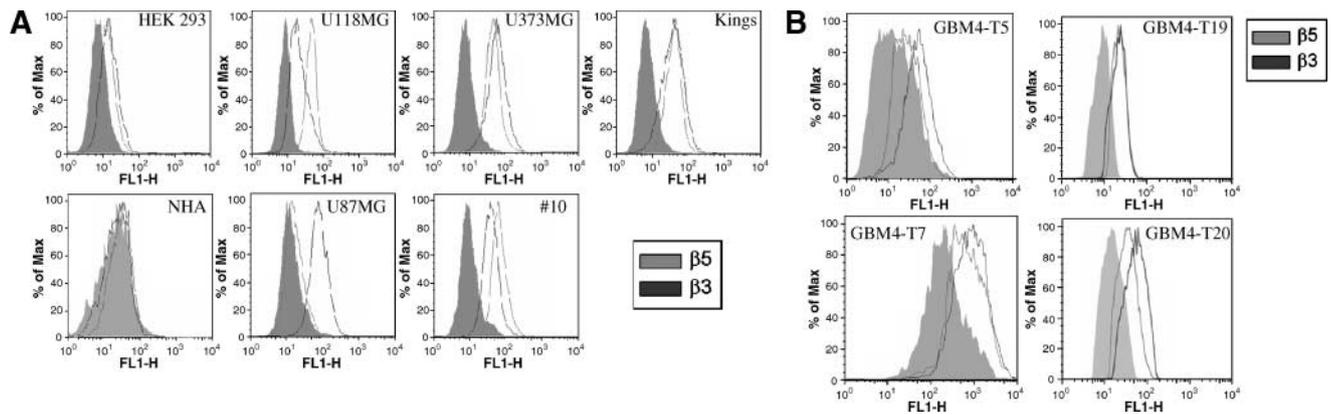


Figure 1. Detection of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin levels in human glioma cells. Passaged human glioma cell lines (A) and patient samples with grade 4 glioma (B) were subjected to fluorescence-activated cell sorting analysis. Gray shading, negative control; blue histogram, $\alpha_v\beta_3$ stained cells; red histogram, $\alpha_v\beta_5$ stained cells. HEK293 cells were used as positive control for both types of integrins. Results represent the means of two independent experiments.

and primary tumor tissue (Fig. 1B) obtained from patients with glioblastoma multiforme. The relative expression of $\alpha_v\beta_3$ in U87MG, U373MG, Kings, No.10, and U118MG was 10.25-, 7.63-, 4.71-, 4.02-, and 1.93-fold greater than in control HEK293 cells. With the exception of U87MG, all tested human glioma cell lines showed elevated levels of $\alpha_v\beta_5$ expression, which were in the range of 5.66-fold (Kings) to 10.27-fold (No.10) higher than that of HEK293 cells. The mean fluorescence intensities detected in primary tumors were also significantly higher than in HEK293 cells. Human glioma cells GBM4-T5, T7, T19, and T20 showed 1.23-, 2.8-, 2.33-, and 3.39-fold increase in expression of $\alpha_v\beta_5$ and 1.81-, 3.75-, 1.76-, and 5.81-fold increase in expression of $\alpha_v\beta_3$, respectively. In contrast, normal human astrocytes did not exhibit expression of $\alpha_v\beta_5$ and showed a relatively lower level of $\alpha_v\beta_3$ integrins (0.69-fold) as compared with HEK293 cells. These findings are summarized in Table 1.

Ad5/3-RGD Shows an Improved Gene Transfer to Glioma Cells *In vitro*

To examine the efficiency of glioma cell transduction with infectivity-enhanced Ad5/3-RGD adenoviruses carrying an RGD peptide in either the HI loop or the COOH terminus, we carried out gene transfer experiments on several cell lines that express different levels of integrin molecules on their surface. As shown in Fig. 2A, both No.10 and Kings tumor cell lines showed a 10-fold increase in luciferase expression with Ad5/3-C-RGD as compared with AdWT or Ad5/3 control viruses ($P < 0.05$). In the case of U87MG cells, Ad5/3-C-RGD showed a significantly enhanced level of cell transduction over AdWT ($P < 0.05$), but not the Ad5/3 vector. In contrast, infection of U118MG and normal human astrocytes with Ad5/3-C-RGD virus led to a decrease in gene transfer as compared with AdWT ($P < 0.05$). An augmented transduction was also observed for Ad5/3-C-RGD in primary tumor specimens (Fig. 2B). In these cells, the luciferase level was 9.7- and 75.2-fold higher than in AdWT (GBM4-T5, $P < 0.05$; GBM4-T7, $P < 0.05$).

To further validate our *in vitro* data, we did immunostaining of infected No.10 glioma cells attached to coverslips (Fig. 3). In these experiments, the Ad5/3-C-RGD vector showed the highest potential to target human glioma cells.

The Double-Modified Viruses Are Capable of Targeting $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrins as Revealed by Antibody Blocking Experiments

Up to this point, we showed that glioma cells were efficiently transduced by Ad5/3-RGD-modified adenoviruses. Furthermore, we hypothesized that the increased infectivity observed in our studies was due to interaction between the RGD-modified fiber knob region and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin molecules of which the abundance on the surface of glioma cells was validated by our fluorescence-activated cell sorting analysis. To test whether $\alpha_v\beta_3$

Table 1. Assessment of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ expression in malignant glioma cell lines by fluorescence-activated cell sorting analysis

	Mean fold increase (%)		Positively stained cells	
	β_3	β_5	β_3	β_5
HEK293	1	1	7.48	6.1
U87MG	10.25	0.622	76.7	3.8
U373MG	7.63	6.62	57.1	40.39
Kings	4.71	5.66	35.27	34.53
U118MG	1.93	6.31	14.5	38.55
No.10	4.02	10.27	30.1	62.7
NHA	0.69	ND	5.21	ND
GBMT4-T5	1.81	1.23	13.54	7.54
GBMT4-T7	3.75	2.8	28.1	17.1
GBMT4-T19	1.76	2.33	13.22	14.22
GBMT4-T20	5.81	3.39	43.5	20.7

NOTE: Values represent the mean fold increase in integrin expression as well the percentage of positive cells.

Abbreviation: NHA, normal human astrocytes.

or $\alpha_v\beta_5$ serve as targets for our RGD-modified adenovectors, we incubated U87MG cells, expressing varying levels of integrin molecules, with different concentrations (1, 10, and 100 $\mu\text{g}/\mu\text{L}$) of anti- $\alpha_v\beta_3$ or anti- $\alpha_v\beta_5$ antibodies for 2 hours on ice before virus infection. As shown in Fig. 4, 100 $\mu\text{g}/\mu\text{L}$ solution of anti- $\alpha_v\beta_3$ antibody reduced the level of Ad5/3-C-RGD transduction by 49.43%. On the other hand, treatment of the cells with anti- $\alpha_v\beta_5$ or anti- $\alpha_v\beta_3$ antibodies resulted in inhibition of the WT-HI-RGD virus-mediated gene transfer by 57.39% and 41.24%, respectively. Thus, infectivity of Ad5/3-HI-RGD and WT-HI-RGD viruses for U87MG cells was more sensitive to $\alpha_v\beta_3$ blocking than that of the control AdWT or Ad5/3 vectors.

Ad5/3-C-RGD Shows Increased Ability to Bind to the Target Cell Surface

Previously, it was shown that the incorporation of the RGD motif in the HI loop of the Ad5 fiber knob results in increased cell binding of the virions as compared with a virus with no RGD modification (43). By using an immunohistochemical staining approach, we have shown an augmented ability of Ad-5/3-C-RGD to target glioma cells and showed involvement of the integrin-dependent pathway in the targeting mechanism.

The observed enhancement of cell transduction could thus be a result of improved kinetics of virus attachment mediated by different regions of the modified fiber knob domain. To address this possibility, we investigated cell binding characteristics of adenoviruses with different RGD modifications. To this end, three cell lines, U87MG, U118MG, and No.10, with different densities of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the surface, were incubated with 1,000 vp/cell of purified viruses on ice for 1 hour. After incubation, total DNA from the cells was isolated and quantitative PCR analysis was done using primers specific for the Ad5 genome *E4* region. The results are presented as ratio of *E4* to human *GAPDH* gene copy number. As shown in Fig. 5, Ad5/3-C-RGD showed the highest potential of binding to the target cells among all Ad5/3 chimeric vectors ($P < 0.05$).

Ad5/3-C-RGD Shows Enhanced Infectivity in Human Glioma Xenografts

To determine whether incorporation of an RGD motif in the knob domain of the chimeric Ad5/3 fiber would further improve adenoviral transduction of glioma xenografts versus the same RGD modification in the Ad5 fiber knob, we injected human glioma xenografts with each virus. Although we did not observe a significant difference in hexon expression (Fig. 6A) between the different viruses, the relative *E4* gene copy number for Ad5/3-C-RGD virus-infected tumor xenografts was at least 10-fold greater than that for the Ad5/3-infected or Ad5/3-HI-RGD-infected ($P < 0.05$) tumors and 100- to 1,000-fold greater than that for AdWT with or without RGD modification ($P < 0.05$; Fig. 6B). However, the AdWT-HI-RGD virus did show a significant increase in the *E4* gene copy number as compared with the wild-type virus or the COOH-terminal modified vector ($P < 0.05$).

Discussion

The use of Ad5 for cancer therapy is limited by the deficiency of its primary cell attachment receptor, or CAR, on brain tumors cells (11–15). Ad5 retargeting to alternate receptors through a fiber genetic modification can be used to circumvent CAR dependence of its natural tropism, and thereby achieve infectivity enhancement. In this study, we used a novel approach of “complex mosaicism” in fiber modification for application in malignant glioma gene therapy (35). This approach combines serotype chimerism with peptide ligand incorporation in a single-fiber molecule. Specifically, we employed Ad vectors with an

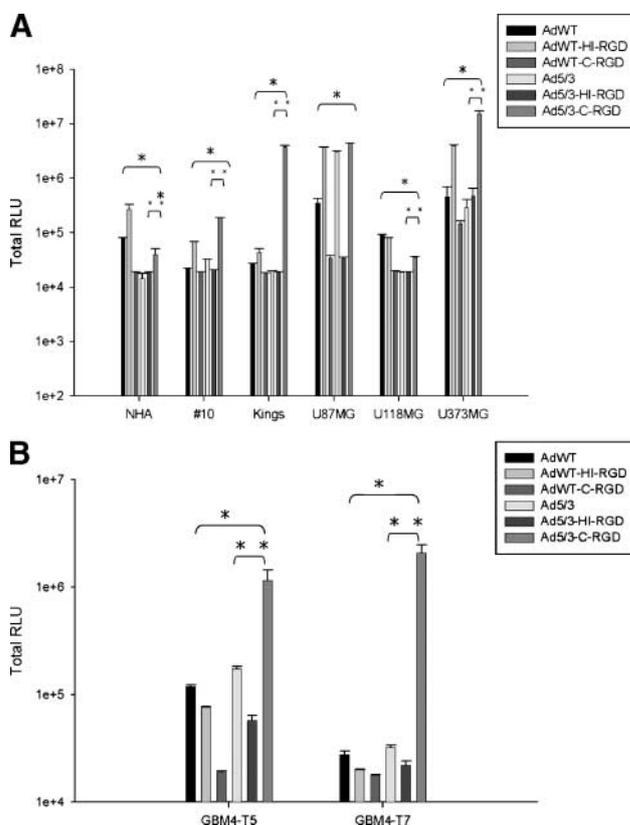


Figure 2. Comparative analysis of adenoviral transduction in human glioma cells mediated by RGD-enhanced vectors. Four human glioma cell lines (No.10, U87MG, U118MG, and Kings), normal human astrocytes (NHA; **A**) and primary brain tumor cells (T5 and T7; **B**) were infected with replication-deficient adenoviruses at 1,000 vp/cell in triplicates. Forty-eight hours after infection, adenoviral transduction efficiency was determined by luciferase assay. Columns, mean total relative light units (RLU) per milligram of total proteins; bars, SD. *, $P < 0.05$, statistically significant difference in gene transfer levels when compared with AdWT. **, $P < 0.05$, statistically significant difference in gene transfer versus Ad5/3. Both No.10 and Kings tumor cell lines showed a 10-fold increase in luciferase expression with Ad5/3-C-RGD as compared with control viruses (AdWT or Ad5/3; $P < 0.05$). In the case of U87MG cells, Ad5/3-C-RGD showed significantly enhanced transduction level over AdWT ($P < 0.05$) but not Ad5/3. In contrast, infection of U118MG and normal human astrocytes with Ad5/3-C-RGD virus led to decrease in gene transfer as compared with AdWT ($P < 0.05$). The enhanced level for Ad5/3-C-RGD transductions was also observed in primary specimens (Fig. 2B). In these cells, the luciferase level was 9.7- and 75.2-fold greater in comparison with AdWT (GBM4-T5, $P < 0.05$; GBM4-T7, $P < 0.05$).

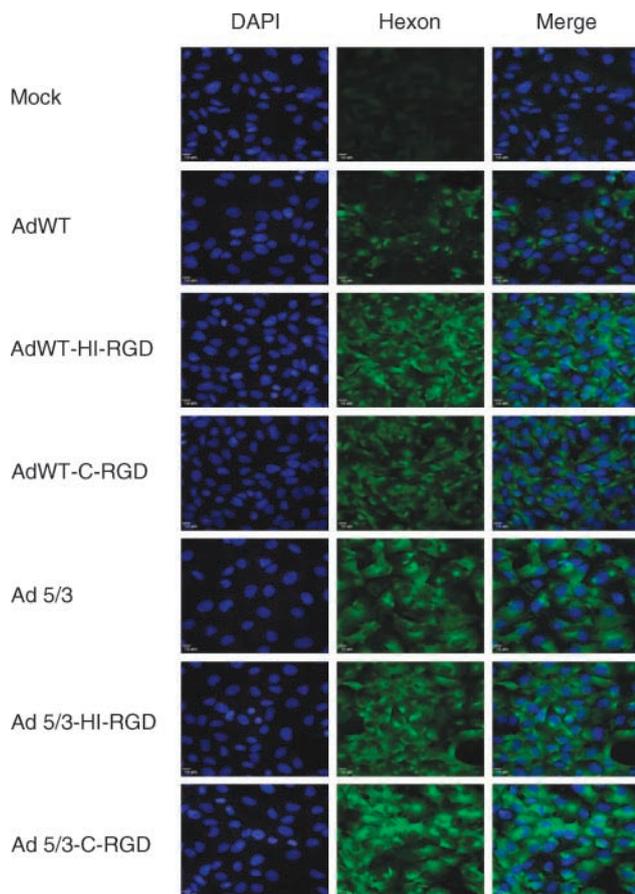


Figure 3. Validation of glioma transduction by an immunohistochemical approach. Human glioma cells (No.10) were grown on coverslips and subsequently infected with Ad vectors at 10 vp/cell in 2% FBS-DMEM. After adsorption, infection medium was replaced by growth medium. Staining was done 48 h postinfection with antibodies that recognize the human Ad5 hexon protein. Ad5/3-C-RGD and, to a lesser extent, AdWT-HI-RGD showed the highest intensity of staining for Ad5 hexon protein at the above time point. Bar, 10 μ m.

integrin-binding RGD peptide genetically incorporated into either the HI loop or the COOH terminus of the Ad3 knob, in the background of Ad5/3 chimera, to achieve simultaneous targeting of cellular integrins and Ad3 receptors, thereby taking advantage of the elevated expression levels of these receptors on malignant gliomas (23, 26–31).⁶

Our results show that the double targeted Ad5/3-RGD vector is capable of more efficiently infecting glioma cells and expressing transgenes than either the wild-type Ad5 or the unmodified Ad5/3 vector. These findings were confirmed in both passaged and primary human glioma cell lines and were subsequently validated by gene transfer blocking studies *in vitro*. Moreover, this enhanced level of transduction was dependent on the RGD-modified fiber, as documented by our binding assay. To quantify the entry of viral genomes into the infected cells, we then analyzed the expression of the E4 protein in an *in vivo* model of human glioma. The results clearly indicate an increase in E4 activity

when using the Ad5/3-C-RGD virus as compared with either Ad5/3 or AdWT control. Taken together, these results suggest that Ad5/3-C-RGD may be a superior vector for further development in the field of neuro-oncology.

Of note, the Ad5/3-C-RGD vector showed an improved profile over Ad5/3-HI-RGD. This finding was at first surprising, given that early studies identified stringent size limitations imposed by the structure of the adenoviral fiber protein on ligands incorporated into its COOH terminus. In fact, published findings (24, 44) strongly suggest that the addition of >25 to 30 amino acid residues of heterologous protein sequence to the COOH terminus of the fiber molecule strongly confounds stability of the fiber trimer and, therefore, is incompatible with the fiber functions. In addition, the three-dimensional structure of the fiber knob (45) clearly indicates that the COOH terminus of the fiber points toward the virion (i.e., away from the cell surface), thereby providing a suboptimal environment for the incorporation of targeting ligands. Moreover, we have previously identified the HI loop of the fiber knob domain as a preferred site for the incorporation of targeting ligands and hypothesized that the structural properties of this loop would allow for the insertion of a wide variety of ligands, including large polypeptide molecules, to improve their performance in receptor targeting relative to the COOH-terminal locale (46).

In the present study, we have tested this hypothesis by deriving a family of Ad vectors of which the fibers contain an RGD ligand in either the COOH terminus or the HI loop of the Ad3 fiber knob. By assessing the levels of infectivity and transgene expression of the resulting viruses, we found that Ad5/3-C-RGD showed an improved profile over Ad5/3-HI-RGD in the setting of malignant glioma. Whereas the precise mechanism underlying this phenomenon is beyond the scope of the current investigation, these results are similar to other studies, where the addition of RGD at the COOH terminus was found to increase adenovirus-mediated gene delivery to bovine endothelial cells *in vitro* and *in vivo* to the kidney vasculature (25, 47). This concept has been further developed by Wickham et al. (25, 48), who have proved the feasibility of this approach by generating several recombinant adenoviruses containing fibers with targeting ligands placed at their COOH termini. It would therefore seem that the constraints relative to the size of the peptide that can be incorporated into the COOH terminus do not affect the function of the RGD ligand and, in fact, enhance the infectivity of the virus as compared with the more favored HI-loop locale in the context of malignant glioma.

Indeed, the data we present in this study suggest that incorporation of an RGD motif into the COOH terminus is compatible with the Ad3 knob binding to its receptor, as Ad5/3-RGD was the only modification of Ad3 knob that significantly increased the vector infectivity for malignant glioma. In contrast, incorporation of the RGD ligand in the HI loop reduced vector infectivity in all cell lines. Of note, the effects of HI loop incorporation of the RGD ligand are markedly different for Ad5 and Ad3 fiber knobs. Whereas HI loop modification in Ad3 knob elicits an inhibition of

vector infectivity, the same modification in the Ad5 knob does not interfere with the intrinsic CAR-binding property of Ad5 knob and can, in fact, display a strong augmentation of viral transduction (~10-fold). Taken together, our

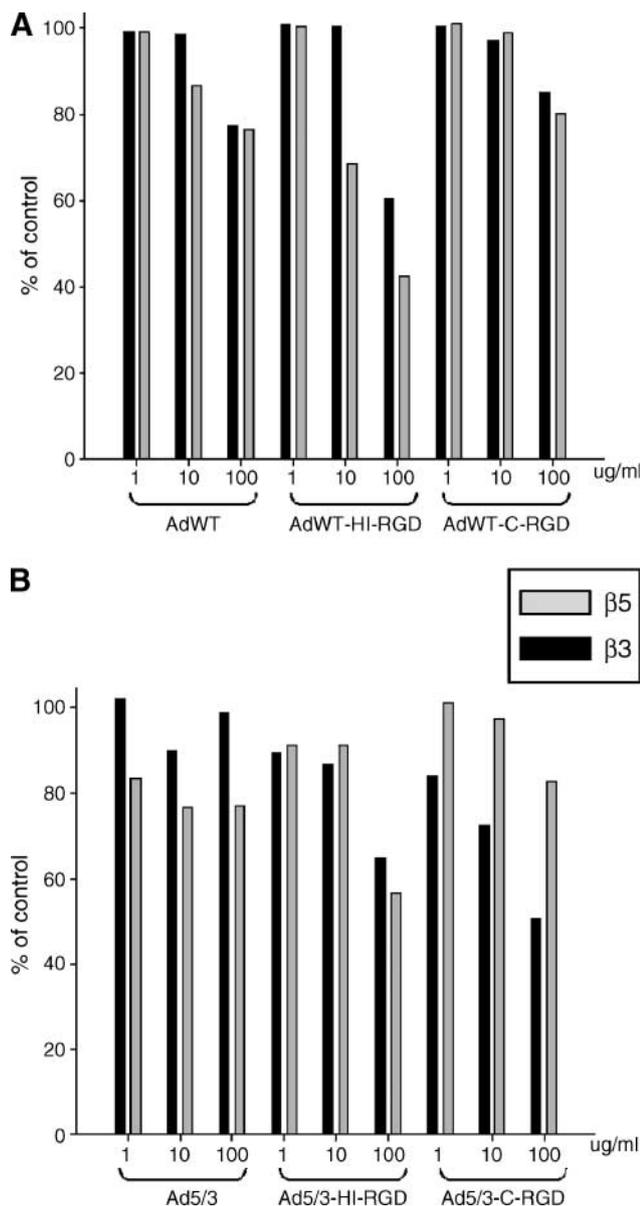


Figure 4. Antibody-mediated blocking of gene transfer in human glioma cells. Human glioma cells (U87MG) were incubated with monoclonal antibodies to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin molecules for 2 h on ice before infection with AdWT, AdWT-HI-RGD, and AdWT-C-RGD (A) and Ad5/3, Ad5/3-HI-RGD, and Ad5/3-C-RGD (B) at 1,000 vp/cell. Results are expressed as percentage of control [(relative light units in cells unblocked with monoclonal antibody/relative light units in cells unblocked with monoclonal antibody) \times 100]. Columns, percent of remaining transduction as a result of inhibition mediated by $\alpha_v\beta_3$ (black) and $\alpha_v\beta_5$ (gray) antibody blocking. The experiment was repeated twice and the data represent the value of two independent experiments. Significant inhibition of transduction by Ad5/3-C-RGD and AdWT-HI-RGD was observed after blocking with anti- $\alpha_v\beta_3$ (49.43%) and anti- $\alpha_v\beta_5$ (57.39%) integrin antibodies, respectively.

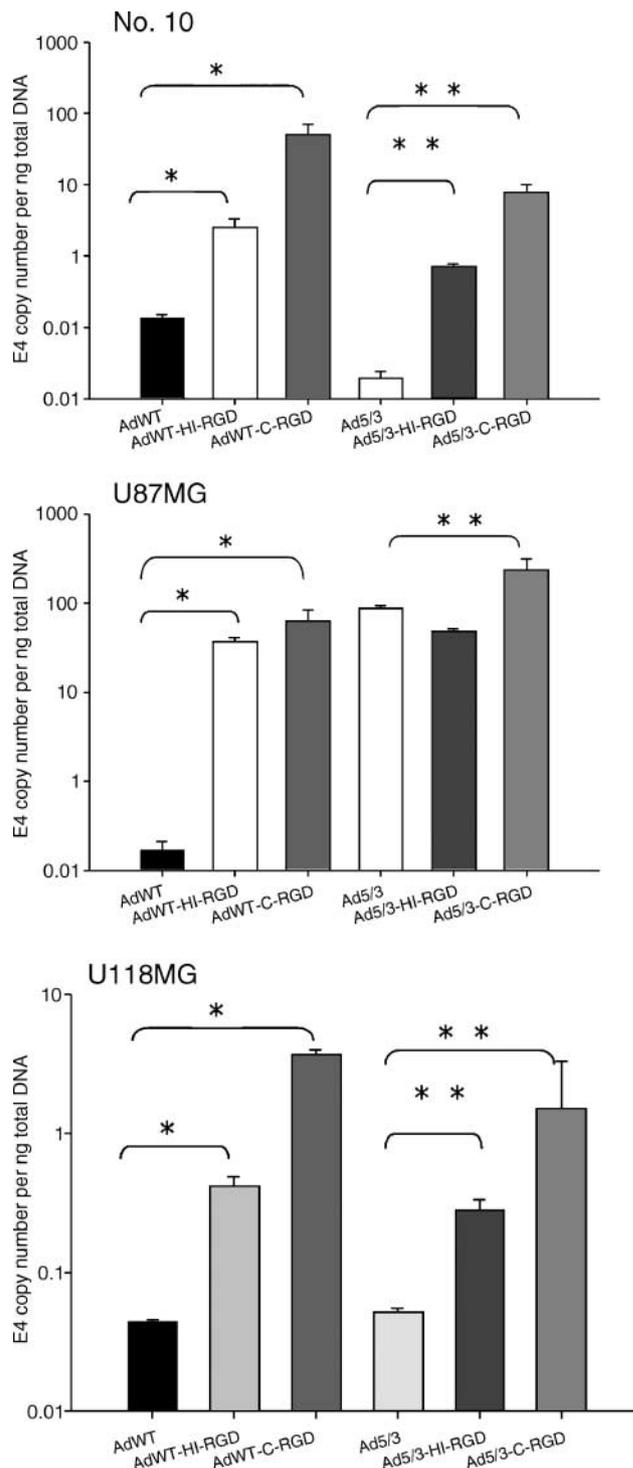


Figure 5. Comparative analysis of the virions binding efficiency to glioma cell surface. U87MG, U118MG, and No. 10 human glioma cells were grown to 60% to 80% confluency for 24 h. Cells were then infected with recombinant adenoviruses (1,000 vp/cell for 1 h at 4°C), washed twice, trypsinized, and resuspended in a small volume of the growth medium. Isolated DNA was analyzed by quantitative PCR using the SYBR Green kit. Columns, mean of two independent experiments done in triplicates; bars, SD. *, **, $P < 0.05$, values with statistically significant difference in gene transfer levels relative to AdWT and Ad5/3, respectively.

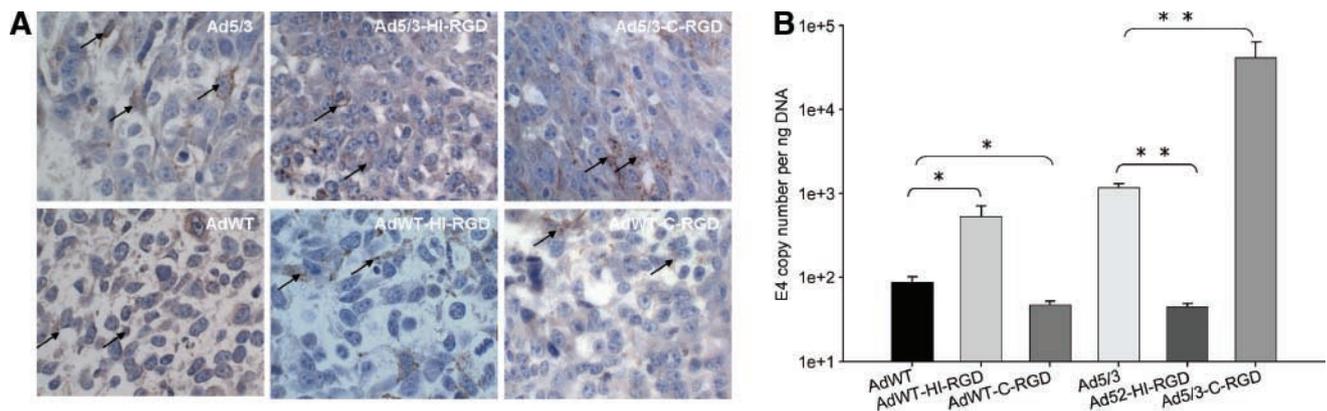


Figure 6. Comparative analysis of transductional efficiency of the RGD modified vectors in glioma xenografts. Mice were s.c. injected with 1×10^7 U373 glioma cells. After 7 d, when tumor reached 0.7 cm in size, the mice were divided into seven groups that received intratumoral injections of AdWT, AdWT-HI-RGD, AdWT-C-RGD, Ad5/3, Ad5/3-HI-RGD, and Ad5/3-C-RGD at 10^8 vp/mouse or were mock infected. Vector distribution was examined in tumors 48 h later by immunohistochemistry using anti-hexon primary and horseradish peroxidase-conjugated secondary antibodies (A) as described in Materials and Methods. The sections were also analyzed by a quantitative PCR with E4 region-specific primers (B). Black arrows, cells that positively stained for hexon. The highest signal was observed for sections of tumors infected with Ad5/3-C-RGD and, to a lesser extent, AdWT-HI-RGD. Viral infectivity, as measured by E4 copy number, supported the immunohistochemical data. *, **, $P < 0.05$, values with statistically significant difference in gene transfer levels relative to AdWT and Ad5/3, respectively. Magnification, $\times 40$.

results suggest that the HI loop of Ad3 knob is less suitable for RGD ligand incorporation than its COOH terminus. This could be due to either the steric hindrance or conformational alterations at the Ad3 receptor-interacting interface of the knob domain caused by genetic incorporation of the RGD-ligand in the particular knob locale.

In conclusion, we present the first evidence to show that the double targeted Ad5/3-RGD virus shows enhanced infectivity and gene transfer in malignant brain tumors. This is a significant finding with important implications for the field of neuro-oncology, where previous attempts in the use of Ad vectors have encountered limitations due to low vector transduction efficiency and limited gene expression (5–10). Our results justify the need to further examine this vector in additional studies, with the ultimate aim of translating this work into a clinical trial for malignant glioma.

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