Preclinical Characterization of the Antiglioma Activity of a Tropism-Enhanced Adenovirus Targeted to the Retinoblastoma Pathway

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Background: Oncolytic adenoviruses are promising therapies for the treatment of gliomas. However, untargeted viral replication and the paucity of coxsackie-adenovirus receptors (CARs) on tumor cells are major stumbling blocks for adenovirus-based treatment. We studied the antiglioma activity of the tumor-selective Delta-24 adenovirus, which encompasses an early 1 A adenoviral (E1A) deletion in the retinoblastoma (Rb) protein-binding region, and of the Delta-24-RGD adenovirus. Delta-24-RGD has an RGD-4C peptide motif inserted into the adenoviral fiber, which allows the adenovirus to anchor directly to integrins. Methods: CAR and integrin expression were examined by flow cytometry in six glioma cell lines and in normal human astrocytes (NHAs). Adenoviral vectors containing green fluorescent protein (GFP) (AdGFP and AdGFP-RGD) were used to infect glioma cell lines with high or low CAR expression. Viability of glioma cells infected with different adenoviruses was assessed by trypan blue staining. Adenovirus replication was quantified with the infection-dose replication assay. Athymic mice carrying glioma xenografts received intratumoral injections of Delta-24-RGD or Delta-24 and were followed for survival, which was analyzed by the Kaplan-Meier method and the log-rank test. All statistical tests were two-sided. Results: Half the glioma cell lines expressed low levels of CAR (defined as <50% of cells expressing detectable CAR); all lines expressed integrins in more than 50% of cells. Infection of U-87 MG cells (a low-CAR-expressing line) with AdGFP-RGD resulted in approximately six times more GFP-positive cells than infection with AdGFP. Delta-24-RGD was more cytopathic to both low- and high-CAR-expressing glioma lines than Delta-24, and it replicated more efficiently in both cell lines. In the xenografted mice, intratumoral injection of Delta-24-RGD was associated with longer survival than intratumoral injection of Delta-24 (P<0.001, log-rank test). Furthermore, 60% of Delta-24-RGD-treated mice but only 15% of Delta-24-treated mice survived more than 4 months (difference = 45%, 95% CI = 21% to 68%). Conclusions: The antitumor activity of Delta-24-RGD suggests that it has the potential to be an effective agent in the treatment of gliomas. [J Natl Cancer Inst 2003:95:652-60]

Malignant gliomas are characterized by infiltrative growth that causes progressive neurologic dysfunction and, almost invariably, death. Glioblastoma multiforme, the most common and aggressive form of glioma, typically recurs despite surgery, radiotherapy, and conventional chemotherapy. Currently there is no effective treatment for glioblastoma, and the median survival time of patients with this disease is approximately 1 year (1). However, recent advances in our understanding of brain tumor biology have suggested that treatment strategies that target the molecular defects of brain tumors may provide effective and selective therapies.

Although a comprehensive compilation of the genetic alterations involved in malignant gliomas is still in progress, the retinoblastoma (Rb) protein or its regulatory proteins (i.e., p16INK4a, cyclin-dependent kinases 4 and 6, and cyclin D1) are deregulated in most malignant gliomas (2). Because the Rb pathway is critical for maintaining a functional G1 checkpoint, inactivation of this pathway results in unregulated cell-cycle progression, abnormal cellular growth and, thus, progression of the malignant phenotype in the majority of cancers (3), including malignant gliomas (2).

Following previous studies on the transforming regions of the early 1 A adenoviral (E1A) protein (4), which identified the regions of the E1A protein that bind to Rb, we developed a tumor-selective adenovirus, Delta-24, that encompasses an eight-amino-acid residue (24 base pairs) deletion in the viral E1A region that is responsible for binding the Rb protein (5). This deletion, which we have shown (5) renders viral replication dependent on inactivation of Rb, generates a tumor-selective, replication-competent virus that induces an anticancer effect in glioma(s). Specifically, Delta-24 did not efficiently replicate in quiescent human lung fibroblasts, but infection of gliomas with Delta-24 inhibited their growth both in vitro and in vivo (5). The anticancer effect of Delta-24 is, however, not equal in every cell line because of the inability of Delta-24 to sufficiently infect a broad spectrum of human glioma cells.

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The ability of adenoviruses to infect tumor cells depends on anchorage to the coxsackie-adenovirus receptor (CAR) (6). Cancer cells express low levels of CAR and, as a result, constitute a difficult target for virus-based therapy (7). Therefore, Delta-24 was modified so that it is theoretically capable of infecting tumor cells via CAR-independent mechanisms. We hypothesized that an ability to circumvent CAR would improve the antitumor effect of Delta-24 and would reduce sequestration of the adenovirus by nontargeted normal cells expressing CAR. Because internalization of the adenovirus into host cells is mediated by a secondary interaction between RGD motifs on penton base protein loops and integrins αvβ5 and αvβ3 (8), Delta-24 was modified to target integrins on the surface of cancer cells as its primary receptor. For this approach, we took advantage of the previously isolated peptide ACDCRGDCFCG (RGD-4C), which binds strongly to the αvβ3 and αvβ5 integrins (9, 10). We inserted the RGD-4C sequence into the HI loop of the fiber knob protein of Delta-24 to generate Delta-24-RGD (11). In this study, we tested the hypothesis that Delta-24-RGD is more infective than Delta-24 in gliomas, but that it is equally selective for cancer cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The glioma cell lines D54 MG, U-251 MG, U-87 MG, T98G, U-138 MG, and Saos-2 were obtained from American Type Culture Collection (Manassas, VA). The SNB 19 glioma cell line was a gift from Dr. Jasti Rao (University of Illinois College of Medicine, Peoria, IL). Cell lines were maintained in Dulbecco’s modified Eagle/F12 medium (DMEM/F12) (1 : 1, vol/vol) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO2 at 37°C. Normal human astrocytes (NHAs) were purchased from Clonetics/BioWhittaker (Walkersville, MD). NHA cultures were maintained in astrocyte growth medium from an AGM-Astrocyte Medium BulletKit obtained from Clonetics/BioWhittaker. For serum starvation conditions, we grew NHAs at a low density (2 x 10^4/per well in a six-well plate) in the kit’s medium with 0.5% fetal bovine serum and no growth supplements. These culture conditions inhibited cell growth without evidence of cell death.

Adenovirus Construction and Infection

Construction of Delta-24 and Delta-24-RGD have been previously described (5, 10). These adenoviral constructs have a 24 base-pair deletion of the E1A region that encompasses the area responsible for binding RB protein (nucleotides 923–946), corresponding to the amino acids L122TCEAGF129. Wild-type adenovirus Ad300 (12), Delta-24-RGD inactivated by UV light, and mock-infected cells (i.e., with DMEM/F12 medium) were used as controls.

For the infectivity analyses, human glioma cells (5 x 10^5) were infected with the replication-deficient adenoviruses AdGFP and AdGFP-RGD (13), which express green-fluorescence protein (GFP). Forty-eight hours after infection, the cells were treated with 0.05% trypsin for 5 minutes and washed twice with phosphate-buffered saline (PBS). The cells were then counted for GFP-positive cells by flow cytometry as described below (14).

Measurement of CAR and αvβ5 Integrin Expression by Flow Cytometric Analysis

Cytometric analysis was used to measure the cell surface expression of CAR and αvβ5 integrins. Briefly, human glioma and NHA cultures (5 x 10^5 cells for both) were incubated with the anti-CAR monoclonal antibody Rmcb (diluted 1 : 2000; provided by Dr. J. M. Bergelson (6). The Children’s Hospital of Philadelphia, Philadelphia, PA), the anti-human integrin mouse anti-β5 antibody (clone P1F6, diluted 1 : 400; Invitrogen, Carlsbad, CA), or the anti-human integrin mouse anti-β3 conjugated monoclonal antibody (diluted 1 : 200; Chemicon International, Temecula, CA) at 4°C for 1 hour. For the visualization of the anti-CAR and anti-β5 antibodies, a second incubation with goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (diluted 1 : 100; Santa Cruz Biotechnology, Santa Cruz, CA) was conducted at room temperature for 1 hour. The cells were then washed twice with PBS and stained with propidium iodide at 50 μg/mL and with RNase at 20 μg/mL for 15 minutes at room temperature. Cell samples were analyzed for fluorescence with an EPICS XL-MCL flow cytometer (Beckman Coulter, Miami, FL) using a 488-nm argon laser for excitation. Fluorescence was detected with a 520-nm band-pass filter, and all cytometric data were analyzed with System II Software (Beckman Coulter). CAR expression was defined as high when it was expressed in 50% or more of the examined cells and as low when it was expressed in less than 50% of the examined cells. This cutpoint was determined a priori based on the distribution of CAR expression shown in Fig. 1.

Competitive Inhibition Assay

Glioma cell lines were incubated in DMEM/F12 media in 96-well plates at 37°C for 24 hours. Recombinant adenovirus fiber knob protein at 100 μL/mL (a gift from Dr. J. Douglas, University of Alabama, Birmingham) or RGD peptide (Gly-Arg-Gly-Asp-Ser-Pro) at 1 mg/mL (American Peptide Company, Sunnyvale, CA) was then added to the culture. RGA peptide (Gly-Arg-Gly-Glu-Ser-Pro) (American Peptide Company) was used as a control for the RGD-based competitive assay. After a 10-minute incubation at 4°C, cultures were infected with either AdGFP or AdGFP-RGD at a multiplicity of infection (MOI) of 50 for 5 minutes at 37°C. Forty-eight hours later, infection efficiency was determined by counting the number of fluorescent cells among 500 cells by phase-contrast microscopy. Each experiment was performed in triplicate.

Cell Viability Assay

Human glioma cells were seeded (at 10^5 cells per well) in DMEM/F12 medium in six-well plates and allowed to grow for 20 hours at 37°C. Cells were then infected with Delta-24, Delta-24-RGD, Ad300, or UV-inactivated Delta-24-RGD at MOIs of 0, 5, and 10 at 37°C for 30 minutes. Experiments were concluded when an MOI of 10 for one of the adenoviral constructs produced a cytopathic effect of more than 50%. The cell monolayers were then washed twice with PBS and were fixed and stained with 0.1% crystal violet in 20% ethanol. Excess dye was removed by rinsing several times with water. Trypan blue experiments were performed to quantitate cell viability, as previously described (14). Briefly, cultures were infected with the
Viral Replication Experiments

Viral replication experiments were performed using human glioma cells (T98G, U-138, U-87, D54, SNB 19, and U-251) and normal human astrocytes (NHA). Cells were incubated with anti-CAR, anti-αvβ3, or anti-αvβ5 integrin monoclonal antibodies, washed to remove unbound monoclonal antibodies, and incubated with fluorescein isothiocyanate-labeled secondary antibodies. After removal of the fluorescein isothiocyanate-labeled antibodies, cells were analyzed by flow cytometry. Data are shown as the percentages of cells expressing CAR (solid bars), αvβ5 integrin (dotted bars), and αvβ3 integrin (open bars). The percentages are the means ± 95% confidence intervals (CIs) of three independent experiments. B) CAR-independent infection of RGD-modified adenoviruses. U-251 MG cells were infected with constructs expressing either the wild-type fiber knob (AdGFP) or a modified fiber knob (AdGFP-RGD) in competition experiments with an excess of fiber knob protein (solid bars), RGD peptide (hatched bars), or RGA peptide (open bars). The infectivity of AdGFP was efficiently blocked by recombinant fiber knob protein compared with bovine serum albumin (100 μg/mL)-pretreated cells (i.e., controls) (*P<.001; two-tailed Student’s t test). In contrast, the infectivity of AdGFP-RGD was not efficiently blocked by recombinant fiber knob protein compared with bovine serum albumin (100 μg/mL)-pretreated cells (i.e., controls) (*, P = .26; two-tailed Student’s t test). Treatment of glioma cells with excess RGD peptide also resulted in reductions in infectivity for both adenoviruses. Data are shown as relative percentage (mean ± 95% CI) of GFP-positive cells scored among 500 cells per treatment group. For clarity, GFP-positive cells in the AdGFP-RGD-infected cultures are presented as 100%.

Infection with Exogenous Wild-Type Rb or p21

The Rb and p21 adenoviruses used in this study and their infectivity have been previously described (16,17). Briefly, after D54 MG and U-251 MG glioma cells (at 2 × 10⁴ cells/well) were seeded in DMEM/F12 medium in six-well plates, the cultures were infected with replication-deficient adenoviral vectors expressing either Rb or p21 or with the control adenoviral vector Ad5CMV-pA (with an empty expression cassette) (16,17) at an MOI of 80 at 37 °C for 30 minutes. Seventy-two hours later, the cultures were treated with either Delta-24-RGD or UV-inactivated Delta-24-RGD at an MOI of 10 at 37 °C for 30 minutes. Cell viability was monitored daily and was quantitated using the trypan blue exclusion test.

Animal Studies

U-87 MG human glioma cells (5 × 10⁵) were engrafted into the caudate nucleus of athymic mice using a guide-screw system, as previously described (18). We performed three independent experiments using 6–10 animals per group in each experiment. On days 3, 6, and 8 after implantation of tumor cells, animals were treated with 5-μL intratumoral injections of Delta-24-RGD, Delta-24, UV-inactivated Delta-24-RGD, or PBS (all 1.5 × 10⁴ pfu/mL). Animals showing general or local symptoms of toxicity were killed. Surviving animals were killed 140 days after tumor implantation. Brains were then removed, fixed in 4% formaldehyde for 24 hours at room temperature, and embedded in paraffin. Hematoxylin-and-eosin-stained sections were evaluated (by Dr. G. Fuller, Department of Neuro-Pathology, M. D. Anderson Cancer Center) for evidence of tumor, necrosis, and viral nuclear inclusions. The largest (a) and the smallest (b) diameters of the tumors were measured, and these measurements were used in the calculation of tumor volume using the formula a × b² × 0.4 (19). All animal studies were performed in the veterinary facilities of the M. D. Anderson Cancer Center in accordance with institutional, state, and federal laws and ethics guidelines for experimental animal care.

Immunohistochemical Analysis

To detect adenoviral E1A and hexon proteins in the tumor xenografts, paraffin-embedded sections of the mouse tumors were deparaffinized and rehydrated with xylene and ethanol following conventional procedures (20). Endogenous peroxidase activity was quenched by incubating the sections in 0.3% hydrogen peroxide in methanol for 30 minutes. The sections were then treated with either goat anti-hexon antibody (diluted 1:100; Chemicon) or goat anti-E1A antibody (diluted 1:100; Santa Cruz Biotechnology) at 4 °C overnight. For immunohistochemical staining, Vectastain ABC kits from Vector Laboratories were used to determine the final viral titer according to the Kärber formula (15). Briefly, cell lysates were clarified by centrifugation at 1000g for 15 minutes at 4 °C, and the supernatants were serially diluted in DMEM/F12 medium for the infection of 293 cells in 96-well plates. The cells were analyzed for an adenoviral cytopathic effect 10 days after infection. Final viral titers were determined as plaque-forming units (pfu)/mL, according to the validated method developed by Quantum Biotechnology (Carlsbad, CA).
(Burlingame, CA) were used according to the manufacturer’s instructions.

**Statistical Analyses**

For the *in vitro* experiments, statistical analyses were performed using a two-tailed Student’s *t* test. Data are expressed as mean ± 95% confidence intervals (CIs). The *in vivo* cytopathic effect of Delta-24-RGD and Delta-24 on human glioma xenografts was assessed by plotting survival curves according to the Kaplan–Meier method (21). Survival in different treatment groups was compared using the log-rank test.

**RESULTS**

**Effect of CAR and RGD-Related Integrins on Adenoviral Infectivity in Human Glioma Cells**

To determine the expression of CAR and RGD-related integrins in human glioma cells, we directly measured the cell surface expression of CAR, $\alpha v\beta 5$, and $\alpha v\beta 3$ by flow cytometric analyses in six human glioma cell lines (Fig. 1, A). CAR was expressed in more than or equal to 50% (high CAR expression) of the examined cells in U-251 MG, SNB 19, and D54 MG cultures and in less than 50% (low CAR expression) of the examined cells in U-87 MG, T98G, and U-138 MG cultures. The examination of the $\alpha v\beta 5$ and $\alpha v\beta 3$ integrin expression in the same set of cell lines showed that the majority of the assayed cells in every cell line expressed one of the integrins. Similar studies showed that NHAs express both CAR (65%, 95% CI = 51.3% to 79.7%) and integrins ($\alpha v\beta 3$: 30.4%, 95% CI = 15.7% to 45% and $\alpha v\beta 5$: 77.4%, 95% CI = 55.7% to 99.1%) (Fig. 1, A).

The pattern of CAR and integrin expression suggests that a retargeted adenovirus that is able to use both CAR and RGD-related integrins for cell anchorage might be able to infect a broader spectrum of glioma cell lines (especially cell lines with lower CAR expression) than would an unmodified adenovirus. To test this hypothesis, we compared the transduction efficiency of two adenoviral vectors: AdGFP-RGD (which contains a GFP expression minicassette and the RGD-4C motif inserted in the fiber knob) and AdGFP (which also encodes the GFP gene but has a wild-type fiber knob). We used U-87 MG and U-251 MG cells to compare infectivity of the two adenoviruses in low- and high-CAR-expressing cultures, respectively. At an MOI of 25, AdGFP-RGD-infected cultures of U-251 MG cells showed 1.48-fold (95% CI = 1.15- to 1.65-fold) more GFP-positive cells than did AdGFP-infected cultures of U-251 MG cells. As expected, the difference in the infectivity of the two adenoviral vectors was more pronounced in the low-CAR-expressing cell line than in the high-CAR-expressing cell line, with 5.7-fold (95% CI = 5.14- to 6.26-fold) more GFP-positive cells in the U-87 MG cultures infected with AdGFP-RGD than in the U-87 MG cultures infected with AdGFP. Thus, the insertion of the RGD-4C motif into the fiber knob of the AdGFP vector increased the ability of the adenovirus to infect human glioma cells. In addition, the difference in the infectivity of the two adenoviral vectors was statistically significantly higher in the low-CAR-expressing cell line than in the high-CAR-expressing cell line ($P<.001$; two-tailed Student’s *t* test). Interestingly, the advantage of the RGD-4C insertion was also evident in the SNB 19 cell line, which has the highest percentage of cells expressing CAR (89.8%, 95% CI = 78.4% to 101.2%) and in which infection with AdGFP-RGD resulted in 1.5-fold (95% CI = 0.94- to 2.06-fold) more GFP-expressing cells than infection with AdGFP.

To determine the mechanism underlying the higher infectivity of AdGFP-RGD adenovirus compared with that of the AdGFP adenovirus, we blocked adenoviral infectivity via CAR by incubating U-251 MG human glioma cells with recombinant fiber knob protein before infection with AdGFP or AdGFP-RGD. Incubation of U-251 MG cells with fiber knob protein reduced AdGFP infectivity by more than 50% ($P<.001$; two-tailed Student’s *t* test), but did not statistically significantly reduce AdGFP-RGD infectivity ($P = .26$; two-tailed Student’s *t* test) (Fig. 1, B). These results indicate that the RGD-4C motif provides an alternative binding site via RGD-related integrins and for CAR-independent glioma infectivity.

**Cytopathic Effect of Delta-24-RGD in Human Glioma Cells**

We next asked whether the increased infectivity of AdGFP-RGD, which suggests an increase in infectivity of Delta-24-RGD, translated into an increased cytopathic effect for Delta-24-RGD. To compare the cytopathic effects of Delta-24 and Delta-24-RGD, we performed dose-dependence assays in which both low- (U-87 MG, U-138 MG, T98G) and high-CAR-expressing cell lines (e.g., U-251 MG, SNB 19, D54 MG) were treated with Delta-24-RGD, Delta-24, or the wild-type adenovirus Ad300. In all six cell lines, Delta-24-RGD required a lower dose to induce a complete cytopathic effect than did Delta-24 (Fig. 2, A). Interestingly, two patterns of Delta-24-RGD- and Ad300-induced cytopathic effects were observed. In U-87 MG, T98G, and U-138 MG cell cultures, all of which had low CAR expression, Delta-24-RGD had a higher cytopathic effect than did Delta-24 or Ad300. However, in U-251 MG, SNB 19, and D54 MG cell cultures, all of which had high CAR expression, Ad300 was similar to or slightly more cytopathic than Delta-24-RGD. Thus, Delta-24-RGD was more potent than Ad300 in cells with low CAR expression. To more precisely quantify the cytopathic effect of Delta-24 and Delta-24-RGD, we performed a trypan blue exclusion test in U-87 MG and U-251 MG cell cultures. The MOI to induce 50% cell death was between 0.1 and 1 for Delta-24-RGD and between 1 and 10 for Delta-24 in both cell lines (Fig. 2, B).

To determine whether the increased cytopathic effect with Delta-24-RGD was related to increased adenovirus replication, we performed a tissue-culture infection dose-replication assay. In two independent experiments, Delta-24-RGD replicated 5.2 and 5.1 orders of magnitude more efficiently than did Delta-24 in U-87 MG cells and 3.1 and 4.4 orders of magnitude more efficiently than did Delta-24 in U-251 MG cells. These experiments also demonstrated that Delta-24-RGD replicated with an efficiency similar to that of Ad300 in U-251 MG cells but rep-
licated 2.1 and 2.9 orders of magnitude more efficiently than Ad300 in U-87 MG cells (Fig. 2, C). The final viral titer of Delta-24-RGD was higher than the viral titer of Delta-24 in lysates from both cell lines.

Because the Rb pathway is disrupted by the homozygous deletion of p16 in the U-87 MG and U-251 MG cell lines, we were also interested in determining whether Delta-24-RGD replicates efficiently in cells lacking expression of Rb. Therefore, we performed replication experiments in Saos-2, an Rb-deficient glioma cell line (5). Two independent experiments showed that Delta-24-RGD at an MOI of 10 achieved titers of $6 \times 10^{11}$ and $5 \times 10^{10}$ pfu/mL 2 days after infection (data not shown). Taken together, these experiments confirm the ability of Delta-24-RGD to replicate in cells with an abnormal Rb pathway due to abnormal expression of an Rb regulator (such as p16) or Rb protein.

**Rb Pathway and the Cytopathic Effect of Delta-24-RGD**

We previously showed that restoration of Rb function in D54 MG cells substantially decreased the cytopathic effects of the Delta-24 adenovirus (5). To determine whether Delta-24-RGD-mediated cell death is also dependent on the cell-cycle regulatory function of the Rb protein, we treated D54 MG cells with replication-deficient adenoviral vectors expressing either Rb or p21 (or an empty vector) and infected the cells with Delta-24-RGD (or UV-inactivated Delta-24-RGD). D54 cell cultures pretreated with Rb or p21 had less cell death (24.1%, 95% CI = 22.8% to 25.3% and 24.5%, 95% CI = 22.4% to 26%, respectively) than did cultures infected with the empty vector (50.2%, 95% CI = 50.1% to 50.3%) (Fig. 3). To confirm these findings, we performed a similar assay with U-251 MG cells. Similarly, U-251 MG cell cultures pretreated with Rb or p21 had less cell death (19.7%, 95% CI = 18.6% to 20.7% and 11.7%, 95% CI = 7.4% to 16%, respectively) than did cultures infected with the empty vector. Interestingly, the rescue of D54 MG cells from the cytopathic effect of Delta-24, which was reported previously (5), and the rescue of Delta-24-RGD by p21 were similar in terms of their inhibition of adenoviral-mediated cell death.

![Fig. 2. A) Cell viability analysis of Delta-24-RGD- and Delta-24-infected human glioma cell cultures. At least three experiments were performed for every cell line. Low-CAR-expressing cell lines (<50% of the cells expressed CAR) are shown in the upper panel, and high-CAR-expressing cell lines (≥50% of the cells expressed CAR) are shown in the lower panel. Delta-24-RGD is more efficient than Delta-24 at inducing a complete cytopathic effect in all cell lines. B) Quantitation of cell death induced by Delta-24-RGD in U-87 MG and U-251 MG human glioma cells. Cell viability was assessed using the trypan blue exclusion test. Data are shown as the percentage (mean ± 95% confidence interval [CI]) of cells alive after infection with Delta-24-RGD or Delta-24 at 0 (solid bars), 0.1 (dotted bars), 1 (hatched bars), or 10 (open bars) multiplicities of infection (MOIs) relative to cells infected with UV-inactivated Delta-24-RGD (control, equal to 100%). The experiments were concluded and the data were analyzed when one of the tested adenoviruses killed more than 50% of the cells at an MOI of 1. C) Analyses of Delta-24 (open bars), Delta-24-RGD (solid bars), and Ad300 (wild-type control) (hatched bars) in U-87 MG and U-251 MG cells. Analysis was performed 2 days after infection at an MOI of 10. Viral titers were determined by the tissue culture infection dose method in 293 cells and expressed as plaque-forming units (pfu) per milliliter. Two independent experiments are shown.

![Fig. 3. Rescue of Delta-24-RGD-mediated cell death by Rb and p21. D54 MG and U-251 MG cells were pretreated with AdCMV-pA (empty vector), AdCMV-p21, or AdCMV-Rb at a multiplicity of infection of 80, followed 3 days later by treatment with Delta-24-RGD or UV-inactivated Delta-24-RGD at a multiplicity of infection of 10. Cell viability was assessed by the trypan blue exclusion test. Data are shown as the percentage (mean ± 95% confidence interval [CI]) of Delta-24-RGD-mediated cell death in Rb or p21-pretreated cells (hatched and open bars, respectively) compared with control AdCMV-pA-pretreated cells (solid bars) from three independent experiments.](https://jnci.oxfordjournals.org/content/95/9/656.f2)
To determine whether pretreatment of glioma cells with an adenovirus expressing Rb would impair the ability of Delta-24-RGD to replicate, we performed a replication assay. In two independent experiments, Delta-24-RGD replicated 4.7 and 5.7 orders of magnitude less efficiently in D54 MG cells pretreated with adenovirus expressing Rb than it did in cells pretreated with the control empty vector (data not shown), indicating that restoration of Rb decreased both cell death and adenoviral replication.

Replication Profile of Delta-24-RGD in NHAs

Reduction of the cytopathic effect of Delta-24-RGD by the restoration of the Rb pathway suggested that Delta-24-RGD might be unable to replicate efficiently in nondoning normal human cells. To confirm this hypothesis, we designed a set of experiments involving the infection of NHAs with Delta-24-RGD. First, we assessed the ability of GFP-expressing adenoviruses to infect NHAs. At an MOI of 100, both AdGFP-RGD and AdGFP infected 80%–100% of the cells. These data were consistent with the observation that a high percentage of NHAs express CAR (Fig. 1, A). Second, we assessed the ability of Delta-24-RGD, Delta-24, Ad300, and UV-inactivated Delta-24-RGD to replicate in serum-starved NHAs. After 3 days at an MOI of 1, wild-type adenovirus Ad300 was able to replicate; however, Delta-24-RGD and Delta-24 were unable to acquire a consistent replication phenotype under these conditions (Table 1). As expected, no virus was detected in the lysates from cultures infected with UV-inactivated Delta-24-RGD. These experiments were consistent with our previous work showing that Delta-24 does not replicate efficiently in nondoning human lung fibroblasts (5).

To determine the therapeutic index (i.e., viral replication in tumor cells/viral replication in normal cells) for Delta-24-RGD, we compared the replication of this adenovirus in serum-starved glioma cells and in NHAs. Delta-24-RGD replication was 1000–10000 times greater in the glioma cells than it was in the NHAs. In contrast, Ad300 replicated with the same efficiency in both the human glioma and NHA cultures (Table 1). These results indicate that Delta-24-RGD replicates more selectively than wild-type adenovirus in human glioma cells.

Effect of Delta-24-RGD on Gliomas In Vivo

To compare the therapeutic efficacies of Delta-24 and Delta-24-RGD, U-87 MG xenografts were grown in the brains of athymic mice. In three independent experiments, the mean survival for the control mice (i.e., mice receiving PBS and UV-inactivated Delta-24-RGD) was 19 days (95% CI = 18 to 20 days) and 18 days (95% CI = 18 to 19 days), respectively. In contrast, the mean survival for the Delta-24-treated mice was 50 days (95% CI = 30 to 70 days), which was statistically significantly longer than the mean survival in control mice (P<0.001; log-rank test). Delta-24-RGD-treated mice survived statistically significantly longer (mean = 131 days, 95% CI = 100 to 162 days) than both the control mice and Delta-24-treated mice (both P<0.001; log-rank test) (Fig. 4). There were no long-term survivors among the control mice; however, 15 of the 25 Delta-24-RGD-treated mice survived more than 4 months after tumor implantation: four of nine in experiment 1, four of six in experiment 2, and seven of 10 in experiment 3. By contrast, there were only four long-term survivors of a total of 26 Delta-24-treated mice: one of 10 in experiment 1, three of six in experiment 2, and zero of 10 in experiment 3. Thus, a higher percentage of mice were long-term survivors in the Delta-24-RGD-treated group than in the Delta-24-treated group (60% versus 15%, respectively; difference = 45%, 95% CI = 21% to 68%).

Table 1. Adenoviral replication in human glioma cells U-251 MG and U-87 MG and in normal human astrocytes (NHAs)*

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<tr>
<td>Exp. 2</td>
<td>5 × 10^4</td>
<td>3.2 × 10^6</td>
<td>2 × 10^7</td>
</tr>
<tr>
<td>Ad300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 1</td>
<td>1 × 10^6</td>
<td>2.5 × 10^7</td>
<td>1.5 × 10^7</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>5 × 10^5</td>
<td>3.1 × 10^4</td>
<td>1.5 × 10^4</td>
</tr>
</tbody>
</table>

*Data represent the final viral titer in plaque-forming units (pfu/mL) from two independent experiments. Viral titers were determined by the tissue culture infection dose method (15) from lysates of cells plated in low confluence, maintained in serum-starved conditions (0.5% fetal bovine serum, no growth supplements), and infected with 2 × 10^5 pfu/mL of Delta-24, Delta-24-RGD, or Ad300 (a wild-type adenovirus used as a control). Exp. = experiment.

Fig. 4. Kaplan–Meier survival curves for overall survival in Delta-24-RGD-, Delta-24-, and control-treated athymic mice with U-87 MG intracranial xenografts. Intracranial implantation of U-87 cells (day 0) was followed by intratumoral injection (days 3, 6, and 8) with Delta-24-RGD (filled triangles; n = 25), Delta-24 (filled squares; n = 26), UV-inactivated Delta-24-RGD (UV-i Delta-24-RGD) (filled circles; n = 26), or phosphate-buffered saline (PBS) (open circles; n = 20). The P values were determined by log-rank test and represent the comparison of survival among Delta-24-RGD-treated mice with that among Delta-24- or UV-i Delta-24-RGD-treated mice. These data represent the combined results of three independent experiments. Note the number of long-term survivors—defined as mice that lived more than 4 months after tumor implantation without showing physical signs of disease—in the Delta-24-RGD group (15 of 25 mice).
Histopathologic Examination of Tumors and Brains

Microscopic examination of the brains of control mice (with U-87 xenografts) revealed noninfiltrative tumors growing in a spherical pattern (Fig. 5, A). Histopathologic characteristics of the tumors included a dense cellular mass, hypervascularity, and no necrotic areas. The mean tumor volume was 81.6 mm³ (95% CI = 81.6 to 102.4 mm³), and all brains of mice that died naturally showed a midline shift and ventricular compression secondary to tumor-mass effects, which are characteristic features of herniation and indicate that tumor growth was probably the cause of death in mice that died naturally.

Fig. 5. Histopathologic examination of the brains of Delta-24-RGD-treated mice. A) Gross morphologic features of a representative tumor (T). Shown is a hematoxylin-and-eosin-stained whole-mount coronal section of the brain of a mouse treated with UV-inactivated Delta-24-RGD that died 19 days after implantation of U-87 MG cells. The mean tumor volume was 81.6 mm³ (95% CI = 81.6 to 102.4 mm³). Note the pronounced mass effect with midline shift, ve = lateral ventricle (arrows). B) High-magnification (x200) cross-sectional view of a Delta-24-RGD-treated U-87 MG xenograft from a mouse that died 25 days after implantation that shows prominent viral inclusion bodies (arrows). C) Cross-sectional view (x100) of a U-87 MG xenograft infected with Delta-24-RGD from a mouse that died 27 days after implantation. The tissue section was incubated with anti-E1A antibody (diluted 1:100), and alkaline phosphatase–diaminobenzidine was used as the chromogen, followed by counterstaining with hematoxylin. Three distinct tumor zones are seen: a region of high E1A protein expression (V), which consists of large numbers of tumor cells with prominent viral inclusions intermixed with apparently intact tumor cells; and a peripheral zone of intact tumor cells with scattered cells with signs of infection (T). Immunohistochemical analyses also demonstrated that Delta-24-RGD was able to transduce late structural genes—that is, genes that encode proteins required for the structure of the adenovirus, such as hexon protein (Fig. 5, D)—which is consistent with active adenoviral replication. Immunohistochemical analyses of normal regions of brain tissue in Delta-24-infected mice were negative for E1A protein and hexon viral proteins (data not shown).

Microscopic examination of the brains of all of the long-term survivors showed complete tumor regression. Sequelae of these tumors were identified, including dystrophic calcification and microcyst formation, at the tumor implantation site in the right caudate nucleus (Fig. 5, E and F). Immunohistochemical analyses of these brains (i.e., long-term survivors) with both anti-E1A and anti-hexon antibodies revealed no viral particles (data not shown). We did not observe either E1A expression or signs of inflammatory reaction in the normal brain tissue.

DISCUSSION

Delta-24-RGD is the first new-generation, replication-competent oncolytic adenovirus with both a high selectivity for glioma tumor cells (versus NHAs) and increased infectivity of a broad spectrum of human gliomas. The high selectivity of Delta-24-RGD for glioma cells is based on the 24 base-pair deletion of the E1A gene, which we have shown (5) renders viral replication dependent on inactivation of Rb. The increased infectivity of Delta-24-RGD results from the addition of the RGD-4C motif within the viral fiber knob of the Delta-24 adenoviral construct. Our current data demonstrate that this modification provides Delta-24-RGD with a powerful and selective antiglioma effect in cells with low CAR expression. Most important, to the best of our knowledge, we have shown for the first time that Delta-24-RGD, in a mouse intracranial glioma model, is capable of spreading centrifugally from the site of injection through an established tumor via renewed replication. Thus, the tropism modification of Delta-24-RGD results in a substantial improvement in outcome by producing a high percentage of long-term survivors that have complete regression of established tumors.

Modification of the Delta-24 fiber knob with the insertion of the RGD-4C motif allowed Delta-24-RGD to overcome the low, heterogeneous expression of CAR in human glioma cells that is believed to limit adenovirus infectivity and, thus, to provide a cytopathic effect in these cells. As is the case in other cancers (7), CAR expression is low in the majority of gliomas (22). The insertion of an RGD-4C motif into the Delta-24 fiber knob has been shown to increase adenoviral infection of a variety of normal and cancer cell lines (23). Our finding that the RGD-4C motif increased the infectivity of Delta-24 supports this concept because the increased infectivity was related specifically to hu-
man glioma cells and because the RGD-4C motif increased infectivity of gliomas with low CAR expression.

The insertion of RGD-4C into replication-deficient adenoviral vectors, which was first described by Dmitriev et al. (24), is believed to enable direct binding and internalization of adenoviruses through a CAR-independent, RGD-related integrin-dependent mechanism (24). However, the relative importance of cell-surface integrins versus CAR expression for entry of an adenovirus into target cells has not been completely defined. Adenovirus-mediated gene transfer is facilitated by an interaction between RGD on the adenovirus penton base and v integrins on the cell surface (18,25,26). Our findings shed light on the role of integrins in the adenoviral infectivity of gliomas. Specifically, they show that if an enhanced fiber-integrin interaction is available through the RGD-4C motif, adenoviruses infect glioma cells efficiently even when CAR expression is low. Therefore, insertion of the RGD motif into Delta-24 should provide substantial improvement in infectivity of gliomas in the clinical setting.

Of note is the fact that the RGD-4C modification of the fiber knob did not alter the ability of the adenovirus to bind CAR. This finding is demonstrated by the fact that, in cells with high CAR expression, the infectivity of adenoviral vectors with the RGD-4C modification was only slightly higher than that of adenoviral vectors without the RGD-4C modification. This finding is in contrast with the finding that adenoviruses in which the native tropism has been reduced after removal of both CAR and integrin interactions and replaced with selective binding motifs bind exclusively to cells expressing the receptors (27,28).

One of the main disadvantages of targeting a surface receptor in gliomas is that no glioma-specific receptor has been found to be expressed in the majority of glioma cells and/or in the majority of gliomas. Unlike the RGD-based approaches, the more selective targeting approaches may find application in a subset of gliomas, but they will probably never be considered a universal method to target the treatment of gliomas. The strategy of combining CAR and integrins as primary receptors might, however, raise the possibility that Delta-24-RGD would be more toxic than Delta-24. Thus, an important finding of our study is that the addition of the RGD-4C motif did not alter the toxicity of the Delta-24 adenovirus in cancer cells.

Our data showed that the E1A deletion safeguards normal cells from adenoviral replication and lysis, rendering Delta-24-RGD replicative only in cancer cells with an abnormal Rb pathway. Notably, like Delta-24 (5), Delta-24-RGD did not replicate efficiently in nondividing NHA5s. Since our initial report (5), the highly selective, conditionally-replicative properties of mutant E1A replication-competent adenoviruses have been confirmed in several independent laboratories, which have consistently demonstrated the low toxicity of oncolytic adenoviruses that target the Rb (29,30) and/or the Rb/E2F pathways (31,32).

We elected to deliver Delta-24-RGD in the in vivo experiments using direct intratumoral injection. Although this approach requires low-risk but sophisticated experimental (18) and clinical techniques (i.e., stereotactic surgery), we believe that the intratumoral approach is the most appropriate method of delivery of adenoviral vectors for brain tumors. In contrast, recent reports (29,31) suggest the possibility of intravenous delivery of Rb-targeted oncolytic adenoviruses to treat solid tumors. However, systemic delivery is unlikely to be successful in treating gliomas because, at least in part, of the blood–brain and blood–tumor barrier. Moreover, most of the systemically delivered therapeutic adenovirus is lost to non-central nervous system tissues, such as hepatocytes and Kupffer cells (33–36). These observations indicate that using the systemic delivery approach for gliomas may require increasing the dose of the therapeutic adenovirus, with the potential for systemic toxicities and low antglioma effect, even if the vector is replication-selective.

In this study, the U-87 MG xenograft intracranial model is highly resistant to a variety of treatments (Table 2). However, treatment of athymic mice that had U-87 xenograft tumors with Delta-24-RGD did result in long-term survival in 60% of animals, and when these animals were killed at 140 days after implantation, there was no evidence of tumor (Fig. 5). These data provide strong evidence for the potential clinical importance of the RGD-4C modification of adenoviral vectors in improving outcome in patients with gliomas.

To our knowledge, detailed examination of the progressive expression of oncolytic adenoviruses in intracranial gliomas has not been reported to date. Specifically, we demonstrate three zones of viral expression that are best explained by a progressive wave-like movement of the vector from the injection site toward the edge of the tumor. This pattern of adenoviral spread appears to underlie the ability of Delta-24 to decrease the size of tumors that are already established. In addition, this well-defined, consistent pattern of centrifugal adenoviral spread with three distinct zones provides researchers with a molecular endpoint for evaluating Delta-24-RGD in clinical trials. Indeed, with these preclinical data, clinical trials using oncolytic adenovirus can be designed in which post-treatment specimens can be analyzed for the presence of these same zones of adenoviral spread after Delta-24-RGD injection. This type of design would allow for more data-driven and less empirically based clinical trials of oncolytic adenoviruses for the treatment of gliomas. Careful examination of adenovirus-treated tumors for the three-zone pattern could be incorporated into clinical trial design as a criterion for disease response to the treatment, biologic activity, and local toxicity (37).

**Table 2. Comparative long-term survival analysis in the U-87 MG xenograft mouse model after different adenoviral treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (route)</th>
<th>N</th>
<th>Long-term survival, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncolytic adenovirus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delta-24-RGD</td>
<td>4.5 × 10⁸ pfu (i.t.)</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>Delta-24</td>
<td>4.5 × 10⁷ pfu (i.t.)</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>PBS</td>
<td>4.5 × 10⁷ pfu (i.t.)</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Adenoviral vectors</td>
<td>Ad-p53‡,§</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Ad-E2F-1¶</td>
<td>5 × 10⁷ pfu (i.t.)</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Chemotherapy†</td>
<td>BCNU</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Temozolomide</td>
<td>7.5</td>
<td>6</td>
</tr>
<tr>
<td>Radiotherapy‡</td>
<td>10 Gy (single-fraction)</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

*pfu = plaque-forming units; i.t. = intratumoral; i.p. = intraperitoneal; PBS = phosphate-buffered saline; BCNU = carmustine.

‡Lang FF: unpublished data.
¶Liu TJ: unpublished data.
§Conrad CA: unpublished data.
In conclusion, oncolytic adenoviruses that target the Rb/E2F pathway are probably among the most efficient and selective oncolytic systems currently available (5,29–32). In this study, we demonstrated that enhanced tropism of this system through genetic incorporation of the RGD-4C peptide into the Delta-24 adenovirus dramatically improved the anti-glioma efficiency and maintained the selectivity of this adenovirus in cancer cells. The anti-glioma activity of Delta-24-RGD suggests that it has the potential to be a much-needed agent in the treatment of gliomas.

REFERENCES


NOTES

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