Gene delivery into malignant glioma by infectivity-enhanced adenovirus: In vivo versus in vitro models

Winan J. Van Houdt, Hongju Wu, Joel N. Glasgow, Martine L. Lamfers, Clemens M. Dirven, G. Yancey Gillespie, David T. Curiel, and Yosef S. Haviv

Department of Neurosurgery, VU University Medical Center, 1007 MB Amsterdam, The Netherlands (W.J.V.H., M.L.L., C.M.D.); Division of Human Gene Therapy, Departments of Medicine, Obstetrics and Gynecology, Pathology, and Surgery (H.W., J.N.G., D.T.C.), Gene Therapy Center (H.W., J.N.G., D.T.C.), Division of Cardiovascular Disease (J.N.G.), and Department of Neurosurgery (G.Y.G.), University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA; and Department of Medicine and Gene Therapy Institute, Hadassah-Hebrew University Medical Center, Jerusalem, Israel, 91120 (Y.S.H.)

Received May 29, 2006; accepted October 12, 2006.

Address correspondence to Yosef S. Haviv, Department of Medicine and the Gene Therapy Institute, Hadassah-Hebrew University Medical Center, P.O. Box 12000, Jerusalem, Israel, 91120 (yhaviv@hadassah.org.il).

Copyright 2007 by the Society for Neuro-Oncology
malignant gliomas, potentially offering both tumor-targeting and novel cell-killing mechanisms. Recombinant adenoviruses appear promising as gene therapy vectors for glioma, on the merit of their ability to infect both dividing and quiescent tumor cells, genome plasticity, mild pathogenicity in humans, and safety shown in gene therapy clinical trials for glioma. While the efficiency of Ad-based gene delivery into glioma cells may depend on the expression levels of the coxsackie-adenoviral receptor (CAR), the primary Ad receptor, other reports have found that capsid-unmodified Ad is a highly efficient vector in the context of glioma. Another factor confounding the use of Ad vectors for glioma involves the dose of vector inoculum, which can be potentially toxic to neighboring brain cells. Clearly, both CAR-dependent and CAR-independent glioma cell infection by Ad vectors needs to be further explored. In this study, we evaluated the utility of CAR-independent genetic retargeting of Ad vectors in glioma models. Genetic retargeting is generally superior to antibody-conjugated Ad with respect to easier administration and the potential for tropism modification in the progeny of conditionally replicative Ad viruses.

Genetic modification of Ad capsid for CAR-independent infection may involve either replacement of the entire Ad fiber or the fiber knob, or the insertion of heterologous ligands into the fiber knob of Ad. Recent genetic approaches developed by our group for CAR-independent Ad infection include insertion of a polypeptide (pK7) motif into the C-terminal end of the Ad5 fiber gene, or insertion of an RGD motif into the HI loop of the fiber knob, or both. Another CAR-independent approach employed display of a xenofiber knob on a human Ad capsid fiber. These genetic approaches to alter vector tropism all proved highly efficient in cancer cells grown in monolayer culture. In this study, we investigated whether the utility of tropism modification of Ad vectors attained in vitro is maintained in solid glioma tumor xenografts. This issue is critical for cancer gene therapy endeavors because tumor components may impose several levels of blockage on vector distribution and gene delivery. In addition to cancer cells, tumors consist of stroma comprising reactive fibroblasts and extracellular matrix, basement membranes, abnormal blood vessels, necrotic regions, and infiltrating cells of the immune system. Standard cancer therapy may further accentuate necrosis, apoptosis, and fibrosis within the tumor, thereby potentially confounding the potential distribution of viral vectors. Our results indicate that while tropism-modified Ad vectors could dramatically enhance infectivity in low-CAR glioma cells in vitro, Ad capsid modification was not the sole determinant of in vivo gene delivery in glioma xenografts.

Materials and Methods

Glioma Cells

Both established and primary human malignant glioma cell lines were used in this study. The glioma cell lines U-118MG and M59K were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). D-65MG, U-251MG, U-87MG, and D-54MG cells were provided by Dr. Darell B. Bigner (Duke University Medical Center, Durham, NC, USA). U87 cells were cultured with Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA, USA) with 15% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml). U251, U118, M59K, D65, and D54 cells were cultured in Dulbecco’s modified Eagle’s/F12 medium (50:50) with 7%–10% FBS, L-glutamine, and antibiotics (Mediatech), as above. All cells were cultured at 37°C in a 5% CO₂ humidified atmosphere. The primary glioma cells VU-15, VU-28, VU-78, and VU-84 were obtained and used before the eighth passage, as previously described. Primary malignant glioma cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS, L-glutamine, and antibiotics as above.

Adenoviral Vectors

All Ad vectors used in this study were replication deficient (E1 deleted) and based on the serotype-5 Ad genome. The construction of Ad, Ad.pK7, Ad5.RGD, and Ad5.RGD.pK7 has been described previously. Ad5 is an untargeted Ad vector, used as an isogenic control for Ad5.pK7, Ad5.RGD, and Ad5.RGD.pK7. Ad5.pK7 and Ad5.RGD differ from Ad5 by the respective insertion of a polypeptide (pK7) or an RGD motif into the C-terminal end of the Ad5 fiber gene or the HI loop of the fiber knob. Ad5.RGD.pK7 contains an RGD motif into the HI loop of the fiber knob in addition to the pK7 motif at the C-terminal end. These Ad vectors all include two identical bicistronic reporter gene cassettes—firefly luciferase and green fluorescent protein (GFP)—each driven by a separate cytomegalovirus promoter.

A different approach for Ad capsid modification used Ad5Luc1-CK (also termed AdCK-2) displaying genetic xenofiber knob replacement via the fiber knob of the canine Ad serotype 2. The cell entry mechanism of Ad5Luc1-CK involves both the putative cellular receptor(s) of the canine Ad serotype 2 and CAR. The control Ad vector for Ad5Luc1-CK was the isogenic, capsid-unmodified Ad5Luc1. Thus, we used two groups of viral vectors, whereby a distinct untargeted Ad5 vector served as an isogenic control to the corresponding retargeted Ad vectors (Fig. 1). All viruses were rescued on 293 cells and purified by using a standard CsCl gradient protocol. The number of viral particles was determined using a conversion factor of 1.1 × 10¹² viral particles (vp) per absorbance unit at 260 nm. The titers of these viruses were as follows: Ad5, 3.1 × 10¹² vp/ml; Ad5.RGD, 2.1 × 10¹² vp/ml; Ad5.RGD.pK7, 7.8 × 10¹¹ vp/ml; Ad.pK7, 1.0 × 10¹² vp/ml; Ad5Luc1, 3.7 × 10¹² vp/ml; Ad5Luc1-CK, 9.7 × 10¹¹ vp/ml.

Flow Cytometry

Glioma cells were rinsed with phosphate-buffered saline (PBS), harvested by versene incubation, and resuspended in
Infection medium. After 1 h, the infection medium was infected at an MOI of 40, 200, and 1,000 in 500 μl of growth medium. After 24 h, cells were seeded in 24-well plates (50,000 cells/well, with each well containing 0.5 ml of growth medium). Cells were seeded in 24-well plates (50,000 cells/well, with each well containing 0.5 ml of growth medium). Cells were seeded in 24-well plates (50,000 cells/well, with each well containing 0.5 ml of growth medium).

Luciferase Assays

Fluorescence Microscopy

Luciferase Imaging

Competitive Inhibition Assays

In Vivo Glioma Xenograft Model and Luciferase Imaging

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and performed according to their guidelines. The glioma xenograft models were established in female athymic nude mice (National Cancer Institute–Frederick Animal Production Area, Frederick, MD, USA) by stereotactical injection of glioma cells into the mouse brain at the region of the caudate-putamen nucleus. Glioma cells that stably express firefly luciferase (Luc^+ U87MG), and regular glioma cells, including high-CAR D54MG cells and low-CAR U87MG cells, were used in the experiments. For each mouse, 5 × 10^3 glioma cells in a 5-μl volume were injected into the brain. Two weeks later, 10^10 vp of each Ad vector was injected into the brain tumors that were established with regular U87MG or D54MG cells. Noninvasive, live luciferase imaging was performed at different days after viral injection to follow the transgene (luciferase reporter) expression. The experimental design of the in vivo glioma xenograft model is depicted in Table 1. Luciferase imaging was performed using a custom-built noninvasive optical imaging system similar to the ChemiPro imaging system (Roper Scientific, Tucson, AZ, USA). For live imaging, the mice were anesthetized with isoflurane inhalation. Following intraperitoneal injection of D-luciferin (2.5 mg/100 μl per mouse), the bioluminescent signals in live mice were captured with a highly sensitive back-illuminated VersArray:1KB charge-coupled device (CCD) camera (Princeton Instruments, Trenton, NJ, USA) that was equipped with a Nikkor f/1.2 lens. Images were acquired with WinView/32 (Roper Scien...
Thus, the novel genetic strategies for tropism modification of Ad vectors could circumvent the resistance of low-CAR glioma cells to Ad5 infection, we employed three retargeted Ad vectors. The first strategy was based on a chimeric Ad vector composed of the human Ad5 shaft flanked by the canine Ad knob (Ad5Luc1-CK). The second Ad retargeting strategy was based on incorporation of a polylsine motif into the C-terminus of the Ad5 fiber (Ad5.pK7). The third Ad vector was based on dual targeting, with both a polylsine motif and an RGD peptide inserted into the Ad5 C-terminus fiber gene and HI loop of the knob, respectively (Ad5.RGD.pK7). Ad5Luc1-CK, Ad5.pK7, and Ad5.RGD.pK7 were designed to target the respective alternative Ad receptors, the putative receptor for canine Ad, HSPG, or both HSPG and cell-surface integrins. Of note, an isogenic Ad.RGD vector was less efficient in enhancing gene delivery into glioma cells and was further used only as a specific control for Ad5.RGD.pK7 (data not shown).

When low-CAR glioma cell lines were tested for susceptibility to Ad infection, the three retargeting strategies proved superior to regular Ad5 vectors with no capsid modification (Fig. 3A–C). Specifically, in the low-CAR U118 and U87 cells, substantially higher levels of gene delivery could be achieved by all three retargeted vectors. In the medium-CAR glioma cell lines D65 and M59K, the infectivity enhancement of the retargeted Ad vectors was lower than in the low-CAR cells. In high-CAR cells, there was no consistent advantage for CAR-independent infection. Of note, the degree of infectivity enhancement of the retargeted Ad vectors varied in the D65 and M59K cell lines, in accordance with the two distinct cell populations in regard to CAR expression, as shown in Fig. 2. While the double-modified Ad5.RGD.pK7 showed more efficient CAR-independent cell entry in the low-CAR primary glioma cells and in U118 cells, Ad5.pK7 infection was more efficient in the low-CAR U87 cells, possibly indicating variable HSPG and integrin cell-surface expression in different glioma cell lines. In low-CAR cells, Ad5.RGD.pK7 was superior to Ad5.RGD.pK7 vector (Fig. 3C), which was previously shown to enhance infectivity in glioma cells.23,25 Thus, the novel retargeted Ad vectors Ad5Luc1-CK, Ad5.pK7, and Ad5.RGD.pK7 augment Ad-based gene delivery into established low-CAR glioma cell lines.

### Results

#### Heterogeneous CAR Expression in Established Glioma Cells

CAR expression is a critical determinant of the efficiency of Ad entry into glioma cancer cells grown in mono-layer.23,25 Therefore, to evaluate the utility of our retargeting strategies, we first measured CAR expression in a panel of established glioma cells. We found that CAR expression in glioma cells was heterogeneous and could be grouped into three major groups: low-, medium-, or high-CAR expression (Fig. 2). Of note, the medium level of CAR expression in the established glioma cell lines D65 and M59K was derived from two distinct populations: low- and high-CAR cells. Thus, within the established glioma cell lines, there was considerable variation in CAR expression.

#### Superiority of Retargeted Ad Vectors in Established Low-CAR Glioma Cell Lines

To evaluate the hypothesis that the novel genetic strategies for tropism modification of Ad vectors could circumvent the resistance of low-CAR glioma cells to Ad5 infection, we employed three retargeted Ad vectors. The first strategy was based on a chimeric Ad vector composed of the human Ad5 shaft flanked by the canine Ad knob (Ad5Luc1-CK). The second Ad retargeting strategy was based on incorporation of a polylsine motif into the C-terminus of the Ad5 fiber (Ad5.pK7). The third Ad vector was based on dual targeting, with both a polylsine motif and an RGD peptide inserted into the Ad5 C-terminus fiber gene and HI loop of the knob, respectively (Ad5.RGD.pK7). Ad5Luc1-CK, Ad5.pK7, and Ad5.RGD.pK7 were designed to target the respective alternative Ad receptors, the putative receptor for canine Ad, HSPG, or both HSPG and cell-surface integrins. Of note, an isogenic Ad.RGD vector was less efficient in enhancing gene delivery into glioma cells and was further used only as a specific control for Ad5.RGD.pK7 (data not shown).

When low-CAR glioma cell lines were tested for susceptibility to Ad infection, the three retargeting strategies proved superior to regular Ad5 vectors with no capsid modification (Fig. 3A–C). Specifically, in the low-CAR U118 and U87 cells, substantially higher levels of gene delivery could be achieved by all three retargeted vectors. In the medium-CAR glioma cell lines D65 and M59K, the infectivity enhancement of the retargeted Ad vectors was lower than in the low-CAR cells. In high-CAR cells, there was no consistent advantage for CAR-independent infection. Of note, the degree of infectivity enhancement of the retargeted Ad vectors varied in the D65 and M59K cell lines, in accordance with the two distinct cell populations in regard to CAR expression, as shown in Fig. 2. While the double-modified Ad5.RGD.pK7 showed more efficient CAR-independent cell entry in the low-CAR primary glioma cells and in U118 cells, Ad5.pK7 infection was more efficient in the low-CAR U87 cells, possibly indicating variable HSPG and integrin cell-surface expression in different glioma cell lines. In low-CAR cells, Ad5.RGD.pK7 was superior to Ad5.RGD.pK7 vector (Fig. 3C), which was previously shown to enhance infectivity in glioma cells.23,25 Thus, the novel retargeted Ad vectors Ad5Luc1-CK, Ad5.pK7, and Ad5.RGD.pK7 augment Ad-based gene delivery into established low-CAR glioma cell lines.
the Ad5/CAR cellular entry pathway with Ad5 knob does not inhibit infection with Ad5Luc1-CK, Ad5.pK7, or Ad5.RGD.pK7. These previous findings, together with our present findings of the specific inhibition of the respective cellular entry mechanisms, seem to indicate that Ad5Luc1-CK, Ad5.pK7, and Ad5.RGD.pK7 infect low-CAR glioma cells via CAR-independent cellular entry pathways. Of note, Ad5.RGD.pK7 seems to have a broader spectrum for cell entry in consideration of its relative resistance to heparin and its promiscuous infection profile, unless both the integrins and HSPG pathways are blocked, as previously demonstrated.21

Retargeting Ad Vectors Enhances Infectivity in Primary, Low-Passage Glioma Cells

Glioma cells derived before the eighth passage from tumor samples of patients (referred here as primary glioma cells) are often considered to better reflect the true nature of tumor cells. Therefore, we further evaluated the utility of the three retargeting strategies in four low-passage, primary glioma cell lines. First, we classified the primary glioma cells on the basis of their CAR expression (Fig. 5A). Next, we evaluated the utility of the various retargeted Ad vectors as gene delivery vehicles into the primary glioma cells. Our findings indicate that in low-CAR primary glioma cells, all three retargeting strategies could augment gene delivery. Of note, the efficiency of Ad5.RGD.pK7 was substantially higher than that of the other vectors (Fig. 5B). In parallel with the established glioma cell lines, infectivity enhancement correlated inversely with the level of CAR expression in primary glioma cell lines.

However, although the level of infectivity enhancement obtained by Ad5.RGD.pK7 in established low-CAR glioma cell lines was approximately 30-fold (Fig. 3A), in primary low-CAR glioma cells it was up to 270-fold (Fig. 5B), thereby showing the relevance of CAR-independent infection in glioma cells in monolayer culture.
Infectivity Enhancement of Ad Vectors Is Compromised In Vivo in Glioma Xenografts

We next examined whether the CAR-independent Ad infection attained in vitro may predict in vivo gene delivery. To this end, we preestablished orthotopic human glioma xenografts in mice, followed by direct intracranial injection of the various Ad vectors. To directly evaluate gene delivery in real time, we studied live animals in which luciferase activity in the tumors was imaged using a CCD camera to quantitatively measure light emission. To validate transcranial measurement of gene expression, we first confirmed intracranial light signaling from glioma xenograft tumors consisting of glioma cells constitutively expressing luciferase (Fig. 6A). The increasing luciferase signals at day 15 compared with day 10 after
tumor inoculation were compatible with active tumor growth, thereby confirming the establishment of the glioma xenograft model. Next, we preimplanted two different types of orthotopic human glioma xenografts (Luc⁺) in nude mice, followed by intratumoral injection of either the three retargeted Ad vectors or the unmodified Ad5 isogenic control vector (Table 1). The in vivo gene delivery efficiency was evaluated by noninvasive luciferase imaging since the vectors contained the firefly luciferase reporter. Of note, the two xenograft types were composed of human glioma cells with either low (U87) or high (D54) levels of CAR expression.

As also found under the in vitro conditions, in high-CAR glioma tumors we observed no infectivity enhancement: the capsid-modified Ad5.pK7 and Ad5.RGD.pK7 vectors showed no significant difference from the control Ad5 vector with regard to in vivo gene delivery efficiency (p = 0.657 and p = 0.372, respectively), whereas the Ad5Luc1-CK vector appeared significantly less efficient than the isogenic control vector (p = 0.020; Fig. 6B). Unlike in the two-dimensional glioma cell monolayers, however, in low-CAR glioma tumors in vivo, the unmodified Ad vectors were not inferior to the capsid-modified Ad (p = 0.239 for Ad5.pK7, p = 0.208 for Ad5.RGD.pK7, and p = 0.447 for Ad5Luc1-CK; Fig. 6C). Thus, in human glioma xenografts, in vitro CAR independence does not necessarily predict enhanced gene delivery in vivo.

Discussion

The use of Ad-based gene therapy against gliomas may be feasible for several reasons. First, local intracranial injection is feasible using surgical and stereotactic techniques. Second, prognosis of aggressive glioma is dismal. Third, clinical gene therapy studies have shown that Ad-mediated brain toxicity is very low, despite the capacity of Ad5 vectors to infect a number of normal brain cell populations, such as neurons, astrocytes, and ependymal cells. It is therefore not surprising that Ad vectors have been previously found to be efficient for gene delivery in several glioma models. However, a major factor limiting the assessment of Ad-based gene therapy strategies for glioma is the inadequacy of currently available human tumor models. For example, data derived from monolayer cell cultures clearly do not reflect the tumor microenvironment.

Previously, genetic strategies to modify the Ad fiber have been reported to augment infectivity in glioma cells. In this study, we revisited the hypothesis that Ad-based gene delivery into gliomas could be enhanced by genetic retargeting of the vectors to alternative cellular Ad receptors. To address this issue, we performed in vitro and in vivo studies with three distinct approaches for Ad retargeting. In vitro, we first showed that CAR expression varies considerably among glioma cell lines. CAR expression was quantified as low, medium, or high. We next demonstrated that in established and primary low-CAR glioma cell lines, all three genetic approaches could enhance Ad infection, independently of CAR.

Of note, in the low-CAR U118 glioma cell line and in primary low-CAR glioma cells, double targeting of HSPG and integrins could augment Ad infectivity (Figs. 3A, 3B), whereas in the low-CAR U87 glioma cell line, double targeting of HSPG and integrins was less efficient than HSPG-based cell entry alone. Furthermore, as shown in Fig. 4A, competitive inhibition of HSPG-mediated cell entry with heparin in U118 cells could efficiently block Ad5.pK7 infection but not Ad5.RGD.pK7 infection. These results indicate that, in U118 glioma cells, integrins may play an important role as mediators of cell entry for Ad5.RGD.pK7 but not for Ad5.
pK7. Because Ad5.RGD.pK7 can potentially utilize both HSPG and integrins for cell entry, variable expression of these cell-surface receptors in distinct cell lines may account for the different capacity to achieve CAR-independent infectivity enhancement. In this regard, it has been suggested that double modification of the Ad fiber may cause conformational ligand changes\(^{21}\) that may affect Ad cell entry differentially in different cell lines.

A major finding of our in vivo studies in glioma xenografts was the lack of infectivity enhancement of Ad5.RGD.pK7 (Fig. 6C), in contrast to results under monolayer conditions. This observation is compatible with results of previous in vivo studies in which, in an intra-peritoneal ovarian cancer model, Ad5.RGD.pK7 was less efficient than Ad5.pK7 and did not enhance infectivity over Ad5. In contrast, in a subcutaneous ovarian tumor xenograft, Ad5.RGD.pK7, but not Ad5.pK7 or Ad5.RGD, enhanced infectivity.\(^{27}\) The variable nature of tumors was also demonstrated in vivo in a syngeneic melanoma tumor model in which another capsid-modified Ad vector incorporating both RGD and pK7 ligands could confer infectivity enhancement in vitro but not in vivo.\(^{24}\) Thus, in addition to the levels of CAR expres-
sion, other factors clearly play significant roles in the susceptibility of glioma tumors to Ad vectors. These other factors may include intratumoral restriction of vector distribution within the tumor mass by (1) high tumor-cell density and intratumoral connective tissue; (2) limited availability of vectors within the “infection zone,” corresponding to up to five layers of tumor cells around the needle track; (3) size of virions; (4) host immune response, augmented by display of charged ligands; (5) tumor inactivation of viral DNA after inoculation; (6) charged ligands promoting binding to extracellular matrix proteins, for example, integrins and HSPGs; (7) a gradient of viral infection that results in a high MOI primarily around the needle track; and (8) attenuation of vectors for safety reasons. Altogether, inefficient intratumoral diffusion probably accounts for the limited distribution of Ad-mediated gene delivery in human glioma tumors, as observed in previous human clinical trials. Some of these obstacles may be addressed by replication-competent oncolytic viruses or via combined therapeutic modalities.

In conclusion, although novel strategies for CAR-independent Ad-mediated cell entry could confer infectivity enhancement of primary and established low-CAR glioma cell lines in vitro, these approaches were less efficient in vivo. Thus, gene delivery into glioma cell monolayers, inclusive of primary glioma cells, cannot predict gene delivery in complex, heterogenic three-dimensional tumors. Further research is required to characterize and overcome the factors impeding gene delivery into glioma tumors.
Acknowledgments

W.J.V.H. was supported by the Dr. Saal van Zwanenberg Foundation, the Dutch Cancer Society, the Brain Foundation of The Netherlands, and the Utrecht University Talma Eykman Award for medical students. M.L.L. is supported by Dutch Cancer Society grant VU2002-2594. Y.S.H. is supported by the Israeli Science Foundation and the B. de Rothschild Foundation. H.W. and G.Y.G. are supported by NIH Brain Tumor SPORE grant P50 CA097247. D.T.C. is supported by NIH grants P01CA104177 and R01CA083821.

References


