A Capsid-Modified, Conditionally Replicating Oncolytic Adenovirus Vector Expressing TRAIL Leads to Enhanced Cancer Cell Killing in Human Glioblastoma Models

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Abstract
Glioblastoma multiforme (GBM) is the most aggressive brain tumor, and patients rarely survive for more than 2 years. Gene therapy may offer new treatment options and improve the prognosis for patients with GBM. Adenovirus-mediated gene therapy strategies for brain tumors have been limited by inefficient gene transfer due to low expression of the adenovirus serotype 5 (Ad5) receptor. We have used an adenovirus vector that specifically replicates in tumor cells and uses an Ad5 capsid and the adenovirus serotype (Ad35) fiber for efficient infection of malignant tumor cells. This vector also expresses adenovirus E1A and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in a tumor-specific manner. Here, we show that this oncolytic vector (Ad5/Ad35.IR-E1A/TRAIL) efficiently infects the GBM tumor cell lines SF767, T98G, and U-87 MG. Tumor cell killing was markedly enhanced with Ad5/Ad35.IR-E1A/TRAIL compared with wild-type Ad5 and Ad35 virus or Ad5/Ad35.IR-E1A- vectors without TRAIL expression in vitro. In vivo experiments using s.c. xenografted U-87 MG cells in NOD/SCID mice showed a significant growth delay of tumors after i.t. injection of Ad5/Ad35.IR-E1A/TRAIL, whereas adenovirus wild-type injections showed only marginal or no effect. Our findings indicate that the use of a capsid-modified adenoviral vector, in combination with TRAIL expression, is a promising novel approach for gene therapy of glioblastoma. [Cancer Res 2007;67(18):8783–90]

Introduction
Glioblastoma multiforme (GBM) is the most aggressive form of human brain tumors (1, 2). The most common therapy of patients with GBM includes surgery, radiation therapy, and chemotherapy. Nevertheless, the median life expectancy of patients diagnosed with GBM is less than 1 year (3). The poor prognosis for patients with GBM is caused by a multitude of resistance mechanisms that start to develop during gliomagenesis and the frequent appearance of recurrent GBM (4–12). Although a number of treatment strategies have been developed, the prognosis of patients with GBM has remained poor. Thus, the development of novel and improved therapeutic approaches is necessary. Adenoviruses-mediated gene therapy strategies have been proposed and studied in clinical trials; however, results with traditional adenovirus serotype 5 (Ad5)–based vectors have been disappointing (13–16). A significant reason for the disappointing outcome with Ad5-based vectors is likely the low expression of the coxsackievirus and adenovirus receptor (CAR) on GBM cells.

Recently, an adenoviral vector system that combines the capsid of the wild-type Ad5 with fiber proteins of the adenovirus group serotype 35 (Ad35) has been described (17–19). This modification changes the viral receptor from CAR to the human CD46, a membrane-bound regulatory protein that protects tissues from complement-mediated damage (20–22), which leads to an important alteration in the tropism of the vector (23). The CAR-independent infection of cells using Ad5/Ad35 vectors is an important feature because CAR expression tends to inversely correlate with the malignant potential of tumor cells, including glioblastoma (24–27). In contrast, CD46 has been shown to result in a potentially increased transduction efficiency of a broad spectrum of different malignant tumor cells (28, 29). Furthermore, we have developed a new concept for tumor-specific gene expression that is based on homologous recombination between inverted repeats (IR) in adenovirus genomes (Ad.IR). This system uses adenovirus vectors deleted for all E1A and E1B genes, which efficiently replicate their DNA in tumor cells. Ad.IR vectors were further modified to express adenovirus E1A and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in a tumor-specific manner. Expression of E1A allows for efficient, tumor-specific viral replication and production of progeny virus, whereas expression of TRAIL upon viral replication and TRAIL-mediated apoptosis confers efficient release of progeny virus and viral spread. A capsid-modified, conditionally replicating, oncolytic adenovirus (Ad5/Ad35.IR-E1A/TRAIL) was able to eliminate preestablished colon carcinoma metastases in xenograft models after a single systemic application and did not cause unspecific toxicity in mice or baboons (30, 31).

In this study, we wished to examine the hypothesis that Ad5/Ad35 tropism-modified adenovirus vectors will result in improved infection of GBM cells and that the combination with tumor-specific TRAIL expression will result in improved tumor cell killing.

Materials and Methods
Cell lines and culture. The human glioblastoma cell lines U-87 MG, T98G, and SF767 were purchased from American Type Culture Collection. DMEM, nonessential amino acids, and sodium pyruvate were purchased from Bethesda Research Laboratories Life Technologies. The 293 cell line was kindly provided by Dr. Miller. The AE25 cell line has been described previously (32). The glioblastoma cell lines were cultured in DMEM supplemented with nonessential amino acids. AE25 cells were cultured in DMEM supplemented with 2 mmol/L l-glutamine and nonessential amino acids. All media were supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.
Virus amplification and purification. Small vector aliquots of the wild-type serotypes Ad5, Ad35, Ad5/Ad35.IR-E1A/GFP and the oncolytic adenoviral vectors Ad5/Ad35.IR-E1A/TRAIL and Ad5/Ad35.IR-E1A/TRAIL were generated as described (17). For large-scale vector preparation, a 15-cm² dish with 15 × 10⁶ 293 cells was infected with a multiplicity of infection (MOI) of 20. After cytopathic effect (CPE) development, cells and virus-containing medium was collected and processed in a triple freeze-thaw procedure before using it to transduce six Petri dishes with 15 × 10⁶ 293 cells. In the second amplification step, cells were again collected in the medium and treated using three freeze-thaw steps, followed by the infection of 30 dishes with 15 × 10⁶ 293 cells per dish. After CPE development, the cells were harvested and pelleted at 2,000 rpm for 10 min. The cells were then resuspended in PBS containing 10 mmol/L MgCl₂ per dish. After CPE development, the cells were harvested and pelleted at 2,000 rpm for 10 min to remove debris, and 5 mL of every lysate were pooled. The lysates were run on CsCl step gradients for purification in two steps. First, the gradients containing 3.5 mL of CsCl (1.35 g/mL) were layered on top of 3.5 mL of CsCl (1.32 g/mL); 5 mL lysate were added on top, and the gradient was ultracentrifuged for 4 to 6 h at 35,000 rpm (SW 41 rotor) at 14 °C. The band containing parental full-length virus was collected from each tube, combined, and transferred on top of the 8-mL CsCl (1.35 g/mL) for the second purification step at 33,000 rpm for 18 h at 14 °C. The full-length band was collected again and dialyzed against 1.1 of the dialysis buffer at 4 °C in the dark for 8 h each. The virus was aliquotted and stored at −70 °C. The titers of recombinant genomes were in the range of 1 × 10¹¹ to 1 × 10¹² per mL. The genome-to-plaque forming unit (pfu) ratios for Ad5, Ad35, and Ad5/Ad35 virus preparations were consistently between 15:1 and 20:1.

Surface protein immunofluorescence analysis. Immunofluorescence analysis of CAR and CD46 expression was done by flow-cytometric analysis. U-87 MG, T98G, and SF767 cells were harvested and incubated in fluorescence-activated cell sorting buffer on ice with a mouse α-human CD46 IgG primary antibody (BD Biosciences) or mouse α-human CAR primary antibody (Abcam, Inc.). The cells were washed, followed by an incubation with a secondary FITC-labeled mouse IgG antibody (BD Biosciences). One well containing cells of the same cell line was harvested but only incubated with the secondary antibody as a negative control. After incubation, the cells were washed, fixed with 4% paraformaldehyde, and analyzed by flow cytometry.

In situ apoptosis detection using the TUNEL assay. Cells of the two human GBM lines U-87 MG and SF767 were grown in 24-well cell culture dishes. After the monocell layer was nearly confluent, the average number of cells per well was estimated using a “Helber” counting chamber. The cells were infected with Ad33 wild-type, Ad5/Ad35.IR-E1A/TRAIL, or the vector Ad5/Ad35.IR-E1A/TRAIL with a MOI of 1 pfu/cell. Wells of each cell line were harvested 48 and 96 h postinfection. Induced apoptosis was detected via visualization of occurred DNA fragmentation by labeling of free 3’-OH termini with FITC and an in situ cell death detection kit (Roche). The cells were collected after trypsin treatment, fixed with 4% paraformaldehyde, and processed following the manufacturer’s protocol. The labeled cells were analyzed using flow cytometry.

Crystal violet cytotoxicity assay. Cells were plated in 24-well dishes and kept in culture until they were nearly confluent. After estimation of the total number of cells per well via Helber counting chamber, the cells were infected with a MOI of 1 or 10. Over a period of 6 days, one well per Ad5, Ad35, Ad5/Ad35.IR-E1A/GFP, and Ad5/Ad35.IR-E1A/TRAIL was stained every 24 h postinfection. Before crystal violet staining, the medium was removed and the cells were fixed for 10 min in 4% paraformaldehyde at room temperature. Fixed cells were washed with PBS and incubated for 5 min in 1% crystal violet solution in 70% ethanol, followed by three rinses with water. Air-dried cells were photographed.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were plated in 48-well dishes and kept in culture until they were nearly confluent. The cells were infected with Ad5, Ad35, Ad5/Ad35.IR-E1A/GFP, and Ad5/Ad35.IR-E1A/TRAIL using a MOI of 1 pfu/cell. After infection over a period of 6 days, 15 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well every 24 h. After an incubation period of 3 h, the supernatant was removed. The cells were dried and dissolved in 200 μL DMSO. The absorbance of the solution was measured using a spectrophotometer and compared with formazan generation in untreated control cells of the same cell line to calculate the percentage of cell death per well.

Animals and treatment protocol. U-87 MG cells were harvested and 1 × 10⁶ cells were resuspended in 100 μL PBS. The cells were mixed with 100 μL Matrigel (BD Biosciences) and injected s.c. into the flanks of 4-week-old to 6-week-old male athymic NOD/SCID mice using a 27-gauge syringe (Becton Dickinson).

For each wild-type virus serotype, viral vector, and one negative control, five mice received injections into both flanks. Tumor growth was measured every other day using a caliper. Once the tumors reached an average size of 55 mm³ (±15%), viral treatment was initiated: 1 × 10⁶ pfu of Ad5, Ad35, Ad5/Ad35.IR-E1A/GFP, or Ad5/Ad35.IR-E1A/TRAIL were injected i.t. The same dose was given 48 h after the first injection, leading to a total application of 2 × 10⁶ pfu. Further increase or reduction of the tumor volume was measured using a caliper. In the second approach, 1 × 10⁵ U-87 MG cells were transduced with the wild-type serotype Ad5, Ad35, or the vector Ad5/Ad35.IR-E1A/TRAIL using a MOI of 100. Twelve hours postinfection, the cells were harvested and resuspended together with 9.9 × 10⁴ uninfected U-87 MG in 100 μL PBS. The cells were mixed with 100 μL Matrigel and injected s.c. into the flanks, leading to an injection of 1 × 10⁷ U-87 MG cells with 1% transduced cells. For each wild-type virus serotype, viral vector, and one negative control containing no previously infected cells, five mice received injections into both flanks. Tumor growth was monitored every 48 h using a caliper. Differences among groups of tumors were compared statistically using the Mann-Whitney U test. All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center.

Detection of apoptotic/necrotic tissue and viral capsid protein in xenografted tumor tissue after viral treatment. U-87 MG cells were harvested, and 2 × 10⁶ cells were resuspended in 200 μL PBS. The cells were mixed with 200 μL Matrigel (BD Biosciences) and injected s.c. into the flanks of NOD/SCID mice. Preestablished s.c. tumors were treated with injections of 1 × 10⁶ pfu of the oncolytic adenovirus Ad5/Ad35.IR-E1A/TRAIL or the Ad535 directly into the xenografted tumor. Mice were sacrificed 2 weeks after viral application, and tumor tissue samples were embedded in OCT and frozen. Microdissections were processed and analyzed for apoptotic/necrotic tissue using the “in situ cell death detection assay” (Roche) following the manufacturer’s protocol. Adenoviral hexon capsid proteins were detected using a goat α-adenovirus hexon capsid protein antibody (Chemicon AB 1056) and a secondary rabbit α-goat IgG H&L (Texas Red) antibody (Abcam, Inc.).

Results

Human GBM cell lines show higher expression of CD46 compared with CAR. A limitation of cancer therapy with traditional adenovirus vectors based on Ad5 has been the low expression of the Ad5 receptor CAR on tumor cells. In contrast to CAR, expression of the group B receptor CD46 was found to be up-regulated in tumors. Thus, we first compared the expression of CD46 and CAR on the GBM cell lines SF767, T98G, and U-87 MG. As shown in Fig. 1, using limiting dilutions of anti-CD46 and anti-CAR antibodies, all three cell lines showed a higher mean fluorescence with anti-CD46 antibody compared with anti-CAR antibody.

The mean fluorescence after incubation with the anti-CD46 antibody in a 1:100 dilution was at least 17-fold higher. Only the cell line SF767 shows higher CAR expression, relative to the isotype antibody in a 1:100 dilution. We next wished to...
determine the ability of Ad5/Ad35.IR-E1A/TRAIL to infect and kill glioblastoma cells. We compared infectivity and killing with the following adenoviral vectors: wild-type Ad5, wild-type Ad35, Ad5/Ad35.IR-E1A/TRAIL, and Ad5/Ad35.IR-E1A/GFP. Human GBM cell lines SF767, U-87 MG, and T98G were infected at a MOI of 1 pfu/cell and observed for 6 days for cell killing. As shown in Fig. 2, in vitro infection at a MOI of 1 pfu/cell with Ad5/Ad35.IR-E1A/TRAIL resulted in tumor cell killing in all three human GBM cell lines examined. However, the cell lines exhibit different sensitivities to Ad5/Ad35.IR-E1A/TRAIL-mediated cell killing.

The assays show that the TRAIL-expressing vector induced cell death of about 40% to 60% of the infected SF767 cell line within 3 days postinfection (dpi), whereas a comparable percentage of cell lysis in U-87 MG cells was not observed until 5 dpi (Fig. 2). The T98G glioblastoma cell line showed the highest resistance to virus-induced cell killing. Cell lysis was first detected at 4 dpi with Ad5/Ad35.IR-E1A/TRAIL by the crystal violet and the MTT assay, and at 6 dpi, cell lysis was <50%. Oncolytics comparable with levels observed in Ad5/Ad35.IR-E1A/TRAIL-infected SF767 and U-87 MG cells was only achieved in T98G cells with a MOI that was 10-fold higher (Fig. 2D). SF767 cells exhibited only marginal cell death at 3 dpi and <40% of cell death at 6 dpi after wild-type Ad35 infection. U-87 MG and T98G cells showed no significant cell death after infection with Ad5 and Ad35 wild-type or Ad5/Ad35.IR-E1A/GFP.

Ad5/Ad35.IR-E1A/TRAIL mediates enhanced induction of apoptosis in human GBM cells compared with infection with adenoviral wild-type virus. Human GBM cell lines U-87 MG, T98G, and SF767 are sensitive to Ad5/Ad35.IR-E1A/TRAIL. GBM cell lines were infected in vitro to examine the apoptosis-inducing potential of the adenoviral vectors (Ad5/Ad35.IR-E1A/TRAIL or Ad5.IR-E1A/TRAIL) compared with wild-type serotype Ad35 (Fig. 3). Ad5.IR-E1A/TRAIL expresses TRAIL in the same manner as Ad5/Ad35.IR-E1A/TRAIL but does not feature the Ad35 fiber and therefore relies on CAR as the primary receptor. The cell lines were infected with a MOI of 1 pfu/cell and observed for 4 days for virus-mediated induction of apoptosis using TUNEL assay. We found that 25% to 50% of the cultured human GBM cells from SF767 or U-87 MG were positive by TUNEL assay 4 days after infection with Ad5/Ad35.IR-E1A/TRAIL, whereas wild-type virus showed no significant increase in TUNEL-positive cells.
compared with mock-infected GBM cultures. Elevated levels of apoptosis were detectable in T98G cells after infection with Ad5/Ad35.IR-E1A/TRAIL but not Ad35 or Ad5.IR-E1A/TRAIL. Direct comparison of Ad5.IR-E1A/TRAIL and Ad5/Ad35.IR.E1A/TRAIL via TUNEL assay shows that, in SF767 and T98G, 20% to 30% more cells seem apoptosis-positive 4 days after infection with Ad5/Ad35.IR.E1A/TRAIL, whereas this difference can be already observed after 2 days in U-87 MG cells.

Injection of Ad5/Ad35.IR-E1A/TRAIL i.t. into xenograft GBM tumors leads to infection of tumor tissue, viral spread, and induction of apoptosis in vivo. To show the effect of Ad5/Ad35.IR-E1A/TRAIL on human GBM cell lines in vivo, the vector was injected directly into s.c. tumor xenografts of U-87 MG cells in NOD/SCID mice. Three weeks after viral injection, dissected tumor tissue was examined for areas of apoptotic tissue around the application site using the TUNEL assay (Fig. 4). Apoptotic tissue areas were found in tumors injected with Ad5/Ad35.IR-E1A/TRAIL and Ad35 wild-type, whereas tumors injected with PBS showed no significant apoptotic areas. Viral capsid hexon proteins were detected in the apoptotic areas using a goat α-adenovirus hexon
capsid protein antibody. The tissue samples of the Ad5/Ad35.IR-E1A/TRAIL vectors showed large apoptotic areas with elevated amounts of viral hexon capsid protein compared with tumor samples injected with Ad35.

S.c. xenografts in NOD/SCID MICE using human GBM U-87 MG show a significant growth inhibition when treated with Ad5/Ad35.IR-E1A/TRAIL. To determine the efficiency of Ad5/Ad35.IR-E1A/TRAIL in human GBM tumor xenografts in a mouse
model, 1 × 10^6 U-87 MG cells were mixed with 1% cells previously infected with Ad5/Ad35.IR-E1A/TRAIL, Ad5/Ad35.IR-E1A/GFP, Ad5, or Ad35 and injected s.c. into NOD/SCID mice. The s.c. growth of the tumors was observed and measured every other day after injection of the cells. The size of the tumors containing virus was compared with the tumor growth of 1 × 10^6 untreated U-87 cells (Fig. 5). The tumors containing cells infected with Ad5/Ad35.IR-E1A/TRAIL started to show growth impairment 8 days after s.c. injection with 40% less tumor volume compared with the negative control. The average growth of the tumors treated with Ad5/Ad35.IR-E1A/TRAIL remained reduced with about 40% to 50% volume size compared with the untreated tumors for the entire 20-day follow-up after injection. Slight impairment was also detectable in the sizes of Ad5-treated, Ad35-treated, and Ad5/Ad35.IR-E1A/GFP-treated tumors. However, the average size of those tumors was only reduced by about 20% to 40%. Control tumor size and the size of Ad5/Ad35.IR-E1A/TRAIL-treated tumors was analyzed using the Mann Whitney U test, which showed that growth impairment was significantly different 8 days after injection (P < 0.01, two-tailed test). A significant difference among untreated tumors and tumors treated with Ad5/Ad35.IR-E1A/TRAIL was confirmed for the entire 20-day follow-up after injection. Ad35-treated and Ad5/Ad35.IR-E1A/GFP-treated tumors showed a significant growth impairment only during the first 4 days (P < 0.01, two-tailed test). The group of Ad5-injected tumors seemed only to be marginally, significantly smaller at two time points during the experiment (day 20 and day 26) compared with the size of untreated tumors (P < 0.05, two-tailed test). Altogether, tumors treated with wild-type virus or Ad5/Ad35.IR vector without TRAIL expression show less effect on tumor growth than Ad5/Ad35.IR-E1A/TRAIL-treated tumors.

The effect of Ad5/Ad35.IR-E1A/TRAIL was further shown in an in vivo experiment, in which 2 × 10^8 pfu of Ad5, Ad35, Ad5/Ad35.IR-E1A/GFP, or Ad5/Ad35.IR-E1A/TRAIL were injected i.t. into xenografted U-87 MG tumors in athymic mice and compared with untreated tumors. After two injections of 1 × 10^8 pfu within 48 h, the tumors were measured every other day over a 14-day period (Fig. 6). Whereas injection with the wild-type virus or the Ad5/Ad35.IR-E1A/GFP led to no change in the tumor growth pattern compared with the untreated negative control, Ad5/Ad35.IR-E1A/TRAIL treated tumors grew <5% during the first 4 days after the second injection. Furthermore, the injection of Ad5/Ad35.IR-E1A/TRAIL led to an average tumor growth that was significantly lower compared with the tumor growth of xenografts treated with wild-type virus Ad5/Ad35.IR-E1A/GFP or untreated tumors until day 10 after viral injections (P < 0.01, two-tailed test).

Discussion

In this study, we show that the oncolytic adenoviral vector Ad5/Ad35.IR-E1A/TRAIL efficiently infects GBM tumor cells and induces TRAIL-mediated apoptosis in infected cells in vitro and in vivo. Significant growth impairment, as well as rapid and efficient induction of apoptosis, is evident in infected GBM cells compared with the adenoviral wild-type virus.

A major limitation for adenovirus-mediated gene therapy has been the low-level expression on tumor cells of CAR, the receptor for the commonly used Ad5. In contrast to CAR expression, most tumor cells have been shown to express high levels of CD46, the receptor for Ad35, Ad21, Ad22, Ad24, and Ad25. Thus, vectors using CD46 as a cellular receptor, like Ad5/Ad35.IR-E1A/TRAIL, transduce malignant tumor cells, such as LoVo, more efficiently (17). In the present study, we analyzed the expression levels of CD46 and CAR in three tumor cell lines, SF767, U-87 MG, and T98G, and confirmed that all three lines are strongly positive for CD46, comparable with findings in other tumor cell lines examined elsewhere (21). Expression of CAR, however, was substantially lower than expression of CD46. These findings support the use of Ad5/Ad35.IR-E1A/TRAIL with the Ad35 fiber protein for infecting GBM cells.

An important feature of the adenoviral vector used here is the expression of TRAIL. The utilization of TRAIL in different therapeutic approaches has been shown to induce apoptosis in a broad spectrum of different cancer types (33–37). Our findings confirmed the ability of enhanced GBM cell killing via TRAIL expression in a viral context by using the Ad5/Ad35.IR-E1A/TRAIL vector.

In contrast, infection with wild-type Ad5 resulted in no or only little cell killing in GBM monolayers in vitro. This was expected because it has been reported that the Ad5 virus does not transduce
neural cells effectively (38). An interesting observation has been the inefficient cell lysis of the serotype Ad35 and the vector Ad5/Ad35.IR-E1A/GFP in T98G cells and the reduced cell killing of U-87 MG and SF767 cells compared directly to Ad5/Ad35.IR-E1A/TRAIL. This does suggest that the increased oncolytic ability of the Ad5/Ad35.IR-E1A/TRAIL vector is not caused by the use of the chimeric Ad5/Ad35 capsid but that the apoptosis-inducing gene TRAIL plays a key role in cell killing of GBM cells after infection with Ad5/Ad35.IR-E1A/TRAIL.

In situ detection of apoptosis induced in SF767 and U-87 MG cells by Ad5/Ad35.IR-E1A/TRAIL using a TUNEL assay supported the findings from the cytotoxicity assay. Separate studies have shown that administration of human TRAIL induces apoptosis in human GBM cells (39, 40). In this study, we show induction of apoptosis in glioma cells by TRAIL expressed in the adenoviral context using the TUNEL assay. Infection of SF767 and U-87 MG cells with Ad5/Ad35.IR-E1A/TRAIL leads to rapid induction of apoptosis compared with cells treated with the wild-type Ad35. These findings show that replication of wild-type adenoviruses alone has only a minor effect on apoptosis induction and cell killing in human GBM cells whereas the additional expression of the transgene TRAIL significantly increases apoptosis. Comparison of the apoptosis inducing features of Ad5/Ad35.IR-E1A/TRAIL and Ad5.IR-E1A/TRAIL show that the switch to the serotype 35 fiber protein generally enhances the transduction efficiency of glioblastoma cells. The increase in induction of apoptosis in SF767 after Ad5/Ad35.IR-E1A/TRAIL infection was comparatively small. Whereas, low-CAR-expressing or non-CAR-expressing cells, like T98G, seem only to be efficiently transduced by adenoviral vectors using the receptor CD46. Efficient spread of Ad35 wild-type virus or Ad5/Ad35.IR-E1A/TRAIL after i.t. injection in vivo was verified by identification of areas expressing adenoviral capsid proteins. TUNEL staining of apoptotic tumor tissue corresponded to areas with expression of adenoviral capsid proteins, suggesting that the virus induces apoptosis in infected cells after in vivo injection. We hypothesize that observed elevated amounts of capsid protein after Ad5/Ad35.IR-E1A/TRAIL injection compared with the wild-type virus indicate an improved effectiveness in replication and spread of Ad5/Ad35.IR-E1A/TRAIL, most likely due to a TRAIL bystander effect as previously described (17). This effect might be due to early release of de novo viral particles after TRAIL-mediated cell killing.

Several studies have suggested that wild-type adenoviral vectors do not allow for efficient virus release and subsequent spread (41). Additionally, it has been shown that inducing viral apoptosis late in viral replication can accelerate the viral spread by de novo produced particles disseminating through endocytosis of apoptotic bodies (42).

The most obvious difference in the oncolytic potential of the vector Ad5/Ad35.IR-E1A/TRAIL compared with wild-type or Ad5/Ad35.IR- vectors that do not express TRAIL was observed using in vivo assays. Growth impairment of xenografted U-87 MG tumors containing Ad5/Ad35.IR-E1A/TRAIL-infected cells was significantly more pronounced compared with wild-type virus or the Ad5/Ad35.IR-E1A/GFP construct. However, when injected directly i.t. in vivo, wild-type virus and a Ad5/Ad35.IR-E1A- construct lacking TRAIL expression failed to affect tumor growth whereas only Ad5/Ad35.IR-E1A/TRAIL significantly inhibited tumor growth. Enhanced induction of apoptosis in GBM cells by Ad5/Ad35.IR-E1A/TRAIL can be explained by a potential synergistic effect of Ad5 protein E1A expression in combination with TRAIL. A similar cell sensitizing effect of the adenoviral E1A protein to TRAIL-induced apoptosis has been shown by the reversion of the resistance of normal primary human lung fibroblast cells to TRAIL (43). We propose that the combined E1A expression with TRAIL does lead to significantly enhanced induction of apoptosis in human GBM tumor tissue.

Our data show that expression of TRAIL in an adenoviral context leads to significant increase in apoptosis induction and cell killing in glioblastoma cell lines compared with wild-type adenovirus or adenoviral vectors lacking TRAIL. We show that the capsid modification of the Ad5/Ad35.IR-E1A/TRAIL vector leads to improved infection whereas the expression of TRAIL in the adenoviral context significantly enhances the ability to induce apoptosis in GBM cells in vitro and in vivo. Additionally, Ad5/Ad35.IR-E1A/TRAIL effectively spreads in GBM tissue after injection. Thus, we believe that the use of Ad5/Ad35.IR-E1A/TRAIL allows for more effective oncolytic therapy of human GBM.

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