Potential of the Conditionally Replicative Adenovirus Ad5-Δ24RGD in the Treatment of Malignant Gliomas and Its Enhanced Effect with Radiotherapy

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ABSTRACT

The use of replication-competent adenoviruses (Ads) for cancer therapy is receiving widespread attention, especially for the treatment of tumors refractory to current treatments such as glioblastoma. AdΔ24, which carries a 24-bp deletion in E1A and replicates in cells with a retinoblastoma-defective pathway, produced a strong antitumor effect in glioma. To improve infection efficiency of primary glialoma cells, which express low levels of coxsackie adenovirus receptor (CAR), the tropism of AdΔ24 was expanded toward αv integrins by insertion of an Arg-Gly-Asp (RGD) motif into the fiber knob (Ad5-Δ24RGD). We show that Ad5-Δ24RGD had a stronger oncolytic effect than the non-RGD-expressing variant on a broad panel of primary glioma cells, in particular on those with low CAR expression. The effects of Ad5-Δ24RGD were also assessed on a panel of primary organotypic glioma spheroids. In all cases, Ad5-Δ24RGD strongly decreased the viability of these small tumor nodules in vitro. In s.c. glioblastoma xenografts expressing low levels of CAR, five intratumoral injections of 1 × 10^7 plaque-forming units Ad5-Δ24RGD resulted in complete tumor regression in 9 of 10 mice and long-term survival in all treated mice. Preclinical evaluations and clinical trials of replication-competent Ad have shown more promising results when combined with conventional therapeutics. Therefore, we assessed the effects of Ad5-Δ24RGD in combination with radiotherapy. Low-dose irradiation before Ad5-Δ24RGD infection decreased viability of glialoma cells more effectively than Ad5-Δ24RGD alone with effects ranging from additive to supra-additive. In addition, combination treatment with Ad5-Δ24RGD and irradiation was studied in glioma xenografts. Five injections of 1 × 10^6 plaque-forming units Ad5-Δ24RGD induced significant tumor growth delay of >119 days compared with untreated controls and led to long-term survival in 6 of 9 mice. When viral treatment was combined with irradiation, tumor regression occurred in all mice resulting in long-term survival without evidence of tumor regrowth in 10 of 10 cases. This study thus provides evidence that Ad5-Δ24RGD has strong antitumor activity in malignant glioma, which can be additionally enhanced by irradiation such that the same therapeutic effect is achieved when a 10-fold lower viral dose is applied. These results support further development of Ad5-Δ24RGD in combination with radiation therapy for treatment of these highly malignant tumors.

INTRODUCTION

Malignant tumors of the central nervous system are generally fatal despite surgery, radiation therapy, and chemotherapy. In particular, the high mortality rate of patients with malignant glioma has led to intensive efforts seeking alternative treatment modalities. Gene therapy approaches have received widespread attention for treatment of glioma. Whereas preclinical studies have shown promising results, clinical trials to date have been disappointing. The main limitations were found to be inefficient tumor cell transduction and lack of penetration of viral vectors into the solid tumor mass.

This has led to the application of replication-competent viruses, with specificity for proliferating or malignant cells, that spread within the tumor and amplify the therapeutic effect (1, 2). This approach is particularly appealing for application in the central nervous system where the tumor cells proliferate amid surrounding normal brain of which the cells are essentially quiescent. Several mutants of herpes simplex virus type I were shown to replicate selectively in dividing cells (3). Ad5 has also emerged as a virus that can be engineered to have oncolytic properties. One strategy to engineer such conditionally replicative adenoviruses (CRAds) is by partial or complete deletion of viral genes that are dispensable in tumor cells only. The first CRAd developed by this paradigm, known as ONXY-015 or dl1520, carries a deletion in the E1B-55kDa coding region that introduces selectivity for tumors with dysfunctional p53 (4). Promising preclinical results led to rapid translation to clinical trials for head and neck cancer, pancreas carcinomas, and recently a trial for malignant glioma (NABTT-9701) was initiated (5).

A second promising type of CRAd, known as AdΔ24 or dl922-947, carries of a partial deletion in the CR2 domain of the adenoviral E1A gene that abrogates the binding of E1A to pRb. The selectivity of these Ad mutants for tumor cells was demonstrated previously (6, 7). Abnormalities of the pRb and p53 pathways have been frequently documented in gliomas (8), and therefore both the E1B55k-deleted and E1A-mutated CRAds are expected to replicate well in these tumors.

The efficacy of these oncolytic agents, however, may be compromised by their limited infection efficiency on gliomas as it has been demonstrated that these tumors express low levels of the primary Ad receptor CAR (9, 10). Insertion of the RGD sequence, known to interact with αv integrins, into the Ad fiber knob was found to strongly enhance glioma cell infection (10). Recently, it was demonstrated that combining this strategy with the AdΔ24 CRAd led to strong oncolytic effects in lung adenocarcinoma, prostate cancer, and ovarian cancer cells (11, 12).

Evidence from preclinical and clinical trials suggests that combining replication-competent Ads with standard anticancer treatments...
such as chemotherapy and radiotherapy results in greater therapeutic benefit (13–18). For glioma, radiotherapy is a standard treatment with demonstrated therapeutic efficacy. Interestingly, additive and even synergistic oncolytic effects on malignant glioma have been found when oncolytic viruses were combined with radiation treatment (19, 20).

In this study, the effect of the RGD insertion on the oncolytic activity of AdΔ24 was assessed on a broad panel of primary glioma cells. Moreover, the efficacy of AdΔ5-Δ24RGD was established in a three-dimensional structure of primary organotypic spheroids and confirmed in vivo in s.c. xenografts. Finally, we present in vitro and in vivo evidence that combined treatment with irradiation potentiates the oncolytic activity of AdΔ5-Δ24RGD in malignant glioma.

MATERIALS AND METHODS

Cell Culture. The Ad5 E1-transformed human embryonal kidney cell line 293, the human lung carcinoma cell line A549, and U373MG (anaplastic astrocytoma grade III) were purchased from the American Type Culture Collection (Manassas, VA). The human glioma cell line U118MG (glioblastoma multiforme) was a kind gift from Dr. Joanne Douglas (UAB Gene Therapy Center, Birmingham, AL) and Gli-6 (glioblastoma multiforme) was obtained from Dr. Sieger Leenstra (Department of Neurosurgery, Academic Medical Center, Amsterdam, the Netherlands). Human glioma cell line SF-763 (University of California at San Francisco Neurosurgery Tissue Bank, San Francisco, CA) and the human glioblastoma xenograft IGRG121 have been described previously (21, 22). Primary glioma cell cultures were derived by mechanical dissociation from fresh tumor material collected during brain tumor surgery at the Departments of Neurosurgery of the VU University Medical Center and Academic Medical Center (Amsterdam, the Netherlands) according to the method of Darling (23) and used before passage 8. A summary of glioma patients and histology of tumor samples used in this study is presented in Table 1. All cells were cultured in DMEM supplemented with 10% FCS and antibiotics (Life Technologies, Inc., Paisley, United Kingdom).

Spheroids. Organotypic multicellular spheroids were prepared from human tumor according to the technique originally described by Bjerkvig et al. (24). Briefly, fresh tumor tissue was obtained at surgery and dissected into small pieces with sterile 21-gauge needles. These explants were cultured individually in 2% agarose-coated 48-well plates in DMEM containing 10% FCS and antibiotics. After confirming viability by morphology, spheroids of similar diameter (300–400 μm) were used for assessment of AdΔ5-Δ24RGD oncolytic activity.

Recombinant Ads. A recombinant E1-deleted Ad expressing the luciferase reporter gene under the CMV promoter, AdCMVLuc, was provided by Dr. Robert D. Gerard (University of Texas Southwestern Medical Center, Dallas, TX). AdΔ24 was constructed using a pXC1 (Microrna Biosystems) derivative, pXC1-Δ24, carrying a 24-bp deletion in the pHb-binding CR2 domain in E1A as described previously (6). Homologous recombination in 293 cells between pXC1-Δ24 and pBHG11 (Microrna Biosystems) led to the formation of AdΔ24. AdΔ5-Δ24RGD was constructed as described previously (11). Briefly, the E1 region from pXC1-Δ24 was cloned into Ccl-A-digested pVK503, an E3-containing rescue plasmid with the RGD-4C modification in the fiber. The virus genome was released by digestion with PacI and transfected into 293 cells to rescue AdΔ5-Δ24RGD.

AdCMVLuc was propagated on 293 cells; AdΔ24 and AdΔ5-Δ24RGD were propagated on A549 cells. Viruses were purified using cesium chloride gradient banding according to standard techniques and titered in parallel by end point dilution titration on 293 cells.

In Vivo Antitumor Efficacy. In vivo antitumor activity was evaluated in s.c. human malignant glioma xenografts derived from a primary tumor, IGRG121, as described previously (22). For each experiment, IGRG121 tumor fragments were transplanted into female SPF-Swiss nude mice. Animals bearing tumors of 150–250 mm3 were pooled and randomly assigned to each treatment group. Total body irradiation (TBI) was performed on day 0 at a dose of 5 Gy (maximum-tolerated dose). X-rays were delivered under a tension of 225 kV and 17 mA using a Philips RT250. After 7 h, the first of five daily intra-tumoral virus or PBS injections was administered. Each day, AdΔ5-Δ24RGD was administered at a dose of 107 pfu (single treatment experiment) or 105 pfu (combined treatment experiment) in 50 μl of PBS. Different sites of the tumor were chosen for each injection and controls were injected with PBS. Two tumor diameters perpendicular to each other were measured three times weekly, and tumor volume was calculated according to the following equation: \( V = \frac{4}{3} \pi \left( \frac{d1 \cdot d2}{2} \right)^2 \), where \( d1 \) and \( d2 \) are the two tumor diameters. The experiments lasted until tumor volumes reached 1500–2000 mm3 or until 120 days.

Immunohistochemistry. Immunohistochemical staining for Ad hexon protein was performed on paraffin-embedded tissue sections using goat anti-Ad hexon antibody 1056 (Chemicon International, Temecula, CA) followed by FITC-conjugated rabbit antismouse antibody (DAKO, Glostrup, Denmark). Negative controls lacked the first antibody. Analysis was performed on a FACScan (Beckton-Dickinson, Erembodegem-Aalst, Belgium). Expression was quantified as the relative median fluorescence intensity compared with the negative control.

Table 1

<table>
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<tr>
<th>Tumor specimen</th>
<th>Male/female</th>
<th>Age</th>
<th>Histology</th>
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<td>Oligodendroglioma</td>
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In Vivo Cytotoxicity Assay. Panels of glioma cell lines or primary cells were seeded in triplicate or quadruplet in 96-well plates at 5 × 103 cells/well. For in vitro studies with irradiation, cells were irradiated with an 80-kV orthovoltage X-ray source (Pantak Therapax SXT 150). Ad infection was performed in DMEM containing 2.5% FCS, which was replaced by culture medium after 1 h. Cell survival was assessed using WST-1 as described by the manufacturer (Sigma, St. Louis, MO). Survival is expressed as a percentage of control uninfected cells.

Primary spheroids were infected with 5 × 107 pfu AdCMVLuc or AdΔ5-Δ24RGD (8–12 spheroids/group), and viability was assessed by WST-1 assay 12 days after infection.

Flow Cytometry. Cells were immunolabeled with either anti-CAR monoclonal antibody RmC8 (25) or with anti-αvβ3 integrin or anti-αvβ5 integrin monoclonal antibodies (Chemicon International, Temecula, CA), followed by FITC-conjugated rabbit antirabbit antibody (DAKO, Glostrup, Denmark). Negative controls lacked the first antibody. Analysis was performed on a FACScan (Beckton-Dickinson, Erembodegem-Aalst, Belgium). Expression was quantified as the relative median fluorescence intensity compared with the negative control.
negative. In contrast, all primary cells displayed high levels of at least one of the integrins tested (data not shown). Therefore, increased efficacy was expected of CRAds targeted toward integrins. To test this hypothesis, U118MG, U373MG, and a panel of primary glioma cell cultures. Viability was assessed 5 days after infection with 5 pfu/cell (cell lines) or 10 pfu/cell (primaries) and is expressed as percentage of uninfected controls ± SD. AdCMVLuc was included as a control for viral particle toxicity. *, P < 0.05 compared with AdΔ24.

Oncolytic Activity of Ad5-Δ24RGD in an Organotypic Spheroid Model. The initial basis for applying replication-competent viral vectors to treat solid tumors was conveyed by the inability of non-replicating viral vectors to penetrate the tumor mass. We therefore sought to validate the oncolytic potential of Ad5-Δ24RGD in an in...
the course of the experiment, a clear cytopathic effect could be observed at the periphery of the spheroids. Whereas the edge of the spheroids infected with a nononcolytic virus remained sharp with attached cells, the Ad5-Δ24RGD-infected spheroids lost their spherical shape with cells rounding up and detaching from the spheroid (Fig. 2A). Viability 12 days after infection was found to be significantly decreased by Ad5-Δ24RGD treatment (Fig. 2B), demonstrating the potent antitumor activity of this agent in a solid tumor mass.

**Oncolytic Activity of Ad5-Δ24RGD in Vivo.** To analyze the effects of Ad5-Δ24RGD in vivo, a human glioma xenograft model was used. The xenograft was derived from primary malignant glioma and expressed low levels of CAR. Treatment at an advanced tumor stage with 10^7 pfu Ad5-Δ24RGD for 5 consecutive days resulted in significant TGD of >117 days compared with untreated controls (five times initial tumor volume at 9.1 days; Fig. 3). Nine of 10 animals experienced complete tumor response and survived tumor-free at 4 months (Table 2). One animal experiencing partial tumor response, which did not regrow thereafter, also survived 4 months. Histological analysis of this tumor revealed a completely calcified tumor remnant without any viable cells.

**Effects of Ad5-Δ24RGD in Combination with Irradiation in Vivo.** In view of the reported synergy between viral oncolysis and irradiation, we investigated the interaction between Ad5-Δ24RGD and irradiation on a panel of glioma cell lines, receiving a dose of 3-Gy irradiation before infection with Ad5-Δ24RGD (1 pfu/cell) (Fig. 4A). In all cell lines tested, we found a significant potentiating effect of irradiation on the oncolytic activity of Ad5-Δ24RGD. In 2 of 5 cell lines (U118MG and SF-763), the cytotoxicity of the two modalities displayed supra-additive effects. Using the same experimental conditions on a panel of primary glioma cell cultures again demonstrated a significant potentiating effect of irradiation on Ad5-Δ24RGD-induced oncolysis (Fig. 4B). In 4 of 7 cell cultures, these effects were supra-additive.

**Effects of Ad5-Δ24RGD in Combination with Irradiation in Vivo.** The effects of Ad5-Δ24RGD in combination with radiotherapy were assessed on IGRG121 xenografts (Fig. 5). To be able to demonstrate additive or even synergistic effects, we used one tenth of the Ad5-Δ24RGD dose that was effective in the single treatment experiment. Mice bearing s.c. tumors received either 5 Gy TBI, intra-...
IRRADIATION ENHANCES Ad5-Δ24RGD ACTIVITY IN GLIOMA

The use of CRAds holds promise for the treatment of cancer, including malignant glioma. In this regard, AdΔ24 has been demonstrated to efficiently replicate in and lyse glioma cells, whereas quiescent cells were resistant to AdΔ24-induced cell lysis (6). Nevertheless, in vivo efficacy may be limited by various factors. First, Ad entry into tumor cells could be inefficient because of lack of CAR expression (9). Second, oncolysis of the tumor may be insufficient because of a reduced intrinsic oncolytic potential of the CRAd or as a result of host immune response. Therefore, we have sought to improve the potential of oncolytic viral treatment in the context of malignant glioma by enhancing AdΔ24 infectivity by targeting to αv integrins, which are highly expressed on intracranial tumors (27, 28), and by combining this virus with irradiation.

Recently, we described the validity of using the organotypic spheroid model for studying Ad infection and spread in a three-dimensional structure in vitro (26). Infection of OMS prepared from glioma tissue with Ad5-Δ24RGD demonstrated the oncolytic potential of this virus in a solid tumor mass. By 12 days after infection, viability of the spheroids was strongly reduced relative to controls. These results could be confirmed in vivo, where Ad5-Δ24RGD treatment led to complete tumor regression and long-term survival in all treated animals bearing a human glioblastoma tumor with low CAR expression. The powerful cytopathic action of Ad5-Δ24RGD on broad panels of primary cell cultures and spheroids, as well as in xenografts, suggests a therapeutic potential for treatment of malignant glioma. However, it should be taken into account that clinical trials using replication-competent viral vectors have to date only demonstrated significant therapeutic efficacy when combined with conventional therapeutics (30). For combination studies in malignant glioma, radiotherapy is the treatment of choice, considering its demonstrated therapeutic efficacy. In vitro experiments on a broad panel of glioma cell lines and primary cell cultures demonstrated the potentiating and even supra-additive effects of irradiation on Ad5-Δ24RGD-induced cytotoxicity.

These findings were corroborated by the enhanced antitumor activity with combination treatment of glioblastoma xenografts in vivo. The synergy observed between Ad5-Δ24RGD and radiotherapy is a
tumoral injections of 10^6 pfu Ad5-Δ24RGD for 5 consecutive days, or the combination of both treatments. Five Gy TBI induced a nonsignificant TGD of 13.8 days compared with untreated controls. Injection of 10^6 pfu Ad5-Δ24RGD for 5 days induced significant TGD of >119 days compared with untreated controls. One of 9 animals experienced CR and 5 of 9 experienced PR. These 6 animals were LTSS. When the viral injections were combined with irradiation, 10 of 10 animals experienced tumor regression (5 PR and 5 CR) and all survived without regrowth of tumors 4 months after treatment (Table 2).

One tumor that continued to grow after low dose virus treatment was resected at day 42. HES staining of paraffin sections of this tumor revealed the classical glioma features with posttherapeutic changes of necrosis and dystrophic and apoptotic figures within viable tumor cells surrounded by fibrosis (Fig. 6A). Immunohistochemistry for hexon Ad capsid protein revealed the presence of viral replication throughout the tumor, in particular surrounding necrotic areas (Fig. 6B).

Histological analysis of tumors retrieved at day 120 from animals with tumor residuals after virus alone or combined treatment showed the absence of viable tumor cells, whereas signs of postnecrotic changes, foreign body reactions, calcium conglomerations, and macrophages were detected (Fig. 6, C and D). This indicated that although the size of the tumor remnants of these LTSSs did not meet the requirements of CR, these animals may be considered as cured. No obvious histological differences were detected between the tumor residuals treated with Ad5-Δ24RGD alone or Ad5-Δ24RGD in combination with irradiation.

DISCUSSION

The use of CRAds holds promise for the treatment of cancer, including malignant glioma. In this regard, AdΔ24 has been demonstrated to efficiently replicate in and lyse glioma cells, whereas quiescent cells were resistant to AdΔ24-induced cell lysis (6). Nevertheless, in vivo efficacy may be limited by various factors. First, Ad entry into tumor cells could be inefficient because of lack of CAR expression (9). Second, oncolysis of the tumor may be insufficient because of a reduced intrinsic oncolytic potential of the CRAd or as a result of host immune response. Therefore, we have sought to improve the potential of oncolytic viral treatment in the context of malignant glioma by enhancing AdΔ24 infectivity by targeting to αv integrins, which are highly expressed on intracranial tumors (27, 28), and by combining this virus with irradiation.

Previously, we demonstrated that replication-deficient Ad vectors, genetically modified to express the RGD peptide in the fiber knob (29) enhance gene transfer into glioma cells by up to 50-fold compared with non-RGD vectors (10). On a panel of glioma cell lines and primary cell cultures, we now demonstrate that the increased infection efficiency of RGD-targeted vectors can be translated into increased oncolysis by CRAds. Interestingly, the extent of increased oncolytic activity was directly and inversely correlated to the level of CAR expression.

Recently, we described the validity of using the organotypic spheroid model for studying Ad infection and spread in a three-dimensional structure in vitro (26). Infection of OMS prepared from glioma tissue with Ad5-Δ24RGD demonstrated the oncolytic potential of this virus in a solid tumor mass. By 12 days after infection, viability of the spheroids was strongly reduced relative to controls. These results could be confirmed in vivo, where Ad5-Δ24RGD treatment led to complete tumor regression and long-term survival in all treated animals bearing a human glioblastoma tumor with low CAR expression. The powerful cytopathic action of Ad5-Δ24RGD on broad panels of primary cell cultures and spheroids, as well as in xenografts, suggests a therapeutic potential for treatment of malignant glioma. However, it should be taken into account that clinical trials using replication-competent viral vectors have to date only demonstrated significant therapeutic efficacy when combined with conventional therapeutics (30). For combination studies in malignant glioma, radiotherapy is the treatment of choice, considering its demonstrated therapeutic efficacy. In vitro experiments on a broad panel of glioma cell lines and primary cell cultures demonstrated the potentiating and even supra-additive effects of irradiation on Ad5-Δ24RGD-induced cytotoxicity.

These findings were corroborated by the enhanced antitumor activity with combination treatment of glioblastoma xenografts in vivo. The synergy observed between Ad5-Δ24RGD and radiotherapy is a
phenomenon that was also observed with other CRAdS, including the E1B-deleted ONX-015 (16) and the prostate-specific antigen promoter-driven CV706 (17, 18). This suggests that the type of viral genome modifications that are introduced to achieve tumor selectivity does not influence the underlying mechanisms leading to synergistic anticancer activity. It has been suggested that irradiation creates an environment that is more conductive to Ad infection or replication (31). Whereas increased viral replication after irradiation has been described for replication-competent herpes viruses (19, 32), two studies on combination treatment of ONX-015 and irradiation reported no significant effects of irradiation on the amount of virus produced in vitro or in vivo (16). However, it cannot be excluded that irradiation accelerates lysis of infected cells, allowing earlier release of progeny virus. This would enhance viral spreading and improve clinical efficacy because immune responses to the Ad allow only for a short time span of replication.

Perhaps the main advantage of combined treatment is the fact that lower viral doses are required to achieve a certain therapeutic effect. This is demonstrated by the results from the two animal experiments. Irradiation enhanced Ad5-Δ24RGD antitumor activity, such that a 10-fold lower viral dose achieved the same therapeutic response. This is an important finding given the fact that sufficient Ad delivery to tumors remains one of the major hurdles in clinical viral (gene) therapy strategies. Furthermore, combined treatment allowing lower viral doses may also lower toxic side effects.

Finally, combination therapy using cytotoxic agents with differing mechanisms of action is an attractive approach to treatment of malignant glioma. It allows cell kill by specific selectivity of each agent, resulting in broader spectrum of oncolytic action. This broader spectrum of oncolysis is expected to be particularly more efficacious in tumors with strong heterogeneity, a hallmark of glioblastoma multiforme. We conclude that the results presented support further development of Ad5-Δ24RGD in combination with radiotherapy for treatment of these highly malignant tumors.

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REFERENCES

IRradiation Enhances Ad5-Δ2ΔRBD Activity in Glioma


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