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Oncolytic Activity of p53-Expressing Conditionally Replicative Adenovirus AdΔ24-p53 against Human Malignant Glioma

Birgit Geoerger,[1,2] Gilles Vassal,[1,2] Paule Opolon,[3] Clemens M. F. Dirven,[4] Jackie Morizet,[2] Lysiane Laudani,[2] Jacques Grill,[1] Giuseppe Giaccone,[5] W. Peter Vandertop,[4] Winald R. Gerritsen,[5] and Victor W. van Beusechem[5]

Department of [1]Pediatrics, [2]Pharmacology and New Treatments in Cancer, and [3]Vectorology and Gene Transfer, Institut Gustave Roussy, Villejuif, France, and [4]Department of Neurosurgery, and [5]Division of Gene TherapyDepartment of Medical Oncology, VU University Medical Center, Amsterdam, the Netherlands

ABSTRACT

Prognosis of malignant glioma is poor, and results of treatment remain mediocre. Conditionally replicative adenoviruses hold promise as alternative anticancer agents for the treatment of malignant glioma. Here, we evaluated the conditionally replicative adenovirus AdΔ24 and its recently developed derivative AdΔ24-p53, which expresses functional p53 tumor suppressor protein while replicating in cancer cells, for treatment of malignant glioma. In comparison to its parent AdΔ24, AdΔ24-p53 killed most malignant glioma cell lines and primary glioblastoma multiforme short-term cultures more effectively, irrespective of their p53 status. Moreover, AdΔ24-p53 caused more frequent regression and more delayed growth of IGRG121 xenografts derived from a glioblastoma multiforme *in vivo*. Five intratumoral injections of 10^7 pfu AdΔ24 gave 24 days median tumor growth delay ($P < 0.01$), 30% tumor regressions, and 30% animals surviving >120 days tumor-free or with a minimal tumor residual. The same dose of AdΔ24-p53 caused >113 days of median tumor growth delay ($P < 0.001$), 70% tumor regressions, and 60% animals surviving >120 days tumor-free or with a minimal tumor residual. Antitumor effects *in vivo* were associated with extensive conditionally replicative adenovirus replication, apoptosis induction, and tumor morphology changes, including dissociation, inflammatory cell infiltration, and necrosis. We conclude that conditionally replicative adenoviruses expressing p53 are promising new agents for treatment of malignant glioma.

INTRODUCTION

Malignant gliomas have a poor prognosis, and patients with glioblastoma multiforme have a median survival of <1 year after diagnosis. Current treatment consists of surgical resection, irradiation, and chemotherapy, which are rather ineffective (1). The search for alternative treatment modalities has revived the concept of using oncolytic viruses to treat cancer (2, 3). In this respect, conditionally replicative adenoviruses appear as attractive anticancer agents that are currently being evaluated in clinical trials (4, 5). Conditionally replicative adenoviruses exert intrinsic anticancer activity through selective replication and lysis in cancer cells. In addition, the release of conditionally replicative adenovirus progeny by infected tumor cells provides a potential to amplify the oncolytic effect by lateral spread through solid tumors.

One strategy to engineer conditionally replicative adenoviruses is by introducing deletions in adenovirus genes that abolish viral functions that are dispensable in cancer cells but not in normal cells, such as interaction of viral E1A and E1B proteins with cellular pRb and p53 tumor suppressor proteins, respectively. Dysfunctions of pRb and p53 pathways are common in malignant glioma (6), making conditionally replicative adenoviruses potentially useful to treat this disease. In this respect, the conditionally replicative adenovirus AdΔ24 or *dl*922–947, which carries a deletion in the CR2 domain of the *E1A* gene that abrogates E1A binding to pRb, has already been shown to efficiently replicate in glioma cells and kill them, whereas quiescent normal cells were resistant (7, 8). These characteristics make conditionally replicative adenoviruses with pRb-binding deficient E1A particularly appealing for treatment of tumors in the central nervous system, where normal cells are essentially quiescent.

An important determinant for the anticancer potency of conditionally replicative adenoviruses is their efficacy in killing host cells and releasing their progeny. This involves multiple processes, including destruction of the cytokeratin network through cytokeratin-18 cleavage (9), cell death brought about by the E3–11.6kDa adenovirus death protein (10), and induction of p53-dependent and -independent apoptosis (11, 12). Notably, rapid adenovirus-induced cell death appears to require functional p53 (13, 14). However, 30–50% of gliomas carry defective p53 mutants, and others have compromised p53 function, *e.g.*, through mdm2 amplification/overexpression or deletion or methylation of the *CDKN2A/p14^{ARF}* tumor suppressor gene (15–17). Hence, most if not all of the glioma cells have dysfunctional p53, which might delay conditionally replicative adenovirus-induced cell death and, thus, limit conditionally replicative adenovirus efficacy. Indeed, we found recently that exogenous expression of p53 during adenovirus replication in cancer cells accelerated cell death and progeny virus release and that an AdΔ24-derivative conditionally replicative adenovirus expressing p53 exhibited enhanced oncolytic potency in the majority of tested cancer cell lines *in vitro* (18).

Here we evaluate the anticancer activity of AdΔ24 and its p53-expressing derivative AdΔ24-p53 on different glioma cell lines and primary glioblastoma multiforme specimens *in vitro*, as well as on primary malignant glioma xenograft models *in vivo*. Furthermore, we investigate conditionally replicative adenovirus replication in normal human brain tissue.

MATERIALS AND METHODS

Cell Lines, Xenografts, and Primary Tumor and Brain Materials. U87MG and U373MG glioma cell lines were obtained from American Type Culture Collection (Manassas, VA), glioma cell line U251MG was a gift of Dr. Peter Sminia (Department of Radiotherapy, VU University Medical Center, Amsterdam, the Netherlands) and glioma cell line SF763 was provided by Dr. Dolores Dougherty (Neurosurgery Tissue Bank, University of California San Francisco, Brain Tumor Research Center, San Francisco, CA). Tumor suppressor gene status of these cell lines is as follows (19): U87MG has wild-type p53, p14^{ARF}/p16 deletion, and PTEN mutation; U251MG has mutated p53 (R273H), p14^{ARF}/p16 deletion, and PTEN mutation; U373MG has mutated p53 (R273H), wild-type p14^{ARF}/p16, and PTEN mutation; and SF763 has mutated p53 (R158L), p14^{ARF}/p16 deletion, and wild-type PTEN. All of the cell lines were maintained in F12-supplemented DMEM with 10% fetal calf serum (FCS) and antibiotics (Life Technologies, Inc., Paisley, United Kingdom).

The xenografts IGRG88 and IGRG121 derived from primary malignant gliomas diagnosed as malignant oligodendroglioma and glioblastoma multiforme, respectively, have been described before (20). IGRG88 expresses high

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Requests for reprints: Victor van Beusechem, Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, the Netherlands. Phone: 31-20-444-8423; Fax: 31-20-444-8168; E-mail: vw.vanbeusechem@vumc.nl.

levels of mutant p53 (F113I) lacking transactivation function and has deleted $p14^{ARF}$; IGRG121 carries wild-type p53 and $p14^{ARF}$ (20). Tumors were passed as s.c. flank xenografts in nude mice, and for *in vitro* experiments cells were dissected from tumors and kept in short-term cultures in DMEM supplemented with 10% FCS and antibiotics (Life Technologies, Inc.).

Fresh tumor material was collected during brain tumor surgery at the Departments of Neurosurgery of the VU University Medical Center and the Academic Medical Center (Amsterdam, the Netherlands) and processed within 3 h after surgical resection. Pathological confirmation of diagnosis was made on tumor material that was processed for cell culture. All of the samples included in this study were diagnosed as glioblastoma multiforme. Primary glioblastoma multiforme cells were obtained after mechanical dissociation of tumor resection material and cultured in DMEM supplemented with 10% FCS and antibiotics. Conditionally replicative adenovirus replication and p53 function experiments were done before passage 10.

Normal brain tissue was obtained as waste material from the corticotomy tract during surgery for low-grade astrocytoma ~2 cm away from the tumor (specimen 1) or by resection of neocortical tissue for treatment of epilepsy (specimens 2 and 3) performed at the Department of Neurosurgery of the VU University Medical Center. The tissue was cut into small pieces of ~2 mm using 21-gauge needles and used immediately for experiments.

Regarding the use of human tissues in this study, ethics, informed consent, and approval comply with the principles stated in the Declaration of Helsinki of the World Medical Association (1989).

Recombinant Adenoviruses. All of the recombinant adenoviruses have been described before. AdGFP is a replication-deficient adenovirus vector expressing cytomegalovirus immediate early promoter-driven enhanced green fluorescence protein (21). AdSVEp53 is a replication deficient adenovirus vector carrying a SV40 early promoter-driven human p53 expression cassette (22). The conditionally replicative adenovirus AdΔ24 (18) carries a 24-bp deletion corresponding to amino acids 122–129 in the CR2 domain of E1A necessary for binding to pRb (7), and AdΔ24-p53 was derived from AdΔ24 by inserting the SVEp53 expression cassette from AdSVEp53 (18). Wild-type adenovirus serotype 5 was kindly provided by Dr. Rob C. Hoeben (LUMC, Leiden, the Netherlands).

Viruses were plaque purified, propagated on 293 cells (American Type Culture Collection) for replication-deficient vectors or A549 cells (American Type Culture Collection) for (conditionally) replicating viruses, and CsCl gradient purified according to standard techniques. The E1Δ24 mutation and p53 expression cassette insertion were confirmed by PCR on the final products. Functional plaque-forming unit (pfu) titers were determined by limiting dilution plaque titration on 293 cells according to standard techniques. In all of the experiments, infections were normalized on the basis of pfu titers.

P53 Reporter Assay. Functional p53 status was determined as described previously (18). Briefly, cells were transfected either with the reporter plasmid PG13-Luc, carrying the firefly luciferase gene driven by a p53-dependent promoter, or with the negative control construct MG15-Luc that has mutated p53-binding elements (23). After 48 h, luciferase expression was measured. The relative luciferase expression in PG13-Luc-transfected cells compared with MG15-Luc-transfected cells was used as a measure for functional p53 expression. Ratios between 0.5 and 2.0 were considered to represent a p53-deficient status, ratios of >2–10 an impaired or heterogeneous p53 activity, and ratios >10 were scored as representing a functional p53 status.

Assays for Adenovirus Oncolytic Potency *in Vitro*. Glioma cells were seeded 5×10^4 cells/well in 24-well plates for cell lines or, depending on cell size, at 2 to 5×10^5 cells/well in six-well plates for short-term glioblastoma multiforme cultures to prepare subconfluent monolayers. Cells were infected with AdΔ24 or AdΔ24-p53 at 1 pfu/cell for 1 h at 37°C. After culture at 37°C for up to 25 days, cell survival was analyzed by WST-1 conversion assay as described (24). WST-1 conversion per hour was expressed either as a percentage of the conversion by uninfected control cells at the start of the culture (Fig. 1) or as a percentage of the conversion by uninfected control cells cultured in parallel (Fig. 2) after subtraction of background values of WST-1 incubated in the absence of cells. Analysis of statistical difference between susceptibility of cells to AdΔ24 and AdΔ24-p53 was done by Wilcoxon signed rank test using InStat software (GraphPad Software, San Diego, CA).

Short-term cultured xenograft-derived cells were seeded 10^4 cells per well in a 96-well plate and the next day infected with AdΔ24 or AdΔ24-p53 at a 10-fold dilution titration ranging from 1 to 0.001 pfu/cell. After 19 days of culture, remaining viable cells were fixed and stained with crystal violet as described (18) and photographed at 25-times original magnification.

Adenovirus Replication in Normal Human Brain Tissue *in Vitro*. Approximately 2-mm small pieces of normal brain tissue were subjected to recombinant adenovirus infection individually at 10^8 pfu/piece in 100 μl of DMEM-F12 with 2% FCS and antibiotics for 1 h at 37°C. Subsequently, pieces were washed once with culture medium, transferred to separate wells in a 96-well plate containing 200 μl of DMEM-F12 with 10% FCS and antibiotics, and cultured for 7 days at 37°C. Successful infection was confirmed 2 days later by inspection of AdGFP-infected tissue pieces under a fluorescence microscope. On day 7, pieces were washed once in PBS and subjected to three freeze-thaw cycles in PBS to release adenovirus. The lysates were cleared by centrifugation and used to determine protein content by BCA Protein Assay (Pierce, Rockford, IL) and adenovirus titer by end point dilution titration on 293 cells. To discriminate between input virus that survived 7 days of culture without replication and progeny released from infected cells, virus output in pfu per microgram protein was compared with the output of pieces infected with replication-deficient AdGFP. Analysis of statistical differences between groups was done by two-tailed nonparametric Kruskal-Wallis test using InStat software (GraphPad Software).

Evaluation of Conditionally Replicative Adenovirus Antitumor Activity *in Vivo*. AdΔ24 and AdΔ24-p53 antitumor activities were evaluated against advanced-stage human malignant glioma IGRG88 and IGRG121 xenografts derived from primary tumors, as described previously (20). All of the animal experiments were carried out under the conditions established by the European Community (Directive 86/609/CCE). Briefly, female SPF-Swiss athymic nude mice bearing s.c. tumors of 100–300 mm^3 were randomly assigned to treatment groups, and intratumoral injections were performed with AdSVEp53, AdΔ24, or AdΔ24-p53 at 10^7 pfu, 10^8 pfu, or 10^9 pfu in 50 μl PBS (Life Technologies, Inc.) or with PBS only for 5 consecutive days. Different sites of the tumor were chosen for each injection. Tumors were measured two to three times weekly and tumor volume calculated according to the equation: V (mm^3) = width^2 (mm^2) × length (mm)/2. The experiment was stopped after 120 days when there were tumor-free survivors. Statistical significance in tumor growth rate (time to reach five times initial tumor volume) between treatment groups and controls was assessed by the two-tailed nonparametric Kruskal-Wallis test. Tumor regression was described using standard terminology including complete tumor regression (*i.e.*, total tumor regression or tumor volume < 15 mm^3 in at least two consecutive measurements) and partial tumor regression (*i.e.*, ≥50% decrease in tumor volume in at least two consecutive measurements; Ref. 25).

Histology and Immunohistochemistry for Adenoviral Hexon Protein and Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay. Glioma xenografts were injected with AdΔ24 or AdΔ24-p53 as described above for 5 consecutive days: IGRG88 at 10^8 pfu/injection and IGRG121 at 10^7 pfu/injection. Tumors were excised at days 10 and 7, respectively, or at day 120 when long-term survivors carried a residual tumor, fixed in Glyo-fixx (Shandon, Pittsburgh, PA), paraffin-embedded, and cut into 4-μm-thick sections. Hematoxylin-eosin-safranin staining was performed on all of the xenografts for analysis of morphology. Masson's trichrome staining for collagen type I using aniline blue (Reactifs RAL, Martillac, France) was done according to the manufacturer's instructions. The polyclonal antiadenovirus hexon protein antibody AB 1056 (Chemicon International, Temecula, CA), diluted 1:300, was detected by a biotinylated rabbit antigoat immunoglobulin antibody streptavidin-horseradish peroxidase conjugate (DAKO), and the chromogen diaminobenzidine, as described before (20). Slides were counterstained with hematoxylin. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed using the *In Situ* Cell Death Detection kit, AP (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions and as described before (26). TUNEL-positive cells, displaying compaction or segregation of the nuclear chromatin or breaking up of the nucleus into discrete fragments, were counted per view at 100-times magnification. Five representative fields were chosen for counting; necrotic fields were excluded.

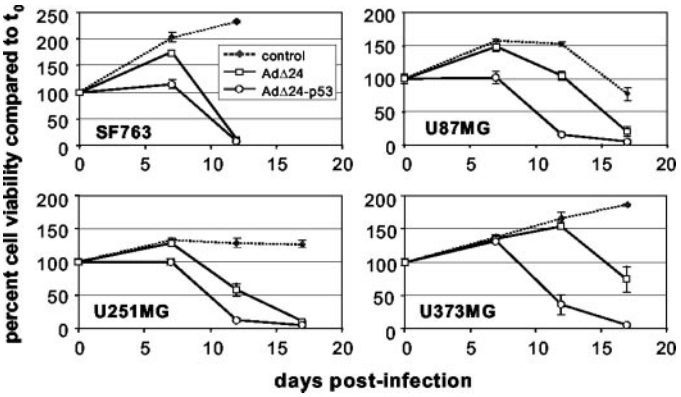

Fig. 1. Oncolytic replication of conditionally replicative adenoviruses on glioma cell lines. Four human glioma cell lines were infected with AdΔ24 (□) or AdΔ24-p53 (○) at 1 pfu/cell or mock-infected (♦) and cultured for up to 17 days. At various time points, relative cell viability compared with culture initiation was determined by WST-1 conversion measurement. Data represent means of triplicate cultures; *bars*, ±SD.

RESULTS

AdΔ24-p53 Exhibits Enhanced Oncolytic Potency against Human Glioma Cell Lines. We first studied the oncolytic potencies of AdΔ24 and AdΔ24-p53 on four human glioma cell lines with different p53 status, *i.e.*, U87MG cells with wild-type p53 sequence and confirmed expression of functional p53 in a p53 transactivation reporter assay (PG13:MG15 ratio 13.7); and U251MG, U373MG, and SF763 cells that carry *p53* genes with missense mutations and are p53-deficient (PG13:MG15 ratios 2.0, 0.8, and 1.6, respectively). Cells were infected with AdΔ24 or AdΔ24-p53 at low multiplicity of infection and cultured for 7, 12, or 17 days, when cell viability was determined by WST-1 assay (Fig. 1). AdΔ24-p53 was more effective in killing glioma cells than AdΔ24 with the most significant efficacy improvement on the most resistant cell line, U373MG. The four human glioma cell lines were eradicated by AdΔ24-p53 with a similar rapid course comparable with AdΔ24-mediated killing of the most sensitive cell line, SF763. Hence, exogenous p53 expression overcame resistance to AdΔ24-induced oncolysis in p53 wild-type and p53-deficient glioma cells.

AdΔ24-p53 Exhibits Enhanced Oncolytic Potency against Human Primary Glioma Specimens *in Vitro*. A panel of eight short-term cultures was established from primary human brain tumor specimens with histologically confirmed diagnosis of glioblastoma multiforme. Reporter assay for p53 transactivation function revealed that one of the samples was p53 positive, four were p53 deficient, and three had low but detectable p53 activity. The latter could indicate presence of functional p53 protein impaired in its activity by, *e.g.*, enhanced degradation. Alternatively, specimens with low p53 activity could be composed of a mixture of p53 wild-type and p53-deficient cells. Short-term cultures were infected with AdΔ24 or AdΔ24-p53 at low multiplicity of infection and cultured until cytopathic effects became evident or for a maximum of 25 days, when cell survival was measured by WST-1 conversion assay (Fig. 2). Duration of culture was dependent on adenovirus receptor coxsackie adenovirus receptor expression level, with coxsackie adenovirus receptor-deficient samples cultured longer than coxsackie adenovirus receptor-positive samples (24). AdΔ24 killed only specimen VU-78 effectively, with >1 log cell kill in 12 days (and >2 logs in 14 days; data not shown). The other seven samples were much more refractory to this conditionally replicative adenovirus, with viability of four specimens not even affected after prolonged culture up to 25 days. In contrast, AdΔ24-p53 exerted detectable cytotoxicity on five of these resistant samples, causing 2–12-fold more cell kill than AdΔ24. The efficacy of AdΔ24-p53 against the panel of primary glioblastoma multiforme specimens was significantly enhanced compared with its parent control AdΔ24 ($P < 0.01$). Oncolysis enhancement did not correlate with p53 status of the cells.

We also compared the oncolytic potencies of AdΔ24 and AdΔ24-p53 *in vitro* on short-term cultures of the human malignant glioma xenografts IGRG88 (p53 mutant) and IGRG121 (p53 wild-type). Cells were infected at a range of multiplicity of infection (1 to 0.001 pfu/cell) and cultured for 19 days to allow multiple conditionally replicative adenovirus replication cycles. After culture, remaining viable cells were stained and photographed. As can be seen in Fig. 3, in both xenograft-derived cell cultures AdΔ24-p53 caused extensive cell death at an estimated 10–100 times lower viral dose than AdΔ24, indicating that AdΔ24-p53 replicated faster than its parental control lacking p53.

AdΔ24 and AdΔ24-p53 Replicate on Normal Human Brain Tissue. To evaluate the selectivity of AdΔ24 and AdΔ24-p53 in the context of malignant glioma treatment, we investigated their replication in normal brain tissue samples *in vitro*. To this end, individual small pieces dissected from three fresh brain explants were infected with AdΔ24 or AdΔ24-p53 at high multiplicity of infection (10^8 pfu per tissue piece). Control samples were infected with wild-type adenovirus serotype 5 or with replication-deficient AdGFP. After 7 days of culture, the content of infectious adenovirus was determined in each tissue piece by titration on 293 cells and normalized by protein content. As can be seen in Fig. 4, significantly more virus was recovered from pieces infected with wild-type adenovirus serotype 5 and the two conditionally replicative adenoviruses than from AdGFP infected controls, confirming that wild-type adenovirus serotype 5 and the conditionally replicative adenoviruses replicated in normal human brain tissue *ex vivo*. However, the amounts of progeny virus produced were very low and highly variable. Median offspring recoveries per microgram tissue were below 10^4 pfu, which corresponds to the amount of wild-type adenovirus serotype 5 produced by a single permissive primary cell (27).

AdΔ24-p53 Exhibits Enhanced Antitumor Efficacy against Human Glioma Xenografts *in Vivo*. We additionally investigated the antitumor activity of AdΔ24 and AdΔ24-p53 on the malignant human glioma xenografts IGRG88 and IGRG121 at an advanced tumor stage *in vivo*. Animals bearing s.c. IGRG88 xenografts on their flank were treated with intratumoral injections of AdΔ24 or AdΔ24-p53. Control animals were injected with PBS and, in one experiment, with the

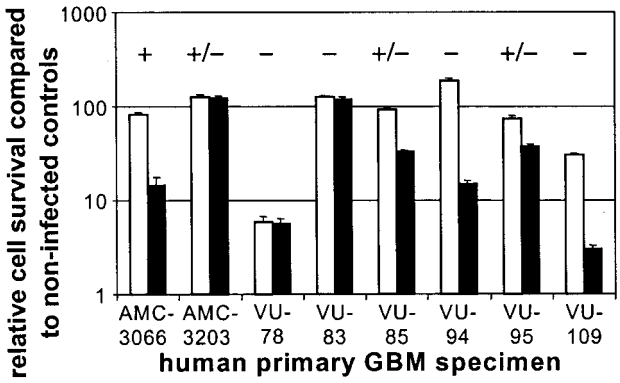

Fig. 2. Oncolytic potency of conditionally replicative adenoviruses against primary short-term cultured human glioblastoma multiforme (*GBM*). Eight primary GBM specimens were infected with AdΔ24 (□) or AdΔ24-p53 (■) at 1 pfu/cell and cultured for 12 (*VU-78*), 14 (*VU-95*), 18 (*VU-94*), or 25 days (all others). Cell viability was determined by WST-1 conversion measurement and is expressed relative to mock-infected cultures. Functional p53 status, *i.e.*, deficient (−), impaired or heterogeneous (+/−), or functional (+) is indicated *above the bars*. Data represent means of a single experiment measured in triplicate; *bars*, +SD.

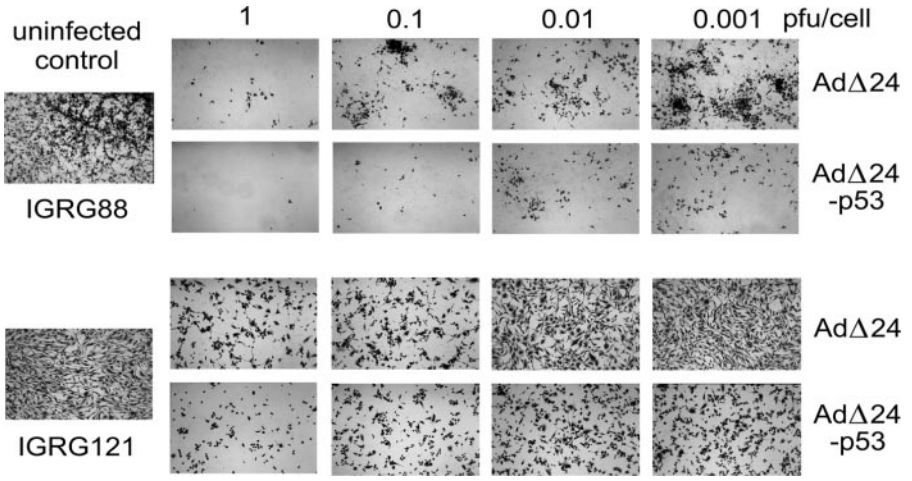

Fig. 3. Oncolytic potency of conditionally replicative adenoviruses against xenograft derived short-term cultured glioma cells. Cells were cultured from IGRG88 and IGRG121 human glioma xenografts and infected with AdΔ24 or AdΔ24-p53 at various multiplicity of infection as indicated. After 19 days culture, remaining viable cells were stained and photographed. On both cultures, AdΔ24-p53 exerted cytotoxicity similar to that of AdΔ24 at a 10–100-fold lower inoculum.

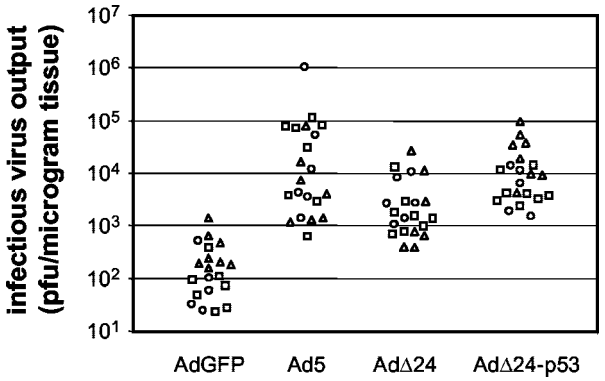

Fig. 4. Adenovirus replication in primary human brain specimens. Small pieces of human brain tissue, obtained from the corticotomy tract of a patient with low-grade astrocytoma (○) and from neocortex of 2 patients treated for epilepsy (□ and △) were infected with Ad5, AdGFP, AdΔ24, or AdΔ24-p53 at high multiplicity of infection, and virus was allowed to replicate for 7 days. Infectious adenovirus output was measured by end point dilution titration on 293 cells and normalized by protein content. Each data point represents the virus output of an individual tissue piece. Ad5 ($P < 0.001$), AdΔ24 ($P < 0.01$), and AdΔ24-p53 ($P < 0.001$) -infected explants contained significantly more virus than AdGFP infected controls. Differences in replication by Ad5, AdΔ24, and AdΔ24-p53 were not significant ($P > 0.05$).

replication-deficient p53-expressing adenovirus vector AdSVEp53. As can be seen in Table 1, the two conditionally replicative adenoviruses AdΔ24 and AdΔ24-p53 caused tumor regressions and significant tumor growth delay compared with PBS controls. In contrast, AdSVEp53 did not result in any tumor regression or significant tumor growth delay. Although in general AdΔ24-p53 treatment tended to achieve longer, more significant tumor growth delay and more frequent tumor regression than treatment with AdΔ24, IGRG88 escaped treatment in most cases, and the differences between the two conditionally replicative adenoviruses were not significant. In contrast, intratumoral treatment of IGRG121 xenografts with AdΔ24 was highly effective, with five injections of 10^8 pfu inducing 90% complete tumor regression and tumor-free survival (Fig. 5*A*). To study the effect of p53 expression, the comparison between AdΔ24 and AdΔ24-p53 on IGRG121 was conducted at a reduced dose of 10^7 pfu daily injections. At this dosage, AdΔ24 induced 30% tumor regression (2 of 10 complete tumor regression and 1 of 10 partial tumor regression) and significant median tumor growth delay of 24 days ($P < 0.01$; Fig. 5*B*). Two animals survived tumor free at day 120; a third one had a residual tumor without viable tumor cells that showed signs of post-necrotic changes, avascular fibrosis, calcium deposits, fatty acid crystals, and macrophages, some of which were multinucleated, characteristic of foreign body reactions, as determined by histology (Fig. 5*D*, *left*). AdΔ24-p53 (Fig. 5*C*) induced more tumor regression (70%; 3 of 10 complete tumor regression and 4 of 10 partial tumor regression) and yielded a more significant median tumor growth delay of >113 days ($P < 0.001$). Three of 10 AdΔ24-p53-injected animals survived tumor-free 120 days after treatment; 3 additional animals had tumor residuals at day 120, 2 of which were without viable tumor cells (data not shown) and the third one with a minimal remnant of tumor cells with atypic nuclei encapsulated in stromal tissue (Fig. 5*D*, *right*). Thus, 60% of AdΔ24-p53-treated animals survived at least 120 days with no or a minimal tumor residual. In conclusion, exogenous p53 expression by AdΔ24-p53 augmented AdΔ24 antitumor efficacy against IGRG121 xenografts *in vivo*.

Histological Analysis of Intratumoral Injected Malignant Glioma Xenografts. We finally evaluated morphological changes, intratumoral viral replication, and induction of apoptotic cell death *in vivo* in IGRG88 (Fig. 6*A*) and IGRG121 (Fig. 6*B*) xenografts injected for 5 consecutive days with 10^8 and 10^7 pfu recombinant adenovirus, respectively. Tumors were excised shortly after treatment to study efficacy of conditionally replicative adenovirus delivery and immediate antitumor effects. As can be seen in the Masson's trichrome and TUNEL panels, PBS-injected IGRG88 and IGRG121 xenografts were highly cellular, rather monotonous tumors with high mitotic activity, vascular proliferation, and some necrosis at baseline. The trichrome staining, furthermore, shows that IGRG88 tumors contained more collagen type I protein than did IGRG121 tumors. Conditionally

Table 1 *Antitumor activity of AdΔ24 and AdΔ24-p53 in subcutaneous IGRG88 malignant glioma xenografts in athymic mice*

	Treatment	*n*	PR	CR	TFS*	5 × vol (days)	TGD (days)	*P*†
Experiment 1								
Control	PBS	9	0	0	0	16		
AdΔ24	10^7 pfu/d × 5	9	1	0	0	30	14	<0.01
AdΔ24-p53	10^7 pfu/d × 5	9	3	0	0	37	21	<0.001
Experiment 2								
Control	PBS	8	0	0	0	15		
AdSVEp53	10^8 pfu/d × 5	8	0	0	0	21	6	NS‡
AdΔ24	10^8 pfu/d × 5	9	1	2	1	39	24	<0.01
AdΔ24-p53	10^8 pfu/d × 5	8	2	3	0	41	26	<0.01
Experiment 3								
Control	PBS	10	0	0	0	14		
AdΔ24	10^9 pfu/d × 5	6	1	0	0	25	11	NS
AdΔ24-p53	10^9 pfu/d × 5	12	3	2	1	40	26	<0.001

* TFS, tumor free survivors were assessed on day 120.

† For each CRAd treatment group, the times to reach five-times initial tumor volume were compared to those of the control group by two-tailed nonparametric Kruskal-Wallis test.

‡ NS, not significant.

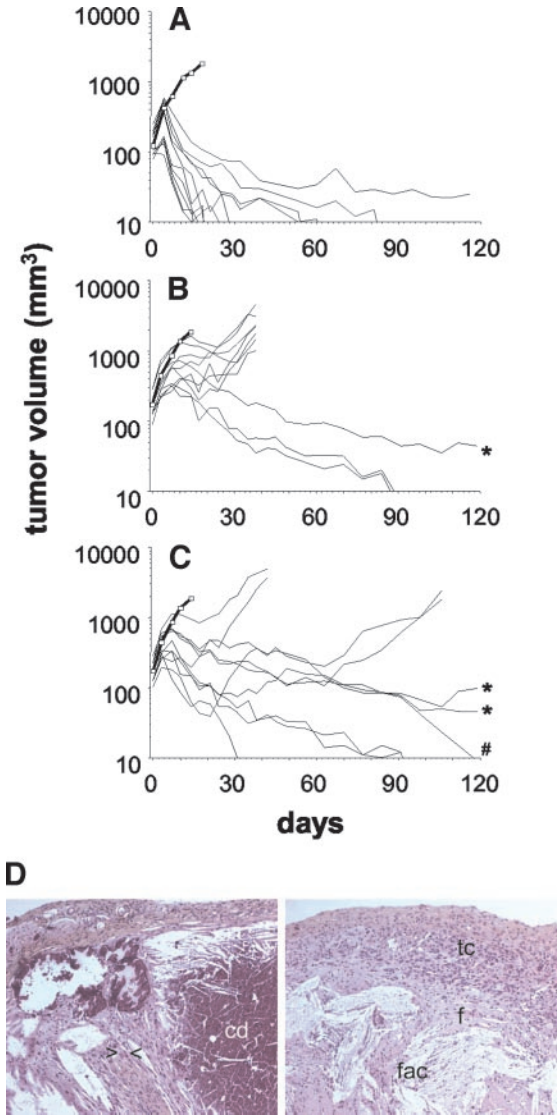

Fig. 5. Antitumor activity of AdΔ24 and AdΔ24-p53 in subcutaneous glioblastoma IGRG121 xenografts *in vivo*. Mice bearing s.c. IGRG121 xenografts were injected for 5 days with AdΔ24 at 10^8 pfu/day (*A*), AdΔ24 at 10^7 pfu/day (*B*), or AdΔ24-p53 at 10^7 pfu/day (*C*). Each *line* represents one treated tumor; lines with □ in each panel show the mean results of the control groups injected with PBS. Tumor residuals at day 120 without viable tumor cells in histology are marked with ∗, the one with a minimal remnant with #. *D*, histology by hematoxylin-eosin-safranin staining on residuals from AdΔ24 (*left*; marked ∗ in *B*) and AdΔ24-p53 (*right*; marked # in *C*) treated tumors showed signs of postnecrotic changes, avascular fibrosis (*f*), calcium deposits (*cd*), fatty acid crystals (*fac*), and spumous multinucleated macrophages characteristic of foreign body reactions (one indicated between > <). One residual tumor contained a few tumor cells with atypic nuclei (*tc*) encapsulated in stromal tissue. Pictures were taken at ×100 magnification.

replicative adenovirus delivery to IGRG88 tumors was very inefficient; hardly any cells in these tumors were infected. Consequently, histological changes were minimal. IGRG88 tumors remained dense without evidence of stromal reaction, although some fibrotic fascicles and infiltration of lymphocytes and macrophages were detected. In contrast, IGRG121 showed much more profound post-treatment changes, with scarce tumor cell nuclei within an edematous tissue; tumors were dissociated, showed lymphatic infiltrations, and extended foci of necrosis, most markedly in AdΔ24-p53-treated tumors. Immunohistochemistry for adenoviral capsid protein confirmed effective conditionally replicative adenovirus delivery by showing well-dispersed infection throughout IGRG121 xenografts injected with either conditionally replicative adenovirus. Replication was mainly detected in tumor borders and around areas of necrosis or fibrosis. TUNEL staining determined in control xenografts a low apoptotic rate in viable tumor areas of only 0.2% in IGRG88 and 0.4% in IGRG121. Consistent with the low infection efficiency, adenovirus treatment caused apoptosis in only few IGRG88 cells, with 0.4%, 0.9%, and 1.7% TUNEL-positive IGRG88 cells after injection of AdSVEp53, AdΔ24, or AdΔ24-p53, respectively. In contrast, in IGRG121, AdΔ24 and AdΔ24-p53 treatment induced much more significant apoptotic cell death with 7.1% and 8.1% TUNEL-positive tumor cells, respectively. Thus, induction of apoptosis correlated with Conditionally Replicative Adenovirus replication and appeared an important determinant for adenovirus mediated cell kill and tumor growth inhibition in malignant glioma. Because AdΔ24-p53 injected tumors contained more lysed cells and larger areas of necrosis, conditionally replicative

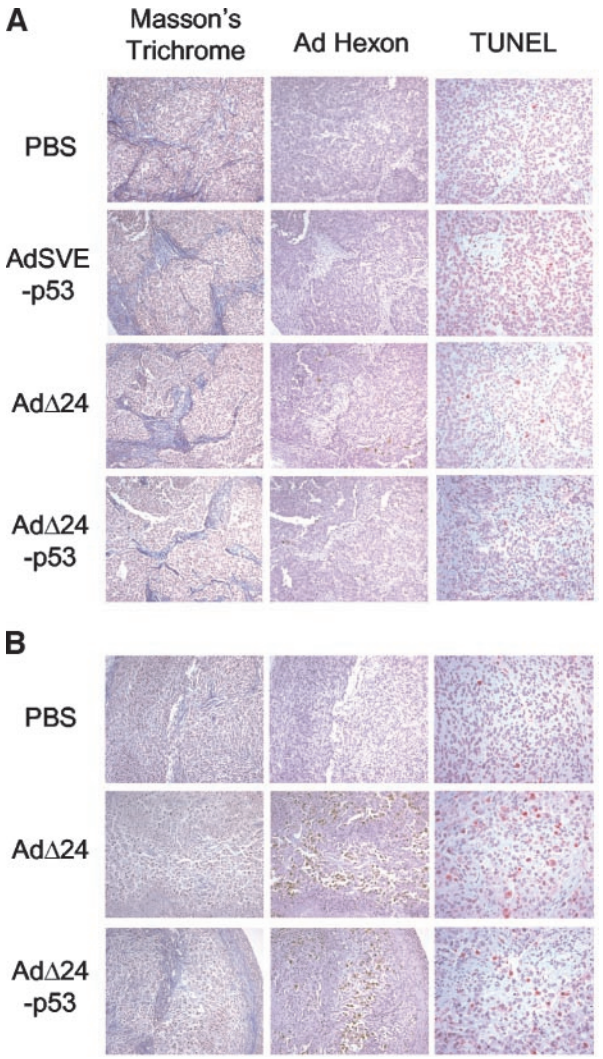

Fig. 6. Histology of conditionally replicative adenovirus-injected malignant glioma xenografts. IGRG88 (*A*) or IGRG121 (*B*) xenografts were injected for 5 days with PBS or with 10^8 and 10^7 pfu recombinant adenovirus, respectively, and excised a few days after virus injection. Masson's trichrome staining shows collagen type I in intense blue, and nuclei counterstained with hematoxilin in violet. Immunohistochemistry for adenoviral hexon protein (stained in *brown*) detected only very few conditionally replicative adenovirus-infected IGRG88 cells, but extensive conditionally replicative adenovirus replication in IGRG121 tumors, mainly at tumor borders and surrounding necrotic or fibrotic areas. Terminal deoxynucleotidyl transferase-mediated nick end labeling (*TUNEL*) staining (TUNEL positive nuclei stained in *red*) reveal only minor post-therapeutic changes and few apoptotic tumor cells in conditionally replicative adenovirus-treated IGRG88 tumors but profound post-therapeutic changes in IGRG121 xenografts, with many apoptotic tumor cells in conditionally replicative adenovirus-treated tumors. Masson's trichrome and immunohistochemistry pictures were taken at ×100 original magnification, TUNEL in viable tumor areas at ×200 original magnification.

adenovirus replication and apoptosis induction were probably even underestimated in these tumors.

DISCUSSION

Conditionally replicative adenoviruses were introduced recently as new agents for cancer therapy. Several preclinical evaluations already showed the potential of these replication-selective adenoviruses for the treatment of malignant brain tumors, such as high-grade glioma (20, 28, 29); and the conditionally replicative adenovirus ONYX-015 has entered into Phase I clinical trials for malignant glioma.

The efficacy of conditionally replicative adenoviruses against cancer including glioma is, however, limited by several factors, including reduced adenoviral entry and adenovirus-induced oncolysis. The former process can be augmented by redirecting infection via alternative molecules expressed on glioma cells (24, 28, 29). The latter process appears to involve the adenoviral E1B55k protein and the p53 tumor suppressor protein (14). The lack of functional p53 in most gliomas might, therefore, reduce sensitivity to conditionally replicative adenovirus-induced cytolysis. To enhance the oncolytic potency of AdΔ24-type conditionally replicative adenoviruses (7, 8), we recently constructed the new conditionally replicative adenovirus AdΔ24-p53 that expresses functional p53 during viral replication in cancer cells (18). Here, we evaluated the efficacy of AdΔ24 and AdΔ24-p53 against human malignant glioma *in vitro* and *in vivo*. Consistent with our previous findings on cancer cell lines of various tissue origins, we found that exogenous expression of p53 by AdΔ24-p53 led to enhanced oncolytic potency compared with its parent AdΔ24 on most glioma cell lines and, importantly, also on primary glioblastoma multiforme cultures tested *in vitro* and on at least one of two glioma xenograft models tested *in vivo*. The superior efficacy of AdΔ24-p53 appeared independent of the cellular p53 genetic background.

Interestingly, the two glioma xenograft models included in our study, which were both sensitive to AdΔ24 treatment and were killed more effectively by AdΔ24-p53 *in vitro*, responded quite differently to conditionally replicative adenovirus treatment at an advanced tumor stage *in vivo*. Conditionally replicative adenovirus injection into IGRG88 tumors resulted in very inefficient infection that allowed these tumors to escape treatment. In contrast, conditionally replicative adenovirus injection into IGRG121 xenografts resulted in dispersed infection of cells throughout the tumor. This allowed both conditionally replicative adenoviruses to cure animals from IGRG121 xenografts, with AdΔ24-p53 being more effective than AdΔ24. Immunohistochemistry confirmed that the adenovirus receptor coxsackie adenovirus receptor was expressed on IGRG88 tumor cells (data not shown), excluding low receptor expression as explanation for the poor infection of these cells. Perhaps the tumor composition played a role, because IGRG88 tumors contained more collagen than did IGRG121 tumors. Clearly, efficient and dispersed delivery of injected adenovirus was important for effective treatment. Because poor tissue penetration of injected adenovirus is recognized as an important limitation in treating patients with malignant glioma (30), our findings underscore the importance of evaluating conditionally replicative adenovirus efficacy in established tumors *in vivo*.

The clear conditionally replicative adenovirus replication and apoptosis induction observed after conditionally replicative adenovirus injection into sensitive IGRG121 xenografts suggested a mechanistic link between oncolysis and apoptosis induction. However, although IGRG121 xenografts were treated more effectively by AdΔ24-p53 we did not detect significant difference in apoptosis induction between AdΔ24- and AdΔ24-p53-injected IGRG121 tumors. We envision three alternative explanations for this. First, the extensive post-treatment changes in IGRG121 cells may have underestimated the apoptosis score in AdΔ24-p53-treated tumors, because TUNEL positive cells can only be determined in viable tumor cell areas. Second, the TUNEL analysis was performed only a few days after conditionally replicative adenovirus injection, when antitumor effects were apparent in both AdΔ24- and AdΔ24-p53-treated tumors, whereas differences in tumor growth only became evident after ~1 month (see Fig. 5), which is consistent with successful treatment requiring multiple cycles of conditionally replicative adenovirus replication and lateral spread. Third, it cannot be excluded that the mechanism for conditionally replicative adenovirus–induced oncolysis enhancement by p53 expression might be distinct from classical p53-dependent apoptosis. The latter would be in agreement with the proposed importance of the p53/E1B55k complex in this process (14).

Finally, we wished to obtain preliminary information on the selectivity of AdΔ24-type conditionally replicative adenoviruses in normal brain tissue. We used a small set of fresh normal human brain specimens cultured *in vitro* and found very low but significant replication of AdΔ24 and of its derivative AdΔ24-p53 in these explants. Although this was somewhat surprising and disappointing, it was not in complete disagreement with earlier findings. Whereas replication of conditionally replicative adenoviruses with pRb-binding deficient E1A was reported attenuated in comparison to wild-type adenovirus serotype 5 in quiescent normal cells including brain astrocytes (7, 8, 28, 31), this was not found for proliferating normal cells (8). In the *ex vivo* experimental setting used herein, the proliferation status of the cells in the cultured brain explants was not assessed. Brain injury can induce proliferation of astrocytes (32), and this might also have happened in the explants. Notably, conditionally replicative adenoviruses with pRb-binding deficient E1A have already been made more selective by combining E1A-CR2 deletion with a second E1A mutation or with the E2F-1 promoter driving early gene expression (31, 33). Because the concept of oncolysis enhancement by p53 expression should also apply to such conditionally replicative adenoviruses with additionally restricted pRb-selectivity, the combination of these favorable attributes to design safe and efficacious conditionally replicative adenoviruses for glioma treatment is warranted. In addition, such conditionally replicative adenoviruses should incorporate the clearly established improvement of glioma cell infection enhancement (24, 28, 29).

In conclusion, we have shown that expression of functional p53 enhances the oncolytic potency of a conditionally replicative adenovirus in malignant glioma cell lines, primary cultures, and xenografts. Hence, enhancement of conditionally replicative adenovirus potency through expression of functional p53 may have utility for more effective treatment of malignant glioma.

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