Differential Effects of Combined Ad5-Δ24RGD and Radiation Therapy in *In vitro* versus *In vivo* Models of Malignant Glioma

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Abstract

Purpose: The integrin-targeted conditionally replicating adenovirus Ad5-Δ24RGD has been shown to possess strong oncolytic activity in experimental tumors and is currently being developed toward phase I clinical evaluation for ovarian cancer and malignant glioma. Previously, we reported that combination therapy of Ad5-Δ24RGD with irradiation led to synergistic antitumor activity in s.c. glioma xenografts. In the current study, the underlying mechanism of action to this synergy was studied and the effects of combined therapy were assessed in an orthotopic glioma model.

Experimental Design and Results: Sequencing studies in U-87 monolayers showed that delivery of irradiation before Ad5-Δ24RGD infection led to a greater oncolytic effect than simultaneous delivery or infection before irradiation. This effect was not due to enhanced virus production or release. Experiments using a luciferase-encoding vector revealed a small increase in transgene expression in irradiated cells. In tumor spheroids, combination therapy was more effective than Ad5-Δ24RGD or irradiation alone. Staining of spheroid sections showed improved penetration of virus to the core of irradiated spheroids. Mice bearing intracranial tumors received a combination of Ad5-Δ24RGD with 1 × 5 Gy total body irradiation or with 2 × 6 Gy whole brain irradiation. In contrast to the *in vitro* data and reported results in s.c. tumors, addition of radiotherapy did not significantly enhance the antitumor effect of Ad5-Δ24RGD.

Conclusions: Combined treatment with Ad5-Δ24RGD and irradiation shows enhanced antitumor activity *in vitro* and in s.c. tumors, but not in an orthotopic glioma model. These differential results underscore the significance of the selected tumor model in assessing the effects of combination therapies with oncolytic adenoviruses.

Standard treatment for malignant glioma (radiochemotherapy with or without gross tumor resection) offers a median survival time for patients with high-grade gliomas of <15 months. New and more efficacious treatment modalities are therefore much sought after. In recent years, oncolytic viral therapy has become a promise for such a new treatment with the development of viruses with tumor-specific targeting and replication properties. One such tumor-specific adenovirus was engineered to replicate selectively in tumor cells with lesions in the retinoblastoma tumor repressor (pRb) pathway. This was accomplished by deleting 24 bp from the *E1A* gene, which abolishes the pRb binding capacity of the E1A protein (1, 2). This adenovirus mutant, known as AdΔ24 or Ad*dl*922-947, showed reduced replication potential in nonproliferating normal cells. The anticancer efficacy of these agents was confirmed *in vitro* and in xenograft animal models (1, 2).

Retargeting of adenovirus toward molecules highly expressed on the tumor cell membranes has been described for various cellular receptors (3–7). Another approach used to target the adenovirus to tumor cells involves the insertion of an integrin binding peptide, Arg-Gly-Asp (RGD), into the fiber of the virus, allowing the virus to make its primary attachment to integrins. Using replication-deficient adenoviral vectors encoding luciferase, it was shown that the RGD modification drastically enhanced infection efficiency of various tumor cells including malignant glioma (3, 8, 9). With an RGD-modified AdΔ24 virus, Ad5-Δ24RGD, we and others showed that improved infection efficiency translates to enhanced oncolysis in (primary) glioma cells and impressive antiglioma activity in s.c. and intracranial glioma xenografts (10, 11).

Previous studies using primary glioma cells and glioma cell lines have also shown a potentiating effect of irradiation on Ad5-Δ24RGD-induced oncolysis (10). The enhancing effect of irradiation on viral oncolysis has been described for other oncolytic adenoviruses as well (12–15); however, an

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underlying mechanism of action has not been elucidated. In the current study, we investigated *in vitro* the optimal sequencing of combined therapy, as well as the effects of irradiation on transgene expression, adenovirus receptor (CAR) and integrin expression, viral replication and release, and penetration of the virus into a three-dimensional tumor model. Moreover, we studied the antitumor activity of Ad5-Δ24RGD in combination with total body irradiation (TBI) or whole brain irradiation (WBI) in an intracerebral xenograft model for 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 the human glioma cell line U-87 MG were purchased from the American Type Culture Collection. All cells were cultured in DMEM supplemented with 10% FCS and antibiotics (Life Technologies).

Recombinant adenoviruses. The recombinant E1-deleted, replication-deficient adenovirus expressing the cytomegalovirus promoter–driven luciferase reporter gene, Ad.Luc, was kindly provided by Dr. R.D. Gerard (University of Texas Southwestern Medical Center, Dallas, TX). The integrin-targeted replication-deficient adenovirus expressing luciferase, Ad5LucRGD, and conditionally replicating adenovirus Ad5-Δ24RGD were a kind gift of Dr. D.T. Curiel (University of Alabama, Birmingham, AL) and have previously been described (9, 16). Viruses were propagated, purified, and titrated as previously described (10).

In vitro *transgene expression.* U-87 cells were seeded in quadruplicates in 48-wells plates at 2×10^4 per well. After 24 h, cells were irradiated with an 80-kV orthovolt X-ray source (Pantak Therapax SXT 150) at a dose rate of 1.3 Gy/min to a total dose of 4 Gy. Infection of cells with the luciferase-encoding vectors Ad.Luc and Ad5LucRGD was done at 6, 12, and 24 h postirradiation. After 48 h, the cells were assayed for luciferase expression using the luciferase chemiluminescent assay system (Promega).

Monolayer experiments. U-87 cells were seeded in quadruplicates in 96-well plates at 10^4 per well. Adenovirus infection was done at indicated times before or after irradiation (4 Gy). Cell survival was assessed using WST-1 reagent (Roche Diagnostics). Viability is expressed as a percentage of control untreated cells. In parallel plates of monolayer viability experiments, supernatants and cell lysates were harvested at various time points to follow viral production and release. Total amount of virus present in cells and media was determined by end-point dilution titration on 293 cells.

Spheroid experiments. Spheroids were produced from U-87 cells using an adaptation of the spinner flask method (17). Briefly, single-cell suspensions were plated in agarose-coated 96-well microplates at 10^4 per well and placed on an orbital shaker at 37°C for 24 h. The rotational forces allow the cells to rapidly aggregate and form spheroids of homogenous geometry and size. Spheroid viability was assessed with the WST-1 assay. Results are presented as percentage of untreated controls. Cryosections were cut from frozen spheroids at 6 μm and the sections from the center of the spheroid (i.e., with maximal diameter) were used for staining for adenovirus hexon proteins.

Flow cytometry. At various time points postirradiation, cultured U-87 cells were immunolabeled with either anti-CAR monoclonal antibody RmcB (18) or anti–$\alpha_v\beta_3$ integrin or anti–$\alpha_v\beta_5$ integrin monoclonal antibodies (Chemicon). Negative controls lacked the first antibody. For detection, FITC-conjugated rabbit anti-mouse antibody (DAKO) was used. Analysis was done on a FACScan (Becton Dickinson). Expression was quantified as the relative median fluorescence intensity compared with the negative control.

In vivo *studies.* For assessing the therapeutic effects of Ad5-Δ24RGD in combination with irradiation, two animal experiments in a mouse model for intracerebral glioma were done, the results of which are presented jointly in Fig. 5. In the first experiment, the effect of Ad5-Δ24RGD in combination with TBI was assessed, and in the second experiment the effect of Ad5-Δ24RGD in combination with WBI was assessed. In both experiments, U-87 cells were stereotactically injected into the right frontal lobe of adult female athymic nude mice (Harlan) as previously described (19). Mice were randomly assigned to each of four treatment groups: PBS, radiotherapy (RT), Ad5-Δ24RGD, and

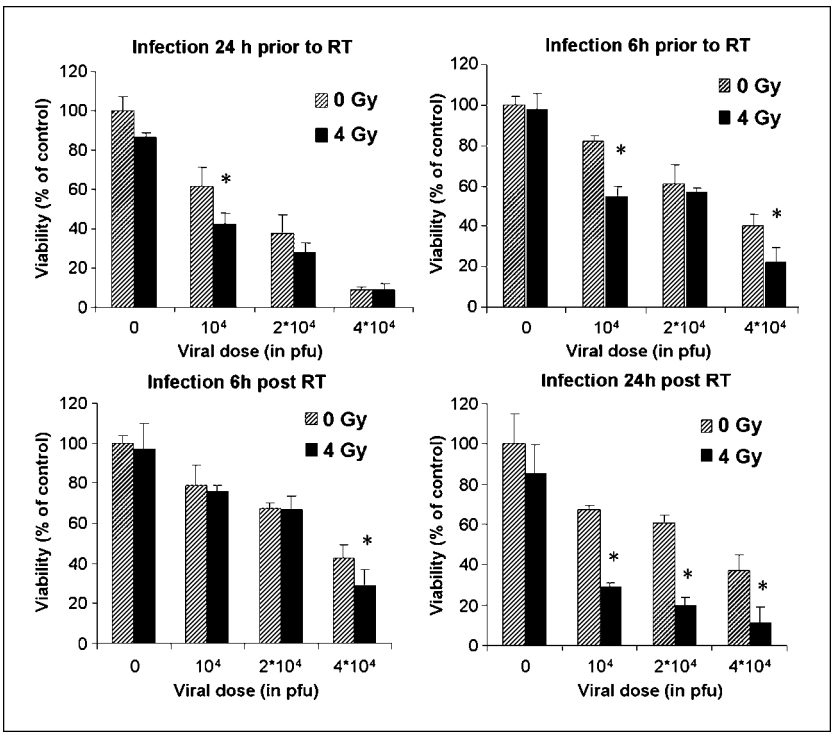

Fig. 1. Dose- and schedule-finding studies on glioma cell monolayers. Viability of U-87 cells treated with increasing doses of Ad5-Δ24RGD in combination with 4 Gy irradiation delivered in various sequences was determined using WST-1 assay. Hatched columns, virus-treated cells; solid columns, virus + RT – treated cells. Columns, percentage of untreated controls; bars, SD. *, $P < 0.01$, compared with nonirradiated cultures.

RT + Ad5-Δ24RGD. For the first experiment, TBI was given once on day 3 at a dose of 5 Gy (maximum tolerated dose). Mice were irradiated with 15-MV X-rays using a linear accelerator Varian Clinac 2300 C/D (Varian Medical Systems). For the second experiment, tumor-bearing mice received WBI. For this, a specific Perspex (polymethyl methacrylate) box was designed, which was constructed such that the mouse heads are positioned in the radiation field whereas the rest of the mouse is positioned outside that field under lead protection. The mice were irradiated on days 3 and 7 after tumor cell injection at a dose of 6 Gy each. This dose was tolerated well in pilot experiments. On day 4, mice in virus groups of both experiments received 2×10^7 plaque-forming units (pfu) of Ad5-Δ24RGD in 3-μL PBS inoculated stereotactically into the same coordinates as the tumor cells and nonvirus groups received 3-μL PBS. In both experiments, animals were monitored daily and sacrificed on appearance of symptoms evident for moribund decline. Brains were removed and frozen for (immuno)histologic analysis.

Immunohistochemistry. Histologic analysis of mouse brains was done on H&E-stained cryosections. Immunohistochemical staining for adenovirus hexon proteins was done on acetone-fixed cryosections of U-87 spheroids or mice brains from *in vivo* experiments with the goat anti-adenoviral hexon protein antibody 1056 (Chemicon) as previously described (19). Bound primary antibodies were detected with horseradish peroxidase–conjugated rabbit anti-goat antibodies and by exposure to the chromogen 3-amino-9-ethylcarbazole or diaminobenzidine.

Scoring of adenovirus staining was done on 5 to 10 cryosections, obtained from different areas of the tumor, of each virus ± RT–treated tumor with exception of the long-term survivors. The total numbers of "hotspots," indicating individual hexon-positive areas within the tumor, were counted in each tumor cryosection using light microscopy and are presented as mean ± SE.

Statistics. Data from *in vitro* experiments are presented as mean ± SD. Statistical analysis between groups was conducted with the two-tailed Student *t* test. For the *in vivo* experiments, a Kaplan-Meier analysis was done and survival in different treatment groups was compared using the log-rank test.

Results

Dose- and schedule-finding experiments. To determine the optimal sequence and schedule for Ad5-Δ24RGD and irradiation therapy, U-87 monolayers were infected 6 or 24 h before irradiation or 6 or 24 h postirradiation. A dose range of Ad5-Δ24RGD and 4-Gy irradiation were used. In parallel experiments, this irradiation dose was found to give greatest enhancement of viral oncolysis relative to controls (not shown). Viability was determined on day 8 postinfection. As shown in Fig. 1, infection of cells 24 h postirradiation led to a greater enhancement of the oncolytic effect than delivery of the therapies on the same day or infection 24 h before irradiation. Under these conditions, all three virus dosages tested showed a significant increase in cell kill compared with Ad5-Δ24RGD treatment alone. Combination therapy reduced U-87 viability dose-dependently by 71% to 89%, compared with 33% to 63% for Ad5-Δ24RGD alone and 15% for radiotherapy alone.

Effects of irradiation on virus production. A time course experiment in which U-87 cells were irradiated 24 h before Ad5-Δ24RGD infection was done, which confirmed the enhanced oncolytic effect of combination therapy, showing significantly enhanced cell killing from day 6 onward (Fig. 2A). In parallel, cells and supernatants were harvested during this experiment to quantify virus production and release during the course of the experiment. As shown in Fig. 2B, no significant effect of 4-Gy irradiation was found on amounts of virus

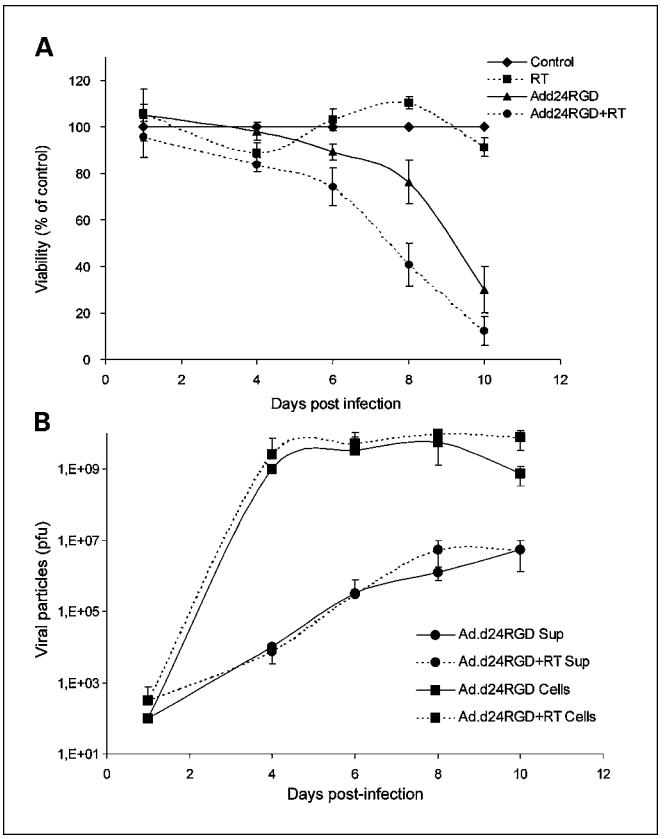

Fig. 2. Oncolysis and virus propagation on glioma cell monolayers. *A,* the effect of 4-Gy irradiation (*RT*), infection with 10^4 pfu Ad5-Δ24RGD, or the combination of Ad5-Δ24RGD + RT on U-87 cell viability was monitored in time using WST-1 assay. Points, percentage of untreated controls; bars, SD. *B,* in parallel, supernatants and cell lysates were harvested from the Ad5-Δ24RGD – and Ad5-Δ24RGD + RT–treated cells at various time points. Total amount of virus present in cells and supernatants (*Sup*) was determined by end-point titration. Points, mean total number of viral particles per lysate; bars, SD.

produced or released by the infected cells at all time points tested. Therefore, the enhanced cell killing effect of combination therapy could not be attributed to enhanced virus production or release by irradiated cells.

Effects of irradiation on infection efficiency. To determine whether the infection efficiency of RGD- targeted viruses was improved in irradiated cells, experiments were done using the luciferase encoding replication-deficient vector Ad.LucRGD. U-87 cells were infected with a dose range of this vector 6, 12, or 24 h after receiving 4-Gy irradiation. When cells were infected 6 h postirradiation, no significant effects on transgene expression were detected. Infection at 12 h postirradiation resulted in a small but significant increase in luciferase levels at a multiplicity of infection of 4, and when the infection was done 24 h postirradiation, significantly enhanced transgene expression was found at all three virus dosages tested (Fig. 3). Parallel experiments using the untargeted Ad.Luc vector showed significantly enhanced luciferase expression in irradiated cells at the 24-h time point only (not shown).

Next, we assessed whether this increase in transgene expression resulted from enhanced infection due to up-regulation of the main binding sites for these adenoviruses on the cell membrane. Flow cytometric analysis for CAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrin expression was done on U-87 cells 6, 12, and

24 h after receiving 4 Gy. At all three time points, a similar expression pattern of CAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins was found, which was not altered in response to irradiation (not shown). This indicates that enhanced binding of the virus to these cell membrane molecules is not a likely explanation for the increased luciferase expression after Ad.LucRGD infection of irradiated cells. Thus, irradiation probably does not facilitate cell entry of oncolytic adenovirus Ad5-Δ24RGD through these adenovirus receptors.

Effects of irradiation on oncolysis and viral penetration in solid tumor structures. The architectural characteristics of multicellular tumor spheroids make these solid tumor structures an excellent *in vitro* model for studying effects of various treatments including radiotherapy and viral oncolysis (20, 21). To study interactions between Ad5-Δ24RGD, irradiation, and glioma cells in these three-dimensional structures, U-87 multicellular spheroids were infected with 10^5 or 10^6 pfu Ad5-Δ24RGD, 24 h after receiving 5-Gy irradiation. As expected, viability of spheroids decreased in time, with combination treatment being more effective than single-agent treatment. On day 8 postinfection, irradiated spheroids of the 10^5 and 10^6 pfu Ad5-Δ24RGD doses were respectively 28% and 19% less viable than nonirradiated counterparts (Fig. 4A).

To assess the kinetics of viral penetration into these tumor structures, spheroids from the 10^6 pfu Ad5-Δ24RGD groups (with and without irradiation) were frozen on days 1, 4, 6, and 8 postinfection. Immunohistochemical staining for adenovirus hexon proteins shows a gradual outside-inside penetration of the virus in time. Irradiated spheroids remained slightly smaller in size than nonirradiated counterparts and viral penetration reached the core of these spheroids, which was not the case in the nonirradiated spheroids (Fig. 4B).

Combination therapy of Ad5-Δ24RGD and irradiation in an intracranial glioma model. Based on these *in vitro* results, we proceeded to study the effect of Ad5-Δ24RGD in combination with irradiation in an intracranial model for malignant glioma. Mice bearing preestablished U-87 tumors received either the maximum tolerated dose for TBI of 5 Gy or irradiation of the head only consisting of two doses of 6 Gy WBI. This dose for WBI was selected based on dose-finding pilot experiments showing a modest antitumor effect. A dose of 2×10^7 pfu Ad5-Δ24RGD was selected based on its therapeutic activity in previous pilot studies and reports by others (11), thereby allowing possible synergistic effects with irradiation to be detectable.

As shown in Fig. 5 and Table 1, treatment with Ad5-Δ24RGD alone led to long-term survival of 17% of animals and median survival was prolonged by 5 days compared with PBS controls ($P < 0.001$). For this aggressive tumor, this corresponds to an increased survival time of 19%. Treatment with 5 Gy TBI was ineffective, increasing median survival by only 3 days and curing none of the animals ($P = 0.06$). The higher dose of 2×6 Gy WBI, however, did prolong median survival by 10 days (37%) and led to long-term survival of 25% of animals ($P < 0.001$).

Surprisingly, combination treatment of Ad5-Δ24RGD with TBI was less effective than Ad5-Δ24RGD alone in terms of long-term survivors (0% compared with 17%), and no significant increase in median survival time was noted (8 versus 5 days; $P = 0.9$). The combination of Ad5-Δ24RGD with WBI did slightly improve long-term survival compared with virus alone (22% compared with 17%) but not compared with WBI alone (22% compared with 25%). Interestingly, this combination prolonged median survival time compared with WBI alone by >100% (21 versus 10 days); however, this effect did not reach statistical significance ($P = 0.45$).

Histologic evaluation of intracranial tumors. Microscopic analysis of brains harvested at time of sacrifice revealed that all symptomatic mice bore massive tumors showing noninfiltrative, spherical growth. Figure 6 depicts representative

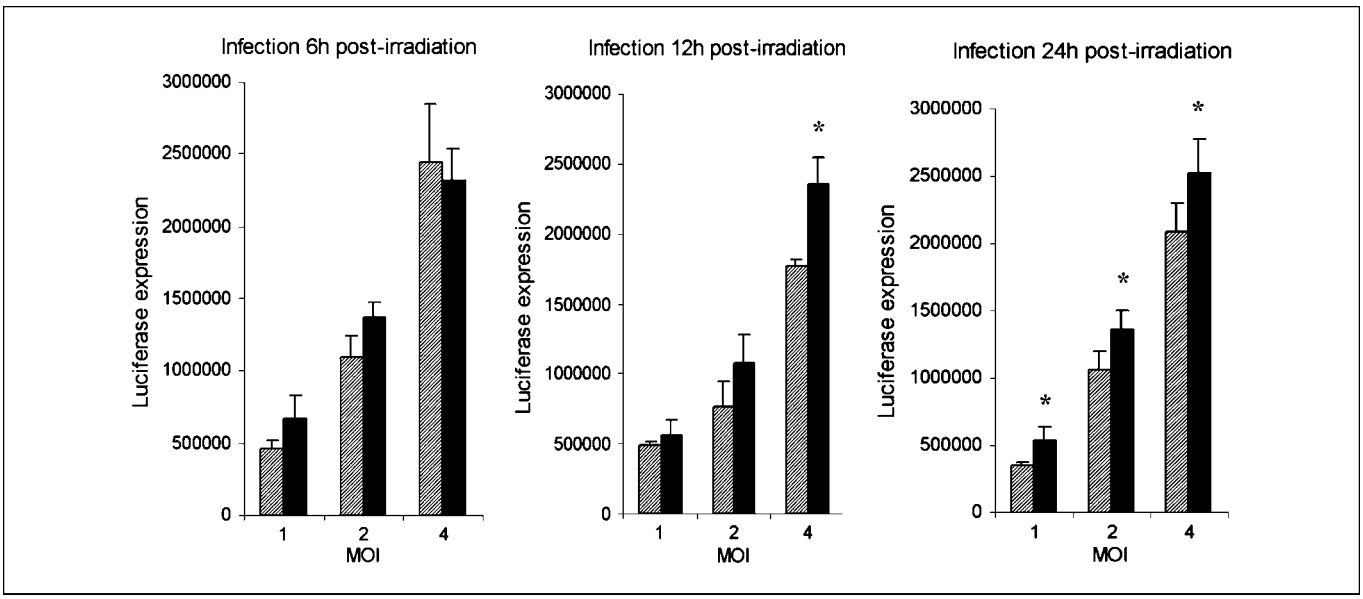

Fig. 3. Effects of irradiation on transgene expression. Control (*hatched columns*) and irradiated (*solid columns*) U-87 monolayers were infected with Ad5.LucRGD at multiplicity of infection (*MOI*) of 1, 2, or 4 at indicated time points postirradiation. Luciferase expression in cell lysates was assessed 48 h postinfection. Columns, luciferase expression expressed in relative light units/50 μL lysate; bars, SD. *, $P < 0.01$, compared with nonirradiated cultures.

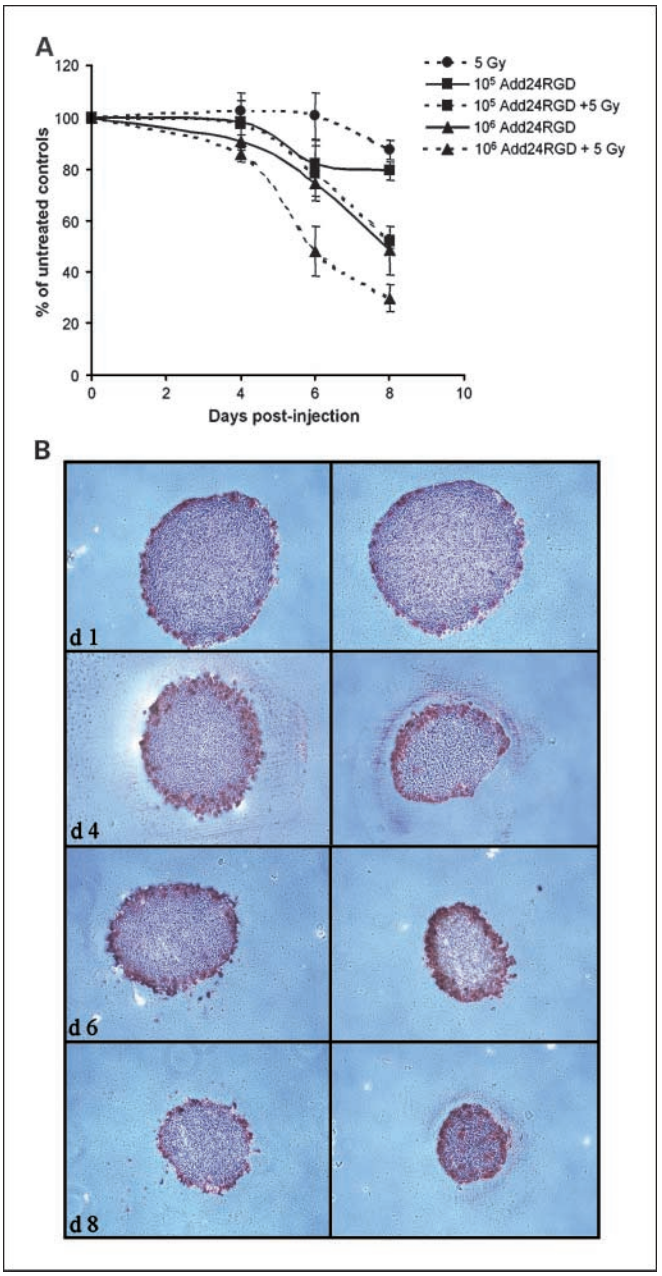

Fig. 4. Oncolysis and virus penetration in multicellular spheroids. *A,* the effect of 5 Gy irradiation, infection with 10^5 or 10^6 pfu Ad5-Δ24RGD, or the combination of Ad5-Δ24RGD with 5 Gy irradiation on the viability of U-87 spheroids was monitored in time using WST-1 assay. Points, percentage of untreated controls; bars, SD. *B,* spheroids from the 10^6 pfu group (*left*) and the 10^6 pfu + 5 Gy group (*right*) were harvested at various time points and cryosections were stained for adenovirus hexon expression (original magnification, ×2).

H&E-stained sections of the different treatment groups. PBS-treated tumors show a dense cellular mass and the absence of necrotic areas (Fig. 6A and B). Tumors of mice that received either TBI or WBI revealed similar histology with areas of lower cell density and secluded foci with infiltrated granulocytes (Fig. 6C and D). Tumors treated with Ad5-Δ24RGD were found to contain multiple regions of dense infiltrates with granulocytes and the presence of apoptotic cells (Fig. 6E and F). Similarly, in both groups of combination-treated tumors, foci of granulocytes and apoptotic cells were observed (Fig. 6G and H). Long-term survivors were sacrificed at 120 days and microscopic analysis of these brains showed complete tumor regression. At the injection site, a glial scar with evidence of some inflammatory cell infiltrates often surrounding a cavity was observed (Fig. 6I and J). No remaining tumor was found in the brains of any of the long-term survivors. In the brains of these responders, no evident histologic differences between virus-treated or virus + irradiation–treated tumors were noted.

Immunohistochemical staining for adenovirus hexon protein, indicative for areas of active viral replication, showed multiple areas of ongoing viral replication in all tumors of animals treated with Ad5-Δ24RGD (Fig. 7). No hexon-positive areas were seen in the brains of the long-term survivors of virus- or WBI + virus–treated animals (Fig. 7D). Importantly, WBI + Ad5-Δ24RGD–treated tumors that did not regress (Fig. 7C) displayed both quantitatively and qualitatively smaller areas of adenovirus replication than tumors from the Ad5-Δ24RGD–treated and TBI + Ad5-Δ24RGD–treated groups (Fig. 7A and B). This was verified by quantitative analysis of the hexon-positive hotspots per tumor cryosection (Fig. 7E) and revealed a decrease from a mean total of 170 ±31 for virus alone to 52 ±14 hotspots per cryosection after addition of WBI ($P < 0.05$). It should be noted that mean time to sacrifice of analyzed brains was 32 days for Ad5-Δ24RGD–treated mice and 45 days for WBI + Ad5-Δ24RGD–treated mice. However, no correlation between time to sacrifice and total number of hexon hotspots was noted within each group. Interestingly, TBI before Ad5-Δ24RGD treatment did not affect total number of hexon-positive hotspots.

Discussion

The replication-competent oncolytic adenovirus Ad5-Δ24RGD has shown potent antitumor activity in various preclinical tumor models (10, 11, 16, 22–24) and is currently being developed toward phase I clinical evaluation for ovarian cancer and malignant glioma.[7] Despite these developments, published preclinical studies evaluating combination treatments of Ad5-Δ24RGD with conventional therapies have thus far been limited to the combination with irradiation. We previously reported synergistic antitumor activity of this combination in a s.c. model for glioma (10). In the current study, we attempted to elucidate the underlying mechanism to this synergy and to validate this effect in a more clinically relevant intracranial animal model.

In vitro combination studies evaluating different dosage and sequence regimens of both therapies showed that delivery of irradiation 24 h before Ad5-Δ24RGD infection led to a greater oncolytic effect than simultaneous delivery of the therapies or irradiating 24 h postinfection. Although this result correlated with a small increase in luciferase expression after infection of irradiated cells with Ad.LucRGD, analysis of CAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrin expression revealed no effect of irradiation on the expression of these primary cell membrane molecules involved in binding of the virus. This would suggest that improved infectivity is not a likely explanation for the found increase in transgene expression in irradiated cells; however, it cannot be excluded that alternative receptors and/or intracellular factors

[7] http://dtp.nci.nih.gov/index.html

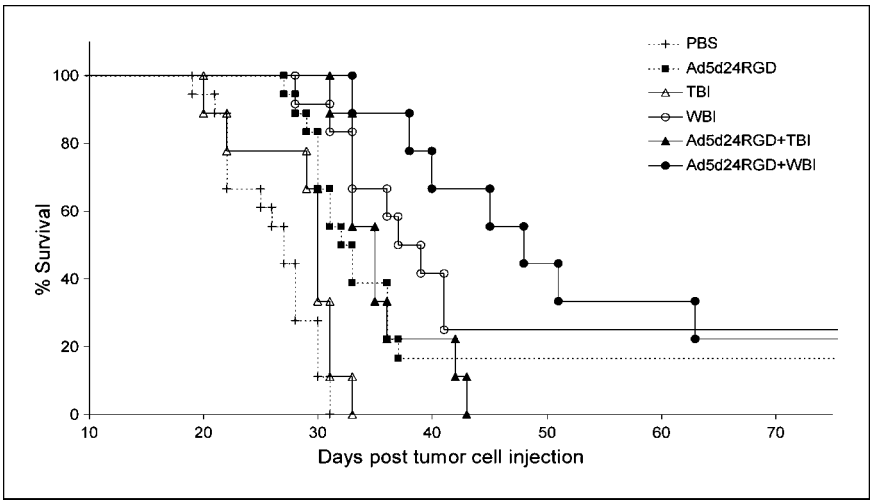

Fig. 5. Therapeutic effects of Ad5-Δ24RGD and radiotherapy on glioma-bearing mice. Combined results of two experiments in which mice bearing intracranial U-87 xenografts received intratumoral injection of PBS or 2×10^7 pfu Ad5-Δ24RGD, or the combination of these with either 5 Gy TBI or 2× 6 Gy WBI. Survival is presented as percentage surviving of initial number of animals per group.

known to play a role in adenovirus binding and entry may be influenced by irradiation. Alternatively, increased transgene expression may be attributed to other factors such as enhanced activity of the cytomegalovirus promoter driving the luciferase expression (25). A time course experiment done in parallel showed that the improved cell killing by Ad5-Δ24RGD in irradiated cells was not due to enhanced virus production or release. Studies by others on the effects of irradiation on adenovirus replication *in vitro* have yielded conflicting results. Increases in viral burst size of the prostate cell–specific oncolytic adenoviruses CV706 and CG7870 in combination with irradiation have been reported (12, 15), as well as decreases in viral DNA synthesis in irradiated cells infected with ADP-overexpressing oncolytic adenoviruses (26). For the E1-B–deleted oncolytic adenovirus dl1520 (ONYX-015), no significant effect of irradiation on virus replication was found (27), consistent with our results. It cannot be excluded, however, that irradiation induces cellular conditions that permit enhanced cell killing by the virus in the absence of enhanced progeny production or release. An alternative explanation for the combination effect of irradiation and Ad5-Δ24RGD infection *in vitro* is the inhibitory effect of irradiation on tumor cell growth. By inducing a temporary growth delay, the virus-to-tumor cell ratio is shifted to the advantage of the virus. This hypothesis is supported by the results of the spheroid experiments, which show the growth inhibitory effect of irradiation on these three-dimensional tumor structures (Fig. 4B), keeping them smaller than their nonirradiated counterparts and, by this means, allowing the virus to penetrate to the core and destroy the spheroid more rapidly. This more general mechanism of enhanced antitumor efficacy of oncolytic adenoviruses in combination with radiotherapy is presumed to be operative in s.c. tumor models as well. Indeed, we and others have shown the potentiating effect of irradiation on the antitumor activity of various oncolytic adenoviruses in a variety of s.c. xenografts (10, 12, 13, 15, 26, 27). Again, both increases (12) and decreases (13) in intratumoral adenovirus replication in irradiated tumors were reported.

Thus far, to our best knowledge, oncolytic adenoviruses have not been combined with irradiation in intracranial models for malignant glioma. Combination therapy using an oncolytic herpes virus and irradiation did show synergistic activity in the U-87 orthotopic tumor model (28). The results of our *in vivo* combination experiments were therefore unexpected. Although 5 Gy TBI has shown therapeutic efficacy in various s.c. glioma models, this dose was found to be ineffective in the U-87 intracranial setting. This may be partly due to the inherent radioresistance of the U-87 tumor cell line, which seems to be greater *in vivo* than predicted by our *in vitro* studies. As a result, no combination effect with Ad5-Δ24RGD and irradiation was found. Increasing the irradiation dose to 2 × 6 Gy WBI did produce a therapeutic effect by itself; however, a significant potentiating effect of the oncolytic activity of Ad5-Δ24RGD could not be shown. This result does not support the hypothesis that inhibition of tumor growth by irradiation leads to synergy between these two treatment modalities. Apparently, other mechanisms are operative in this glioma model. Discrepancies between results from *in vitro* and s.c. tumor models on one hand, and intracerebral tumor models on the other hand, may lie in the

Table 1. Median survival times for *in vivo* experiments

Treatment	*n*	Median survival (d)	Increase in median survival (d)	Increase in median survival (%)	Percentage long-term survivors
PBS	18	27 ± 1.1	0	0	0
Ad5-Δ24RGD	18	32 ± 1.4	5	18.5	16.7
TBI	9	30 ± 0.5	3	11.1	0
WBI	12	37 ± 2.6	10	37.0	25
Ad5-Δ24RGD + TBI	9	35 ± 1.4	8	29.6	0
Ad5-Δ24RGD + WBI	9	48 ± 4.5	21	77.8	22

location and surroundings of the tumor cells. Indeed, Camphausen et al. (29) showed that the experimental growth conditions of glioma cells, including U-87, quantitatively and qualitatively influenced radiation-induced changes in gene expression in these cells. Whereas relatively few genes were affected by irradiation when grown in monolayer culture, an increase was seen in s.c. growth of glioma cells and an even further increase was found in orthotopic grown cells. Apparently, the normal brain environment has a profound effect on radiation-induced changes in gene expression, and it is not implausible that these changes in gene expression may counteract the oncolytic activity of the virus (e.g., by hampering the adenovirus replicative cycle). This is supported by the results of the hexon staining of virus-treated tumors. Delivery of a therapeutic dose of 2 × 6 Gy WBI quantitatively and qualitatively decreased the number of hexon-positive hotspots in these tumors, indicating that viral replication was diminished in these tumors. This effect was not seen in animals treated with the lower dose of 5 Gy TBI. Possibly, this irradiation dose on the tumor was too low to induce the antagonistic effect, or the effect was counteracted by the temporary hematologic consequences of TBI, which results in a decline in circulating immune cells. Indeed, immunosuppression before intratumoral adenovirus injection in orthotopic gliomas has been shown to improve adenoviral replication (19). Finally, the inhibitory effect of irradiation on (orthotopic) intratumoral virus replication seems to be specific for adenovirus, as Bradley et al. showed a 2- to 5-fold enhancement of herpes virus replication using the same animal model (28).

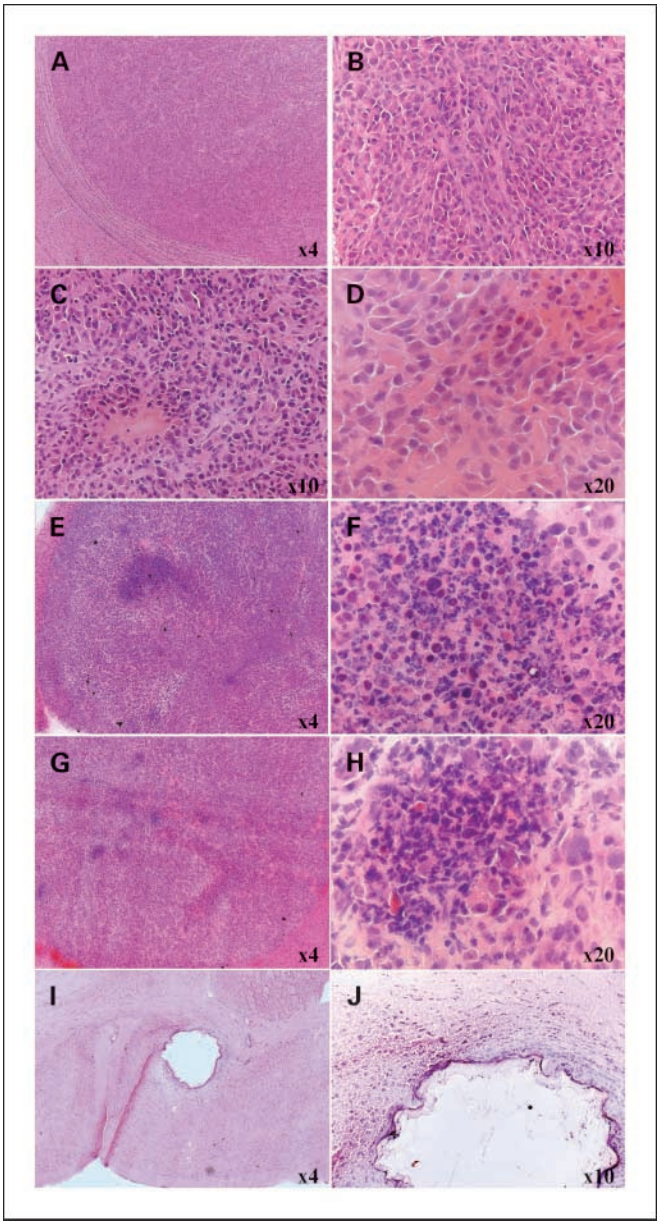

Fig. 6. Histopathologic analysis of treated tumors. H&E staining of representative cryosections of brains harvested at time of sacrifice showing growth of large, noninvasive tumors (*A* and *B*). Tumors from the TBI and WBI groups (*C* and *D*) showing secluded foci with infiltrated granulocytes and tumors treated with Ad5-Δ24RGD containing regions of dense infiltrates with granulocytes and apoptotic cells (*E* and *F*). Combination-treated tumors receiving either TBI + virus or WBI + virus also showing foci of granulocytes and apoptotic cells (*G* and *H*), and long-term survivors showing complete tumor regression with a glial scar and inflammatory cell infiltrates surrounding a cavity (*I* and *J*). Original magnifications are indicated in the figures.

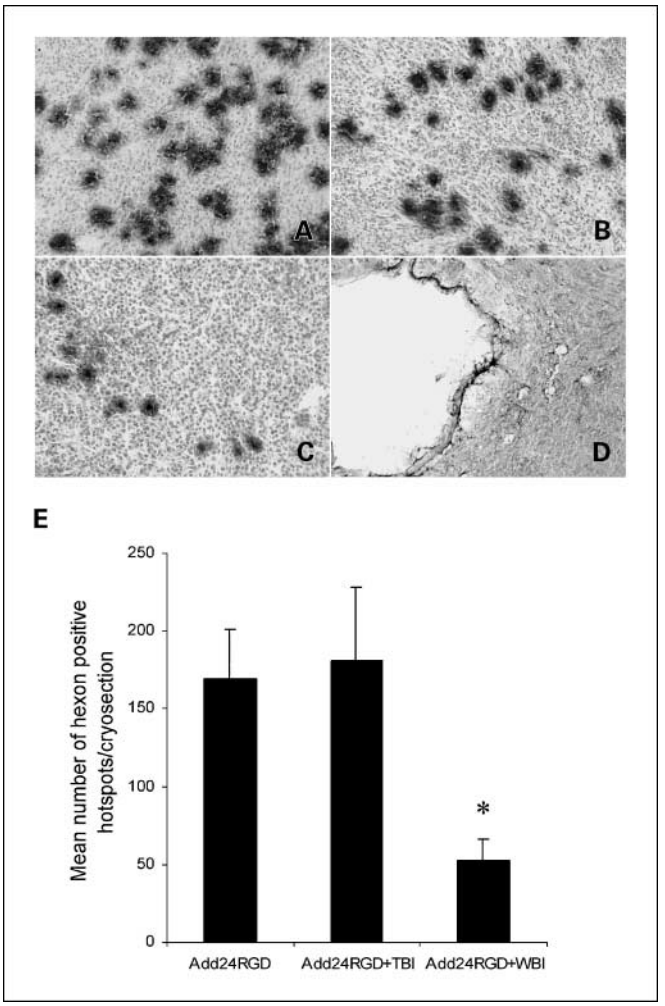

Fig. 7. Intratumoral virus replication. Representative cryosections showing immunohistochemical staining for adenoviral hexon expression in tumors treated with Ad5-Δ24RGD (*A*), Ad5-Δ24RGD + TBI (*B*), or Ad5-Δ24RGD + WBI (*C*) and in brains of long-term survivors (*D*). Original magnification, ×10. E, quantitative analysis of hexon expression in cryosections of Ad5-Δ24RGD – and combination-treated tumors. Columns, mean number of hexon-positive hotspots per cryosection; bars, SD. *, $P < 0.01$, compared with Ad5-Δ24RGD group.

Combination therapy using cytotoxic agents with differing modes of action is gaining increasing attention in oncology treatment. A wider range of antitumor activity and the opportunity to augment cell kill within the range of toxicity for each agent offer significant advantages compared with single-agent treatment. Our data show that results obtained in preclinical models combining oncolytic adenovirus with radiotherapy may differ depending on the experimental growth conditions of the tumor cells. Results obtained *in vitro* or in s.c. tumor models for malignant glioma may be less valuable for predicting the therapeutic efficacy of combination therapies for this tumor. As the natural environment of a glioma (i.e., the brain) may be of great influence on the response of the tumor to certain agents, orthotopic animal models may therefore be essential for preclinical evaluation of novel therapies. This may be even more important when combining different treatment modalities of which the antitumor effects may be additional but the influence of the environment may act antagonistically.

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