Combined effects of radiotherapy and angiostatin gene therapy in glioma tumor model

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The objective of the present study was to evaluate the antitumor effect of a defective adenovirus expressing a secretable angiostatin-like molecule (AdK3) in combination with radiotherapy in rat C6 gliomas s.c. preestablished into athymic mice. *In vitro*, the combination regimen was significantly ($P < 0.001$) more cytotoxic for human microcapillary endothelial cells than either treatment alone, whereas survival of C6 glioma cells was not affected in the conditions used. Radiotherapy and AdK3 gene delivery was then studied on well established C6 xenografts (165 ± 70 mm^3). In these tumors, AdK3 intratumoral injections had only a marginal effect. Interestingly, when experimental radiotherapy was added, significantly higher ($P < 0.005$), and possibly synergistic, antitumoral effects were observed that tightly correlated a marked decrease of intratumoral vascularization. The combination of radiotherapy and AdK3 intratumoral injections also revealed a significant ($P < 0.05$) inhibition of tumor growth as compared with either treatment alone for larger tumors (467 ± 120 mm^3). Altogether, these data emphasize the potential of combining a destructive strategy directed against the tumor cells with an anti-angiogenic approach to fight cancer.

angiogenesis | recombinant adenovirus | cancer

Malignant gliomas, which account for one-third of primary brain tumors, are typically characterized by rapid cell proliferation and a marked propensity to invade and damage surrounding tissues, and are among the most vascularized tumors. Their prognosis in patients remains dismal despite the use of combinations of surgery, radiotherapy, and chemotherapy. More than 90% of patients experience local recurrence, and the 5-year survival rate is only 9% (1), emphasizing unmet therapeutic needs. In this regard, inhibition of tumors angiogenesis has recently emerged as a promising therapeutic strategy by shifting the balance from pro-angiogenic toward an angiostatic state (2).

Intratumoral angiogenesis has proved to be useful as a prognostic marker for brain tumors (3). In the histopathological classification of these tumors, both the density and morphology of tumor microvasculature are important grading criteria. Whereas the microvasculature of low-grade tumors resembles that of normal brain, the more anaplastic tumors like glioblastoma show an increased vessel density. Indeed, malignant glioma progression is often correlated with an up-regulation of angiogenesis because of an increase in vascular endothelial growth factor (VEGF) and its tyrosine kinase receptor flt-1 by glioma cells, and to an up-regulation of flk-1/KDR in endothelial cells (3).

The dependence of high-grade gliomas on VEGF-mediated angiogenesis has been exploited for the development of anti-angiogenic strategies using anti-VEGF antibodies or a recombinant retrovirus encoding a truncated VEGF receptor-2 to antagonize its biological function *in vivo* (4–6). Neutralization of only one angiogenic factor such as VEGF is nevertheless not sufficient to completely suppress angiogenesis in high-grade tumors as additional pro-angiogenic cytokines (e.g., basic fibroblast growth factor, hepatocyte growth factor) are often up-regulated by human glioma tumors (3).

The recent discovery of specific inhibitors of endothelial cell proliferation such as angiostatin, an internal proteolytic cleavage product of plasminogen, offers a promising strategy for cancer management and/or treatment. For example, repeated bolus injections of the angiostatin peptide has been shown to suppress subsequent tumor growth in different murine models, including experimental gliomas (7–9). However, such an approach may be restricted to particular settings as there was no anti-tumoral effects when recombinant angiostatin was delivered to mice bearing end-stage experimental carcinomas (10).

Angiostatin gene transfer has also been shown to exert potent anti-tumoral effects in mice after regional delivery with a retroviral (11) or an adenoviral vector (12). In particular, we previously reported that a single, local injection of a defective adenovirus encoding a secretable angiostatin-like molecule (AdK3) into small rat C6 xenografts (20 mm^3) could suppress subsequent tumor growth and neovascularization (12). On the other hand, the effects were only marginal when AdK3 was intratumorally injected into larger C6 tumors (see this report). We undertook the present study to evaluate whether association of adenovirus-mediated angiostatin gene delivery with local x-ray irradiation could enhance the overall efficacy in an experimental setting quite refractory to either treatment alone.

Materials and Methods

Recombinant Adenovirus. AdK3 is a defective recombinant E1E3-deleted adenovirus directing the expression and secretion of an angiostatin-like molecule (i.e., the N-terminal fragment of human plasminogen up to residue 333) from the human cytomegalovirus (CMV) immediate-early promoter (12). AdK3 was constructed and amplified by using 293 cells, and CsCl-purified material was recovered and titrated as plaque-forming units (pfu) as described (13). AdCMV-GFP (V. Randrianarison, Unité Mixte de Recherche 1582, Villejuif, France) is an E1E3-deleted adenoviruses expressing the green fluorescent protein (GFP) from the CMV immediate-early promoter. AdCO1 is an "empty" control E1E3-deleted that does not display any expression cassette in place of the E1 genes (12).

Abbreviations: VEGF, vascular endothelial growth factor; CMV, cytomegalovirus; pfu, plaque-forming unit; GFP, green fluorescent protein; HMEC, human microcapillary endothelial cell; moi, multiplicity of infection.

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Culture and Adenovirus Infection. Rat C6 glioma cells (American Tissue Culture Collection CCL-107) were grown in DMEM (GIBCO/BRL) supplemented with 10% FCS, 1 mM L-glutamine, and 1× non-essential amino acids (GIBCO/BRL). 293 cells (14) were cultured in MEM (GIBCO/BRL) supplemented with 10% FCS, 1 mM L-glutamine, and 1× non-essential amino acids. Human microcapillary endothelial cells (HMEC-1) were obtained from E. W. Ades (Emory University Hospital, Atlanta). They were grown in MCDB 131 (GIBCO/BRL) supplemented with 20% FCS, 1 mM glutamine, 1 mg/ml hydrocortisone, and 10 ng/ml epithelial growth factor as described (15). For both cell types, adenovirus infection was carried out on exponential growing cells. In all experiments, the cells were counted the day of infection and were infected at a given multiplicity of infection (moi) in culture medium supplemented with 2% FCS.

Western Blot and FACS Analysis. Subconfluent cultures in 6-well tissue culture dishes were infected with AdK3 and AdCO1 at a moi of 300 pfu/cell, and the culture supernatants were collected 24, 48, and 72 h postinfection. Twelve microliters of supernatant were run on a Nu/PAGE 10% Bis·Tris precasted gel (NOVEX, San Diego) before transfer onto a nitrocellulose membrane (Hybond ECL, Amersham). The membrane was then incubated for 2 h in TBS/5% skimmed milk/0.05% Tween 20, followed by 1 h of incubation with anti-human plasminogen mAb A1D12 (16) and 1 h of incubation with a peroxidase-conjugated goat anti-mouse serum (Jackson ImmunoResearch). Angiostatin detection was then carried out by chemiluminescence using the ECL Plus kit (Amersham). Sensitivity of the cells to adenovirus infection was assessed by flow cytometry analysis (FACScan, Becton Dickinson) 48 h postinfection with the AdCMV-GFP reporter virus at a moi of 10, 50, 150, and 300 pfu/cell.

***In Vitro* Effect of Virus Infection and/or Irradiation.** HMEC-1 and C6 cells were respectively seeded at a density of 5×10^4 and 10^5 cells in 24-well tissue culture dishes on the day before infection. Virus infection was carried out in triplicate in 250 μl of medium for 2 h at a moi ranging from 10 to 700 pfu/cell, before addition of 500 μl of culture medium. The number of cells that survived infection was then determined after 4 days (see below). Internal controls (i.e., cells that were mock-infected) were included in each culture plate.

To evaluate the radiosensitivity of C6 and HMEC-1 cells, photon x-irradiation was carried out in quintuplate with a Phillips 200-kV apparatus to deliver doses of 1, 2, 5, and 7.5 Gy using a ^{137}Cs source at a dose-rate of 1.4 Gy/min, and cell survival was determined after 9 days. Non-irradiated cells were included in each culture plate as controls.

Cell survival was assessed by quantifying at the indicated time the number of cells that stained positive after incubation with MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide]. In this assay, the culture medium was removed, and the cells were incubated for 2 h at 37°C with 250 μl of PBS and 25 μl of a 5 mg/ml MTT solution. The cells were then lysed overnight with 250 μl of lysis buffer (10 g of SDS in 50 ml DMF/H_2O 1:1, pH 4.7) at 37°C. A 100-μl aliquot of the soluble fraction was then transferred into 96-well microplates, and the 570-nm optical density was measured. The number of cells that stained positive for MTT was then determined by comparing the OD of the sample with those from standard cell suspensions. The ED_{50} (efficacy dose) value was defined as the moi triggering a 50% inhibitory effect.

For the combination treatment, HMEC-1 and C6 cells were first irradiated with a dose of 5 Gy and immediately were infected with AdK3 or AdCO1 at a moi of 300 pfu/cell. Non-infected and non-irradiated cells were used as controls. In a separate experiment, HMEC-1 cells were irradiated with a dose of 5 Gy. They were then infected with AdCO1 or AdK3 at a moi of 100, 200, 300, 400, 500, 600, or 700 pfu/cell. In both experiments, cell survival was assessed after 4 days. A Student's *t* test was used for statistical analysis.

Experimental *in Vivo* Model. Cultured C6 glioma cells were harvested, washed, and resuspended in PBS at 5×10^6 cells/ml. Two hundred microliters of the cell suspension was then injected intradermally into the flank of 6- to 7-week-old male nude mice. In a first experiment, randomization of the animals was carried out 10 days after grafting: i.e., at a time when tumor volume had reach a mean value of 165 ± 70 mm^3. Five experimental groups were attributed, each with five animals: one group was subjected to irradiation alone, two groups were subjected to AdCO1 or AdK3 injection alone, one group was subjected to irradiation and AdCO1 injection, and the last group was subjected to irradiation and AdK3 injection. Treatment was initiated at this time.

Irradiation consisted in a single 7.5-Gy dose delivered with a Philips RT250 radiation source operating at 200 kV with a 0.2 mm Cu-filter. Irradiation was locally confined to the tumors by shielding the rest of the body with lead. Irradiation was performed on days 10, 12, and 14 postgrafting whereas virus injection was performed intratumorally on days 11, 13, and 15 postgrafting (5×10^9 pfu per injection in a volume of 150 μl). Tumor growth was then monitored until day 26 postgrafting, at which time intratumoral vascularization was assessed (see below).

In a separate experiment, the three treatment cycles were initiated 14 days after grafting, at a time when tumor had reached a mean volume of 467 ± 120 mm^3. Five experimental groups were defined as above, and irradiation was performed on days 14, 16, and 18 postgrafting, whereas virus injection was performed on days 15, 17, and 19 postgrafting. Tumor growth was monitored until day 30 postgrafting, at which time mice from the control groups started to die because of tumor burden. Mice survival was evaluated until day 54 postgrafting, at which time all animals were killed. A Student's *t* test was performed for statistical analysis.

Assessment of Tumor Vascularization. Immunohistochemistry was used to assess the importance of the intratumoral vascularization within the different experimental groups. Tumor tissues were fixed (in 5% acetic acid, 75% absolute ethyl alcohol, 2% formalin, and 18% water), were transferred into 100% ethanol, and were embedded in paraffin, and 5-μm sections were prepared. After xylene treatment and rehydration, endogenous peroxydase activity was quenched by 3% H_2O_2 for 5 min. The sections were washed in distilled water, were immersed in citrate buffer (Dako), and were placed in a microwave oven for 20 min at 750 W, then for 15 min at 250 W. Tumor sections were incubated with blocking serum (BioGenex Laboratories, San Ramon, CA) (1:10) for 10 min and were incubated for 1 h with a murine monoclonal anti-human smooth muscle actin antibody (Dako, 1:100). After treatment with Optimax wash buffer (BioGenex), the sections were incubated with Dako's mouse EnVision visualization system for 30 min, were treated with AEC chromogenic substrate for 5 min, and were counterstained with hematoxylin. Tumor vasculature was quantified by expressing the surface ratio within 18–42 fields that stained positive by using a CCD camera (resolution 768 × 576 pixels) at a 200-fold magnification. Image processing algorithms using the Matrox INSPECTOR 2.2 software (Matrox, Dorval, Canada) were specifically developed for quantification, and reproducibility was ensured by keeping the same settings all along the process.

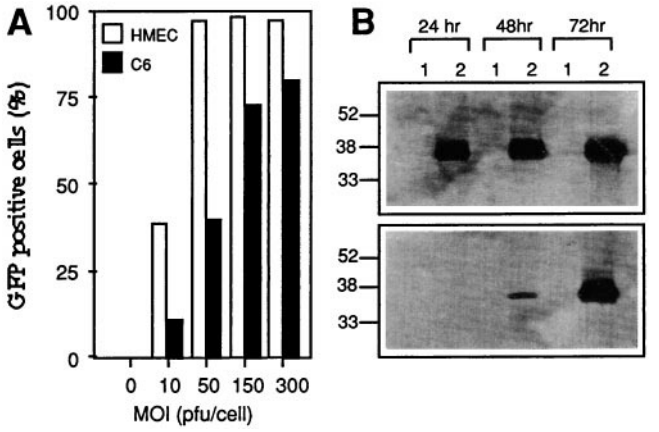

Fig. 1. (*A*) Efficacy of adenovirus-mediated gene delivery into HMEC-1 and C6 cells. Both cell lines were infected with increasing mois of Ad-CMVGFP reporter virus, and cells expressing GFP were assessed by flow cytometry 48 h postinfection. (*B*) Analysis of angiostatin secretion from virally infected cells. HMEC-1 (*Upper*) and C6 cells (*Lower*) were infected with AdCO1 (lane 1) or AdK3 (lane 2) at a moi of 300 pfu/cell, and culture mediums were collected 24, 48, and 72 h postinfection and submitted to Western blot analysis with anti-human plasminogen mAb A1D12.

Results

Consequences of AdK3 and Irradiation *in Vitro*. AdCO1 is an E1E3-deleted empty adenovirus that was used as a control virus throughout this study. AdK3 is "isogenic" to AdCO1 except that it contains a CMV/angiostatin expression cassette in place of the E1 genes (12). AdCMV-GFP is isogenic to AdK3 except that it directs expression of the green fluorescent protein instead of angiostatin.

Efficacy of adenovirus-mediated gene delivery was first evaluated for C6 glioma cells and compared with cells of endothelial origin (HMEC-1). For this purpose, we used FACS analysis to quantify after 48 h the proportion of cells that had been infected with the AdCMV-GFP reporter virus. As shown in Fig. 1*A*, a dose-dependent increase of GFP-positive cells was observed for both cell lines, with an ID40 (the moi required to transduce 40% of the cell population) of 10 and 50 pfu/cell for HMEC-1 and C6 cells, respectively. That HMEC-1 cells are slightly more susceptible to adenovirus infection than C6 cells is supported by specific anti-angiostatin Western blot analysis of the cell supernatants after infection with AdK3. As shown in Fig. 1*B*, the 38-kDa angiostatin molecule was secreted more readily into the culture medium of HMEC-1 infected cells (*Upper*) as compared with C6-infected cells (*Lower*).

The ability of adenovirus-mediated delivery of angiostatin to specifically block proliferation of endothelial cells *in vitro* was then analyzed (Fig. 2*A*). To this end, the number of cells that survived infection with AdK3 or its empty control AdCO1 at a moi ranging from 10 to 700 pfu/cell was quantified after 4 days (see *Material and Methods*). In this assay, HMEC-1 cells were found sensitive, in a dose-dependent manner, to infection with AdK3, with an ED_{50} (defined as the moi leading to a 50% inhibitory effect) in the range of 400 infectious particles per cell (pfu/cell). In contrast, infection of HMEC-1 cells with the control empty virus had only marginal effect, even at the highest tested dose (Fig. 2*A Left*). The dose-dependent inhibitory effect specifically observed after HMEC-1 infection with AdK3 is thus a direct consequence of angiostatin expression, with little or no participation of adenovirus infection *per se*. In sharp contrast, C6 glioma cells were found completely refractory to AdK3 infection in this assay, whatever the moi used (Fig. 2*A Right*).

We then documented the sensitivity of HMEC-1 and C6 cells

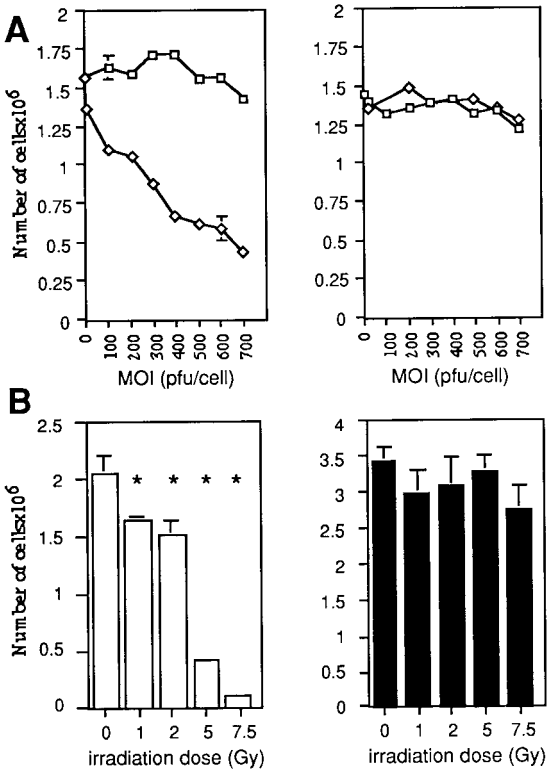

Fig. 2. (A) Inhibition of endothelial cell proliferation *in vitro*. HMEC-1 (*Left*) and C6 cells (*Right*) were infected with AdK3 (◇) or AdCO1 (□) at a moi ranging from 10 to 700 pfu/cell. The cells that survived to AdK3 or AdCO1 infection were quantified after 4 days by an MTT test (see *Material and Methods*). (*B*) Sensitivity of HMEC-1 and C6 cells to ionizing radiations *in vitro*. HMEC-1 (*Left*) and C6 cells (*Right*). Cells were irradiated at a dose ranging from 1 to 7.5 Gy, and cell survival was assessed after 9 days. Non-irradiated cells were included in each culture plate as controls.

to ionizing radiations *in vitro* (Fig. 2*B*). To this end, the cells were irradiated at a dose ranging from 1 to 7.5 Gy, and cell survival was assessed after 9 days. HMEC-1 and C6 again reacted very differently in this assay: at low dose (e.g., 2 Gy), a significant ($P < 0.01$) inhibitory effect was observed for HMEC-1 cells, and there was very little survival at higher doses (Fig. 2*B Left*). In sharp contrast, C6 glioma cells were found quite refractory to irradiation, at all irradiation doses (Fig. 2*B Right*).

We then wondered to what extent the combination of angiostatin gene delivery and irradiation could enhance cytotoxicity when applied to cells sensitive (HMEC-1), or refractory (C6), to either treatment separately. In a first experiment, HMEC-1 and C6 glioma cells were irradiated at 5 Gy before infection with AdK3 or its control (AdCO1), at a moi of 300 pfu/cell. As shown in Fig. 3*A*, the combination treatment still had no obvious effect on C6 cell (*Right*). Interestingly enough, however, in the case of HMEC-1 cells, the combination treatment led to a significantly higher inhibitory effect ($P < 0.001$) as compared with control cells that had been subjected to either irradiation or AdK3 infection (Fig. 3*A Left*). In these cells, the higher efficacy of the combination treatment was also evident when a 5-Gy irradiation dose was associated with virus infection at a moi ranging from 100 to 700 pfu/cell (Fig. 3*B*). An ED_{50} in the range of 400 pfu/cell was again observed after HMEC-1 infection with AdK3 (see above), and this value dropped to 100 pfu/cell when a 5-Gy irradiation dose was also performed.

Anti-Tumoral Effects of AdK3 and Irradiation. Because C6 gliomas cells are somehow resistant to x-ray irradiation, experimental C6

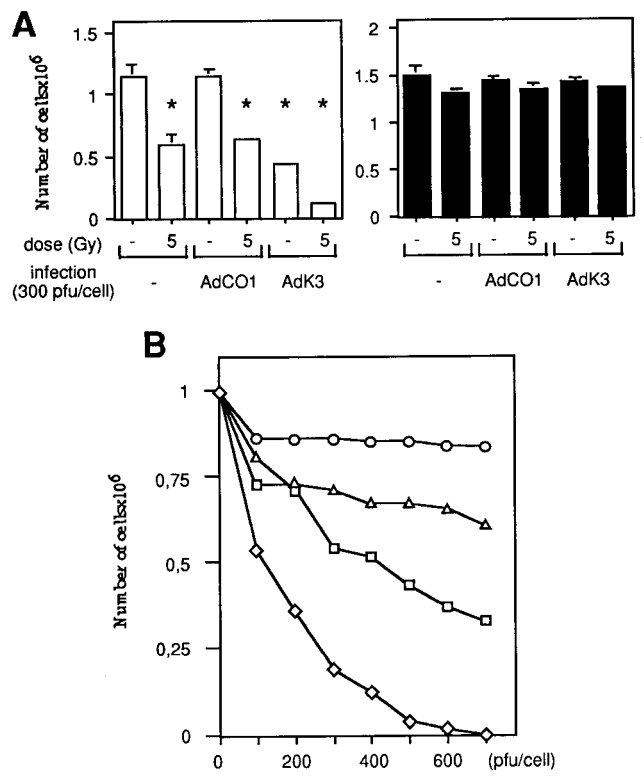

Fig. 3. *In vitro* effect of adenovirus infection and/or irradiation. (*A*) *In vitro* surviving of HMEC-1 (*Left*) and C6 cells (*Right*) irradiated with a dose of 5 Gy before being infected with 300 pfu/ml of AdK3 or AdCO1. Cell survival was assessed 96 h after x-ray and/or adenovirus infection. Non-infected and non-irradiated cells were used as controls. (*B*) In a separate experiment, HMEC-1 cells were irradiated with a dose of 5 Gy and immediately were infected with AdK3 (◇) or AdCO1 (△) at a dose ranging from 100 to 700 pfu/cell. The combined treatments were compared with HMEC-1 infection with AdCO1 (○) or AdK3 (□) alone. Cell survival was assessed after 4 days.

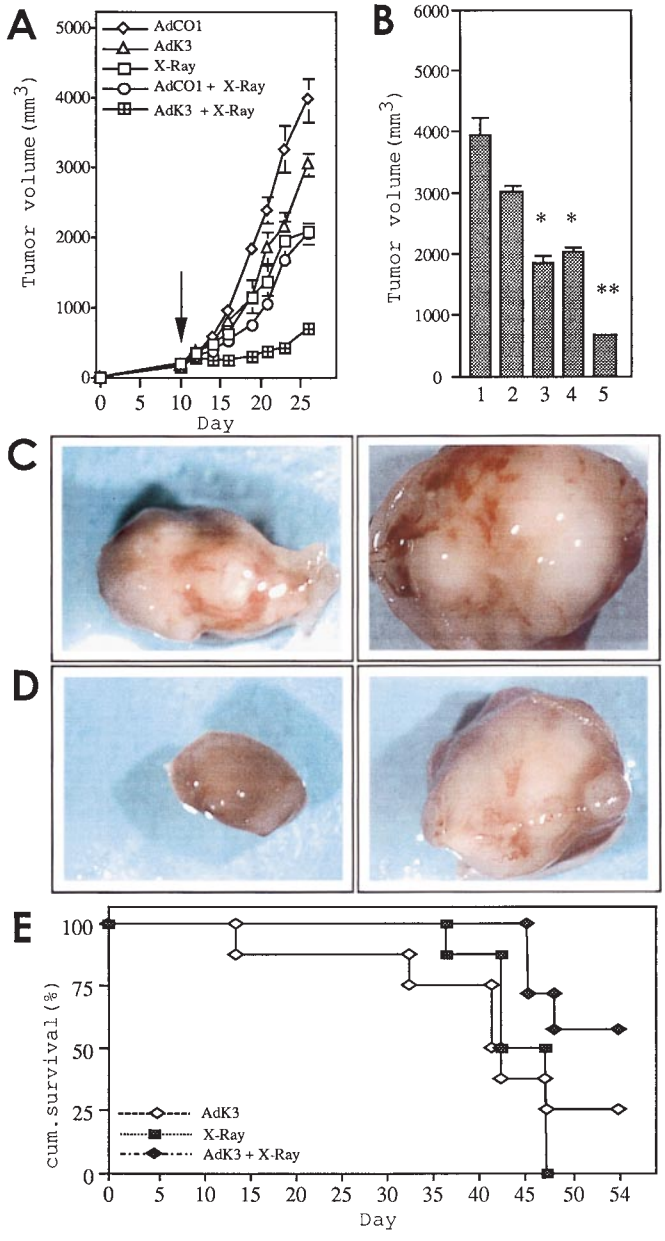

Fig. 4. Tumor growth after combined treatment with radiation and AdK3. (*A*) Athymic mice with C6 glioma xenografts (165 ± 70 mm^3) were treated with three locally fractioned doses of 7.5 Gy each and three intratumoral injections of 5 × 10^9 pfu of AdK3 or AdCO1 24 h after each irradiation. Treatment was initiated at day 10 (arrow) postgrafting (see *Materials and Methods*). Control animals were treated with three injections of AdK3 or AdCO1, or with three irradiations of 7.5 Gy each. Tumor volume was monitored until day 26 postgrafting (*A*), at which time they were extracted and analyzed (*B*). Representative tumors from the AdCO1-injected group (*C Right*), the AdCO1 + x-ray combination group (*C Left*), the AdK3-injected group (*D Right*), and the AdK3 + x-ray combination group (*D Left*) are shown at the same magnification. (*E*) Survival of AdK3-, x-ray-, or AdK3 + x-ray -treated mice 54 days after grafting. All mice were killed at day 54 for bioethical reasons.

xenografts grown onto nude mice were subjected to three consecutive cycles of irradiation at a sublethal dose (7.5 Gy). In a first experiment, mice were randomized 10 days after grafting, when the tumors had reached a mean volume of 165 ± 70 mm^3. The treatment cycles were initiated at this time and consisted of a local irradiation at days 10, 12, and 14 postgrafting, whereas virus was injected intratumorally (5 × 10^9 pfu per injection) on the day that followed irradiation (i.e., days 11, 13, and 15 postgrafting). Control animals were treated the same way except that irradiation was omitted. Tumor growth was then monitored until day 26 postgrafting. As shown in Fig. 4*A*, a very significant inhibition ($P < 0.01$) of tumor growth was observed very early on within the animal group that had been subjected to the AdK3/irradiation combination. That angiostatin gene delivery by itself had no significant effect on tumor growth ($P = 0.23$) outlines the restriction of angiostatin-based anti-angiogenic approaches to the treatment of small experimental C6 tumors (1). At completion of the experiment (i.e., 26 days postgrafting), AdK3-injected tumors that had also been irradiated were much smaller (690 ± 58 mm^3) than tumors solely injected with the AdCO1 control virus (3,952 ± 312 mm^3) or solely injected with the AdK3 virus (3029 ± 163 mm^3), or tumors subjected to the irradiation/AdCO1 group (2,035 ± 155 mm^3) (Fig. 4*B*; also see *C* and *D*).

When treatment was initiated at a later stage (i.e., 14 days after grafting when the tumor volume had reached a mean value of 467 ± 120 mm^3), the AdK3/irradiation combination was less effective: a less significant ($P < 0.05$) inhibition of tumor growth was observed 30 days after grafting (mean tumor volume 1,641 ± 124 mm^3). No or very little effect was observed within the group only subjected to irradiation (3,024 ± 395 mm^3), or AdK3 injection alone (3,051 ± 455 mm^3). Combining angiostatin gene delivery and irradiation also extended survival in this experiment: As shown in Fig. 4*E*, although 29% survival rate was

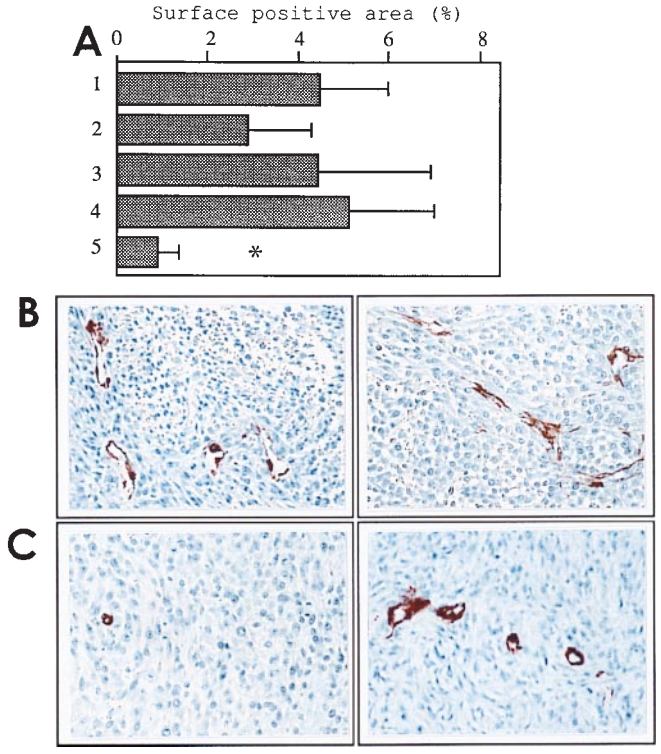

Fig. 5. Inhibition of intratumoral vascularization. (*A*) The importance of intratumoral vascularization was assessed by smooth muscle actin-immunostaining at day 26 postgrafting and quantified (see *Materials and Methods*). Lanes: 1, AdCO1-injected group; 2, AdK3-injected group; 3, irradiation alone; 4, combination of AdCO1 and irradiation; 5, combination of AdK3 and irradiation. Immunostaining of representative tumors from the AdCO1-injected group (*B Right*), the AdCO1 + x-ray combination group (*B Left*), the AdK3-injected group (*C Right*), and the AdK3 + x-ray combination group (*C Left*) are shown at the same magnification.

observed within the AdK3-treated group, and all mice were dead within the irradiated group, more than 50% of animals from the AdK3/irradiated group were alive 54 days after grafting. All mice were then killed for bioethical reasons.

Combination of AdK3 and Radiotherapy Inhibits Tumor Angiogenesis. Intratumoral vascularization was assessed immunohistologically by using human anti-smooth muscle actin antibody because this antibody displays a good reactivity on rodent myofibroblasts and pericytes. It was quantified by assessing the surface area per field that scored positive in this assay (see *Materials and Methods*). As shown in Fig. 5*A*, the data indicated a marked reduction of intratumoral vascularization within the tumor sections from the AdK3/irradiated group ($1.0 \pm 0.6\%$, $P < 0.001$), as compared with tumor sections from the AdCO1-injected group ($4.44 \pm 1.6\%$), tumors from the AdK3-injected group ($3.0 \pm 1.5\%$), tumors that were only subjected to irradiation ($4.4 \pm 2.6\%$), or tumors treated by the AdCO1/irradiation combination ($5.11 \pm 2\%$).

Discussion

Combining an angiostatic approach, which typically targets non-tumor cells (i.e., angiogenic endothelial cells), with tumoricide drugs may increase the overall efficacy of the treatment. In this regard, regional radiotherapy appears better suited than chemotherapy because angiostatin-mediated impairment of the tumor vasculature may *in fine* restrict access of the cytotoxic drugs to the tumor mass. This may be particularly true for brain tumors for which the bloodtumor interface may not allow efficient delivery of the drug as previously suggested (17). Radiotherapy is indeed the treatment typically used in patients diagnosed with brain tumors (18). We thus undertook the present study to document whether regional radiotherapy and intratumoral angiostatic gene delivery would synergize in their ability to treat experimental gliomas in mice.

For this purpose, we used a first-generation adenoviral vector (AdK3) that constitutively expresses the N-terminal domain of human plasminogen, including its leader signal sequence, allowing it to achieve secretion of the angiostatin peptide after virus infection. As a model, we choose rat C6 gliomas s.c. implanted into immunodeficient mice because this p53-deficient tumor model is somehow resistant to ionizing radiations (19) (also see this study). Furthermore, we previously reported that this model could respond to adenovirus-mediated angiostatin delivery (12), at least in certain experimental conditions (this study).

We first demonstrated that C6 glioma cells were not affected *in vitro* by angiostatin gene delivery in infection conditions as high as 700 virus per cell (i.e., a multiplicity of infection allowing to transfer the gene in nearly all cells) (see Fig. 1*A*). These cells were also found refractory to irradiation, alone (Fig. 2*B*) or in combination with AdK3 infection (Fig. 3*A*). In contrast, HMEC-1 cells were found sensitive to either treatment applied separately (Fig. 2 *A* and *B*), and this inhibitory effect was exacerbated when treatments were combined (Fig. 3 *A* and *B*). That cells of endothelial origin such as HMEC-1 cells are sensitive to ionizing radiations *in vitro* is in complete agreement with previous studies (20, 21). Furthermore, a single irradiation dose has also been reported to be effective in inhibiting angiogenesis *in vivo* in two different models (22, 23).

Because neovascularization is required for tumor growth *in vivo*, we wondered whether the marked responsiveness of endothelial cells to irradiation or angiostatin would translate into better anti-tumoral effects when C6 xenografts were s.c. implanted in immunodeficient mice. For this purpose, well developed C6 xenografts were subjected to a three-cycle treatment consisting of local x-ray irradiation at a dose of 7.5 Gy and/or three intratumoral injections of 5×10^9 infectious virus particle. In this experimental setting, we confirmed that C6 xenografts are quite resistant to irradiation, especially when carried out at a late stage of tumor development. For example, C6 xenografts with a mean volume of 165 ± 70 mm^3 were only marginally responsive after three cycles of irradiation whereas no significant effects were observed when larger tumors were treated (data not shown). When the tumors were solely infected with the AdK3 adenovirus, there was also very little or no effect on tumor growth (Fig. 4). However, a marked inhibition of tumor growth became apparent when angiostatin gene delivery was associated with irradiation, and this antitumoral effect tightly correlated with a marked inhibition of intratumoral vascularization (Fig. 5). Distinct mechanisms may have contributed to the anti-tumor effect specifically associated with the radiation-AdK3 combination regimen reported here. For example, irradiation by itself may have exerted some anti-angiogenic effects as the proliferation rate that characterizes angiogenic endothelial cells may have sensitized them to ionizing radiations, as previously suggested (24). Independent studies in murine models also suggested that the vessel networks within brain and mammary experimental tumors can be significantly affected by fractionated irradiation (25–27).

An additional important conclusion that can be made from this study is the lack of efficacy of angiostatin gene delivery within well established C6 xenografts. Although a single local injection of 10^9 pfu of AdK3 into small tumors (i.e., tumors with a mean volume of 20 mm^3) could specifically inhibit subsequent neovascularization and tumor growth (1), there was little, if any, effect when higher doses of virus were intratumorally injected into larger tumors (Fig. 4). Several explanations could explain

this apparent discrepancy. For example, the threshold effective dose may not have been reached in the case of larger tumors to achieve sufficient secretion of the angiostatin inhibitor within the tumor mass. On the other hand, it is also possible that an angiostatin-based anti-angiogenic approach may be especially effective in antagonizing the growth of endothelial cells during the early stage of tumor angiogenesis. This statement is supported by a recent study in which bolus injections of the angiostatin protein were reported to exert very different antitumor effects depending on the development stage of experimental carcinogenesis in a pancreatic murine model (10). Altogether, these observations emphasize the restricted action of angiostatin to hold only small tumors or metastasis into a dormant state and that the association of angiostatin or other anti-angiogenic agents with cytotoxic therapies such as regional radiotherapy is particularly potent to improve the clinical outcome of malignant diseases.

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