

Potential of the Antitumor Effect of Ionizing Radiation by Brief Concomitant Exposures to Angiostatin¹

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Abstract

Angiostatin, a proteolytic fragment of plasminogen, inhibits the growth of primary and metastatic tumors by suppressing angiogenesis. When used in combination with ionizing radiation (IR), angiostatin demonstrates potent antitumor synergism, largely caused by inhibition of the tumor microvasculature. We report here the temporal interaction of angiostatin and IR in Lewis lung carcinoma (LLC) tumors growing in the hind limbs of syngeneic mice. Tumors with an initial mean volume of $510 \pm 151 \text{ mm}^3$ were treated with IR alone (20 Gy \times 2 doses on days 0 and 1), angiostatin alone (25 mg/kg/day divided twice daily) on days 0 through 13, or a combination of the two as follows: (a) IR plus angiostatin (days 0 through 13); (b) IR plus angiostatin (days 0 and 1); and (c) IR followed by angiostatin beginning on the day after IR completion and given daily thereafter (days 2 through 13). By day 14, tumors in untreated control mice had grown to $6110 \pm 582 \text{ mm}^3$, whereas in mice treated with: (a) IR alone, tumors had grown to $2854 \pm 338 \text{ mm}^3$ ($P < 0.05$ compared with untreated controls); and (b) angiostatin alone, tumors had grown to $3666 \pm 453 \text{ mm}^3$ ($P < 0.05$ compared with untreated controls). In combined-treatment groups, in mice treated with: (a) IR plus longer-course angiostatin, tumors reached $2022 \pm 282 \text{ mm}^3$ ($P = 0.036$ compared with IR alone); (b) IR followed by angiostatin, tumors reached $2677 \pm 469 \text{ mm}^3$ ($P > 0.05$ compared with IR alone); and (c) IR plus short-course angiostatin, tumors reached $1032 \pm 78 \text{ mm}^3$ ($P < 0.001$ compared with IR alone). These findings demonstrate that the efficacy of experimental radiation therapy is potentiated by brief concomitant exposure of the tumor vasculature to angiostatin.

Introduction

Tumor cells express pro-angiogenic factors that include vascular endothelial cell growth factor (1), basic fibroblast growth factor (2), and certain angiogenesis-promoting peptides (3). Experimental evidence from transgenic mouse models suggest that angiogenesis is necessary early in tumor development (4, 5). The angiogenic "switch" hypothesis is based on a change in the balance between pro- and anti-angiogenic factors toward a pro-angiogenic state as being essential to tumor progression (3, 5). Further support for the importance of angiogenesis in human tumor progression comes from correlations of increasing tumor vascularity with more aggressive clinical behavior in human tumors (6) and the demonstration that pro-angiogenic factors are up-regulated during tumor progression (3, 4, 7).

Inhibition of angiogenesis has emerged as a promising strategy to treat both primary and metastatic tumors by shifting the balance from a pro-angiogenic toward an anti-angiogenic state (3, 4, 7). Strategies

using monoclonal antibodies or soluble receptors to pro-angiogenic cytokines such as vascular endothelial cell growth factor show promise in inhibiting tumor growth in experimental models (8–11). Also, antisense strategies targeting basic fibroblast growth factor and its receptor have demonstrated antitumor effects (2). Recently, AS³ and endostatin, enzymatic cleavage products of plasminogen and collagen type XVIII, respectively, have been isolated from tumor-bearing mice (12, 13). AS is generated from plasminogen by enzymatic activity derived from the tumor itself (14–16) or from host macrophages infiltrating the tumor (17). Treatment with AS or endostatin causes significant tumor regression and can cause tumor dormancy in mouse tumor model systems (18, 19). Recently, it has been reported that gene transfer of a cDNA encoding the AS peptide in a viral vector can inhibit tumor growth (20, 21). Importantly, these anti-angiogenic proteins exhibit no detectable toxicity in experimental animals (12, 13).

Anti-angiogenic proteins, although effective at shrinking tumors, are not tumoricidal. Tumor regrowth frequently occurs once treatment with the angiogenesis inhibitor is terminated (18, 19). One strategy to overcome this therapeutic limitation is to combine angiogenesis inhibitors with cytotoxic therapies. In this context, we recently combined AS with IR and demonstrated a marked increase in antitumor effects (22). Here, we report that there is a temporal interaction between AS and IR. AS delivered concomitantly with IR for 2 days is as effective at suppressing primary tumor growth as a 14-day course of AS administration combined with an identical course of IR. By contrast, AS is not as effective if delivered after IR. Taken together with previous data demonstrating that AS and IR fail to exhibit an interactive killing effect in tumor cells (22), these data suggest that AS and IR interact primarily on the tumor microvessels. This finding is especially important in that adequate supplies of AS are not yet available for prolonged human administration.

Materials and Methods

Cell Lines and Cell Culture. LLC cells (low metastatic strain, LM), a gift of J. Folkman (Dept. of Surgery, Children's Hospital, Harvard Univ. Medical School, Boston, MA; Ref. 12), were grown at 37°C in 7% CO₂ in DMEM with 10% heat-inactivated (56°C for 20 min) fetal bovine serum. Cells were subcultured no more than ten times before being used in animal experiments.

Animals and Tumor Model. Eight-week-old female C57BL/6 mice (Frederick Cancer Research Institute, Frederick, MD) were housed in accordance with the University of Chicago's institutional guidelines. Depending on the experimental protocol, 5×10^5 to 2×10^6 cells suspended in PBS were injected s.c. into the right hind limb. Tumors were allowed to attain a volume of approximately 500 mm³ when treatment with either AS, IR, or a combination of the two was begun. All of the experiments were replicated a minimum of two times.

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³ The abbreviations used are: AS, angiostatin; IR, ionizing radiation; LLC, Lewis lung carcinoma.

Tumor volume was estimated by direct measurement and then calculated using the formula for the volume of an ellipsoid, as described previously (22). Measurements were made three times a week for the duration of the experiments. Depending on the experiment, five to eight mice were assigned to each experimental group on day 0. Because of tumor burden, usually three to five mice per group remained at the conclusion. At various time points, mice were anesthetized using Metaflane inhalation and killed by cervical dislocation to obtain tissue for histology.

Tumor Irradiation. Mice were irradiated using a GE Maxitron X-ray generator operating at 150 kV, 30 mA, using a 1-mm aluminum filter at a dose rate of 188 cGy/min. Mice were shielded with lead except for the tumor-bearing right hind limb. The specific dosage and schedule for IR exposure is described when that experiment is discussed.

AS Production and Dosage. Human AS was generated from human plasminogen as described previously (15). AS was suspended in PBS and administered as i.p. injections twice daily at a total dose of 25 mg/kg/day (0.5 mg/day per mouse) or 50 mg/kg/day (1 mg/day per mouse). The duration of AS treatment and its administration relative to radiation therapy is described for each individual experiment.

Data Analysis. Mean tumor volumes for each experimental group \pm the SE were calculated. Differences between treatment groups were determined by a one-way ANOVA using the SigmaStat 2.0 statistics software package (Jandel Scientific). Differences between individual pairs of treatment groups were determined using Student's *t* test. Differences between treatment groups were considered statistically significant when $P = 0.05$.

Results and Discussion

Effect of AS Dose on LLC Growth. To characterize the effects of human AS alone on LLC, we tested two different doses, 25 mg/kg/day and 50 mg/kg/day, divided twice daily (Fig. 1). Untreated controls received PBS. Neither of the two doses caused shrinkage or growth arrest of these primary tumors, which are large compared to the tumors used in earlier investigations of the effects of AS on LLC growth (12, 18, 19). By day 9, the difference between tumor volumes in the untreated control group and the group receiving 25 mg/kg/d of AS was statistically significant ($P = 0.033$), as was the difference between untreated controls and those receiving 50 mg/kg/d of AS

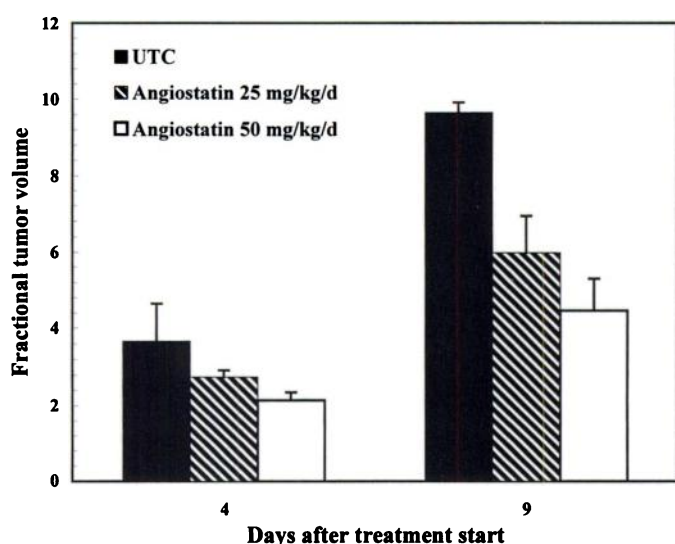


Fig. 1. The effect of different doses of AS on the growth of LLCs implanted in the hind limbs of mice. Mice bearing LLCs in their right hind limb (mean volume = 499 ± 188 mm³) were treated with AS at a dose of either 25 mg/kg/day or 50 mg/kg/day, and the growth of the tumors was measured relative to their initial volumes at days 4 and 9 after treatment began. At day 9, the difference between tumor volumes in the untreated control group and the group receiving AS at 25 mg/kg/day was statistically significant ($P = 0.033$), as was the difference between tumor volumes in the untreated controls and the group receiving AS at 50 mg/kg/day ($P = 0.002$), but in these experiments, AS at 50 mg/kg/day did not produce statistically significantly more tumor volume reduction than AS at 25 mg/kg/day ($P = 0.27$).

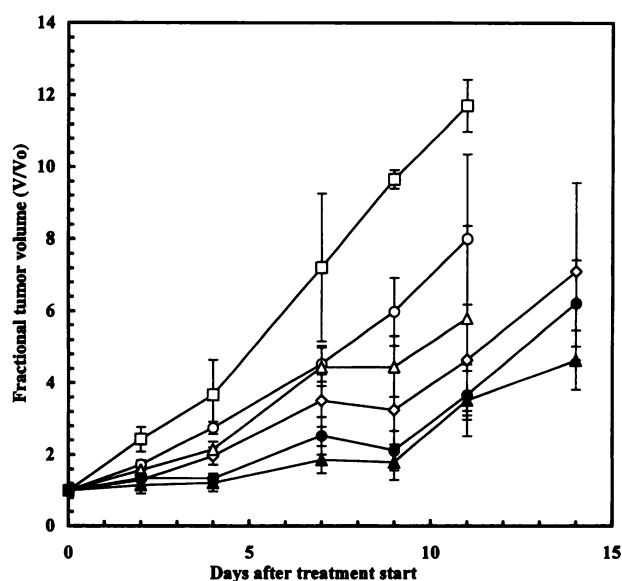


Fig. 2. The effect on tumor growth of differing AS dosages combined with IR. Mice bearing LLCs in their hind limbs were treated with varying combinations of IR and AS. IR was administered as two doses of 20 Gy on days 0 and 1. AS was administered at a dose of either 25 mg/kg/day or 50 mg/kg/day on days 0 through 13. Mean tumor volume on day 0 was 499 ± 188 mm³. □, untreated controls; ◇, IR alone; ○, AS at 25 mg/kg/d; ●, IR + AS at 25 mg/kg/d; △, AS at 50 mg/kg/d.

group ($P = 0.002$). There was no significant difference in tumor volume between the two groups receiving different doses of AS.

Effect of Combined Therapy with IR and AS on LLC Growth.

Next we investigated the effect of combining IR with these two different doses of AS. In initial studies, we treated LLC tumors with differing dose schedules of IR to define a regimen that produced growth delay (data not shown). Because the LLC is a rapidly growing tumor that can increase in volume 10-fold within 14 days, we chose a regimen with two IR fractions consisting of 20 Gy given on each of the first 2 days of the experiment. Mice were treated either with AS alone at doses of 25 or 50 mg/kg/d for the duration of the experiment or with IR alone at a dose of 40 Gy given as two 20 Gy doses on consecutive days. (Fig. 2.) The combination of AS and IR was more effective as an antitumor therapy than either of the therapies alone, although there was no significant difference between IR plus 25 mg/kg/day AS and IR plus 50 mg/kg/day AS. For experiments investigating the temporal interaction of IR and AS, we, therefore, used the lower dose, 25 mg/kg/day.

IR Plus a 2-Day Course of AS Is as Effective as IR Plus a 14-Day Course of AS.

To determine whether the interactive effect of AS and IR on tumor growth inhibition could be achieved with a short concomitant course of AS, we asked which temporal sequence of AS relative to IR is most effective: (a) IR and AS administered concomitantly; (b) AS administered after IR is completed; or (c) IR and AS delivered concomitantly, with AS continued after IR is completed. We hypothesized that, if the interaction of AS with IR requires the presence of AS at the time of IR administration, then there should be a superior antitumor effect when AS is administered concomitantly with IR than when AS is administered after the completion of the course of IR.

Mice bearing LLC tumors in their hind limbs were treated with combinations of IR and AS at a dose of 25 mg/kg/day according to the following: (a) IR (20 Gy \times 2 doses administered on day 0 and 1) plus a long course of AS, which was administered for the duration of the experiment (days 0 through 13); (b) IR (20 Gy \times 2 doses administered on day 0 and 1) plus a short course of AS, which was administered only during the 2 days when the IR was administered (days 0 and 1);

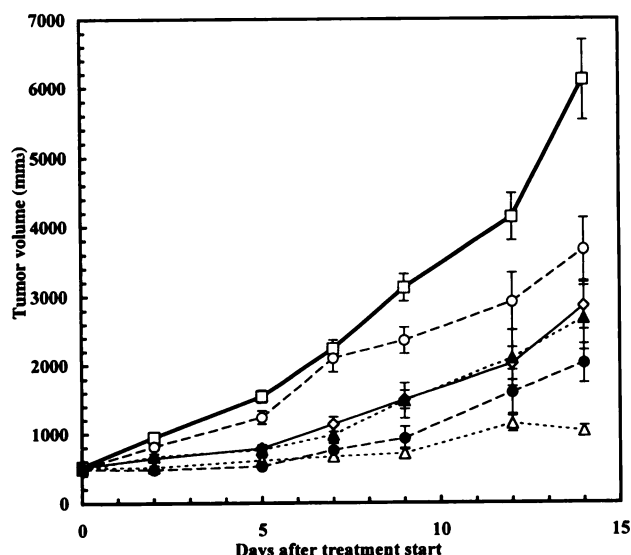


Fig. 3. The effect on tumor growth of varying the timing of AS doses relative to treatment with IR. Mice bearing LLCs in their hind limbs were treated with varying combinations of IR and AS. IR and AS were administered either alone or in combination. IR (20 Gy) was administered on days 0 and 1 for a total of 40 Gy. AS was administered at a dose of 25 mg/kg/day in all of the groups receiving AS. Mean tumor volume on day 0 was $510 \pm 151 \text{ mm}^3$. See text for further comments. □, untreated controls; ◇, radiation (20 Gy on days 0 and 1, total = 40 Gy); ○, AS for 14 days (day 0 through 13); △, radiation (20 Gy on days 0 and 1, total = 40 Gy) administered concurrently with AS for two days (day 0 and 1); ●, radiation (20 Gy on days 0 and 1, total = 40 Gy) administered with AS, with the AS continued on days 2 through 13 for a total of 14 days of AS (days 0 through 13); ▲, radiation (20 Gy on days 0 and 1, total = 40 Gy) followed by AS beginning on the day after IR is completed (day 2) and continued through day 13.

and (c) IR (20 Gy \times 2 doses administered on day 0 and 1) followed by AS beginning on the day after IR and continuing for the duration of the experiment (days 2 through 13). These experimental groups were compared with mice treated with either IR alone or AS alone and to untreated controls (Fig. 3).

By day 9, from an initial volume of $510 \pm 151 \text{ mm}^3$, tumors in the untreated control mice had grown to $3123 \pm 195 \text{ mm}^3$; in the IR-alone group to $1491 \pm 135 \text{ mm}^3$ ($P < 0.05$ relative to untreated control); and in the AS-alone group to $2357 \pm 194 \text{ mm}^3$ ($P > 0.05$ relative to untreated control). By day 9 of treatment, in the combined-treatment groups, tumors treated with IR plus continuous AS reached a volume of $932 \pm 165 \text{ mm}^3$ ($P < 0.05$ relative to IR alone); in the group receiving IR followed by days 2 through 13 of AS, $1480 \pm 259 \text{ mm}^3$ ($P > 0.05$ relative to IR alone, not significant); and in the group receiving IR plus 2 days of AS, $705 \pm 81 \text{ mm}^3$ ($P < 0.001$ relative to IR alone). By day 14, the tumors in the untreated control group had achieved a mean volume of $6110 \pm 582 \text{ mm}^3$; IR alone, $2854 \pm 338 \text{ mm}^3$ ($P < 0.05$ relative to untreated control); AS alone, $3666 \pm 453 \text{ mm}^3$ ($P < 0.05$ relative to untreated control); IR plus short-course AS, $1032 \pm 78 \text{ mm}^3$ ($P < 0.001$ relative to IR alone); IR plus long-course AS, $2022 \pm 282 \text{ mm}^3$ ($P = 0.036$ relative to IR alone); and IR followed by AS, $2677 \pm 469 \text{ mm}^3$ ($P > 0.05$ relative to IR alone).

There is a trend toward increased tumor regression in all of the experiments in the IR-plus-short-course-AS group, even when compared with mice receiving IR plus long-course AS, although the difference is not statistically significant. The observations that IR combined with a short concomitant course of AS is more effective in causing tumor regression than a longer course of AS administered after IR and that further AS administered after IR combined with concomitant AS does not improve tumor shrinkage (Fig. 3) demonstrate a temporal requirement for the interaction of AS and IR.

Inhibition of tumor angiogenesis provides a therapy directed at a target common to the growth and metastasis of all tumors, regardless

of histological type (3, 7). Studies of endogenous inhibitors of angiogenesis such as AS and endostatin demonstrate potent antitumor effects in murine tumor models (12, 13, 18, 19). Because these agents are not tumoricidal, primary treatment of malignancies with anti-angiogenic agents may require lifelong therapy. We previously reported that such limitations might be overcome by a strategy of combining AS with a cytotoxic agent (22). Although the combination of IR and AS produced more tumor volume reduction in the combined-treatment group, the dosing schedule we tested required relatively large quantities of AS.

Our results support an interaction between IR and AS in tumor growth inhibition that occurs only during IR treatment and is dependent upon the action of AS on tumor blood vessel endothelium at the time IR is administered. This interaction does not occur unless AS is present at the time of IR administration, and tumor volume shrinkage is not increased by the continuation of AS treatment after IR has been completed (Fig. 3). AS does not affect the exponential growth of LLC *in vitro* (data not shown). Also, AS does not increase the killing of LLCs by IR in clonogenic killing assays but does enhance vascular endothelial cell killing by IR (22). These observations, when combined with the present study demonstrating a distinct temporal interaction between IR and AS, suggest that the combination of AS and IR does not target the tumor itself but, instead, targets the tumor blood vessels. Furthermore, the observation that additional treatment with AS beyond a brief, concomitant exposure of the tumor to AS and a cytotoxic agent such as IR produces no additional benefit in tumor regression is important because it suggests that limited AS supplies may be most effectively and efficiently used in combination with cytotoxic therapies such as IR, rather than as a primary therapy. These results suggest a new approach in the design of clinical trials with AS and other anti-angiogenic agents.

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