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Endostatin Improves Radioresponse and Blocks Tumor Revascularization after Radiation Therapy for A431 Xenografts in Mice

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Abstract

Purpose: Clinical trials of antiangiogenic agents used alone for advanced malignancy have been disappointing but preclinical studies suggest that the addition of radiation therapy could improve anti-tumor efficacy. To test the hypothesis that antiangiogenic therapy combined with radiation therapy can overcome the limitations of antiangiogenic monotherapy, we studied the effects of endostatin combined with radiation on the growth and vascularization of A431 human epidermoid carcinomas growing intramuscularly in the legs of mice.

Methods and Materials: Mice with established A431 human epidermoid leg tumors were treated with radiation, endostatin, both radiation and endostatin, or vehicle control. The experiment was repeated and mice from each group were killed at 2, 7, and 10 days after irradiation so that tumor tissue could be obtained to further analyze the kinetics of the anti-tumor, anti-vascular, and antiangiogenic response to therapy.

Results: Endostatin enhanced the anti-tumor effects of radiation and prolonged disease free survival was observed in the combined treatment group. Endothelial cell proliferation was increased in tumors after irradiation but was blocked by the concurrent administration of endostatin, and the combination of endostatin with radiation enhanced endothelial cell apoptosis within 48 hours after irradiation. Expression of vascular endothelial growth factor, interleukin-8, and matrix metalloproteinase-2 were increased in tumors after irradiation, and this increase was blocked by concurrent administration of endostatin.

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Conclusions: These data indicate that endostatin can block tumor revascularization after radiation therapy and thereby augment radioresponse.

Keywords

Endostatin; Radiation; Revascularization; Angiogenesis; Mice

INTRODUCTION

Under physiological conditions that require neovascularization, angiogenesis is tightly regulated (1). In malignant angiogenesis, new vessels are markedly disordered (2) and are characterized by a lack of pericytes and other support cells, impaired flow (3), the presence of plasma proteins that function as a neostroma (4), and increased vascular permeability. Many antiangiogenic agents have been described and several are now in clinical trials in cancer patients (5) and a few have been approved for clinical use (6). However, although antiangiogenic agents offer great therapeutic potential, preclinical and early clinical trial results suggest that these agents will have a delayed onset of activity and may induce stabilization of disease, and not regression, in patients with advanced disease (7-10). For example, preclinical studies with antiangiogenic agents demonstrate that antiangiogenic monotherapy is associated with a delayed onset of activity with tumors progressing by as much as 400% over a period of several days prior to response to therapy (9). Given that the doubling time of murine tumors is several folds higher than is observed in the presentation of human cancer, this delay in onset of activity could translate to several months in patients. For patients with advanced malignancies, this delay in onset of activity may make the use of these agents impractical. Further, microscopic residual disease persists in tumor-bearing mice treated with antiangiogenic monotherapy even after prolonged administration at high dose (10) and recent studies suggest that angiogenesis in various capillary beds may be differentially regulated, suggesting that antiangiogenic therapy may require organ-specific optimization (11,12). Thus, before antiangiogenic agents can be successfully incorporated into clinical strategies to treat cancer and other angiogenesis dependent diseases, a greater understanding of the process of angiogenesis and of the interaction between the endothelial cell and its microenvironment is required.

Angiogenesis provides both a perfusion effect and a paracrine effect to a growing tumor (5) and in addition to providing nutrients and oxygen and removing catabolites, proliferating endothelial cells produce multiple growth factors that can promote tumor cell growth, invasion, and survival (13). These observations led Folkman to propose a two-compartment model of malignancy consisting of endothelial and tumor cell populations (14) and it will be prudent to target both by combining radiation and other conventional therapies with antiangiogenic agents. Although traditional therapies for cancer are associated with a number of toxicities and therapeutic challenges, their limitations are distinct from those that have been observed and might be expected with angiogenesis inhibition. Thus, we hypothesized that the use of angiogenesis inhibitors in combination with other modalities could help to overcome the limits of each and that angiogenesis inhibitors will block tumor revascularization after radiation therapy. To test this hypothesis, we studied the effects of endostatin (8), an endogenous angiogenesis inhibitor that is highly specific for microvascular endothelial cells and has not caused any significant toxicity in animal models or clinical use, combined with radiation on the growth and revascularization of A431 human epidermoid carcinoma xenografts in the legs of nude mice.

METHODS AND MATERIALS

Cell lines and reagents

A431 cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, vitamin solution, and penicillin-streptomycin at 37°C in 5% CO₂ and 95% air. Tumor cells were free of Mycoplasma and pathogenic murine viruses. Human recombinant endostatin was provided by EntreMed, Inc. (Rockville, MD).

Tumor radioresponse studies

Male 6 – 8 week old athymic nu-nu/Ncr (nu/nu) mice were maintained in the specific pathogen-free mouse colony of the Department of Experimental Radiation Oncology approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture and the Department of Health and Human Services. The musculature of the right proximal hind legs of the mice were injected with 1×10^6 A431 cells. When tumors reached a diameter of 6 mm, mice were randomly assigned to receive sham treatment, a single fraction of radiation (15 Gy), endostatin (50 mg/kg subcutaneously once daily for 21 days), or the combination of radiation and endostatin. Radiation was delivered to mice that were immobilized in a jig with the tumor centered in a circular 3 cm diameter radiation field using a dual-source Cs¹³⁷ irradiator at a dose rate of 6 Gy/min. Endostatin treatment was initiated 2 days prior to irradiation to allow for an effective plasma concentration prior to the delivery of irradiation. Three mutually orthogonal tumor diameters were measured every 2 days with a vernier caliper. Regression and regrowth of tumors were followed until tumor diameter reached 14 mm, at which point mice were sacrificed in accordance with institutional guidelines. The treatment experiment was repeated, and mice from each group were sacrificed at 2 days, 7 days, or 10 days after irradiation (4 mice from each group for each of the three time points) to provide tumor tissues for immunohistochemical analyses.

Immunohistochemistry

For vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), matrix metalloproteinase-2 (MMP-2), and matrix metalloproteinase-9 (MMP-9) staining, sections of formalin-fixed, paraffin-embedded tissue specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. For VEGF, bFGF, and IL-8 staining, the slides were treated with pepsin for 20 minutes at room temperature. For CD31 staining, sections of frozen tissues (8 µm) were fixed with cold acetone and transferred to PBS. The slides were then rinsed with PBS, and endogenous peroxidase was blocked by use of 3% hydrogen peroxide in PBS (IL-8 and MMP-9) or methanol (VEGF, bFGF, MMP-9, and CD31). The samples were then washed with PBS and incubated for 10 minutes at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 hours at 4°C with a 1:400 dilution of rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 dilution of rabbit anti-human bFGF antibody (Sigma, St. Louis, MO), 1:25 dilution of rabbit polyclonal anti-IL-8 antibody (Biosource, Camarillo, CA), 1:100 dilution of rabbit polyclonal anti-MMP-2 antibody (Chemicon International, Temecula, CA), 1:400 dilution of rabbit polyclonal anti-MMP-9 antibody (Chemicon International) or 1:700 dilution of monoclonal rat anti-CD31 antibody (PharMingen, San Diego, CA). The samples were then rinsed with PBS and incubated for 60 minutes at room temperature with the appropriate dilution of peroxidase-conjugated anti-rabbit IgG or anti-rat IgG. For MMP-2 and MMP-9, slides were incubated with biotinylated anti-rabbit IgG (Biocare Medical, Walnut Creek, CA) for 30 minutes and then washed and incubated with streptavidin HRP (DAKO, Carpinteria, CA) for 30 minutes. The

slides were rinsed with PBS and incubated for 5 minutes with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were then washed three times with distilled water and counterstained with Gill's hematoxylin. Sections (4 μm thick) of formalin-fixed, paraffin-embedded tumors were also stained with hematoxylin-eosin for routine histologic examination.

Immunofluorescence double staining for endothelial cell proliferation and apoptosis

For CD31 and bromodeoxyuridine (BrdU) double staining, mice were injected intravenously with 250 μg of BrdU. One hour later, the mice were killed, and tumors were collected and cut into two pieces, which were placed into either buffered 10% formalin solution or OCT compound (Miles Laboratories, Pittsburg, PA) to be snap-frozen in liquid nitrogen. Frozen tissues were sectioned (8 μm), and then monoclonal rat anti-CD31 and secondary goat anti-rat antibody conjugated to Texas Red were used for CD31 staining. For BrdU staining, the slides were incubated with 1% Triton X-100 for 8 minutes. After washing, the slides were treated with 2 N HCl in PBS for 30 minutes at 37°C. The slides were washed twice with 0.1 M Tris for 5 minutes, then treated with protein-blocking solution and incubated for 18 hours at 4°C with monoclonal mouse anti-BrdU Ab (Becton, Dickinson, Franklin Lakes, NJ). The samples were then incubated with goat anti-mouse antibody conjugated to Alexa488. The TUNEL assay was performed using an apoptosis detection kit (Promega Corp., Madison, WI) following the manufacturer's instructions. After CD31 staining with Texas Red, samples were fixed with 4% paraformaldehyde (methanol free) for 10 minutes at room temperature, washed twice with PBS for 5 minutes, and then incubated with 0.2% Triton X-100 for 15 minutes at room temperature. After again being washed twice with PBS for 5 minutes, the samples were incubated with equilibration buffer (from kit) for 10 minutes at room temperature. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was added to the tissue sections. The sections were incubated in a humid atmosphere at 37°C for 1 hour in the dark. The reaction was terminated by immersing the samples in 2X standard saline citrate for 15 minutes. Samples were washed 3 times for 5 minutes to remove unincorporated fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 $\mu\text{g}/\text{ml}$ Hoechst stain for 10 minutes at room temperature. Immunofluorescence microscopy was performed using an $\times 20$ or $\times 40$ objective to individually select for green and red fluorescence. Endothelial cells were identified by red fluorescence and proliferating and apoptotic cells were identified by localized green nuclear staining. The number of proliferating endothelial cells was determined in 18-25 random fields at $\times 200$ magnification and the number of apoptotic events was counted in 7-9 random fields at $\times 400$ magnification.

Quantification of microvessel density, vascular area and immunoreactivity

Blood vessels in solid tumors were counted under a light microscope after immunostaining for CD31. Areas containing the highest number of capillaries and small venules were identified by scanning the tumor sections at low power ($\times 40$). Individual vessels were counted in 10 random fields at $\times 100$ magnification (0.159- mm^2/field). Vascular area was determined by summing the whole vascular areas in 10 random 0.159- mm^2 fields at $\times 100$ magnification.

For quantification of the immunohistochemical reaction intensity, the absorbance of 100 bFGF-, VEGF-, IL-8, MMP2-, and MMP9-positive cells from each of the differently treated tumor tissues was measured in 10 random fields at $\times 200$ magnification using Optimas Image Analysis software (Bioscan, Washington, DC). The cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and was expressed as a ratio of tumor cell expression to normal muscle cell expression multiplied by 100.

Data Analysis

Statistical significance was determined using Student's t test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Endostatin treatment improves tumor radioresponse

To determine the effect of endostatin on tumor radioresponse, A431 tumor xenografts of 6 mm in diameter were treated with 15 Gy single dose local tumor irradiation alone, endostatin at a modest dose of 50 mg/kg administered subcutaneously once daily for 21 consecutive days or the combination of endostatin and irradiation. For the combined treatments, endostatin therapy was initiated 2 days before irradiation to the tumor. Tumor growth delay was the primary treatment endpoint. Each treatment was effective in delaying tumor growth, as compared to control ($p < 0.005$), with the combined treatment with endostatin and irradiation being the most highly effective (Fig. 1). In the group treated with endostatin and irradiation together, 2 of 5 mice still had locally controlled tumors 185 days after irradiation at which time the experiment was terminated.

To further quantify the anti-tumor effects of the different treatments and to determine the enhancement factor for tumor radioresponse, we calculated the time in days for tumors needed to triple in volume (Table 1). This time was 8.0 ± 1.4 days for the control (untreated) group, 50.4 ± 14.8 days for the irradiation group, 16.0 ± 2.2 days for the endostatin group, and greater than 118 days for the endostatin plus irradiation group ($p < 0.05$). Endostatin greatly enhanced radioresponse of A431 xenografts with an enhancement factor of more than 2.4 calculated by dividing the normalized tumor growth delay (the time for tumors treated with both endostatin and radiation to grow from 8 to 12 mm in diameter minus the time in days for tumors treated with endostatin alone to reach the same size) in mice treated with endostatin plus irradiation with the absolute tumor growth delay in mice treated with irradiation alone (see Table 1).

Endostatin treatment enhances tumor cell apoptosis and inhibits tumor cell proliferation after irradiation

The anti-tumor effects of irradiation at the cellular level and its modification by endostatin were assessed by quantifying tumor cell apoptosis and proliferation at 2, 7, and 10 days after irradiation delivered in a single fraction. As shown in Figure 2, tumor cell apoptosis was relatively low (Fig. 2a) while proliferation was relatively high (Fig. 2b) in tumors from untreated mice. Endostatin alone had little or no effect upon tumor cell apoptosis at the time points studied whereas radiation alone significantly increased ($p < 0.001$) tumor cell apoptosis as compared to the control at day 7 that was increased by a factor of about 2 when radiation was combined with endostatin (Fig. 2a).

During the observation period of growth of untreated tumors tumor cell proliferation was constant until day 10 after irradiation when it was increased (Fig. 2b). At this time point, tumor cell proliferation, assessed by the labeling index with BrdU, was reduced by endostatin or radiation as compared to control and especially by the combination of these two treatments ($p < 0.02$).

Microvessel density and vascular area do not predict for tumor response

High microvessel density (MVD) correlates with poor prognosis in many different types of cancer (15,16) and the efficacy of antiangiogenic therapy is frequently evaluated by determining MVD after tumor response to therapy. However, MVD can vary as a function of the rate of tumor response and other variables and does not always correlate with tumor

response to therapy (17). To study the feasibility of using MVD as a surrogate marker for response to combined radiation therapy and antiangiogenic therapy, MVD was sequentially measured in tumor tissues obtained from mice 2, 7, or 10 days after treatment with endostatin and/or irradiation (Fig. 3a and b). MVD in the control group increased from 15.7 ± 1.3 per high-power field at day 2 to 39 ± 2.0 per high-power field at day 10. At day 10, the endostatin group had significantly lower ($p < 0.001$) MVD than the control group yet there was no significant difference in MVD between the endostatin plus radiation group and the control group. Vascular area was also measured (Fig. 3c) and no significant difference between the control and endostatin treatment groups was observed and as with MVD there was no significant difference between the endostatin plus radiation group and the control group. The radiation alone group had a significantly lower vascular area than the control group at day 10 ($p < 0.01$). When taken together, these data suggest that even though MVD is useful as a prognostic indicator for the risk of metastatic disease in patients with early stage cancer (15, 16), it is not necessarily useful as a surrogate for predicting tumor response to combined antiangiogenic and radiation therapies.

Endostatin blocks tumor revascularization after irradiation

It has recently been reported (18) that endothelial cell apoptosis precedes crypt stem cell apoptosis in a gastrointestinal radiation damage model and that the endothelial cell may be a primary target of irradiation (19) in some tumor models where endothelial cell apoptosis precedes tumor cell apoptosis after radiation. To characterize the effects of endostatin upon tumor revascularization after irradiation in A431 tumors we sequentially assessed both endothelial cell apoptosis and proliferation using immunofluorescence double staining for endothelial cells (CD31) and apoptosis (TUNEL) or proliferation (BrdU incorporation). Within 2 days of irradiation given in a single fraction endothelial cell apoptosis was increased in the endostatin and endostatin plus radiation groups ($p < 0.05$ versus control). However, 10 days after tumor irradiation, endothelial cell apoptosis was not increased in any of the treatment groups (Fig. 4a).

In contrast to apoptosis, endothelial cell proliferation in treated tumors increased with time having the highest level at day 7 (Fig. 4b and c) after irradiation. Endostatin alone inhibited endothelial cell proliferation throughout the observation period and when endostatin and radiation were combined, the inhibition of endothelial cell proliferation was virtually complete ($p < 0.005$ relative to control) suggesting that the effect of endostatin is particularly prominent in inhibiting endothelial cell proliferation.

To further define the impact of endostatin and/or irradiation upon tumor revascularization, we calculated the ratio of endothelial cell proliferation and apoptosis as an angiogenesis index. As shown in Figure 5, the angiogenesis index was lowest for the combined endostatin and radiation treatment group and was close to 0 at day 10. Given that tumor angiogenesis is directly dependent upon both endothelial cell proliferation and apoptosis it will be prudent to include both parameters as part of the assessment of the impact of antiangiogenic agents as part of the combined modality treatment of cancer.

Endostatin suppresses the upregulation of proangiogenic and invasive molecules induced by irradiation

To further characterize the inhibition of tumor revascularization that was observed after irradiation in mice treated with concurrent endostatin, the expression of proangiogenic and invasive molecules was sequentially studied by optical density analysis of stained tissue sections. For each of the time points studied (2, 7, and 10 days after the single 15 Gy fraction of irradiation in the radiation group) no significant difference in the expression of basic fibroblast growth factor or matrix metalloproteinase 9 was observed between the different

treatment groups (data not shown). However, within 7 days of radiation therapy VEGF, IL-8, and MMP-2 were all upregulated in the irradiation group as compared to control (Fig. 6, $p < 0.0001$). The increased expression of VEGF after irradiation was offset only marginally (Fig. 6a) by the addition of endostatin ($p < 0.005$) whereas the increased expression of both IL-8 (Fig. 6b) and MMP-2 (Fig. 6c) after irradiation was substantially offset by the addition of endostatin ($p < 0.001$). These data show that endostatin can selectively offset proangiogenic and invasive factors that are increased in response to radiation therapy of tumors.

DISCUSSION

It had long been assumed that an angiogenesis inhibitor would impair the effect of ionizing radiation by inducing tumor hypoxia until Teicher and colleagues (20) observed that antiangiogenic agents given in combination with radiation therapy improved tumor oxygenation and anti-tumor efficacy. Hansen-Algenstaedt and colleagues studied androgen-dependent Shinogi male mouse mammary carcinomas treated with anti-VEGFR2 antibodies, hormone ablation and/or chemotherapy (21). They found that in the groups that received combined modality therapy, pO_2 initially decreased with therapy but then subsequently increased and tumor growth arrest was observed. Enhancement of tumor response to radiation therapy has been demonstrated when it is combined with anti-VEGF agents (22) even for tumors that were markedly hypoxic (23,24) with an increase in tumor growth delay and an augmentation of tumor curability in the combined modality groups. More recently, it has been shown that the combination of agents that prevent VEGF signaling and radiotherapy reduced tumor resistance to radiation response and improved response (25) and SU5416, a small molecule antagonist of VEGFR signaling, had an additive effect when combined with irradiation and permanent vascular changes were observed after combined treatment resulting in complete remission (26). An increase in tumor growth delay and an augmentation of tumor curability was observed when radiation therapy was combined with a selective inhibitor of the cyclooxygenase-2 enzyme (27) providing the basis for clinical trials. In patients with poor prognosis lung cancer, celecoxib at high dose was administered concurrently with thoracic radiotherapy. The treatment resulted in actuarial local progression-free survival of 66.0% at 1 year and 42.2% at 2 years (28). However, given the cardiac toxicity that has now been observed with this class of agents, further assessment and additional studies are required and more specific inhibitors of angiogenesis may be needed to minimize the potential of toxicity.

In the current study, the highly selective inhibitor of angiogenesis endostatin significantly enhanced radioresponse of A431 human epidermoid carcinoma xenografts in mice and was associated with long term tumor control in some animals. Weichselbaum and colleagues also demonstrated an enhancement of the effects of radiotherapy when the angiostatin (29) or endostatin (30) and ionizing radiation were combined using low doses of each modality. In our studies, we found that the enhancement of the effects of radiotherapy by endostatin is due in part to a prevention of the tumor revascularization that was observed shortly after irradiation mediated by sustained endothelial cell apoptosis and a prevention of endothelial cell proliferation. In clinical trials, endostatin therapy was associated with increased endothelial cell apoptosis (31,32). In the current study, endostatin increased endothelial cell apoptosis and the effect was enhanced by radiation. These data provide the basis for a strategy to use endostatin and other antiangiogenic agents to maximize and sustain the endothelial apoptosis that can occur within hours of radiation therapy (19) and to then prevent endothelial cell proliferation driven by proangiogenic factors secreted by tumors after radiation therapy (22).

The increased expression of proangiogenic factors after irradiation may function to protect the tumor vasculature and thereby mediate a rapid recovery of endothelial cell proliferation accounting for the rapid regrowth of tumors that can be observed after radiation therapy. The current studies indicate that revascularization after radiation therapy can be blocked by

endostatin and that the combination of antiangiogenic therapy and radiation therapy may therefore overcome the limitations of each therapy resulting in superior and more durable anti-tumor activity. Prior reports have shown that endostatin can impact the endothelial cell at multiple molecular levels (33) and endostatin treatment can decrease tumor cell expression of several proangiogenic and invasive molecules (34-37). To further analyze the mechanism of the blockade of tumor revascularization by endostatin after radiation therapy we evaluated changes in expression of the proangiogenic or invasive molecules VEGF, bFGF, and IL-8 and MMP-2 and MMP-9. In our studies, endostatin only marginally offset VEGF expression but had a substantial impact upon both IL-8 and MMP-2.

In conclusion, our studies show that multimodality therapy that includes radiation therapy and angiogenesis inhibitors offers the potential for improved efficacy in the treatment of cancer and other angiogenesis dependent diseases. However, additional work is still needed to provide a rational basis for the combination of angiogenesis inhibitors with other modalities and to identify potential problems that might arise from these combinations so that these agents will be successfully incorporated into existing therapy in a timely and rational manner.

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Figure 1.

Growth of A431 tumor xenografts. The musculature of the right proximal hind legs of male nude mice were injected with A431 human epidermoid carcinoma cells and when tumors reached a diameter of 6 mm, mice were randomly assigned to receive sham treatment (control), a single fraction of radiation (15 Gy), endostatin (50mg/kg subcutaneously daily for 21 days), or the combination of radiation and endostatin. Each group consisted of 5 mice and data were expressed as mean $\pm$ standard error.

Figure 2.

Time course analyses of tumor cell apoptosis and proliferation. (a) Tumor cell apoptosis as evaluated by the TUNEL assay. Apoptotic tumor cells were counted in 7-9 random fields at $\times 400$ magnification, excluding necrotic areas. (b) Tumor cell proliferation as evaluated by BrdU incorporation. Mice were injected intravenously with 250 μg of BrdU and killed 1 hour after injection. The number of proliferating tumor cells was determined in 3-6 random fields at $\times 200$ magnification.

Figure 3.

Time course analyses of tumor MVD and vascular area. (a) Representative CD31 staining in tumor tissue harvested from mice killed 7 days after irradiation. (b) MVD (CD31 staining) was determined in 10 random 0.0159 mm² fields at $\times 100$ magnification. (c) Vascular area was determined by summing the whole vascular area in 10 random 0.159-mm² fields at $\times 100$ magnification.

Figure 4.

Time course analyses of endothelial cell apoptosis and proliferation. (a) Endothelial cell apoptosis as determined by CD31-TUNEL double-staining. The number of apoptotic endothelial cells was counted in 7-9 random fields at $\times 400$ magnification. (b) Endothelial cell proliferation as determined by CD31-BrdU double-staining. The number of proliferating endothelial cells was determined in 18-25 random fields at $\times 200$ magnification. (c) Representative staining for CD31 (red fluorescence), BrdU (green fluorescence), or both CD31 and BrdU (yellow fluorescence) in tumor tissue harvested from mice killed 7 days after irradiation.

Figure 5.
The ratio of endothelial cell proliferation and apoptosis.

Figure 6.

Time course analyses of the expression of proangiogenic and invasive molecules. The expression of VEGF, IL-8, or MMP-2 was determined by the absorbance intensity for each of the differently treated tumor tissues was measured in 10 random fields at $\times 200$ magnification using Optimas Image Analysis software (Bioscan). The cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and was expressed as a ratio of tumor cell expression to normal muscle cell expression multiplied by 100. (a) Expression of VEGF. (b) Expression of IL-8. (c) Expression of MMP-2.

Table 1

Treatment	Time required for tumor to volume to triple (days)	Absolute growth delay (days)	Normalized growth delay (days)	Enhancement factor
Control	8 ± 1.4			
XRT	50.4 ± 14.8	42.4 ± 14.8		
Endostatin	16 ± 2.2	8 ± 2.2		
Endostatin + XRT	NA (>118)	>110	>102	>2.4