

Blockade of the Vascular Endothelial Growth Factor Stress Response Increases the Antitumor Effects of Ionizing Radiation¹

David H. Gorski, Michael A. Beckett, Nora T. Jaskowiak, Douglas P. Calvin, Helena J. Mauceri, Rabih M. Salloum, Saraswathy Seetharam, Ann Koons, Danielle M. Hari, Donald W. Kufe, and Ralph R. Weichselbaum²

Departments of Radiation and Cellular Oncology [D. H. G., M. A. B., D. P. C., H. J. M., S. S., A. K., D. M. H., R. R. W.] and Surgery [N. T. J., R. M. S.], University of Chicago, Chicago, Illinois 60637, and Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 [D. W. K.]

Abstract

The family of vascular endothelial growth factor (VEGF) proteins include potent and specific mitogens for vascular endothelial cells that function in the regulation of angiogenesis. Inhibition of VEGF-induced angiogenesis, either by neutralizing antibodies or a dominant-negative soluble receptor, blocks the growth of primary and metastatic experimental tumors. Here we report that VEGF expression is induced in Lewis lung carcinomas (LLCs) both *in vitro* and *in vivo* after exposure to ionizing radiation (IR) and in human tumor cell lines (Seg-1 esophageal adenocarcinoma, SQ20B squamous cell carcinoma, T98 and U87 glioblastomas, and U1 melanoma) *in vitro*. The biological significance of IR-induced VEGF production is supported by our finding that treatment of tumor-bearing mice (LLC, Seg-1, SQ20B, and U87) with a neutralizing antibody to VEGF-165 before irradiation is associated with a greater than additive antitumor effect. *In vitro*, the addition of VEGF decreases IR-induced killing of human umbilical vein endothelial cells, and the anti-VEGF treatment potentiates IR-induced lethality of human umbilical vein endothelial cells. Neither recombinant VEGF protein nor neutralizing antibody to VEGF affects the radiosensitivity of tumor cells. These findings support a model in which induction of VEGF by IR contributes to the protection of tumor blood vessels from radiation-mediated cytotoxicity and thereby to tumor radioresistance.

Introduction

Tumors influence the surrounding host stroma by inducing angiogenesis to supply their oxygen and nutrient needs. In normal tissues, angiogenesis is tightly regulated by the balance between angiogenic and antiangiogenic factors (1). One of the most important angiogenic proteins, VEGF,³ is expressed by diverse human tumors (2–4). Evidence for the importance of VEGF-induced angiogenesis in tumor growth includes the observation that inhibition of VEGF action by neutralizing antibodies or a dominant-negative soluble receptor blocks the growth of primary and metastatic experimental tumors (5–9) and that, in a mouse fibrosarcoma model, disruption of tumor-specific VEGF expression results in decreased tumor growth and vascularity, as well as increased tumor cell apoptosis (10). Moreover, it has been observed that serum VEGF levels are elevated in certain patients with malignant tumors after radiation therapy (11). These observations suggest that the paracrine relationship between the tumor and its

vasculature represents a potential target for a strategy to enhance IR-induced antitumor activity.

In the present study, we demonstrate that exposure of LLC and human tumor xenografts to IR is associated with induction of VEGF expression. We also show that blocking the action of this IR-mediated increase in VEGF using neutralizing anti-VEGF antibodies results in increased endothelial cell killing by IR and produces greater than additive antitumor effects in murine tumor model systems. These findings support a model in which induction of VEGF by IR contributes to the protection of tumor blood vessels from radiation-mediated cytotoxicity and thereby to tumor radioresistance, suggesting that an effective use of anti-VEGF antibodies in clinical antitumor therapy is in combination with a cytotoxic therapy such as IR.

Materials and Methods

Cell Culture. LLC cells (a gift of J. Folkman, Children's Hospital, Harvard Medical School, Boston, MA) and SQ20B cells were maintained as described previously (12, 13). HUVECs were maintained in EGM-2 medium (Clonetics). U87 and T98 human glioblastoma cells were maintained in RPMI 1640 (Life Technologies, Inc.) + 10% FBS (Intergen); U1 melanoma cells, DMEM (75%) + F12 (25%) + 10% FBS; Seg-1 esophageal adenocarcinoma cells (a gift of D. Beer, Section of General Thoracic Surgery, Department of Surgery, University of Michigan Medical School, Ann Arbor, MI; Ref. 14), DMEM + 10% FBS.

Tumor Models and Administration of IR. To establish tumors, LLC cells were injected s.c. into the right hind limb (1×10^6 cells in PBS) of C57BL/6 female mice (Frederick Cancer Research Institute). To establish human tumor xenografts, SQ20B human squamous cell carcinoma cells (Ref. 15; 5×10^6), Seg-1 esophageal adenocarcinoma cells (Ref. 14; 3×10^6), and U87 glioblastoma cells (3×10^6) were injected s.c. into the hind limb of female athymic nude mice (Frederick Cancer Research Institute). Tumor volume was determined by direct measurement with calipers and calculated by the formula (length \times width \times depth/2) and reported as the mean volume \pm SE, as described previously (12, 13). Tumors were allowed to attain a mean size between 350–450 mm³ (LLC, 442 ± 14 mm³; SQ20B, 372 ± 16 mm³; Seg-1, 407 ± 20 mm³; U87, 453 ± 32 mm³), after which mice were divided into experimental groups and treated. Tumors 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. LLC tumors received 40 Gy (20 Gy on days 0 and 1); SQ20B, 40 Gy (10 Gy on days 0, 1, 2, and 3); Seg-1, 20 Gy (5 Gy on days 0, 1, 2, and 3); and U87, 40 Gy (5 Gy on days 0, 1, 4, 5, 7, 8, 11, and 12). Mice were shielded with lead except for the tumor-bearing right hindlimb. The care and treatment of animals were in accordance with institutional guidelines.

Neutralizing Antibodies against VEGF. For experiments with LLC tumors, neutralizing polyclonal goat antibody (IgG) against recombinant mouse VEGF-164 (R & D Systems) was suspended in PBS and administered via i.p. injection (10 μ g/mouse, 3 h before each IR fraction). For experiments with human tumor xenografts, a neutralizing monoclonal antibody to recombinant human VEGF-165 (R & D Systems) was used (10 μ g/mouse, 3 h before each IR fraction). Control mice in experiments with LLC and human tumor xenografts received nonimmune goat IgG (Sigma) or mouse IgG (Sigma), respectively. Monoclonal anti-VEGF antibody was used for most *in vitro* exper-

Received 4/14/99; accepted 6/1/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹This work was supported by National Cancer Institute Grants CA42596 and CA41068, as well as Grant DE/CA11921 from the Chicago Oral Cancer Center and a grant from the Lederer Foundation. Dr. Calvin is supported in part by National Cancer Institute Training Grant T32CA09675. We also acknowledge support from the Center for Radiation Therapy, The Chicago Tumor Institute, and the Gerald-Norton Foundation.

²To whom requests for reprints should be addressed, at Department of Radiation and Cellular Oncology, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637. Phone: (773) 702-0817; Fax: (773) 834-7233; E-mail: rrw@rover.uchicago.edu.

³The abbreviations used are: VEGF, vascular endothelial growth factor; LLC, Lewis lung carcinoma; IR, ionizing radiation; HUVEC, human umbilical vein endothelial cell; MMT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide.

Table 1 VEGF levels in Lewis lung carcinoma tumors after exposure to 40 Gy

Day	Untreated controls		Ionizing radiation (40 Gy)	
	Mean tumor volume (mm ³ ± SE)	VEGF (pg VEGF/mg total protein)	Mean tumor volume (mm ³ ± SE)	VEGF (pg VEGF/mg total protein)
2	947 ± 43	69 ± 21	641 ± 22	234 ± 79 ^a
5	1545 ± 93	46 ± 18	786 ± 52	135 ± 32 ^a
14	6110 ± 582	90 ± 23	2854 ± 338	194 ± 47 ^a

^a P < 0.05 relative to VEGF levels in untreated controls.

iments with HUVECs, but in some *in vitro* experiments, a polyclonal goat anti-human VEGF-165 neutralizing antibody (R & D Systems) was used.

Measurement of VEGF Levels in Tumor Extracts and Conditioned Media. Tumor extracts were prepared by homogenizing tumors in a buffer containing 150 mM NaCl, 10 mM Tris, 5 mM EDTA, 0.5% Triton X-100, and 1 μM dithiothreitol (pH 7.5), 50 μM PMSF, 1 μg/ml leupeptin, and 2 μg/ml aprotinin. The homogenate was subjected to three freeze-thaw cycles in liquid nitrogen to lyse cells and then spun at 5000 × g at 4°C to pellet debris. VEGF levels were measured in tumor extract supernatants by ELISA (R & D Systems). VEGF levels were normalized to total extract protein concentration as measured by Lowry assay and expressed as pg VEGF/mg total extract protein. For *in vitro* studies, cells were plated in six-well plates, allowed to attach overnight, and exposed to IR. At various time points, VEGF levels in conditioned media were measured by ELISA and normalized to cell number in each well.

Northern Blots. Total RNA was isolated from cultured cells and tumor tissue by the guanidine thiocyanate method (16) using Trizol LS (Life Sciences, Inc.). Twenty-five μg of total RNA was fractionated on 1.2% agarose gels containing formaldehyde and blotted onto nylon membranes, then hybridized with a ³²P-labeled cDNA probe encoding human VEGF (17). Hybridizations were carried out at 60°C in 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate, 1 mM EDTA, and 1% bovine serum albumin (18), and blots were washed to a stringency of 0.2× SSC. After autoradiography, blots were stripped of probe and rehybridized to a labeled cDNA encoding rat glyceraldehyde-3-phosphate dehydrogenase to demonstrate message integrity.

MTT Assays. HUVECs were plated (1 × 10³ cells/well in 96 well plates) in EGM-2 media (Clonetics) containing 2% fetal calf serum and allowed to attach overnight. Medium was replaced with EGM-2 plus 2% fetal calf serum containing different concentrations of recombinant human VEGF-165 (R & D Systems). In other experiments, the concentration of VEGF-165 was kept constant (10 ng/ml), and either a neutralizing polyclonal or monoclonal anti-human VEGF-165 antibody (R & D Systems) was added before treatment with IR. Ninety-six h after IR, cells were pulsed with MTT (Sigma; Ref. 19) at 0.5 mg/ml culture volume for 4 h, after which the medium was removed, and the dye was solubilized in dimethyl sulfoxide. Except where otherwise noted, the dose of IR used was 10 Gy. Absorbance was measured at 515 nm and normalized to untreated control cells by the following equation:

$$P = \frac{A - A_0}{A_{\text{control}} - A_0}$$

where P is the proliferation relative to control, A is absorbance at 515 nm (A₅₁₅), A₀ = A₅₁₅ at T = 0 h, and A_{control} = A₅₁₅ for control cells (unirradiated, grown in 10 ng/ml VEGF-165).

Clonogenic Assays. Clonogenic assays were performed as described previously (13). Briefly, HUVECs and LLCs were plated in EGM-2 medium. Eighteen h after plating, HUVEC medium was replaced with serum-free medium containing no bFGF, to which a defined amount (0–100 ng/ml) of recombinant VEGF-165 (R & D Systems) had been added. Four h later, cells were irradiated with doses of 0–1000 cGy using a cobalt source. Cells were incubated for 48 h, after which medium was replaced with complete EGM-2 containing 10 ng/ml VEGF. After 14–17 days, cells were stained with crystal violet. Colonies were counted, and surviving fractions were determined. Colonies containing >50 cells were scored as positive.

Data Analysis. Statistical significance was determined using one-way ANOVA or Student's t test, as appropriate.

Results and Discussion

We examined the production of VEGF by LLC tumors *in vivo* after exposure to IR. LLC tumors were established in the hindlimbs

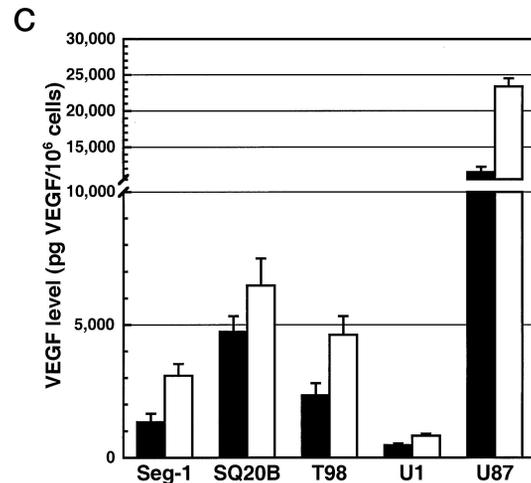
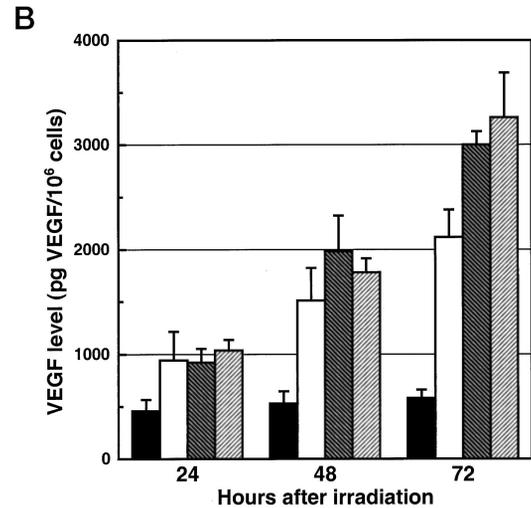
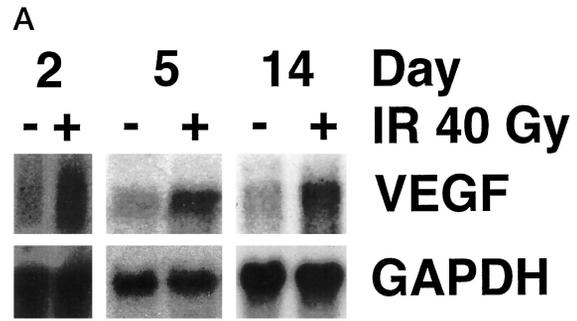


Fig. 1. VEGF levels in LLC and human tumor xenografts. A, VEGF mRNA levels in LLC tumors after IR exposure. LLC tumors were exposed to 40 Gy (two 20-Gy fractions). Total RNA was isolated from irradiated tumors and probed with a cDNA encoding human VEGF-165, after which they were stripped of probe and re probed with a cDNA to glyceraldehyde-3-phosphate dehydrogenase to demonstrate message integrity. Blots from a representative experiment are displayed. B, VEGF protein levels in LLC conditioned medium after IR exposure. LLCs were plated in six-well plates at 25% confluence, allowed to attach overnight, and then irradiated with 0, 5, 10, or 20 Gy. Conditioned medium was collected every 24 h, and VEGF levels were normalized to cell number. A dose-dependent increase in VEGF secretion was observed for all doses of IR (P < 0.05). ■, 0 cGy; □, 500 cGy; ▨, 1000 cGy; ▩, 2000 cGy. Data are presented as means; bars, SE. C, VEGF expression in human tumor cell lines. Subconfluent human tumor cells (Seg-1 esophageal adenocarcinoma, SQ20B squamous cell carcinoma, U1 melanoma, and U87 and T98 glioblastoma) were exposed to 10 Gy. Conditioned medium from irradiated and unirradiated cells was collected 24 h later. VEGF levels in conditioned media were measured by ELISA and normalized to cell number. An IR-dependent increase in VEGF secretion was observed in each cell line: Seg-1 (P = 0.02), SQ20B (P = 0.08), T98 (P = 0.02), U1 (P = 0.009), and U87 (P = 0.0009). No VEGF was detectable in medium unconditioned by cells. ■, no IR; □, 1000 cGy. Data are presented as means; bars, SE.

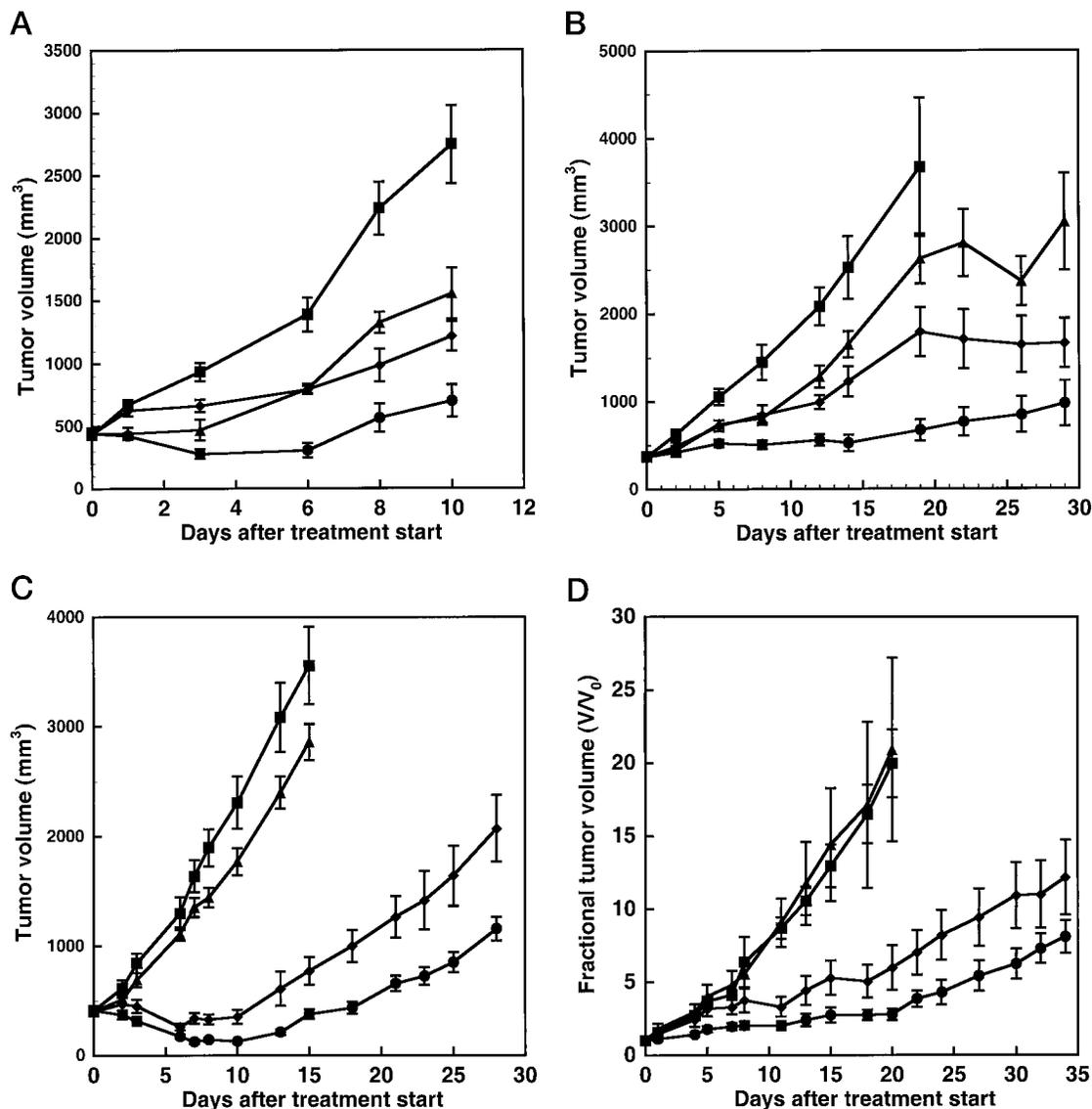


Fig. 2. Effect of VEGF blockade before treatment with IR in murine tumors and human xenografts. Tumor-bearing mice were treated with neutralizing anti-VEGF antibody, IR, or a combination of the two, and tumor volumes were measured (see "Materials and Methods"). ■, control; ◆, IR alone; ▲, anti-VEGF antibody alone; ●, IR combined with anti-VEGF antibody. Data are presented as the means; bars, SE; *P*s are for combined therapy relative to IR alone. A, LLC. Mice were divided into experimental groups ($n = 5/\text{group}$) and treated with: 40 Gy administered as two 20-Gy fractions on days 0 and 1; 40 Gy plus polyclonal goat anti-mouse VEGF-164 antibody administered 3 h before each fraction; or goat anti-mouse VEGF-164 antibody alone. The experiment was repeated three times. There was a statistically significant difference between the IR alone and combined treatment groups for all days after day 3 ($P < 0.02$ for all days). B, SQ20B squamous cell carcinoma xenografts. Mice were divided into experimental groups ($n = 6\text{--}8/\text{group}$) and treated as follows: 40 Gy administered as four 10-Gy fractions on days 0, 1, 2, and 3; 40 Gy plus monoclonal anti-human VEGF-165 antibody administered 3 h before each fraction; or monoclonal anti-human VEGF-165 antibody alone. There was a statistically significant difference between the IR alone and combined treatment groups for all days after day 5 ($P < 0.03$ for all days). C, Seg-1 esophageal adenocarcinoma xenografts. Mice were divided into experimental groups ($n = 8/\text{group}$) and treated as follows: 20 Gy administered as four 5-Gy fractions on days 0, 1, 2, and 3; 20 Gy plus monoclonal anti-human VEGF-165 antibody administered 3 h before each fraction; or monoclonal anti-human VEGF-165 antibody alone administered identically to the combined treatment group. There was a statistically significant difference between the IR alone and combined treatment groups for all days after day 6 ($P < 0.02$ for all days). D, U87 glioblastoma xenografts. Mice were divided into experimental groups ($n = 6/\text{group}$) and treated as follows: 40 Gy administered as eight 5-Gy doses on days 0, 1, 4, 5, 7, 8, 11, and 12; 40 Gy plus monoclonal anti-human VEGF-165 antibody administered 3 h before each dose of IR; or monoclonal anti-human VEGF-165 antibody alone. Results are reported as fractional tumor volumes, with an initial tumor volume of $453 \pm 32 \text{ mm}^3$. There was a statistically significant difference between the IR alone and combined treatment groups for all days after day 11 ($P < 0.05$ for all days).

of female C57BL/6 mice and allowed to grow to a volume of $510 \pm 11 \text{ mm}^3$ (2.5% body weight). They were then irradiated with 20 Gy on days 0 and 1 and harvested at days 2, 5, or 14. VEGF levels in extracts from control tumors remained relatively constant ($46\text{--}90 \text{ pg/mg}$ total protein) for 14 days (Table 1). By contrast, on day 2, the mean VEGF level in irradiated tumors was increased more than 3-fold compared with that in unirradiated tumors ($234 \pm 79 \text{ pg/mg}$ total protein, $P = 0.032$). The mean VEGF level in irradiated tumors remained 2.2-fold higher than that in unirradiated tumors at day 14 ($194 \pm 47 \text{ pg/mg}$ total protein, $P = 0.027$). Plasma VEGF levels remained low or undetectable in both control and irradiated animals (data not shown). To confirm the effects of

IR, VEGF mRNA levels were assessed in the same tumors by Northern blot analysis. VEGF transcripts were induced 3-fold 2 days after exposure to IR and remained elevated for 14 days (Fig 1A).

To determine whether IR induces VEGF in tumor cells, subconfluent LLC cells were exposed to different doses of IR. Conditioned medium was harvested at various intervals for measurement of VEGF levels by ELISA. VEGF levels in LLC-conditioned media exhibited an IR dose-dependent increase within 24 h. By 72 h, VEGF levels were elevated 6-fold over control in media from LLC irradiated with 20 Gy (Fig. 1B). VEGF expression *in vitro* was also measured in irradiated human tumor cell lines: Seg-1 (esophageal adenocarci-

Table 2 Effect of combining anti-VEGF antibody and ionizing radiation

Tumor	Day ^b	Fractional tumor volume relative to untreated controls ^a				
		Ionizing radiation	Anti-VEGF antibody	Combined (expected) ^c	Combined (observed)	Observed/expected ^d
LLC	6	0.570	0.574	0.327	0.220	0.673
Seg-1	13	0.198	0.778	0.154	0.069	0.448
SQ20B	19	0.512	0.715	0.366	0.182	0.497
U87	18	0.302	1.000	0.302	0.166	0.550

^a Obtained by dividing the mean volume of treated tumors by that of untreated controls.

^b The day at which tumors treated with both anti-VEGF and IR demonstrated the most pronounced greater than additive growth inhibition.

^c Obtained by multiplying the fractional tumor volumes obtained by each treatment modality individually to yield the fractional tumor volume expected if the effects of each treatment modality were additive.

^d Obtained by dividing the observed fractional tumor volume by the expected fractional tumor volume. A ratio <1 indicates a greater than additive effect, and a ratio >1 indicates a less than additive effect. Effects were greater than additive for LLC beginning on day 3; for Seg-1, day 6; for SQ20B, day 12; and for U87, day 3.

noma; Ref. 14); SQ20B (a radioresistant squamous cell carcinoma line; Ref. 15); U1 (melanoma); and T98 and U87 (glioblastoma). Under basal conditions, these tumor cell lines secreted widely differing quantities of VEGF, but all demonstrated an IR-dependent increase in VEGF production within 24 h of treatment with 10 Gy (Fig. 1C). These findings demonstrate that IR induces VEGF expression in diverse tumor cell types.

To determine whether induction of tumor VEGF secretion by IR affects the antitumor response, we treated LLC tumors with neutralizing antibodies against murine VEGF-164 before IR exposure. Female C57BL/6 mice bearing LLC tumors were divided into experimental groups and treated as follows: IR alone, 20 Gy on consecutive days (40 Gy total); anti-VEGF (10 μ g 3 h before each fraction); and IR plus anti-VEGF (Fig. 2A). By day 6, consistent with previous observations (5, 6, 8, 9), treatment with anti-VEGF alone produced a 42.6% reduction in tumor volume (796 ± 41 mm³; $P = 0.004$). IR alone produced 43.0% reduction (792 ± 30 mm³; $P = 0.006$). However, the combination of IR and anti-VEGF resulted in a 78.0% reduction (305 ± 58 mm³; $P = 0.001$ relative to IR alone), a greater than additive effect.

To extend these findings to other tumor models, we combined neutralizing anti-VEGF antibody with IR in human squamous cell carcinoma and esophageal adenocarcinoma xenografts. Athymic nude mice bearing radioresistant human head and neck squamous cell carcinoma xenografts (SQ20B; Ref. 15) were treated with IR and a monoclonal neutralizing antibody against human VEGF-165. SQ20B xenografts were treated with IR alone (40 Gy administered as four 10-Gy fractions), anti-VEGF alone (10 μ g i.p. 3 h before each fraction), or combined IR and anti-VEGF (10 μ g administered 3 h before treatment with IR). As observed for LLC, VEGF blockade markedly increased the efficacy of IR in inhibiting tumor growth (Fig. 2B). Next, we examined a xenograft model for a human esophageal adenocarcinoma. Female athymic nude mice bearing Seg-1 xenografts (14) were treated with IR alone (20 Gy in four 5-Gy fractions), anti-VEGF (10 μ g administered 3 h before each fraction), or combined therapy. Again, the antitumor effect of IR was enhanced by anti-VEGF (Fig. 2C). As shown for LLC, the antitumor effects of combined therapy were greater than additive in both human xenografts (Table 2).

Finally, to determine whether this combined approach is effective against a tumor that secretes large quantities of basal and IR-stimulated VEGF, we treated athymic mice bearing U87 glioblastoma xenografts with combined IR (eight 5-Gy fractions, see "Materials and Methods") and anti-VEGF therapy (10 μ g administered 3 h before each IR fraction). By day 18, anti-VEGF produced no detectable tumor growth inhibition, and IR alone inhibited tumor growth by 68.8% relative to untreated controls (Fig. 2D). Combined therapy, however, produced an 83.4% inhibition ($P = 0.046$), also a greater than additive effect.

We examined tumor volume data for all tumors on the day of maximum tumor regression in the combined treatment group relative to the tumor regression observed on the same day for groups treated with each

therapy alone. In each tumor type, the effect of combined therapy was greater than additive (Table 2) beginning early in treatment (for LLC, day 3; Seg-1, day 6; SQ20B, day 12; and U87, day 2) and continued for the course of the experiment. We also examined the growth data in terms of number of days for tumors to regrow to twice the original volume. The time for untreated LLC tumors to grow to twice their initial volume was 2.6 ± 0.5 days. IR alone produced a growth delay of 4.0 ± 1.0 days, whereas anti-VEGF delayed growth to twice initial volume by 3.4 ± 0.7 days. However, combined treatment produced a greater than additive growth delay (9.2 ± 1.4 days, $P = 0.02$). Even greater combined effects were observed for tumor xenografts. For U87 xenografts, time to tumor volume doubling was 2.4 ± 0.6 days and was delayed 1.1 ± 0.3 days by IR and only 0.3 ± 0.1 days by anti-VEGF alone. However, combined treatment produced a growth delay of 9.0 ± 4.3 days ($P = 0.03$). Of note, of the tumors we examined, U87 glioblastoma produced the most VEGF *in vitro*. Similar greater than additive effects of a magnitude between that observed for LLC and U87 were observed for SQ20B ($P = 0.003$) and Seg-1 xenografts ($P = 0.001$; data not shown). These findings demonstrate that blocking the effects of VEGF enhances the tumoricidal effects of IR in diverse tumor models of human malignancies for which IR is a major therapeutic modality, an observation that may have implications for human therapy. Importantly, this effect was observed using a dose of anti-VEGF that by itself had little to no effect on tumor growth, indicating that even a slight inhibition of VEGF action can result in a marked increase in the antitumor effect of IR.

To investigate the mechanism of the effects observed *in vivo* and assess the effects of VEGF on IR-mediated killing of tumor cells and endothelial cells *in vitro*, we measured the survival of HUVECs after exposure to IR. The effect of exogenous VEGF protein on IR-mediated cell killing of HUVECs was assessed by MTT proliferation assay (19) and clonogenic assay (13; Fig. 3). As measured by MTT assay, pretreatment with VEGF protected HUVECs against the cytotoxic effects of 10 Gy in a dose-dependent fashion (Fig. 3A). Clonogenic survival after IR was also increased in a dose-dependent fashion when VEGF was added to the HUVEC culture medium (Fig. 3B). By contrast, adding anti-VEGF to the culture medium before IR exposure decreased HUVEC proliferation in a dose-dependent fashion (Fig. 3C). Neither SQ20B nor LLC proliferation was affected by VEGF or anti-VEGF (Fig. 3C and data not shown). These results demonstrate that IR-induced VEGF production by tumors specifically inhibits the lethal effects of IR on endothelial cells, and that blocking VEGF action increases endothelial cell killing by IR. The effect of manipulating VEGF levels on the radiosensitivity of vascular endothelial cells *in vitro* is on the order of 3–4-fold and therefore relatively modest (Fig. 3). However, the observation that pretreatment of tumor-bearing animals with neutralizing antibody against VEGF at doses that alone are insufficient to cause significant tumor growth inhibition before exposing tumors to IR suggests that this effect is biologically significant *in vivo*.

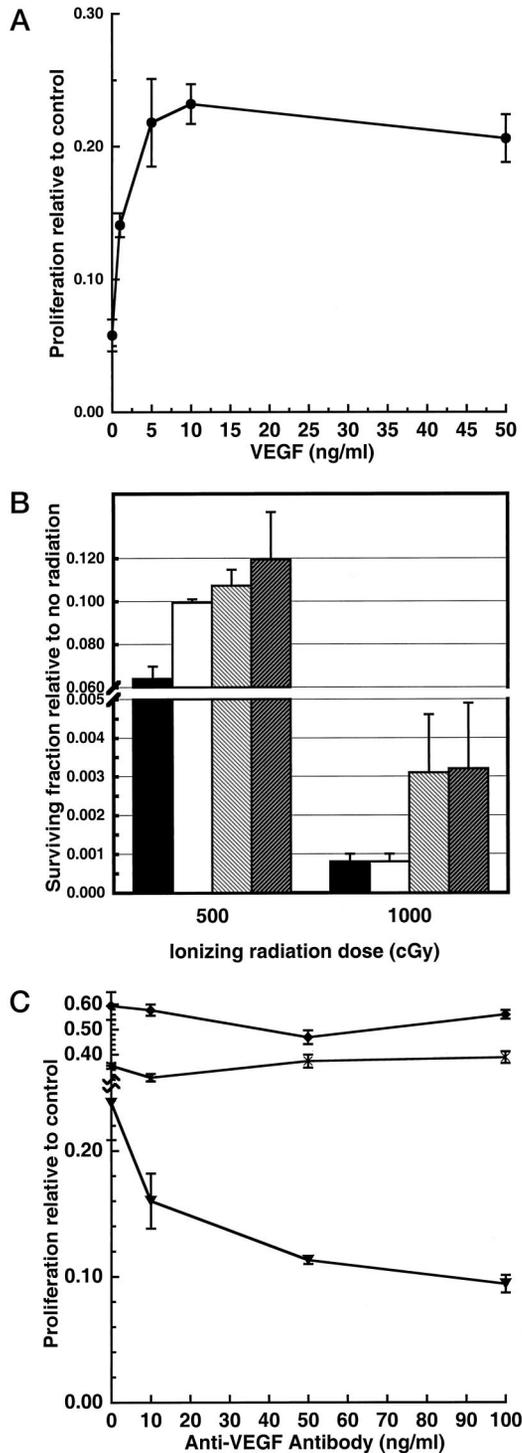


Fig. 3. Effect of manipulating VEGF levels on IR-mediated vascular endothelial cell killing *in vitro*. For MTT assays, HUVECs were plated in 96-well plates at 1×10^3 cells/well and treated with either differing concentrations of recombinant human VEGF-165 or monoclonal anti-human VEGF-165 antibody before exposure to IR, and absorbance readings measured 96 h after IR were done (see "Materials and Methods"). For clonogenic survival assays, HUVECs were treated with different concentrations of VEGF 4 h before irradiation (see "Materials and Methods"). A, MTT assay for HUVECs pretreated with varying concentrations of recombinant human VEGF-165 4 h before IR treatment. Absorbance measurements are normalized to those obtained under standard conditions (no IR treatment and VEGF = 10 ng/ml). B, clonogenic survival assay for HUVECs pretreated with 0, 1, 10, or 100 ng/ml VEGF-165 \pm 500 and 1000 cGy. Surviving fraction is normalized to plating efficiency for unirradiated cells. VEGF protected HUVECs from IR-mediated killing at 500 cGy ($P < 0.05$ for all doses of VEGF) and 1000 cGy (10 ng/ml VEGF, $P = 0.06$; 100 ng/ml VEGF, $P = 0.07$). ■, 0 ng/ml VEGF; □, 1 ng/ml; ▨, 10 ng/ml; ▩, 100 ng/ml. C, MTT assay for HUVECs and SQ20B cells pretreated with monoclonal anti-VEGF-165 antibody 4 h before treatment with 1000 cGy. Absorbance measurements were taken at 96 h after IR and normalized to those obtained with no pretreatment with antibody. VEGF = 10 ng/ml for both cell types. ◆, SQ20B; X, LLC; ▼, HUVECs.

IR is a major therapeutic modality that is primarily effective in the treatment of relatively small tumors, whereas large tumors respond only with considerable toxicity to normal tissues. Our findings demonstrate that IR can induce VEGF expression by experimental tumors, and that this VEGF induction may represent a tumor response to radiation stress. Importantly, blocking the effects of VEGF production by irradiated LLC and human tumor xenografts results in greater than additive antitumor effects *in vivo* for very large experimental tumors (Fig. 2 and Table 2). In addition, VEGF abrogates the killing of endothelial cells by IR, whereas blocking the action of VEGF increases IR-induced killing. These findings support a model in which the disruption of the paracrine relationship between tumor and endothelium enhances the efficacy of IR in killing tumor cells. Our present results, combined with previous results showing that angiostatin and IR also produce greater than additive antitumor effects (12, 13), emphasize the potential importance of combining irradiation with an angiogenesis inhibitor because the tumor cells, tumor stroma, and their interactions are targeted by this combined therapy. Depriving the tumor endothelium of VEGF may thus represent an important strategy to increase the antitumor effects of IR and may indicate that the best use of antiangiogenic therapy is in combination with cytotoxic antitumor therapies such as IR.

Acknowledgments

We thank Drs. Judah Folkman and David Beer for supplying tumor cell lines and Drs. Samuel Hellman, Daniel Haraf, and Mitchell Posner for their support.

References

- Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, 86: 353-364, 1996.
- Ferrara, N., and Davis-Smyth, T. The biology of vascular endothelial growth factor. *Endocr. Rev.*, 18: 4-25, 1997.
- Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.*, 13: 9-22, 1999.
- Thomas, K. A. Vascular endothelial growth factor, a potent and selective angiogenic agent. *J. Biol. Chem.*, 271: 603-606, 1996.
- Asano, M., Yukita, A., Matsumoto, T., Kondo, S., and Suzuki, H. Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor-121. *Cancer Res.*, 55: 5296-5301, 1995.
- Borgstrom, P., Hillan, K. J., Sriramarao, P., and Ferrara, N. Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. *Cancer Res.*, 56: 4032-4039, 1996.
- Goldman, C. K., Kendall, R. L., Cabrera, G., Soroceanu, L., Heike, Y., Gillespie, G. Y., Siegal, G. P., Mao, X., Bett, A. J., Huckle, W. R., Thomas, K. A., and Curriel, D. T. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc. Natl. Acad. Sci. USA*, 95: 8795-8800, 1998.
- Kanai, T., Konno, H., Tanaka, T., Baba, M., Matsumoto, K., Nakamura, S., Yukita, A., Asano, M., Suzuki, H., and Baba, S. Anti-tumor and anti-metastatic effects of human-vascular-endothelial-growth-factor-neutralizing antibody on human colon and gastric carcinoma xenotransplanted orthotopically into nude mice. *Int. J. Cancer*, 77: 933-936, 1998.
- Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth-factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature (Lond.)*, 362: 841-844, 1993.
- Grunstein, J., Roberts, W. G., Mathieu-Costello, O., Hanahan, D., and Johnson, R. S. Tumor-derived expression of vascular endothelial growth factor is a critical factor in tumor expansion and vascular function. *Cancer Res.*, 59: 1592-1598, 1999.
- Gridley, D. S., Lored, L. N., Slater, J. D., Archambeau, J. O., Bedros, A. A., Andres, M. L., and Slater, J. M. Pilot evaluation of cytokine levels in patients undergoing radiotherapy for brain tumor. *Cancer Detect. Prev.*, 22: 20-29, 1998.
- Gorski, D. H., Mauceri, H. J., Salloum, R. M., Gately, S., Hellman, S., Beckett, M. A., Sukhatme, V. P., Soff, G. A., Kufe, D. W., and Weichselbaum, R. R. Potentiation of the antitumor effect of ionizing radiation by brief concomitant exposures to angiostatin. *Cancer Res.*, 58: 5686-5689, 1998.
- Mauceri, H., Hanna, N., Beckett, M., Gorski, D. H., Staba, M. J., Stellato, K. A., Bigelow, K., Heimann, R., Gately, S., Dhanabal, M., Soff, G., Sukhatme, V. P., Kufe, D., and Weichselbaum, R. R. Interaction of angiostatin and ionizing radiation in anti-tumour therapy. *Nature (Lond.)*, 394: 287-291, 1998.
- Hughes, S. J., Nambu, Y., Soldes, O. S., Hamstra, D., Rehemtulla, A., Iannettoni, M. D., Orringer, M. B., and Beer, D. G. Fas/APO-1 (CD95) is not translocated to the cell membrane in esophageal adenocarcinoma. *Cancer Res.*, 57: 5571-5578, 1997.
- Weichselbaum, R. R., Dahlberg, W., and Little, J. B. Inherently radioresistant cells exist in some human tumors. *Proc. Natl. Acad. Sci. USA*, 82: 4732-4735, 1985.
- Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, 162: 156-159, 1987.
- Mukhopadhyay, D., Knebelmann, B., Cohen, H. T., Ananth, S., and Sukhatme, V. P. The von Hippel-Lindau tumor suppressor gene product interacts with Sp1 to repress vascular endothelial growth factor promoter activity. *Mol. Cell. Biol.*, 17: 5629-5639, 1997.
- Church, G. M., and Gilbert, W. Genomic sequencing. *Proc. Natl. Acad. Sci. USA*, 81: 1991-1995, 1984.
- Erlich, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Res.*, 47: 943-946, 1987.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Blockade of the Vascular Endothelial Growth Factor Stress Response Increases the Antitumor Effects of Ionizing Radiation

David H. Gorski, Michael A. Beckett, Nora T. Jaskowiak, et al.

Cancer Res 1999;59:3374-3378.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/59/14/3374>

Cited articles This article cites 19 articles, 11 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/59/14/3374.full#ref-list-1>

Citing articles This article has been cited by 100 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/59/14/3374.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/59/14/3374>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.