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SU5416 and SU6668 Attenuate the Angiogenic Effects of Radiation-induced Tumor Cell Growth Factor Production and Amplify the Direct Anti-endothelial Action of Radiation *in Vitro*

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ABSTRACT

In recent decades, radiation research has concentrated primarily on the cancer cell compartment. Much less is known about the effect of ionizing radiation on the endothelial cell compartment and the complex interaction between tumor cells and their microenvironment. Here we report that ionizing radiation is a potent antiangiogenic agent that inhibits endothelial cell survival, proliferation, tube formation and invasion. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor were able to reduce the radiosensitivity of endothelial cells. Yet, it is also found that radiation induces angiogenic factor production by tumor cells that can be abrogated by the addition of antiangiogenic agents. Receptor tyrosine kinase inhibitors of Flk-1/KDR/VEGFR2, FGFR1 and PDGFRβ, SU5416, and SU6668 enhanced the antiangiogenic effects of direct radiation of the endothelial cells. In a coculture system of PC3 prostate cancer cells and endothelial cells, isolated irradiation of the PC3 cells enhanced endothelial cell invasiveness through a Matrigel matrix, which was inhibited by SU5416 and SU6668. Furthermore, ionizing radiation up-regulated VEGF and basic fibroblast growth factor in PC3 cells and VEGFR2 in endothelial cells. Together these findings suggest a radiation-inducible protective role for tumor cells in the support of their associated vasculature that may be down-regulated by coadministration of angiogenesis inhibitors. These results rationalize concurrent administration of angiogenesis inhibitors and radiotherapy in cancer treatment.

INTRODUCTION

Tumor growth is dependent on angiogenesis (1, 2). Consequently, there has been significant growth in interest in prevention of angiogenesis using endogenous inhibitors of angiogenesis (3) or inhibitors of RTKs[2] involved in angiogenesis (4, 5). Recent studies have revealed the antiangiogenic potential of conventional chemotherapeutic agents (6–8). Low, metronomic dosing of these therapeutics have been found to increase damage to proliferating ECs and, thereby, act as an antiangiogenic treatment.

Radiotherapy is the most important nonsurgical treatment for cancer. Interestingly, for many tumors, the standard radiation treatment schedule can, in fact, be considered radiotherapy with low metronomic dosing. For approximately 50 years, radiotherapy has typically consisted of a daily dose of 2 Gy, 5 times weekly, for a total dose of up to 60 Gy over 6 weeks or so. Historically, this schedule is not the result of a theoretical formulation, but rather the empirical result of clinical studies seeking to optimize clinical outcome. Important questions arising from these observations are whether microvascular ECs function as important targets of ionizing radiation and, more generally, how radiation affects communication between a tumor and its microenvironment.

Radiation research has primarily concentrated on the cancer cell compartment. Relatively little attention has been paid to the effect of radiation on the EC compartment and the complex interaction between the tumor and its microenvironment, consisting of extracellular matrix, cytokines, and ECs. Three objectives, therefore, warrant greater focus:

1. Examination of effects of radiotherapy on microvascular ECs and the role of VEGF and bFGF in this interaction.
2. Determination of how the two-compartment system, consisting of tumor and ECs, intercommunicates in response to radiation therapy.
3. Elucidation of the mechanisms behind the effective combination of antiangiogenic agents and radiotherapy on the ECs.

The conventional explanation of the effectiveness of radiotherapy is that tumor cell DNA is the principal target of ionizing radiation. Similarly, the side effects of radiotherapy are presumed to be attributable primarily to radiation damage to normal cell DNA, although this scenario has recently been questioned by the observation that a single large dose of radiation selectively damaged the ECs of the gut microvasculature (9), leading to the death of epithelial stem cells as a secondary event.

VEGF and bFGF, potent angiogenic cytokines, appear to be important in modulating the effects of radiation on ECs (10–13). VEGF is a known survival factor for ECs and selectively induces EC proliferation, migration, and tube formation (14). In clinical settings, high VEGF levels have been associated with poor prognosis and poor therapeutic outcome in various human cancers, such as malignant gliomas (15). Interestingly, these types of cancers are often described as highly resistant to radiation treatment. There is also evidence that proangiogenic factors such as bFGF may inhibit radiation-induced damage to normal tissue (9, 16).

Recently, several groups have shown in preclinical studies that combining antiangiogenic agents with ionizing radiation improves the antitumor effect of radiation (11, 17–21), in particular, by VEGF signaling inhibitors. The first clinical trial to use an antiangiogenic agent and radiation has been reported recently (22).

Here we examine the ability of IR to inhibit EC proliferation, tube formation, migration, and clonogenic survival. We also analyze the ability of VEGF and bFGF to act as radioprotectors in ECs. Additionally, we show how the RTK inhibitors SU5416 and SU6668 can reverse these protective effects. In an effort to better mimic *in vivo* conditions, we use a coculture system to demonstrate how selective IR of the tumor compartment may activate ECs via VEGF and bFGF release. Accordingly, we show that the VEGFR2 is up-regulated in ECs, and SU5416 and SU6668 were able to prevent radiation-

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[2] The abbreviations used are: RTK, receptor tyrosine kinase; EC, endothelial cell; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IR, ionizing radiation; HUVEC, human umbilical vein endothelial cell; HDMEC, human dermal microvascular endothelial cell; MPM, modified Promocell medium; TK, tyrosine kinase; FACS, fluorescence-activated cell sorting; PE, plating efficiency; GF, growth factor.

dependent tumor-cell induction of EC invasiveness. By combining these findings, we propose a tumor/endothelium communication model for the tumor-survival mechanism in radiotherapy and suggest how concurrent antiangiogenic therapy may ameliorate the process by interfering with this communication.

MATERIALS AND METHODS

Reagents and Cell Culture. Primary isolated HUVECs and HDMECs (Promocell, Heidelberg, Germany) were cultured up to passage 9. Cells were maintained in culture at 37°C with 5% CO_2 and 95% humidity in serum reduced (5% FCS) MPM supplemented with 2 ng/ml VEGF and 4 ng/ml bFGF (Promocell). This combination of GFs (VEGF and bFGF) optimized growth kinetics. Human prostate tumor cells (PC3; Tumorbank DKFZ, Heidelberg, Germany) were cultured in DMEM medium (10% FCS). Human recombinant VEGF and bFGF proteins were purchased from Promocell.

The angiogenesis inhibitors SU5416 and SU6668 were synthesized at SUGEN Inc. (South San Francisco, CA) as described previously (4, 5). SU5416 is an ATP-competitive inhibitor of the Flk-1/KDR/VEGFR2 RTK, with a K_i value of ~160 nM. It also inhibits PDGFRβ TK with a K_i value of 320 nM, but is significantly less potent for inhibition of FGFR1, with a K_i value of 19.5 μM. SU5416 blocks VEGF stimulated HUVEC proliferation with an IC_{50} value of 40 nM *versus* an IC_{50} value of >50 μM for bFGF stimulated HUVEC proliferation (4). SU5416 inhibited tumor metastases, microvessel formation, and cell proliferation. SU6668 is a potent inhibitor of PDGFRβ, with a K_i value of 8 nM. It also inhibits the kinases of VEGF receptor 2 (Flk-1/KDR) and FGFR1, with K_i values of 2.1 and 1.2 μM, respectively (5).

Endothelial and Tumor Cell Proliferation Assay. HUVEC and HDMEC passages 6–9 were grown to confluence in MPM supplemented with 5% FCS, containing 2 ng/ml VEGF and 4 ng/ml bFGF. PC3 cells were cultured in DMEM supplemented with 10% FCS. Cells were harvested by trypsinization at 37°C and neutralized with trypsin-neutralizing solution. A suspension of 50,000 cells in MPM/DMEM was added to 25 cm^2 flasks (Becton Dickinson, Heidelberg, Germany). The cells were incubated for 24 h at standard conditions, irradiated with doses of 0, 1, 2, 5, and 10 Gy, and incubated for another 72 h. Cells were then dispersed in trypsin, resuspended, and counted in a Coulter counter.

Clonogenic Assay. Endothelial cells (HUVECs and HDMECs) and PC3 cells were grown in MPM (ECs) and DMEM (PC3), respectively. To account for radiation mortality, increasing numbers of cells (10^2 to 5×10^4) were plated in 25-cm^2 flasks. Cells were irradiated with doses of 0–10 Gy using 6 MeV X-rays from a linear accelerator (Primus, Siemens, Erlangen, Germany) at a dose rate of 118 cGy/min. Cultures were returned to the incubator for 14–17 days, after which they were stained with crystal violet (Sigma, Germany), colonies were counted and the surviving percentage was determined for clonogenic survival after correcting for PE.

EC Morphogenesis Assay: Tube Formation. To examine the ability of the ECs to produce tubular structures *in vitro*, 24-well plates were coated with 300-μl Matrigel (Becton Dickinson). This extract of the Engelbreth-Holm-Swarm murine sarcoma, which contains basement membrane components, is liquid at 4°C and forms a gel when warmed to 37°C. When plated on Matrigel, HUVECs (48,000 cells/well) undergo differentiation into capillary-like tube structures in MPM medium (10% FCS) supplemented with VEGF (2 ng/ml) and bFGF (4 ng/ml). Angiogenesis inhibitors were added before radiation. Six hours after the incubation on the Matrigel at 37°C/5% CO_2, the media were aspirated, the cells were fixed and stained with Diff-Quik II reagents (Dade Behring AG, Germany), and the slides examined for EC alignment in pictures taken with a microscope.

Matrigel Invasion Assay. A Matrigel assay was used to assess the migration/invasion ability of ECs after direct radiation of ECs and combined treatment with SU5416. Transwell inserts with an 8-μm pore size were coated with Matrigel (0.78 mg/ml; Becton Dickinson). HUVEC were trypsinized and 200 μl of cell suspension (3×10^5 cells/ml) per condition were added in triplicate transwells. Chemoattractant medium containing various concentrations of VEGF and bFGF (500 μl) was added to the lower wells.

To assess the effects on ECs after selective radiation of adjacent tumor cells, we developed a modified coculture model of the Matrigel invasion assay. PC3 cells were first seeded in 24-well plates. After IR of the PC3 cells, Matrigel-coated transwells with HDMEC were added in the upper compartment and were allowed to migrate toward the PC3 cell compartment. HDMECs were used in the coculture because these cells are microvascular ECs and, thus, resemble as close to *in vivo* conditions as possible. After 18 h of incubation, ECs that had invaded to the underside of the membrane were fixed, stained in thiazine and eosin solution using Diff-Quik II solution, and sealed on slides. Migrating cells were counted by microscopy.

Quantitative Reverse-transcription PCR. HDMECs and PC3 cells were raised and treated as indicated. Total RNA was extracted (Qiagen, RNeasy) and treated with DNase 1 (DNA-free, Ambion) to remove contaminating genomic DNA. First-strand cDNA was reverse-transcribed from total RNA using the cDNA Archive Kit (ABI) and stored at −20°C at a concentration equivalent to RNA at 20 ng/μl until use. cDNA was used at a final concentration of 0.1 ng/μl. The 18S subunit was used as the endogenous control because of its low variability across all samples. The gene cluster VEGFR2 was profiled against treated HDMECs. The primers used were 5′-GTGTAC-CGGTTGGCAAAA-3′ and 5′-ACGATGCAACTGAGGTGG-3′ with the TaqMan probe 5′-CCACAAGGTATTTCAAG-3′. The clusters VEGF-A and bFGF were profiled against treated PC3. The consensus sequences derived from all accession numbers within the UniGene code were used for primer and probe design. The primers used for VEGF-A were 5′-ACGAGGGCCTG-GAGTGTGT-3′ and 5′-CATCACACCATGCAGATTATGCG-3′ with the TaqMan probe 5′-CCCACTGAGGAGTCC-3′. The primers used for bFGF were 5′-CCGACGGCCGAC-3′ and 5′-TCAAGCTACAACTTCAAGCA-GAAGA-3′ with the TaqMan probe 5′-AGAAGAGCGACCCTC-3′. A fluorogenic 5′-nuclease assay and the ABI Prism 7900HAT sequence detection system were used for real time quantitation. Quantitation of relative expression levels is achieved by using standard curves for the threshold cycle amplification reaction of targets and endogenous controls.

Flow Cytometry. At various times, up to 72 h after therapy, FACS analysis (FACScans; Becton Dickinson, San Jose, CA) was performed. Cells were fixed in Hank's solution and 70% ethanol. After concentrating the cells by centrifugation and removing the supernatant, the cells were washed in PBS. Cells were again pelleted and the supernatant was discarded. Next, the cells were resuspended in the staining solution of PBS, RNase, and propidium iodide, and FACS measurement for apoptotic cells was performed.

Statistical Analysis. Student's *t* test was used to compare means. For multiple comparisons ANOVA was used with Fisher's least-significant difference method. All tests were two-tailed.

RESULTS

VEGF and bFGF Abrogate Radiation-induced Proliferation Inhibition in EC. Because EC proliferation is required in tumor angiogenesis, our first series of experiments was designed to study the effects of radiation and the potential modifications of different combinations of VEGF and bFGF on proliferation in our EC system. For proliferation assays without radiation, the cells were plated in collagen I-coated flasks and allowed to adhere for 24 h in MPM supplemented with VEGF (2 ng/ml) and bFGF (4 ng/ml). Then media was changed as indicated, and cells were counted after 72 h. As shown in Fig. 1, VEGF and bFGF induce EC proliferation in a dose-dependent manner. The combination of VEGF and bFGF resulted in a greater effect on EC proliferation than each cytokine alone.

To explore the effect of radiation on HUVEC proliferation, we irradiated cells in the same modified Promocell media as used above, supplemented with both GFs, with single radiation doses (0, 1, 2, 4, and 10 Gy. Immediately after IR, media were changed to different final concentrations and combinations of VEGF and bFGF. The cells were counted 72 h after radiation.

Show in Fig. 1*B* is the radiation-dose dependent inhibition of EC proliferation for all combinations of VEGF and bFGF. The maximum inhibition was 60% at 10 Gy radiation if the 72 h incubation media was GF free. IR with 2 Gy resulted in 30% inhibition in the absence of added GFs. Thus, radiation doses used in the clinic have antiproliferative activity on ECs *in vitro*.

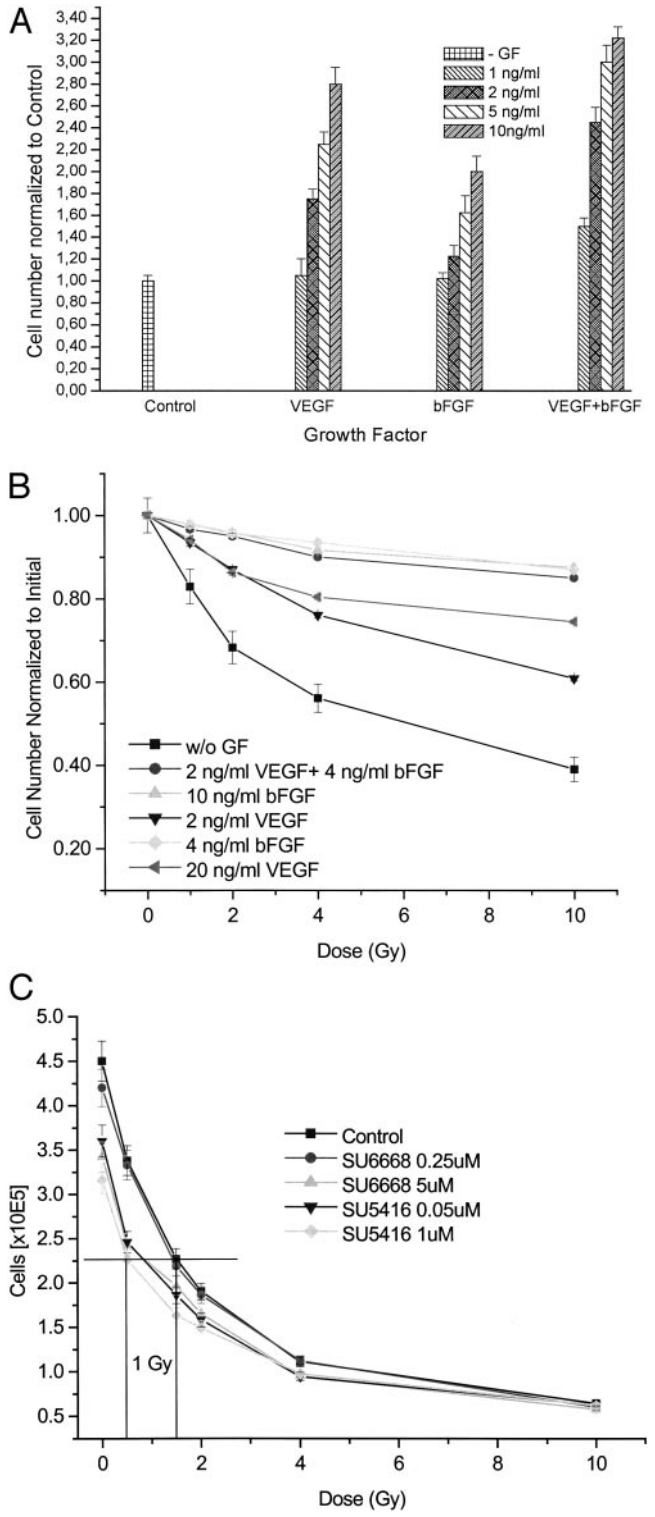

Fig. 1. The effect of VEGF, bFGF, SU5416, and SU6668 on radiation-induced inhibition of HUVEC proliferation. Human recombinant VEGF (2 ng/ml) and bFGF (4 ng/ml) were added to the media until HUVECs were 60% confluent. The cells were then treated as indicated and the number of cells were counted after a 72-h incubation period. *A*, effects of VEGF and bFGF and their combination on proliferation (72-h incubation). *Bars* represent cell numbers from 5–8 plates normalized against control without GFs (mean ± SD). *B*, cell proliferation normalized to initial cell number in response to ionizing radiation. Cells were irradiated in MPM in the presence of VEGF (2 ng/ml) and bFGF (4 ng/ml). Thereafter, different concentrations of GFs (*w/o GF*: no GF supplement) were used and cells were counted after a 72-h incubation (mean ± SD; $n = 5$). *C*, cell proliferation in response to ionizing radiation combined with RTK inhibitors SU5416 and SU6668. SU5416 or SU6668 was added in GF-free medium, and cells were irradiated with 0 to 10 Gy. After 1-h incubation, medium was changed to the final concentration of VEGF (2 ng/ml) and bFGF (4 ng/ml; *Control*: no RTK inhibitor; mean ± SD; $n = 5$), and cells were counted after 72 h.

Increasing doses of VEGF and bFGF in the media during the 72-h incubation period after radiation enhanced endothelial proliferation (Fig. 1*B*). When both GFs were combined, protection against radiation-induced proliferation inhibition was higher than with either factor alone. This is demonstrated by the observation that 2 ng/ml VEGF combined with 4 ng/ml bFGF yielded higher cell numbers than 20 ng/ml VEGF or 10 ng/ml bFGF alone. bFGF appeared to be more effective (based on concentration) in protecting against radiation-induced proliferation inhibition than VEGF, but VEGF alone was also able to markedly decrease the antiproliferative effect of radiation compared with media without GF over the entire radiation dosage range (*e.g.*, 20% more cells at 2 Gy; $P < 0.01$). Thus, both bFGF and VEGF markedly decreased the proliferation radiosensitivity of ECs *in vitro*.

RTK Inhibitors Enhance the Antiproliferative Effect of Radiation on EC. To further characterize the modulatory effect of GF signaling in response to radiation, the RTK inhibitors SU5416 and SU6668 were used. For proliferation assays, the cells were plated in collagen I-coated flasks and allowed to adhere for 24 h in MPM supplemented with 2 ng/ml VEGF and 4 ng/ml bFGF as shown in Fig. 1*B*. Then, SU5416 or SU6668 was added in GF-free medium and incubated for 1 h. Cells were then irradiated in GF-free medium with up to 10 Gy. Thereafter, the medium was removed and new medium added with a final concentration of 2 ng/ml of VEGF and 4 ng/ml of bFGF. As shown in Fig. 1*B*, radiation inhibited EC proliferation in all groups with a maximum inhibition of 90% at 10 Gy (Fig. 1*C*). Both SU5416 (0.05 and 1 μM) and SU6668 (5 μM, but not 0.25 μM) markedly enhanced the antiproliferative effect of radiation over the entire dose range. This additional effect was diminished toward greater radiation doses with a maximum inhibiting effect of 90% with either 10 Gy alone or 10 Gy with RTK inhibitors (Fig. 1*C*). As expected, the more potent VEGFR2 inhibitor, SU5416, was active at a lower concentration than SU6668 (0.05 μM *versus* 5 μM, respectively). The shift to the left of the radiation dose proliferation response curve induced by the addition of RTK inhibitors resulted in a therapeutic gain of ~1 Gy in a clinically relevant dosage range. The observation that 0.5 Gy with RTK inhibitor were isoeffective to 1.5 Gy without RTK inhibitor is shown in Fig. 1*C*. The comparison of Fig. 1*B* and *C* also shows that the presence or absence of GFs during IR itself had also influenced EC proliferation: when GFs were absent during IR, proliferation inhibition was higher (maximum of 90%; Fig. 1*C*) than in the presence of GFs (60%; Fig. 1*B*), irrespective of GFs in the media during the 72-h incubation period after radiation. Thus, it is conceivable that GFs cannot rescue ECs if given after radiation.

GFs Promote PE of EC Reversed by RTK Inhibition. PE and clonogenic survival are criteria considered to be the standard determination of cell survival in response to ionizing radiation. Therefore, we first examined the effects of GFs on HUVEC PE without radiation. The combination of VEGF and bFGF, as well as each factor alone, increased PE compared with control cells without GF ($P < 0.01$; Fig. 2*A*). The combination of both factors increased PE compared with VEGF alone ($P < 0.02$). SU6668 exhibited a modest, but not significant, reduction of PE of cells supplemented with a combination of VEGF and bFGF ($P < 0.1$). SU5416 significantly reduced the PE of cells supplemented with VEGF only ($P < 0.01$).

RTK Inhibitors Increase Radiosensitivity of EC in Clonogenic Survival Assay. The clonogenic survival of HUVEC in the presence of VEGF and bFGF yielded the typical shouldered radiation dose-response curve (Fig. 2*B*). The absence of either VEGF or bFGF (Fig. 2*C*) significantly reduced the survival fraction compared with radiation alone (40% survival *versus* 25% at 2 Gy, 15% *versus* 6% at 4 Gy; $P < 0.02$). No significant difference in clonogenic survival was detected between VEGF withdrawal and bFGF withdrawal ($P > 0.5$).

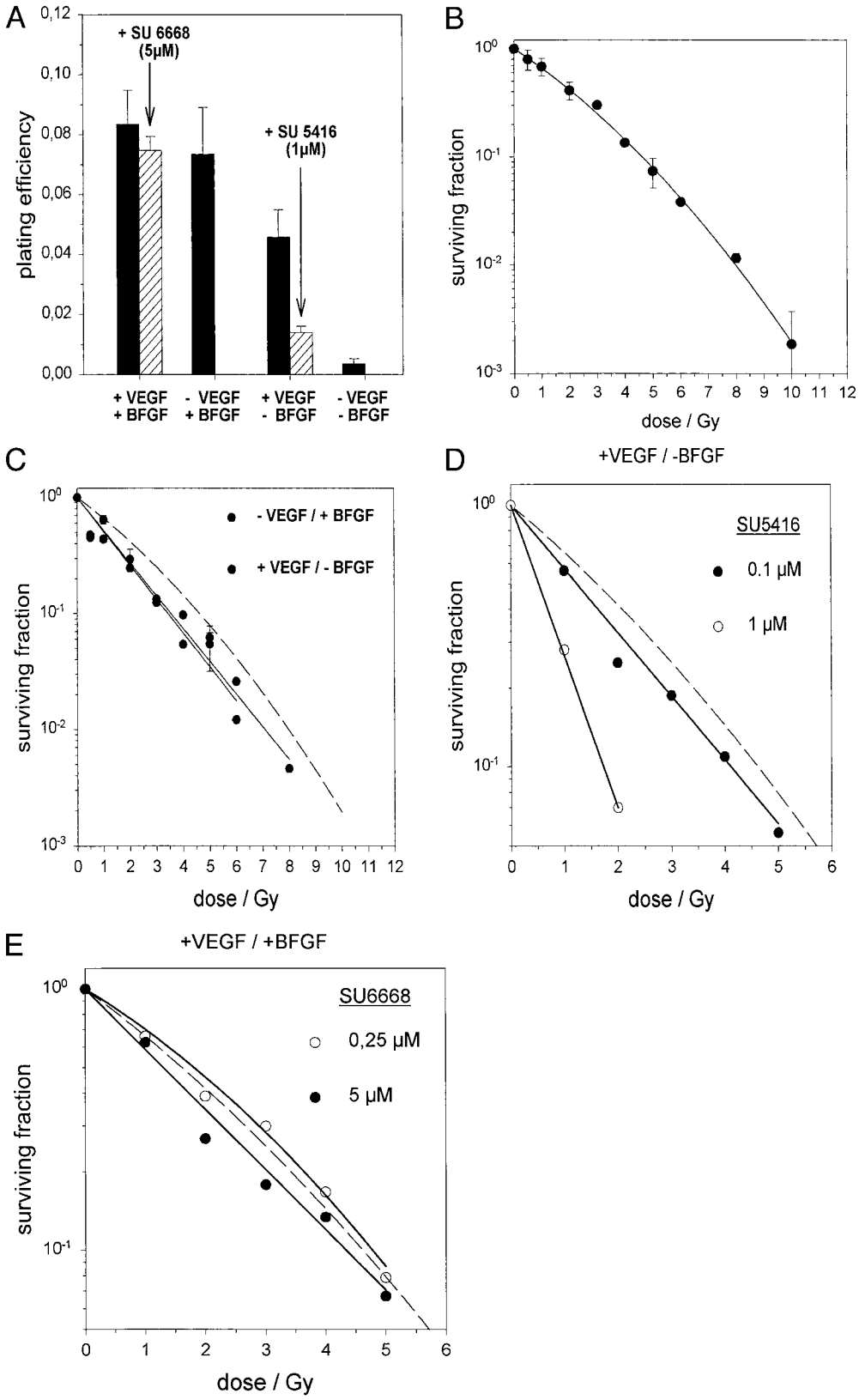

Fig. 2. Clonogenic survival and PE of HUVECs in response to ionizing radiation modulated by VEGF (2 ng/ml), bFGF (4 ng/ml), SU6668, and SU5416. Cells were plated in 25-cm^2 flasks, incubated 2 h with the indicated concentration of medium, and irradiated with single doses of 0 to 10 Gy using 6 MeV X-rays from a linear accelerator (Primus, Siemens, Erlangen, Germany) at a dose rate of 200 cGy/min. PE and surviving percentage were determined. Data represent the mean ± SD of two to three separate experiments, each plated in triplicate. *A*, VEGF and bFGF both increased PE compared with the control without GF ($P < 0.01$). SU5416 significantly reduced PE of cells containing VEGF ($P < 0.01$), but SU6668 reduction of the PE promoted by both factors was not significant ($P > 0.1$). *Solid bars* represent no RTK inhibitors, *hatched bars* represent the presence of RTK inhibitors. *B*, control curve of clonogenic survival of HUVECs. In *C–E*, control curves of clonogenic survival of HUVECs are represented as *dashed lines*. *C*, clonogenic survival in response to combined addition of VEGF and bFGF, or bFGF or VEGF alone. If one factor was missing, the survival fraction was lowered. *D*, clonogenic survival after treatment with SU5416 in the presence of VEGF, demonstrating decreased clonogenic survival at 0.1 and 1 μM ($P < 0.05$ and $P < 0.01$ *versus* control at 1 Gy). *E*, clonogenic survival after treatment with SU6668 in the presence of VEGF and bFGF, demonstrating decreased clonogenic survival at 5 μM, but not at 0.25 μM ($P < 0.05$ and $P > 0.5$ *versus* control at 2 Gy).

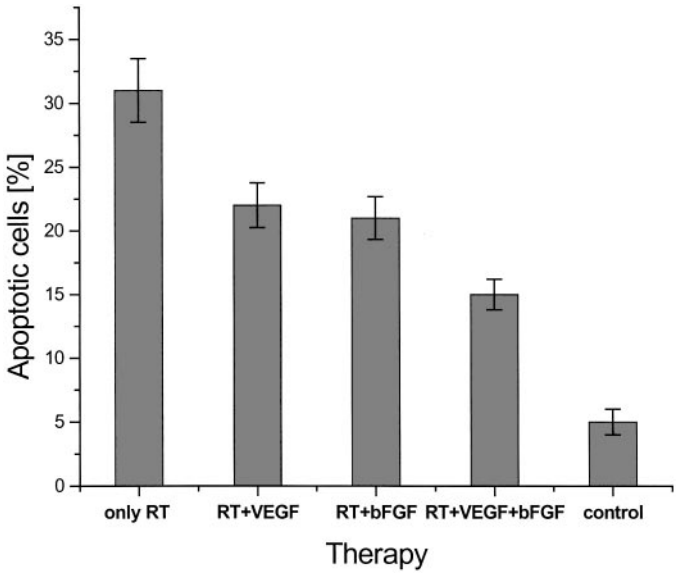

Fig. 3. Percentage of apoptotic HUVECs after treatment with radiation and with or without GF supplement. After growing in MPM, cells were allowed to adhere for 24 h in 25-cm^2 flasks and irradiated with 6 Gy in the presence of VEGF (2 ng/ml) and bFGF (4 ng/ml). Immediately after IR, the medium was changed to different concentrations of GFS (no GF, 2 ng/ml VEGF, 4 ng/ml bFGF, or both GFs). Cells were removed after 24 h, stained with propidium iodide, and analyzed by FACS for apoptotic cells. *Bars* represent means ± SD, $n = 6$. ($P < 0.02$ for comparison between RT only and each GF combination, $P < 0.05$ for comparison between combined GF and single GF, $P > 0.5$ for comparison between VEGF and bFGF.)

Thus, the withdrawal of either VEGF or bFGF enhances the radiosensitivity of HUVEC (Fig. 2*C*). Because of the very low PE (Fig. 2*A*) and short survival of HUVEC in the absence of both VEGF and bFGF, no meaningful curve for clonogenic survival could be generated under this condition.

That the impact on clonogenic survival by withdrawal of GFs can be imitated by the addition of an RTK inhibitor to control media is shown in Fig. 2*D* and *E*. The addition of SU5416 decreases the surviving fraction compared with radiation alone in a SU5416 dose-dependent manner. At 2 Gy, clonogenic survival decreased from 40% with control media to 25% at 0.1 μM SU5416 and further to 6% at 1 μM SU5416. This result indicates that SU5416 markedly increases the clonogenic radiosensitivity of HUVEC (Fig. 2*D*). The effects seen with SU6668 were milder, demonstrating significantly reduced clonogenic survival at 5 μM, but not at 0.25 μM in the presence of both VEGF and bFGF, compared with radiation only (Fig. 2*E*). If neither VEGF nor bFGF were added to the media, no clonogenic survival could be determined, emphasizing the importance of the GF for EC survival.

GFs Reduce Radiation-induced Apoptosis in EC. To investigate potential mechanisms of the protective effect of GFs against radiation damage, the apoptosis rate of irradiated HUVECs was analyzed in the presence or absence of GFs. The observation that 6 Gy radiation significantly induced apoptosis compared with untreated control cells (31% apoptotic cells *versus* 5%, $P < 0.01$) is demonstrated in Fig. 3. If the cultures were supplemented with GF immediately after IR, the apoptotic rate decreased to 22% (VEGF), 21% (bFGF), and 15% (VEGF + bFGF), respectively. No significant difference was seen between the addition of VEGF or bFGF ($P > 0.5$), although the combination protected against radiation-induced apoptosis better than each single factor ($P < 0.05$). Qualitatively similar results were obtained with Hoechst staining of HUVEC cytospins and counting the apoptotic cells under the microscope (data not shown). These findings indicate that both VEGF and bFGF may protect ECs from radiation-induced cell killing, at least in part, by blocking radiation-induced apoptosis.

SU5416 Enhances Radiation-induced Inhibition of EC Tube Formation. The ability of ECs to produce tubular structures is an important step in angiogenesis. Therefore, the effect of radiation and SU5416 on EC tube formation was examined. As shown in Fig. 4, control HUVEC plated on Matrigel and incubated with control media containing VEGF and bFGF aligned to form lumen-like structures and

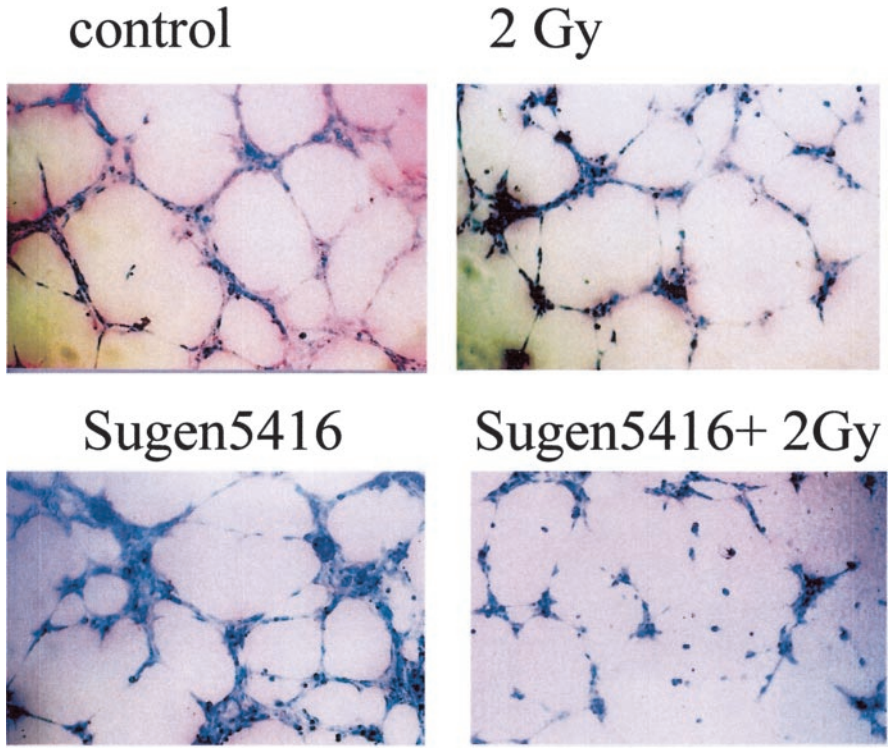

Fig. 4. Radiation and RTK inhibitors reduce EC tube formation. HUVECs (4.8×10^4 cells) were resuspended for 1 h in control MPM supplemented with VEGF (2 ng/ml) and bFGF (4 ng/ml; *A*), or, in addition, during this hour irradiated with 2 Gy (*B*), or, in addition, treated with 1 μM SU5146 (*C*), or, in addition, irradiated and treated with 1 μM SU5416 (*D*). Cells were plated on 24-well plates coated with Matrigel as described in "Materials and Methods." After 6 h, the media were gently aspirated, and the cells were fixed and stained. The slides were examined for EC tube formation by microscopy (×60–100). Representative figures are shown.

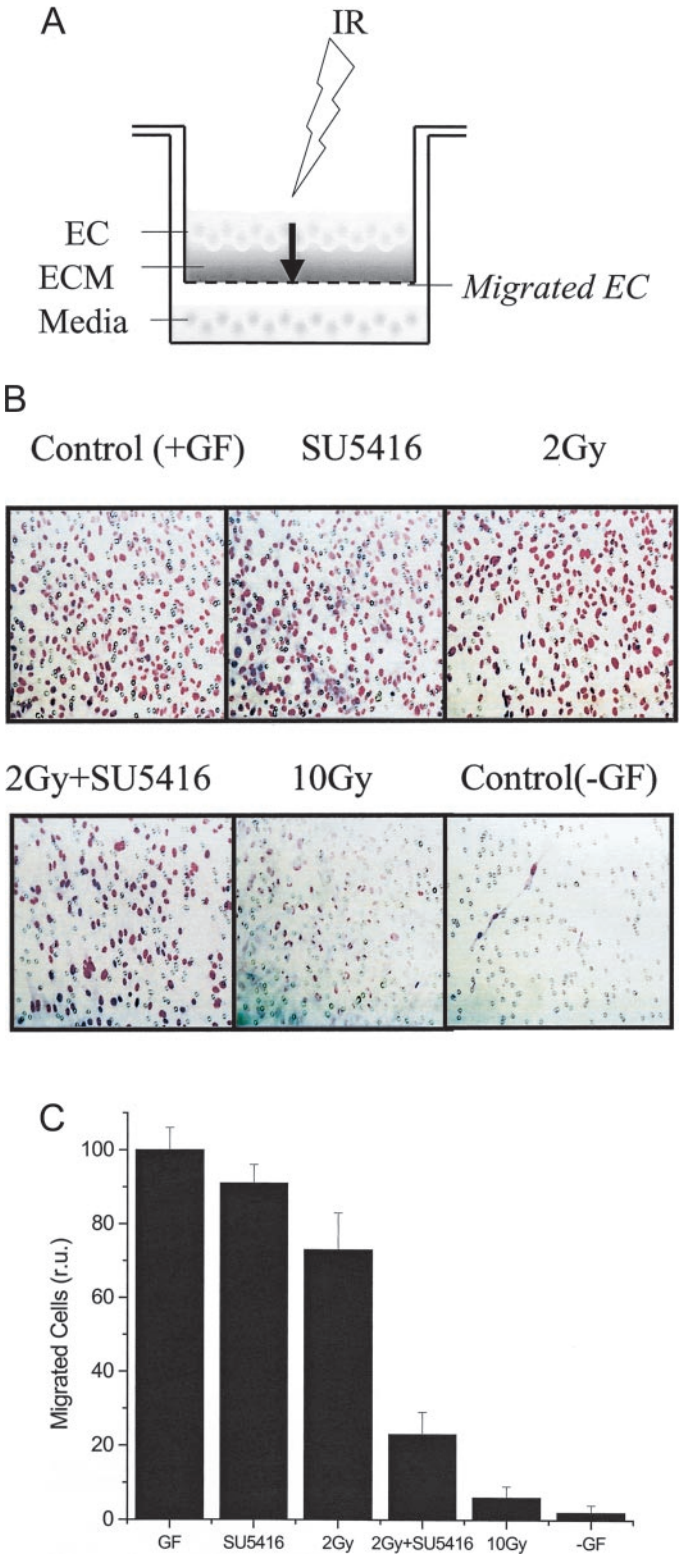

Fig. 5. *A*, schematic diagram of the Matrigel assay. Radiation and SU5416 inhibit EC migration and invasion. ECs were pipetted into inserts of Matrigel-coated transwells, with the lower chambers containing either MPM control medium with or without GFs (GF: 2 ng/ml VEGF, 4 ng/ml bFGF), GF +1 μM SU5416, GF +2 Gy radiation, GF +2 Gy +1 μM SU5416, GF +10 Gy, or media without GFs (*-GF*). Chemotaxis assays (n = 6/sample) were performed as described in "Materials and Methods." Representative figures of migrating HUVECs are shown (*B*). The number of cells that migrated were counted by microscopy and the data are presented as the mean ± SE per field (×60), and were normalized to the media with GF (*C*).

anastomosing tubes with multicentric junctions. HUVEC cultures irradiated with 2 Gy formed fewer tubes, as well as fewer and weaker anastomoses. SU5416 similarly inhibited EC morphogenesis. When both treatments, SU5416 and 2 Gy radiation, were combined, the resulting capillary-like network of ECs appeared less developed and weaker than that induced after each treatment alone, suggesting additive inhibition of tube formation.

SU5416 Enhances Radiation-induced Inhibition of EC Migration and Invasion. Because EC migration and invasion are critical for tumor angiogenesis, the invasion of cells through Matrigel-coated transwell inserts was examined (Fig. 5a). As shown in Figs. 5 b and c, radiation decreased HUVEC invasion in a dose-dependent manner, with 27% reduction at 2 Gy ($P < 0.02$, *versus* control) and an almost complete inhibition, with 94% reduction, at 10 Gy ($P < 0.01$, *versus* control). SU5416 (1 μM) reduced invasion by 9% ($P < 0.05$). Interestingly, a combination of 2 Gy and SU5416 (1 μM) showed a more than additive reduction of HUVEC invasion (expected 34% reduction, observed 77% reduction; $P < 0.02$ compared with 2 Gy alone) (Table 1).

SU5416 and SU6668 Reverse EC Invasion Induced by Radiation of Tumor Cells. It has been observed for many tumor types that radiation increases the secretion of cytokines such as VEGF and bFGF by tumor cells, which, in turn, may increase the angiogenic response of the tissue (14). This effect may be considered the self-protection of the tumor cells or a "survival mechanism" for radiation damage. To model this *in vitro*, a two-compartment coculture invasion assay was used, with the human prostate cancer cell line, PC3, as the chemotactic "agent." After IR of the PC3 cells, Matrigel-coated transwells with HDMECs were added in the upper compartment, and the ECs were allowed to migrate toward the PC3 cell compartment (Fig. 6*A*). The data indicate that radiation rendered PC3 tumor cells chemotactic and invasion promoting for ECs in a radiation dose-dependent manner (Fig. 6*B*). The addition of either SU5416 or SU6668 to the EC media resulted in a marked reduction of EC invasion, suggesting an involvement of the VEGF pathway in radiation-induced PC3 activation. As shown in Fig. 6*B*, the increase of migrating cells was almost completely suppressed by both RTK inhibitors at the lower radiation dose of 2 Gy, but not at the higher dose of 10 Gy. Because 2 Gy is the clinically relevant dose in radiotherapy, this finding may further support a concurrent application of RTK inhibitors and radiotherapy to prevent tumor angiogenesis by suppressing EC migration and invasion induced by radiotherapy itself.

Comparison of Tumor *versus* EC Radiosensitivity. To begin to unravel the effects of ionizing radiation in the complex tumor system, it is important to examine its effects on each component. Therefore, the relative radiosensitivity of tumor and ECs used in the coculture studies above was compared using proliferation and clonogenic assays with HDMECs and PC3. IR decreased proliferation and survival of both cell types. In both tests, the ECs appeared to be more radiation sensitive than the tumor cells (Fig. 6, *C* and *D*).

IR Up-regulates VEGF and bFGF in Cancer Cells, As Well As VEGFR2 in ECs. To provide direct evidence that VEGF and bFGF signal transduction are important in IR-induced, tumor cell-mediated modulation of EC behavior, we used real-time PCR to measure RNA expression of VEGF-A and bFGF in the PC3 cell line. A dose- and time-dependent up-regulation of VEGF of up to 2.4-fold and of bFGF of up to 1.7-fold was observed in PC3 cells after 2 Gy radiation, with a peak expression at ~12 h after 2 Gy radiation (Fig. 7, *A–D*).

To further support the communication model between tumor and endothelium, we used the same method to determine the expression level of VEGFR2 in HDMECs in response to radiation. A time- and dose-dependent up-regulation of VEGFR2 was observed in the HDMECs with a peak expression of 1.5-fold at 8 h after 2 Gy radiation

Table 1 *Migrated endothelial cells as a function of radiation dose delivered to PC3 cells and as a function of media composition for ECs*

Radiation causes PC3 tumor cells to attract ECs in a dose-dependent manner. If the RTK inhibitors SU5416 and SU6668 are added to the endothelial control media, EC invasion can be markedly reduced. The increase of migrated cells by radiation can be suppressed by both RTK inhibitors at the low dose of 2 Gy, but not at the high dose of 10 Gy. Data are mean ± SE ($n = 6$) of migrated cells per field normalized to 100 for control media.

	0 Gy	2 Gy	10 Gy	2 Gy vs. 0 Gy	10 Gy vs. 0 Gy
Control media	100 ± 13	141 ± 16	173 ± 22	$P < 0.05$	$P < 0.01$
+5 μM SU5416	42 ± 5	45 ± 6	75 ± 8	$P > 0.5$	$P < 0.01$
+10 μM SU6668	35 ± 5	39 ± 5	71 ± 9	$P > 0.5$	$P < 0.01$
Each treated *vs.* control	$P < 0.001$	$P < 0.005$	$P < 0.005$		

(Fig. 8*A*). The dose-response curve determined at 4 h after radiation yielded a maximum up-regulation of 1.9-fold at 10 Gy (Fig. 8*B*).

DISCUSSION

This report demonstrates that ionizing radiation is a potent antiangiogenic agent with typical effects for angiogenic inhibitors, including dose-dependent inhibition of EC proliferation, migration/invasion, tube formation, and PE/clonogenic survival. The data further show that VEGF and bFGF inhibition ameliorates these responses *in vitro*. Significantly, radiation protection was enhanced when GFs were used in combination, suggesting a complementarity in their downstream pathways. The ability of radiation to markedly suppress human microvascular EC proliferation at doses relevant to clinical radiotherapy raises the prospect that EC killing likely contributes to the overall tumor-suppressive effect of radiation (9).

Our proliferation studies also suggest that the presence or absence of GFs during radiation influence the inhibitory effects of radiation on proliferation. Without GF supplementation during radiation, the inhibitory effects of radiation on proliferation were more pronounced than with GFs. Likewise, there was a heightened protective effect of GF supplementation to the media after IR when VEGF and bFGF were present during radiation. The extent to which these angiogenic factors spare tumor reduction *in vivo* may be a rough reflection of the component of radiation response that is attributable to vascular targeting.

These present studies have also demonstrated that the abrogation of downstream GF signaling by RTK inhibitors SU5416 and SU6668 reversed the VEGF- and bFGF-induced protective effects. Taken together, the combination of radiation and the RTK inhibitors SU5416 and SU6668 was found to yield greater antiangiogenic effects than each treatment alone. These results support the idea that combined use of radiotherapy and VEGFR inhibitors (or angiogenesis inhibitors in general) may potentially allow a lowering of the radiation doses necessary to achieve local tumor control (17, 21).

The radioprotective effects of VEGF and bFGF in other studies have been argued to arise from reduced EC apoptosis (9, 10, 12, 16) and increased stem cell survival (23). The effects of VEGF on EC survival may be mediated through several different pathways. VEGF may up-regulate the antiapoptotic proteins Bcl-2 and A1 and may also prevent apoptosis by activating the antiapoptotic kinase Akt/PKB via a phosphatidyl-inositol-3 kinase dependent pathway (24). In addition, VEGF was found to maintain survival signals in ECs by tyrosine phosphorylation of focal adhesion kinase or through direct interaction with extracellular matrix components such as $\alpha_V\beta_3$ integrin (25). Mitotic cell death, rather than apoptosis, is often considered to be the dominant form of cell mortality caused by ionizing radiation (26). If apoptosis plays an important role in radiation-induced death in ECs and, subsequently in the vasculature, the radiation effects on the vasculature could account for the major contribution of apoptosis to radiation-induced effects on tumors.

In a coculture invasion model, endothelial invasion was enhanced by selectively irradiating the tumor cell compartment, suggesting that IR has indirect angiogenic properties. This resulted, at least in part, from IR-induced up-regulation of VEGF and bFGF in the PC3 human prostate cancer cell line. This model of elevated paracrine release of VEGF and bFGF may account for the *in vivo* observations that

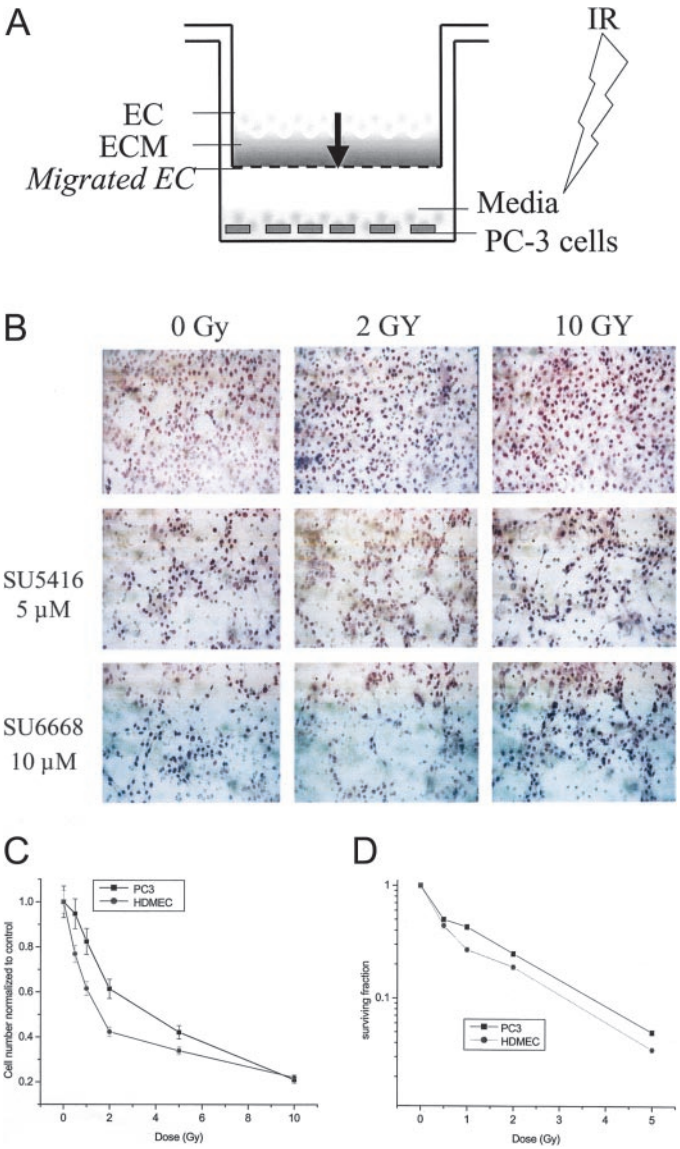

Fig. 6. *A*, schematic diagram of the coculture study. Visualization of migrating ECs that were chemically attracted by irradiated PC3 tumor cells. HDMECs were pipetted into inserts of Matrigel-coated transwells with the lower chambers containing PC3 cells that were irradiated immediately before seeding. ECs were supplemented with either modified Promocell control media or MPM containing 5 μM SU5416 or 10 μM SU6668. *B*, representative figures of migrating HDMECs are shown. Comparison of radiosensitivity of HDMEC and PC3. *C*, proliferation, normalized to untreated control cells, in response to ionizing radiation (mean ± SD; $n = 5$). *D*, HDMEC and PC3 clonogenic survival. In both the proliferation and clonogenic assay the ECs were more sensitive to radiation than the tumor cells.

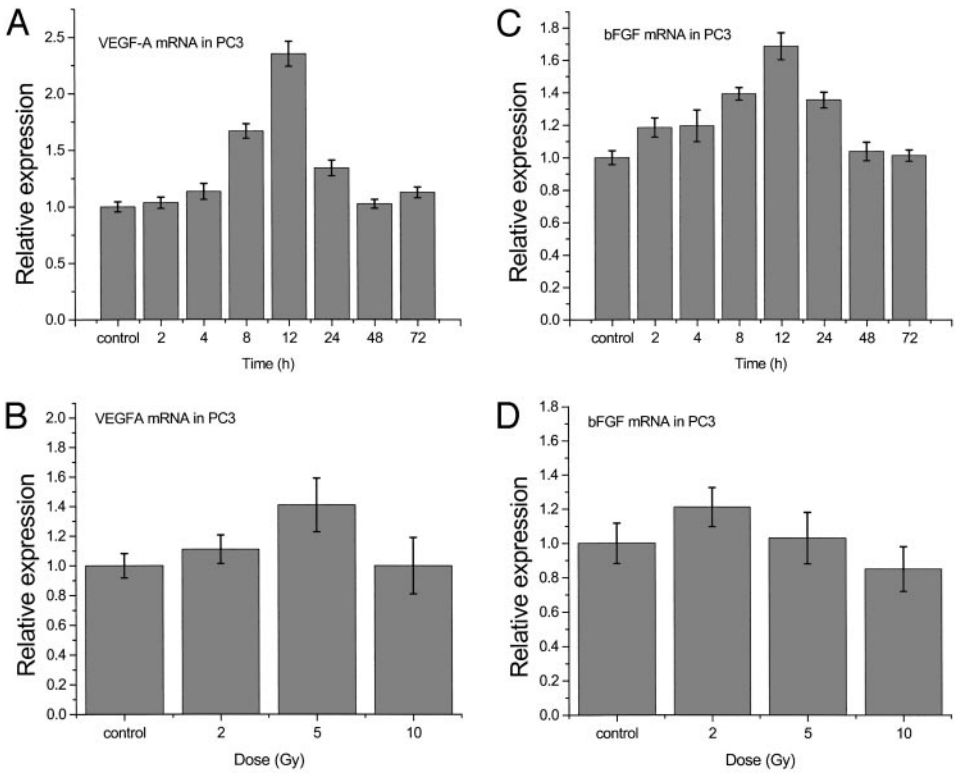

Fig. 7. Relative level of VEGF-A and bFGF mRNA expression in response to radiation in PC3 human prostate cancer cells (mean ± SD). *A*, time course of VEGF-A mRNA after 2 Gy. *B*, dose response of VEGF-A mRNA at 4 h after radiation. *C*, time course of bFGF mRNA after 2 Gy. *D*, dose response of bFGF mRNA at 4 h after radiation.

sublethal doses of tumor IR promote migration and invasiveness of glioblastoma in rats (27, 28).

IR also up-regulated the receptor VEGFR2 in EC. VEGFR2 is considered the key VEGF receptor in tumor angiogenesis. The observation that IR increased the expression of VEGFR2 mRNA in human microvascular ECs and VEGF and bFGF in a human tumor cell line suggests that the tumor cells may transmit an angiogenic stimulus to its associated vasculature in response to radiation. Paracrine GF release by the tumor and the corresponding receptor up-regulation in the endothelium may represent a coordinated mechanism by which primary radiation-induced antivascular effects are attenuated. SU5416 and SU6668 were also able to decrease EC invasion in response to tumor radiation in the coculture model, thus decreasing the indirect angiogenic effects of radiation offers another rationale for the combined use of angiogenesis inhibitors and radiation in cancer therapy.

These results enable us to speculate about the well-known discrepancy between tumor radiosensitivity *in vitro* and tumor response *in vivo* (13). For example, sets of cell lines from the clinically radiosensitive Hodgkin's lymphoma and the clinically radioresistant glioblastoma have similar or overlapping *in vitro* radiosensitivities (29). This would suggest that the clinical radioresistance could result from the fact that, unlike the *in vitro* situation, where the tumor cells are the only radiation targets, different types of supporting cells, including ECs, interact with the tumor compartment *in vivo*. Perhaps certain ECs are more sensitive to ionizing radiation than cancer cells in agreement with our *in vitro* findings in HDMEC endothelial and PC3 prostate cancer cells and as reported by others (9). Likewise, it has been reported that ECs are more susceptible to the chemotherapeutic agent vinblastine than cancer cells (6). Furthermore, our tumor-endothelium communication data suggest that the tumor compartment can produce survival factors for ECs by paracrine signaling from radiation damage. The implication is that clinically radioresistant and radiosensitive tumors may differ, at least in part, because of differences in their ability to protect their vasculature. With respect to the consequences of clinical radiotherapy, the coculture data may also suggest that radiation effects may not necessarily be restricted to the site of physical radiation dose distribution, but can enhance tumor angiogenesis and tumor promotion outside the directly irradiated fields. The effects of radiotherapy can extend beyond the death of the target

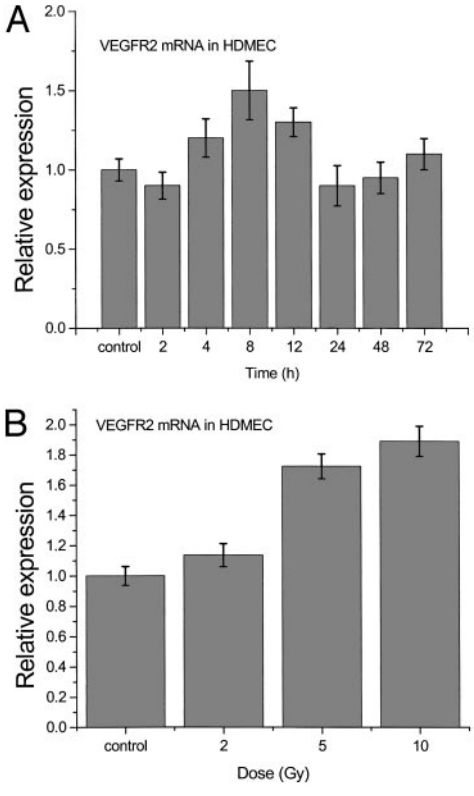

Fig. 8. Relative level of VEGFR2 mRNA in response to radiation in HDMECs in MPM supplemented with 2 ng/ml VEGF and 4 ng/ml bFGF (mean ± SD). *A*, time course after 2 Gy. *B*, dose response at 4 h after radiation.

tumor cells, in that factor production by such cells has been observed to influence the local environment for some time thereafter (30).

In summary, our results have established the basis for a salvage model of how tumors protect their vasculature from radiation-induced damage. It simultaneously rationalizes the use of angiogenesis inhibitors that interrupt VEGF and bFGF signaling concomitantly with radiotherapy in cancer treatment. Our data support the idea of additive effects being obtained when radiotherapy is combined with antiangiogenic therapy. Further experimentation is necessary to determine whether systemic application of VEGF or bFGF (to protect normal tissue) will produce more radioresistant tumor phenotypes that will then require higher radiation doses.

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