Inhibition of Vascular Endothelial Growth Factor Receptor Signaling Leads to Reversal of Tumor Resistance to Radiotherapy

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Abstract

Certain refractory neoplasms, such as glioblastoma multiforme (GBM) and melanoma, demonstrate a resistant tumor phenotype in vivo. We observed that these refractory tumor models (GBM and melanoma) contain blood vessels that are relatively resistant to radiotherapy. To determine whether the vascular endothelial growth factor receptor-2 (Flk-1/KDR) may be a therapeutic target to improve the effects of radiotherapy, we used the soluble extracellular component of Flk-1 (ExFlk), which blocks vascular endothelial growth factor binding to Flk-1 receptor expressed on the tumor endothelium. Both sFlk-1 and the Flk-1-specific inhibitor SU5416 eliminated the resistance phenotype in GBM and melanoma microvasculature as determined by both the vascular window and Doppler blood flow methods. Human microvascular endothelial cells and human umbilical vein endothelial cells showed minimal radiation-induced apoptosis. The Flk-1 antagonists sFlk-1 and SU5416 reverted these cell models to apoptosis-prone phenotype. Flk-1 and sFlk-1 antagonists also returned GBM and melanoma tumor models to radiation-sensitive phenotype after treatment with 3 Gy. These findings demonstrate that the tumor microenvironment including the survival of tumor-associated endothelial cells contributes to tumor blood vessel resistance to therapy.

Introduction

The response of tumor microvasculature to radiation is dependent on the dose and time interval after treatment (1–6). Tumor blood flow does not decrease unless high doses in the range of 20–45 Gy are used (1). Studies of the radiation response in tumor vasculature showed an increased blood volume if doses below 500 rads were administered (6). Blood flow studies (interstitial Xe clearance) of irradiated mouse sarcoma showed that blood flow increased within 3–7 days of irradiation (4). Recent studies suggest that this delayed increase in tumor blood flow may be, in part, due to radiation-induced VEGF expression (7).

VEGF is found to increase in tumors that are resistant to treatment such as malignant gliomas and melanomas, and expression is also associated with worsened prognosis (8). Blocking antibodies to VEGF have been shown to reduce angiogenesis in these tumors (9). VEGF binds to endothelial receptors Flt-1 and Flk-1 to activate a signal transduction cascade, whereas neutralizing antibodies to Flk-1 inhibit angiogenesis (9). This inhibition of angiogenesis was most effective in glioblastoma xenografts and also reduced tumor growth in several tumor models. Endothelial cell proliferation and survival after in vitro irradiation were enhanced by supplementation of VEGF (7), whereas anti-VEGF antibody enhanced cytotoxic effects of irradiation in endothelial cells.

Models to study the role of Flk-1 signaling in radioresistance include melanomas and GBM. GBM has a universally fatal clinical outcome in both children and adults (10–12). In contrast, in vitro studies show that 58 human GBM cell lines have radiosensitivity that is in the range of cell lines from more curable human tumors (13, 14). In contrast, studies of the radiation response in GBM tumors in animal models have shown that the radiosensitivity of GBM tumors in vivo did not correlate with the radiosensitivity of the same cell lines in vitro (13–17). Gliomas are also among the most vascular neoplasms (18, 19). VEGF is one of the angiogenic growth factors that is expressed within GBM. The VEGF-survival phenotype in endothelial cells is mediated by binding to the Flk-1/KDR receptor (20, 21). SU5416 is a potent and selective inhibitor of Flk-1/KDR that blocks tyrosine kinase catalysis and inhibits tumor vascularization and growth (22). The chemical structural formula is 3-[2,4-dimethyl pyrrol-5-yl] methyl indenyl]-2-indolinone with a Kᵢ of 0.16 μM for Flk-1. The effectiveness of this Flk-1 inhibitor to inhibit glioma-associated neo-vascularization has been studied (22). In addition, the soluble Flk-1 receptor (sFlk-1 and sFlt-1) sequesters unbound VEGF, thereby preventing Flk-1 activation (23–27). In this study, we used these antagonists in tumor models and found that the VEGF receptor-signaling pathway enhances the radiation-induced response in tumor blood vessels.

Materials and Methods

Tumor Model. The B16F0 cell line was obtained from American Type Culture Collection (Manassas, VA). B16F0 cells were maintained in HyClone (VWR) MEM/Earle’s balanced salt solution with 10% FCS and 0.5% penicillin-streptomycin from Life Technologies, Inc. GL561 cells were maintained in DMEM with Nutrient Mixture F-12 1:1 (Life Technologies, Inc.) with 1% sodium pyruvate. The GL261 cell line was obtained from Dr. Yancie Gillespie (University of Alabama–Birmingham, Birmingham, AL; Refs. 16 and 28). This cell line forms tumors in C57BL6J mice following s.c. injection into either hind limb (28) or the dorsal skin fold window chamber. Cells were trypsinized and counted by hemocytometer. Cells were washed in complete medium, and 10⁶ cells were injected s.c. into the hind limb or into the dorsal skin fold window. After tumor formation, tumors were irradiated as described previously (28).

Tumor Vascular Window Model. Life Technologies, Inc. penicillin-streptomycin solution (200 μl) was injected into the hind limb of the mouse before the procedure. The dorsal ventral window is a 3-g plastic frame applied to the skin of an animal and remains attached for the duration of the study. The chamber was screwed together, whereas the epidermis was cut and remains open with a plastic covering. The midline was found along the back, and a clip was placed to hold the skin in position. A template equivalent to the outer diameter of the chamber was traced, producing the outline of the incision. A
circumcision. The chamber was lined with a layer of moistened gauze to maintain a moist environment. The top of the chamber was fixed using surgical tape. To ensure the skin was not pinched, the template was removed, and the four flaps were then removed using a scalpel.

Tumor blood vessels developed in the window within 1 week. We studied the time- and dose-dependent response of tumor blood vessels to radiation using the window model. Five mice were entered into each of the treatment groups. The window frame was marked with coordinates, which were used to photograph the same microscopic field each day. Vascular windows were photographed using a ×4 objective to obtain a ×40 total magnification. Color slides were used to catalogue the appearance of blood vessels on days 0–7.

Color slides were scanned into Photoshop software and analyzed by Optimas software. Vascular center lines were positioned by Optimas software and verified by an observer blinded to the treatment groups. Tumor blood vessels were quantified by the use of Optimas software, which quantifies the vascular length density of blood vessel within the microscopic field. The mean and SEM of vascular length density for each treatment group was calculated, and the variance was analyzed by the Kruskal-Wallis test (29, 30).

**Power Doppler Sonography.** Murine hind limb tumors were imaged after irradiation. Tumors were imaged with a 10–5 MHz linear Entos probe attached to an HDI 5000 (probe and HDI 5000 from ATL/Philips, Bothell, WA). Power Doppler sonography images were obtained with the power gain set to 82%. Care was taken to minimize motion artifact. A 20-frame cine-loop sweep of the entire tumor was obtained with the probe perpendicular to the long axis of the lower extremity along the entire length of the tumor. The raw data were transferred over the Radiology network to a personal computer. The color area was quantified using HDI-lab software (ATL/Philips). This software allows direct evaluation of power Doppler cine-loop raw. The color area was recorded for the entire tumor. Five mice were entered into each treatment group. Values for the color area were averaged for each tumor set, and treated groups were compared with controls with the unpaired Student’s t test.

Power Doppler was used to quantify blood flow in 1-cm diameter tumors treated with Flk-1 inhibitors and radiation. Tumor-bearing mice were treated with a minimally effective dosage of SU5416 (0.75 mg) administered by i.p. injection at 1 h before irradiation (2 Gy). SU5416 was injected every other fraction for a total of three doses. Tumors were treated with minimally effective dose of 3 Gy per day for 7 days. Blood flow was quantified by power Doppler on days 4 and 7.

**Adoption of Flk-1 Antagonists.** The VEGF receptor Flk-1 is a receptor tyrosine kinase that is specifically inhibited by the dominant negative expression of the mutant form of the receptor (soluble Flk-1), as described previously (23). The soluble Flk-1 receptor was constructed by fusing the extracellular domain of murine flk-1 to a histidine tag at the COOH terminus (ExFlk.6His; Ref. 23). ExFlk.6His blocked activation of Flk-1 and formed heterodimers with endogenous cell surface Flk-1 in the presence of VEGF (23). ExFlk.6His also inhibited VEGF-induced DNA synthesis and migration in HUVECs. To determine whether ExFlk.6His can inhibit angiogenesis in our dorsal tumor vascular window model, the Ad·ExFlk.6His (2 × 10⁸ pfu) was administered to mice by tail vein.

To determine whether the Flk-1 receptor inhibition alters the radiation response in tumor blood vessels, we used Ad·ExFlk and both the window and Doppler blood flow analysis. We studied the tumor blood vessels in GL261 and B16F0 by use of the dorsal skin fold window model in C57BL6 mice. Tumors were implanted within the window, and tumor blood vessels developed within 1 week. Ad·ExFlk.6His and control Ad·LacZ vectors were administered to tumor-bearing mice at 2 × 10⁸ pfu by tail vein injection. Vascular regression was visualized by use of the dorsal skin fold window model. We combined subtherapeutic doses of radiation (2 Gy) given 24 h after a minimally therapeutic dosage of Ad·ExFlk.6His (2 × 10⁸ pfu). We studied the time course of vascular regression after treatment.

To determine whether Flk-1 kinase inhibitors enhance radiation cytotoxicity, we used the kinase inhibitor SU5416 (22, 31, 32). SU5416 alone, given as a single 0.75-mg i.p. injection, achieved a dose-dependent reduction in tumor blood vessels. We combined subtherapeutic doses of radiation (2 Gy) with a minimally therapeutic dosage of SU5416 to determine whether radiation responses can be modified by Flk-1 inhibition. B16F0 and GL261 tumors were implanted in the dorsal skin fold window in C57BL6 mice, and angiogenesis was observed for 1 week.

**Tumor Volume Analysis.** C57BL6 mice received s.c. injections in the right thigh of 10⁵ viable cells of a murine glioblastoma (GL261) suspended in 0.2 ml of a 0.6% solution of agarose. Each set of six mice was stratified into four groups to control for mean tumor volume. An equal number of large and intermediate-size tumors were present in each group. Mouse tumors were stratified into groups, so that the mean tumor volume of each group was comparable. The mean volume of the tumors in mice at the time of treatment (day 1) with radiation, SU5416, SU5416 + radiation, and control were 0.52 cm³, 0.56 cm³, 0.59 cm³, and 0.49 cm³, respectively. The first group received no treatment (control group) and contained four mice. Each of the other three groups (treatment groups) contained six mice. The second group received radiation therapy. Irradiated mice were immobilized in lucite chambers, and the entire body was shielded with lead, except for the tumor-bearing hind limb. A total dose of 24 Gy was administered in eight fractionated doses on consecutive days. The third group received SU5416 administered by i.p. injection of 0.75 mg. The fourth group received SU5416 on days 1, 3, 5, and 7 of radiation therapy. The radiation therapy was administered in the same manner as the radiation-only group. A second experiment was performed to confirm these findings. Ten mice were stratified into the four treatment groups. Treatment was begun 14 days after the tumor cells had been injected. This experiment has been repeated with 10 mice in each of the treatment groups. The mean volume of the tumors in mice at the time of treatment (day 1) with radiation, SU5416, SU5416 + radiation, and control were 0.90 cm³, 0.99 cm³, 0.90 cm³, and 0.91 cm³, respectively.

Twice weekly tumor volumes were measured using skin calipers, as described previously (15, 16, 29). Tumor volumes were calculated from a formula (a × b × c/2) that was derived from the formula for an ellipsoid (mr³/6). Data were calculated as the percentage of original (day 0) tumor volume and graphed as fractional tumor volume ± SD for each treatment group, and the variance was analyzed by the Kruskal-Wallis test (30).

**Results**

**Tumor Vascular Window Model.** The vascular window model allowed direct measurement of the vascular response to ionizing radiation in tumor blood vessel models. Fig. 1 shows blood vessels within GL261 (A and B) and B16F0 (C and D) implanted into the dorsal skin fold window model. Blood vessels developed over 1 week, at which time they were treated with ionizing radiation (0 h, Fig. 1). Radiation induced dose- and time-dependent reduction in tumor vasculature within the window. GL261 and B16F0 tumor vascular length density has minimal change after irradiation with a standard 2-Gy radiation dose. GL261 vessels have minimal response to 6 Gy (Fig. 1B), and microvasculature of B16F0 tumor requires a dose of 3 Gy to achieve regression (Fig. 1D).

We studied the dose-dependent response of tumor blood vessels to radiation using the window model in C57BL6 mice. Tumor blood vessels were quantified by the use of line morphometry measurements, which sums the length of vessels (24). We quantified tumor blood vessels within B16F0 melanoma tumors, which showed increased vasculature after treatment with 2 Gy. Treatment with 3 Gy reduced tumor vascular length density within 48 h (P < 0.05), whereas 6 Gy induced vascular obliteration within 24 h. Blood vessels within the GL261 glioma showed increased vascular length density in response to 3 Gy (Fig. 1E), whereas 6 Gy reduced vascular length density within 72 h (P = 0.04).

**Doppler Blood Flow Analysis of Tumor Vasculature.** We used power Doppler sonography to study the response of tumor blood vessels to ionizing radiation. This method uses amplitude to measure...
flow in microvasculature. B16F0 melanoma and GL261 glioma tumors were implanted in the hind limb of C57BL6J mice. Tumors were grown to 1 cm in diameter and then irradiated with 2, 3, 6, or 10 Gy. Doppler analysis of tumor blood flow was measured on days 0, 3, and 7. Fig. 2 shows representative color Doppler images at day 3 of GL261 and B16F0 tumors treated with 0, 3, and 10 Gy. After low-dose irradiation (3 Gy) blood flow increased, whereas blood flow decreased after high-dose irradiation. The flow in each pixel (power weighted pixel density) was summed to compare dose- and tumor-dependent changes in tumor blood flow over time (Fig. 2B). Blood flow throughout the entire tumor was measured after treatment with 6 Gy. Tumor models showed a decrease in blood flow by day 3 \((P = 0.045)\). At day 7, B16F0 blood flow began to return and GL261 showed blood flow that was greater than before treatment (Fig. 2B).

**Flk-1 Antagonists Eliminate Radioresistance in Tumor Blood Vessels.** The importance of signal transduction through the VEGF receptor is illustrated by use of sFlk-1. This protein binds to VEGF and sequesters this ligand before its binding to cellular receptor (23–25). sFlk also functions as dominant negative by forming inactive heterodimers with membrane-spanning VEGF receptors (26, 27). This specific inhibitor of VEGF prevents the mitogenic and migratory response of endothelial cells to VEGF. To determine whether the Flk-1 receptor inhibition alters the radiation response in tumor blood vessels, we used Ad.ExFlk and both the window and Doppler blood flow analysis. We studied the tumor blood vessels in GL261 and B16F0 by use of the dorsal skin fold window model in C57BL6 mice. Tumors were implanted within the dorsal skin fold window. Ad.ExFlk.6His, \(2 \times 10^8\) pfu, was administered by tail vein injection to tumor-bearing mice. We combined subtherapeutic doses of radiation (2 Gy) given 16 h after a minimally therapeutic dosage of Ad.ExFlk.6His \(2 \times 10^8\) pfu. We studied the time course of vascular regression after treatment. No vascular regression was achieved after treatment with 2 Gy and control vector Ad.lacZ. Ad.ExFlk.6His alone at \(2 \times 10^8\) pfu achieved minimal regression of tumor blood vessels. The Ad.ExFlk.6His administered 16 h before irradiation resulted in regression of tumor blood vessels at a rate that is analogous to treatment with 6 Gy.

To further determine whether Flk-1 kinase inhibitor eliminates resistance to therapy, we used the Flk-1 selective kinase inhibitor SU5416 (22, 31, 32). SU5416 given alone as a single 0.75-mg i.p. injection achieved a modest reduction in tumor vascularity. We combined subtherapeutic doses of radiation (2 Gy) with a minimally therapeutic dosage of SU5416 to determine whether radiation response can be modified by Flk-1 inhibition. B16F0 and GL261 tumors were implanted in the dorsal skin fold window in C57BL6 mice, and angiogenesis was observed for 1 week. GL261 and B16F0 tumors treated with 2 Gy alone showed no vascular response to radiation. SU5416 alone resulted in a minimal regression of the tumor blood
vessels and rapid restoration of vasculature (Fig. 3B). SU5416 administered 1 h before irradiation markedly enhanced the radiation effect with complete destruction of tumor blood vessels within 24 h of treatment ($P = 0.02$, as compared with radiation alone; Fig. 3B). This enhancement of the vasculitic effects of radiation was observed in both tumor models (Fig. 3B). These findings indicate that the treatment with SU5416 led to a suboptimal antivascular effect in this model whereas in combination with radiation lead to a more pronounced and persistent antiangiogenic response.

To determine whether inhibition of Flk-1-mediated signal transduction also enhances the cytotoxic effects of radiotherapy in established tumors, we used power Doppler to quantify blood flow in 1-cm tumor models treated with Flk-1 inhibitors and radiation. Tumor-bearing mice were then treated with a minimally effective dosage of SU5416 (0.75 mg) administered by i.p. injection at 1 h before irradiation (2 Gy). SU5416 was injected every other fraction for a total of three doses. Tumors were treated with minimally effective dose of 3 Gy per day for each of 7 days. Blood flow was quantified by power Doppler on days 4 and 7. Radiation alone and SU5416 alone achieved minimal reduction in GL261 and B16F0 tumor blood flow (Fig. 4A). Tumors treated with SU5416 1 h before irradiation showed significant reduction in tumor blood flow as compared with either agent alone ($P = 0.004$; Fig. 4B), consistent with the observations in the window model after the addition of SU5416.

Flk-1 Inhibition Abrogates a Survival Mechanism in Irradiated Endothelial Cells. To determine whether these inhibitors enhance radiation-induced intrinsic cell death, we studied apoptosis in HUVEC primary culture endothelial cells, 3B11 murine endothelial cells, and HMECs. Endothelial cells showed minimal radiation-induced apoptosis in complete medium (Fig. 5). Induction of apoptosis required the inhibition of Flk-1 by use of the soluble Flk-1 receptor (ExFlk) or SU5416. Apoptosis was quantified by counting the number of apoptotic bodies in 100 cells. Fig. 5 shows the percentage of HUVECs undergoing apoptosis after irradiation is 2% as compared with no apoptotic cells in the untreated control cells. SU5416 (50 μM) induced apoptosis in 4% of cells, and ExFlk induced apoptosis in 5% of cells. When these Flk-1 inhibitors were added before irradiation, an increase in apoptosis was observed. HUVECs stained positive for apoptosis in 14% of SU5416 ($P = 0.005$, compared with radiation alone) and radiation and 12% of ExFlk and radiation ($P = 0.01$, compared with radiation alone). 3B11 cells and HMECs treated with Flk-1 antagonist and radiation showed a similar enhancement of radiation-induced apoptosis.

Flk-1 Antagonist Enhances Regression of Tumor Volume. To determine whether inhibition of Flk-1 enhances radiation-induced tumor volume regression, we measured tumor volumes by use of calipers, as described previously (15, 16, 29). GL261 tumor-bearing mice were treated with radiation using 3 Gy/day and three daily
fractions/week for 2 weeks. Mice received SU5416 0.75 mg by i.p. injection, administered 1 h before irradiation during fractions 1, 3, 4, and 6. Control mice received identical doses and schedule of either SU5416 alone or radiation alone. GL261 tumors treated with radiation alone showed minimal growth delay (Fig. 6). No growth delay was observed in tumors treated with SU5416 alone. However, the combination of SU5416, followed by radiation caused initial tumor regression and significant reduction in tumor volumes as compared with radiation alone \((P = 0.01)\) or SU5416 alone \((P < 0.009)\). A second experiment was performed to confirm these findings. Ten mice were randomly assigned into the four treatment groups. Fig. 6B shows the mean and SE of four plates treated in each of the treatment groups.

**Discussion**

The role of VEGF-mediated endothelial cell survival has also been implicated in the radiation response in tumors (7). Of the four tumor models studied here, the glioblastoma model was found to be radiation resistant and expressed high levels of VEGF. Whereas VEGF inhibition achieved additive growth delay in the three epidermoid carcinoma tumors, a greater than additive effect was achieved in the glioblastoma tumors when VEGF blocking antibody was combined with radiation (7). This heterogeneity between VEGF-dependent tumor models is, in part, related to the increased expression of VEGF in...
gliomas (33–35). Previous use of the vascular window to study the response of tumor microvasculature to radiation have shown heterogeneity in the response to radiation (23–25). We found very little variation in radiation response in tumor blood vessels between individual mice. In contrast, other studies have found heterogeneity in tumor vascular reaction to radiation (36, 37). The present study used minimal therapeutic doses of radiation (3 Gy), which produced little to no response in GL261 and a marked response in B16F0 in tumor blood vessels, which consequently resulted in little variation between mice. The present tumor models include GI261 and B16F0 tumors in C57BL/6 mice, which have reduced variability between individual animals. The response of tumor microvasculature to radiation is dependent on the dose and time interval after treatment (1–6). Studies of the radiation response in tumor vasculature conducted two to three decades ago measured 3Cr-labeled RBCs to determine blood volume showed an increase if doses below 500 rads were administered. Blood flow studies (interstitial Xe clearance) of irradiated mouse sarcoma showed that blood flow decreased within hours, which was followed by an increase within 3–7 days (4).

In the present study, we found that Flk-1 antagonists enhanced the vasculitic effects of ionizing radiation on tumor microvasculature. The VEGF-survival phenotype in endothelial cells is mediated by binding to the Flk-1/KDR receptor (20, 21). A potent and selective inhibitor of Flk-1/KDR inhibits tyrosine kinase catalysis and inhibits tumor vascularization in growth (31, 32). The effectiveness of this Flk-1 inhibitor was studied in gliomas (22). This study showed that inhibition of Flk-1 controls growth and progression of the angiogenesis-dependent gliomas. A second Flk-1 antagonist is soluble Flk-1 receptor, which sequesters unbound VEGF, thereby preventing Flk-1 activation as we have shown (23). Taken together, these models show that signaling through Flk-1 contributes to radiation response in tumor blood vessels.

VEGF prolongs the survival of human endothelial cells, which is associated with increased expression of the antiapoptotic protein Bcl-2 (38, 39). Enhanced endothelial cell survival was associated with a dose-dependent increase in Bcl-2 expression and decrease in expression of the processed forms of caspase-3 (39). In addition to a 5.2-fold induction of Bcl-2 by VEGF, there is a 2.4-fold induction of A1 (38). Overexpression of Bcl-2 in HUVECs prevented apoptotic cell death in the absence of VEGF (38). Thus, a potential mechanism of action of Flk-1 antagonists during the radiation response is through the regulation of apoptosis. Our schedule of administration of SU5416 was based on the assumption that Flk-1 activity predetermines the tumor blood vessel survival phenotype, as determined by window and was based on the assumption that Flk-1 activity predetermines the tumor vascular response to radiation (36, 37). The present study used Flk-1 inhibition reverses radioresistance.

References

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