Review

Tumor Response to Ionizing Radiation Combined with Antiangiogenesis or Vascular Targeting Agents: Exploring Mechanisms of Interaction

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Abstract

Recent preclinical studies have suggested that radiotherapy in combination with antiangiogenic/vasculature targeting agents enhances the therapeutic ratio of ionizing radiation alone. Because radiotherapy is one of the most widely used treatments for cancer, it is important to understand how best to use these two modalities to aid in the design of rational patient protocols. The mechanisms of interaction between antiangiogenic/vasculature targeting agents and ionizing radiation are complex and involve interactions between the tumor stroma and vasculature and the tumor cells themselves. Vascular targeting agents are aimed specifically at the existing tumor vasculature. Antiangiogenic agents target angiogenesis or the new growth of tumor vessels. These agents can decrease overall tumor resistance to radiation by affecting both tumor cells and tumor vasculature, thereby breaking the codependent cycle of tumor growth and angiogenesis. The hypoxic microenvironment of the tumor also contributes to the mechanisms of interactions between antiangiogenic/vasculature targeting agents and ionizing radiation. Hypoxia stimulates up-regulation of angiogenic and tumor cell survival factors, giving rise to tumor proliferation, radioresistance, and angiogenesis. Preclinical evidence suggests that antiangiogenic agents reduce tumor hypoxia and provides a rationale for combining these agents with ionizing radiation. Optimal scheduling of combined treatment with these agents and ionizing radiation will ultimately depend on understanding how tumor oxygenation changes as tumors regress and regrow during exposure to these agents. This review article explores the complex interactions between antiangiogenic/vasculature targeting agents and radiation and offers insight into the mechanisms of interaction that may be responsible for improved tumor response to radiation.

Introduction

Ionizing radiation is an effective modality for the treatment of many tumors. It is a widely used treatment for cancer, with over half of all cancer patients receiving radiation therapy during their course of treatment (1). Although widely used, a need remains to improve the cure rate by radiation therapy alone. The most frequent treatment is to combine cytotoxic chemotherapeutic agents with radiation (2, 3). The cytotoxicity of chemotherapeutic agents, however, is not limited to tumor cells because treatment of tumors with these agents can result in significant normal tissue toxicity.

There has been a recent rapid development of two new classes of drugs termed antiangiogenesis and vascular targeting agents that target the relatively genetically stable tumor-associated vasculature (endothelial cells) rather than the genetically unstable tumor cell (4). The importance of targeting tumor vasculature development and function first became apparent in the 1970s through the seminal studies of Judah Folkman (5), who demonstrated that angiogenesis is important for the growth and survival of tumor cells. The relationship between angiogenesis and tumor growth suggests that both tumor cells and their supporting endothelial cells are potential targets for cell killing and should be considered when planning cancer treatment (6).

At least four theoretical advantages exist for considering tumor endothelial cells as targets for cancer therapy: (a) endothelial cells are more easily accessed by antiangiogenic/vascular targeting agents compared with drugs that act on tumor cells directly and have to penetrate large bulky masses; (b) antiangiogenic/vascular targeting agents may avoid tumor drug resistance mechanisms because they are directly cytotoxic to endothelial cells; (c) angiogenesis occurs in very limited circumstances in adults (wound healing and ovulation), thus antiangiogenic therapies targeting specific receptors on proliferating tumor endothelium potentially are safe and should avoid normal tissue toxicities; and (d) because each tumor capillary potentially supplies hundreds of tumor cells, targeting of the tumor vasculature should lead to a potentiation of the antitumorogenic effect (7).

Investigations of antiangiogenic/vascular targeting agents that have been conducted in preclinical and clinical trials indicate that tumor cures are limited when these agents are used as...
the sole method of treatment (8). Some recent preclinical studies suggest that the combination of radiotherapy and angiogenic blockade enhances the therapeutic ratio of ionizing radiation by targeting both tumor cells and tumor vessels (9–12). At present, there is great interest in combining antiangiogenic/vascular targeting strategies with conventional cytotoxic therapies such as radiotherapy to improve therapeutic gain. The mechanisms by which tumor response to radiation is enhanced by these new agents, however, are not currently understood. In light of the known fact that oxygen is a potent radiosensitizer, the combination of ionizing radiation and antiangiogenic/vascular targeting agents would appear to be a counterintuitive approach to tumor cure because a reduction in tumor vasculature would be expected to reduce tumor blood perfusion and reduce oxygen concentration in the tumor. Some recent studies, however, indicate that oxygen levels may actually increase after treatment with antiangiogenic agents and ionizing radiation (13–16).

Because the mechanisms of interaction between ionizing radiation and antiangiogenic/vascular targeting agents are not fully understood, the ideal way to use this potentially powerful combination for tumor cure has yet to be determined. The purpose of this review is to examine possible mechanisms of interaction between antiangiogenic/vascular-induced cell death and cell death resulting from ionizing radiation, in the expectation of aiding in the design of rational protocols for treatment with antiangiogenic/vascular targeting agents and radiation.

Role of Angiogenesis in Primary Tumor Growth and Metastasis

A competent and expanding vascular supply is a necessary component of the progressive growth of solid tumors because cells in solid tumors, like normal tissue, must receive oxygen and other nutrients to survive and grow (17). The connection between oxygen supply and tumor growth was first made in the 1950s by radiobiologists who were aware of oxygen as a radiosensitizer and the fact that hypoxic cells in tumors are resistant to radiation therapy (18). Histological analyses of human and rodent tumors performed by Thomlinson and Gray (18) were the first studies to suggest that regions of viable cells exist close to tumor blood vessels and that these walls or cords of viable tumor cells correspond in thickness to the distance that oxygen can diffuse (1–2 mm3). The “tumor cord” model implied that hypoxic cells exist in a state of oxygen and nutrient starvation at the limits of the diffusion range of oxygen, and it was hypothesized that tumor cells could proliferate and grow only if they were close to a supply of oxygen from tumor stroma. In the 1970s, Folkman (5) corroborated the earlier findings of radiobiologists and proposed the importance of tumor vasculature as a viable target for anticancer therapy. He reported that a tumor without an adequate blood supply would grow only to a few thousand cells in size or around 1–2 mm3, which is the distance that nutrients can enter tumor cells by passive diffusion (5). To increase in size beyond this passive diffusion-limited state, the growing tumor mass must acquire new blood vessels. A switch to the angiogenic phenotype allows the tumor to expand rapidly. This so-called “angiogenic switch” (19) is regulated by environmental factors and by genetic alterations that act to either up-regulate proangiogenic factors (i.e., VEGF3 and bFGF) and transforming growth factors (TGF-α and TGF-β) and/or down-regulate inhibitors of angiogenesis [i.e., angiostatin, endostatin, thrombospondin, and IFN-α (20)]. Tumor angiogenesis is a multistep process of degradation of the extracellular matrix, migration and proliferation of endothelial cells from postcapillary venules, and, finally, tube formation (21). More than 20 endogenous activators and inhibitors have been identified in this process (7). The initial step in the process is the activation of quiescent endothelial cells by binding of tumor-produced or stromal-produced growth factors to endothelial receptors. VEGF is the most potent and specific growth factor for endothelial cell activation (22). Evidence for the importance of VEGF-induced angiogenesis in tumor growth was demonstrated by use of neutralizing antibodies or a dominant-negative soluble receptor to inhibit VEGF action and growth of primary and metastatic experimental tumors (10, 23). VEGF also functions as a powerful antiapoptotic factor for endothelial cells in new blood vessels (24). VEGF is secreted by almost all solid tumors (25). Leukemias are now also considered to be angiogenesis-dependent malignancies and have been shown to express VEGF (26–28). Factors that lead to up-regulation of VEGF expression and secretion include external stresses such as ionizing radiation produced in the application of radiation therapy and tumor microenvironment factors such as hypoxia or a decrease in pH (29, 30). Hypoxia is the most potent stimulus for induction of VEGF, which occurs by activation of Src kinase. Src kinase activation leads to an increase in HIF-1α and consequent up-regulation of VEGF expression (31, 32). Growth factors stimulating VEGF production include IGF-I and -II, epidermal growth factor, and PDGFs. Two specific signal pathways are known to mediate the up-regulation of VEGF: (a) the phosphatidylinositol 3′-kinase/Akt (protein kinase B) signal transduction pathway, which leads to stabilization of HIF-1α (33, 34); and (b) the MAPK pathway, in which activation of extracellular signal-regulated kinase increases transcription of the VEGF gene (see Fig. 1 for summary of signaling mechanisms; Ref. 35). Mutant ras oncogenes and loss or mutation of the tumor suppressor gene, p53, can also result in increased VEGF expression and angiogenesis (36, 37).

Although the growth of solid tumors depends on angiogenesis for generation of a vascular network, the amount of newly formed vessels in the tumoral stroma does not necessarily lead to increased blood flow (38). This inequality exists because newly formed microvessels in most solid tumors are abnormal when compared with the morphology of the host tissue vasculature (39). Endothelial cells lining tumor blood vessels differ in many respects with regard to gene expression from those of normal vasculature (40). Because of lack of adequate vascular

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; bFGF, basic fibroblast growth factor, TGF, transforming growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; IFP, interstitial fluid pressure; HIF-1α, hypoxia-inducible factor 1α; PG, prostaglandin; EBRT, external beam radiation therapy; EGFR, epidermal growth factor receptor; COX, cyclooxygenase; rhAngiostatin, recombinant human angiostatin.
maturation (41), vessels are often dilated and tortuous (42), have an incomplete or missing endothelial lining, and have interrupted basement membranes that result in an increased vascular permeability with extravasation of RBCs and blood plasma expanding the interstitial fluid space (39). Moreover, blood flow is often erratic, with stasis or even reversal of blood flow within individual vessels (43). Extravasation of macromolecules and development of high IFP often results in vascular collapse (42, 44, 45). Consequently, oxygen availability to the tumor cells shows great variability. Many human tumors exhibit hypoxic regions that are heterogeneously distributed within the tumor mass (46). Low perfusion rates and hypoxia may then coexist with high nonfunctional vascular density, creating hypoxic regions (41). In these regions of hypoxia, endothelial cells may up-regulate survival factors to maintain their integrity and prevent apoptosis (47). Thus, so-called “angiogenic hot spots” or localized regions of intense angiogenesis may be created and may be associated with failure of radiotherapy (48). However, it is not clearly known whether hypoxia in the tumor produces angiogenic hot spots or whether these regions of intense angiogenesis are a property of the genomic instability of the tumor cells themselves, resulting in up-regulation of proangiogenic molecules.

**Ionizing Radiation and Hypoxia**

Radiation-induced cell death is usually attributed to DNA damage to tumor cells, thus triggering cell death by apoptosis and/or necrosis. Oxygen is known to be a potent radiosensitizer and, through interaction with the radicals formed by radiation, is essential for the induction of radiation-induced DNA damage. Cells irradiated in the presence of air are about three times more sensitive than cells irradiated under conditions of severe hypoxia (49). Hypoxia, then, contributes to radiation resistance. As already discussed in an earlier section, the “tumor cord” model of early radiobiologists implied that hypoxic cells exist in a state of oxygen and nutrient starvation at the limits of the diffusion range of oxygen, and it was hypothesized that tumor cells could proliferate and grow only if they were close to a supply of oxygen from tumor stroma [i.e., 1–2 mm³ (18, 50)]. Today, however, it is known that the width of viable regions of tumor cells can vary quite widely, depending on tumor microenvironmental factors such as pO₂ and respiratory metabolism of viable cells (51). Experimental and clinical studies have provided both direct and indirect evidence for the presence of hypoxic cells in tumors (52, 53). Regions of viable cells exist in proximity to tumor blood vessels, whereas regions of necrosis are observed at increased distances from blood vessels. Viable hypoxic cells, at very low oxygen tensions, are believed to exist at the edges of necrotic regions. Hypoxia resulting from diffusion-limited processes is known as chronic hypoxia. It is also known that in addition to these radiobiologically hypoxic cells, there is a distribution of tumor cells at intermediate oxygen levels that can influence response to low-dose fractionated radiotherapy (54, 55). Hypoxia can also be the result of intermittent blood flow arising from the abnormal tumor microvasculature (56–58). This acute hypoxia is distinct from that of chronic hypoxia because the affected cells would be expected to have intermittently perfused areas that could give rise to tumor regrowth (55). The effect of hypoxia on tumor control is that the existence of viable cells in a microenvironment at low oxygen tensions can give rise to populations of tumor cells that are not only radioresistant but also highly angiogenic and are potentially resistant to antiangiogenic therapy as well (59, 60).
 Radiation and Angiogenesis: A Vicious Cycle

Tumor vasculature is abnormal, and the endothelial cells lining tumor blood vessels have different phenotypic properties from those of normal vasculature (41). Consequently, increased tumor angiogenesis, as indicated by increased microvessel density or by increased VEGF expression, does not necessarily correlate with increased blood flow and oxygen availability. This situation, together with the existence of heterogeneous hypoxic regions within tumors, makes it difficult to predict how tumor angiogenesis will affect response to radiation therapy in a particular tumor. As already noted, hypoxia leads to radiation resistance because of lack of oxygen to facilitate DNA damage by radiation-induced free radicals. Hypoxic conditions also create a microenvironment in which tumor cells become less angiogenesis dependent, more apoptosis resistant, more capable of existing under hypoxic conditions, and more malignant because of the development of genomic instability and mutant genotypes impacting on apoptosis/survival signaling pathways (61). Hypoxic tumor cells are particularly known to up-regulate HIF-1α, which increases the expression of VEGF (30, 62). All these factors suggest diminished sensitivity to antiangiogenic therapy as well as radiation therapy.

Strong evidence exists that cytotoxic therapy alone, such as radiation, can result in intensification of angiogenic processes (48). Direct up-regulation of VEGF after irradiation of various cancer cell lines has been reported (10). This response is part of the overall cellular response to stress and is associated with the induction of a variety of transcription factors that can activate transcription of cytokine, growth factor, and cell cycle-related genes. The products of these genes regulate intracellular signaling pathways through tyrosine kinases, MAPKs, stress-activated protein kinases, and ras-associated kinases. These multiple pathways affect tumor cell survival or alter tumor cell proliferation. With regard to angiogenesis, radiation exposure can result in activation of the EGFR, which, in turn, can activate the MAPK pathway (63). MAPK signaling is linked to increased expression of growth factors, such as TGF-α and VEGF. It is possible that radiation therapy itself contributes to radioresistance by up-regulating and intensifying angiogenic pathways. The increased tumor cell proliferation that is often seen after radiation may be the result of up-regulated angiogenic pathways (48) as well as increased proliferation in the tumor stem cell compartment (64). Although many tumors reoxygenate after radiation, in tumors that are unresponsive to radiation therapy, up-regulated angiogenesis after radiation may lead to factors contributing to radiation resistance such as increased vascular permeability, increased IFP, decreased tumor perfusion, increased oxygen consumption, increased hypoxia, and up-regulated survival pathways (14, 45, 65). These factors all contribute to making radiation therapy less effective in some tumors (62, 66, 67).

 Radiation and Antiangiogenic Interactions

The existence of tumor microenvironmental factors, such as hypoxia, that can promote the up-regulation of angiogenic and survival pathways and hence resistance to radiation therapy has prompted studies combining antiangiogenic agents with radiation in an effort to overcome this resistance. Teicher et al. (15) were the first to show an increased response to single-dose radiotherapy with antiangiogenic agents. A number of preclinical studies have since indicated that antiangiogenic agents can enhance the tumor response to radiation [see Tables 1 and 2 (9–11, 13, 14, 68–75)]. However, it is difficult to compare and interpret relative efficacies of different antiangiogenic agents with radiation because of the variability in experimental conditions reported in the literature such as tumor models, tumor host strain, starting tumor size, final tumor volume measured, and dosing and scheduling (see Table 2). As expected, different tumor models can vary a great deal in their responses to antiangiogenesis treatment. The differences in tumor response to antiangiogenic agents may occur because of differences in angiogenic growth patterns arising from differences in the relative levels of angiogenic growth factors such as VEGF that stimulate angiogenesis. For example, the U87 human glioblastoma xenograft model that appears in many studies is known to be a highly vascularized aggressive tumor and exhibits high levels of VEGF, which would theoretically make it more resistant to therapy compared with tumors with lower VEGF expression, and thus it would require higher concentrations of antiangiogenic agents for efficacy (76). Differences in tumor response to angiogenic agents may also result from the presence of different patterns of multiple angiogenic factors among different tumors influencing tumor growth and angiogenesis. This would explain

<table>
<thead>
<tr>
<th>Agent</th>
<th>Target</th>
<th>Activity</th>
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<tbody>
<tr>
<td>TNP-40</td>
<td>Protein myristoylation and dependent signaling pathways in endothelial cells</td>
<td>Up-regulation of apoptotic pathways in endothelial cells</td>
</tr>
<tr>
<td>Angiostatin</td>
<td>Endothelial cell membrane receptor (possibly membrane proton pump)</td>
<td>Inhibition of pH regulation and up-regulation of apoptotic pathways in endothelial cells</td>
</tr>
<tr>
<td>VEGF/VEGFR inhibitors</td>
<td>Neovascularization processes; endothelial cell survival pathways; tumor cell survival pathways indirectly and possibly directly through autocrine signaling loops</td>
<td>Inhibition of endothelial cell migration, tube formation, etc.; up-regulation of apoptosis in endothelial cells through down-regulation of key signaling molecules, <em>i.e.</em> survivin, BCL-2</td>
</tr>
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<td>COX-2 inhibitors</td>
<td>Prostaglandin synthesis</td>
<td>Inhibition of VEGF synthesis in tumor cells; up-regulation of apoptotic signaling pathways in tumor cells and endothelial cells</td>
</tr>
<tr>
<td>EGFR inhibitors</td>
<td>VEGF synthesis, apoptotic signaling in tumor cells and endothelial cells</td>
<td>Inhibition of tumor angiogenesis; up-regulation of apoptosis in both tumor cells and endothelial cells</td>
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<tr>
<td>Vascular targeting agents</td>
<td>Microtubular cytoskeleton in endothelial cells</td>
<td>Vasculature shutdown leading to tumor necrosis</td>
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*i.e.* ZD6126
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<tr>
<th>Antiangiogenic agent</th>
<th>Tumor model</th>
<th>Tumor host</th>
<th>Tumor diameter at start of treatment (mm)</th>
<th>Total radiation dose</th>
<th>Effect of RT + drug (end point: tumor growth delay)</th>
<th>Reference no.</th>
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<tr>
<td>Fumagillin analogue</td>
<td>C3H/He mice</td>
<td>Mouse mammary carcinoma</td>
<td>4</td>
<td>10 Gy/5 fractions</td>
<td>Additive effect with 2–4 doses of 100 mg/kg drug after RT</td>
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<td>TNP-40</td>
<td>Athymic nude mouse</td>
<td>Human glioblastoma multiforme (U87)</td>
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<td>Greater than additive effect with daily dose of 6.7 mg/kg before RT</td>
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<td>Angiostatin</td>
<td>C57BL/6 mice</td>
<td>Recombinant Adk3 (expressing angiostatin)</td>
<td>5</td>
<td>12 Gy/2 fractions</td>
<td>Significant tumor regression with 25 mg/kg drug given</td>
<td>12</td>
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<tr>
<td>Angiostatin</td>
<td>Athymic nude mouse</td>
<td>Murine Lewis lung carcinoma (LLC)</td>
<td>9</td>
<td>30 Gy/5 fractions</td>
<td>Significant tumor regression with 25 mg/kg drug given</td>
<td>12</td>
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<td>Human malignant glioma (D54)</td>
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<td>50 Gy/10 fractions</td>
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<td>Human squamous cell carcinoma (SQ-20B)</td>
<td>11–12</td>
<td>40 Gy/10 fractions</td>
<td>Significant tumor regression with 25 mg/kg drug given</td>
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<td>VEGFR-2 blockade</td>
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<td>Human small cell lung carcinoma (54A)</td>
<td>6</td>
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<td>C57BL6 mice</td>
<td>Murine glioblastoma (GL261)</td>
<td>10–11, 11–12</td>
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<td>A/J mice</td>
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<td>Greater than additive effect with 100 mg/kg injected daily for 2 days before and after RT</td>
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<td>40 Gy/4 fractions</td>
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<td>VEGF mAb A.4.6.1</td>
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<td>20 Gy, 30 Gy/ single fraction</td>
<td>Greater than additive with 100 μg of drug given 6× on alternate days prior to RT</td>
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<td>Greater than additive with 100 μg of drug given 6× on alternate days prior to RT</td>
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<td>ZD1839 (Iressa)</td>
<td>SCC-1</td>
<td>Human squamous cell carcinomas</td>
<td>~5</td>
<td>21 Gy/7 fractions</td>
<td>Synergistic effect with 0.5 mg (orally) in 12 doses during RT</td>
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<td>SCC-6</td>
<td>Human squamous cell carcinomas</td>
<td>~5</td>
<td>21 Gy/7 fractions</td>
<td>Additive effect with 0.5 mg drug (orally) in 12 doses during RT</td>
<td>71</td>
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<td>C225 antibody</td>
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<td>Human head and neck carcinoma (A431)</td>
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<td>18 Gy/single fraction</td>
<td>Greater than additive effect with 1 mg of drug in 3 doses before and after RT</td>
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<td>C3Hf/Kam mice</td>
<td>Mouse sarcoma (NFSA)</td>
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<td>25–80 Gy/single fraction</td>
<td>Greater than additive effect with 6 mg/kg drug before and during and after radiation for 10 consecutive days</td>
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the lack of efficacy of the single agent in antiangiogenesis trials. Differences in tumor response to antiangiogenic agents may also be influenced by the organ microenvironment in which the tumor is growing during the study. Studies of ectopic versus orthotopic tumor implantations have revealed differences in response to antiangiogenic treatments (73, 77).

The size of transplantable tumors at the start of an experiment is also an important factor to consider when examining tumor response to radiation in murine systems. Tumor growth delay studies reported in the literature vary greatly with regard to tumor size at start of treatment as well as tumor volume that is permitted to be reached during treatment until a statistically meaningful effect is seen. Tumor size can affect oxygen tension, nutrient supply, and pH, which are all factors in determining radiation response. Radiosensitivity is directly proportional to tumor size (Fig. 1). As tumor size increases, oxygen tension and pH decrease because of a greater demand for oxygen and nutrients. Many murine tumors, even tumors as small as 6 × 6 mm, have low oxygen tension (1–10 mm Hg) and are considered hypoxic. As tumors become increasingly large, oxygen is unavailable, and glycolysis dominates, leading to acidosis. Eventually, the lack of oxygen and nutrients and chronic exposure to low pH lead to tumor necrosis. When a tumor is allowed to reach a size beyond 2000 mm³ in volume, a volume large enough to create hypoxia, necrosis, and consequent radioresistance, the comparison of antiangiogenic treatment plus radiation with radiation alone under these conditions may result in an underestimation of the tumor growth delay. In some tumor models, for example, radiation resistance emerges at ~250 mm³. In tumors larger than 500 mm³, tumor oxygen tension is less than 2 mm Hg, and radiation resistance is significant (Fig. 2).

Another factor that may influence tumor growth delay is the tumor bed effect, which can prolong tumor growth delay indirectly through the late effects of radiation on tumor stroma [vasculature/connective tissue (78)]. Other factors such as the type of anesthetic or restraint used to irradiate the animal, as well as animal and tumor temperature, should also be considered because they all could affect perfusion. Special attention should be paid to generalizations about relative efficacies of treatments based on individual in vivo tumor growth delay studies. With these caveats in mind, some of the antiangiogenic strategies that have been used in combination with radiation are reviewed below.

The list of antiangiogenic agents used in combination with radiation include angiotatin (9, 12), anti-VEGF or VEGFR antibodies and tyrosine kinase inhibitors (10, 13, 14, 68, 72), TNP-470 (15, 73, 79), COX-2 inhibitors (80–83), and anti-EGFR inhibitors (71, 84). The mechanisms of enhancement are believed to involve both direct and indirect targeting of tumor cells and/or endothelial cells (see Table 1). The antiangiogenic and antitumor effects have been reported to be additive as well as synergistic. Possible mechanisms of enhanced tumor response include (a) indirect inhibition of vessel formation by inhibition of vascular growth factors and endothelial receptors (14), (b) direct radiosensitization of endothelial cells [endothelial cell apoptosis (72, 85, 86), (c) direct radiosensitization of tumor cells (i.e., tumor cell apoptosis) (87), and (d) decrease in number of hypoxic cells [improved oxygenation (14, 15, 88)].

Radiation and VEGF/VEGFR Signaling Pathway Inhibitors

Some studies targeting the VEGF/VEGFR signaling pathway in conjunction with radiation have been reported. Gorski et al. (10) found that antibodies to VEGF, when combined with ionizing radiation in vitro, resulted in increased endothelial cell death without affecting tumor cells. Greater than additive antitumor effects were noted in a variety of tumor model systems in vivo. It was then proposed that enhancement of radiation response occurs through inhibition of VEGF-induced protection against radiation damage to endothelial cells. This suggestion was supported by a recent study showing that SU5416, an inhibitor of VEGFR kinase, increased the radiation-induced apoptosis in endothelial cells (68). It has also been reported that SU668, which inhibits VEGF, bFGF, and PDGF receptor kinases, which are angiogenic growth factors, caused apoptosis in endothelial cells in vivo (89). It was then proposed that VEGF, fibroblast growth factor, and PDGF may be required not only for neoangiogenesis but also for survival of existing endothelial cells and for maintenance of existing tumor vasculature.

In addition to growth factors and their receptors, other factors associated with endothelial cell survival exist such as maintenance of association with perivascular cells (pericytes and vascular smooth muscle cells), integrin interaction with the extracellular matrix, and antiapoptotic proteins such as survivin and BCL2 (7, 47, 60). Endothelial cells, like other cells, also depend on intracellular pH-regulatory mechanisms to survive. If
they fail, metabolism ceases, and apoptosis ensues (90). It has been proposed that angiotatin may compromise endothelial intracellular pH and hence survival by inhibiting a major proton pump in the cell membrane of endothelial cells (91).

Because most endothelial survival factors interact with VEGF function in complex pathways, VEGF seems to be the predominant stabilizing factor (47). More recently, Gupta et al. (86) demonstrated that genetically engineered VEGF-producing xenografts were more resistant to the cytotoxic effects of ionizing radiation than xenografts that do not produce VEGF (86). Because the VEGF-null and the VEGF-producing xenograft cell lines exhibited identical radiosensitivities in vitro, their observations support the hypothesis that radiation targets the tumor endothelial cells as well as the tumor cells under conditions in which protective survival factors are diminished. These observations further confirm earlier studies that suggest that VEGF mediates tumor radioresistance by directly affecting the radiosensitivity of the endothelial cells.

Effects of COX-2 Inhibitors on Angiogenesis and Radiation Response of Tumors

Although the exact mechanisms for improved response in tumors are not known, the rationale for using COX-2 inhibitors with ionizing radiation is based on the angiogenic tumor environment and the biochemical responses of tumors to radiation (92). The COX-2 enzyme is induced in cells after injury or stress, such as radiation, and catalyzes the synthesis of PGs from arachidonic acid. PGs serve to protect cells and tissue from radiation damage (92) and promote angiogenesis by the up-regulation of factors such as VEGF (93). Therefore, pretreatment with COX-2 inhibitors before exposure to stress may inhibit the inflammation response induced by PGs. Furthermore, COX-2 inhibitors at clinically relevant doses, such as those achieved during the treatment of arthritis, may act directly on endothelial cells to inhibit proliferation and to increase their intrinsic sensitivity to radiation (80). The ability of COX-2 inhibitors to enhance radiation therapy has been demonstrated in murine and human tumor models (83, 74, 94–96). For example, it was demonstrated that the radiation response of a murine sarcoma was increased if tumor-bearing mice were pretreated with a COX-2 inhibitor before radiation therapy (74). Tumor growth delay by drug plus radiation was significantly increased over drug or radiation alone, and the TCD 50 (dose of radiation required for 50% tumor cure) was reduced by almost half in the drug plus radiation group. To elucidate the mechanism of sensitization, an intradermal injection of tumor cells was used to visualize and count newly forming vessels. Neovascularization was shown to precede tumor growth, and pretreatment with a COX-2 inhibitor effectively reduced neovascularization, resulting in reduced tumor growth.

There is also direct evidence that down-regulation of PGs by COX-2 inhibitors reduces angiogenesis and increases radiosensitivity. In another study with a murine fibrosarcoma expressing high levels of PGE2, radiosensitization was again observed by pretreatment of mice with a COX-2 inhibitor (95). COX-2 expression was not affected by treatment, and no increase in the amount of tumor cell apoptosis was observed, but PGE2 was significantly decreased. PGE2 is a vasoactive compound that can affect tumor perfusion as well as stimulate tumor growth and induce angiogenesis. The effect of the COX-2 inhibitor on PGE2-mediated angiogenesis was determined to be a key mechanism in the increased radiation response.

The concept that COX-2 inhibitors can reduce the radiation dose required to achieve tumor control caused great excitement among radiation oncologists and stimulated the writing of many clinical protocols. However, preclinical studies on human tumor xenografts showed that, although COX-2 inhibitors increased the effectiveness of radiation, inhibition of angiogenesis was not a factor in the improved response. Authors concluded that in human tumors, the intrinsic sensitivity to radiation was increased (96), possibly via a cell cycle mechanism (97). An increase in the incidence of apoptosis was also a factor (83). Experimental evidence in murine tumor systems indicates that inhibition of angiogenesis by COX-2 inhibitors is a factor in the increased radiation response. However, in human tumor models, other factors may play a predominant role in the increased radiation sensitivity.

The existing approval of these agents for noncancerous conditions has allowed clinicians to use COX-2 inhibitors in a large number of clinical trials in combination with radiation and chemoradiation. To date, these trials are still too immature to yield meaningful outcome data.

Effect of Radiation and Antiangiogenic Agents on Tumor Oxygenation

Enhancement of tumor response to radiation plus antiangiogenic agents has also been explained by an increase in tumor oxygenation after treatment. Tumor oxygenation is a function of perfusion and consumption of oxygen, including consumption of oxygen by both tumor and endothelial cells. Antiangiogenic agents can theoretically improve tumor oxygenation by reducing the number of oxygen-consuming tumor cells and endothelial cells, which, in turn, will reduce the overall demand for a given amount of oxygen. Antiangiogenic agents can also theoretically increase perfusion by reducing the number of immature, inefficient vessels (41, 98–100) and by decreasing IFP (38, 101). However, IFP is related to many factors, including vascular leakage and vascular resistance. High vascular resistance may reduce tumor IFP and, at the same time, reduce perfusion. Thus, a reduction of tumor IFP may not necessarily improve perfusion or oxygenation (102).

The belief that there is an improved tumor response to angiogenesis inhibitors and radiation is derived from animal tumor models. Fig. 3 provides a hypothetical model of how oxygen tension could be increased by treatment with antiangiogenic drugs and radiation.

Tumor oxygen tension is a function of oxygen consumption (by both tumor cells and endothelial cells) and tumor perfusion. In nonmalignant tissue, the blood supply is organized and efficient. Perfusion and oxygen consumption are balanced so that the tissue is in an oxygenated steady state. In contrast, malignant tissue contains dividing tumor cells that outgrow their blood supply (Fig. 3a). The overgrowth causes
Fig. 3 Hypothetical model for the rationale of combining antiangiogenic agents with radiotherapy. Nonmalignant tissue has a mature organized blood supply. Perfusion and oxygen consumption are balanced so that the tissue is in an oxygenated steady state. In normal tissue, the IFP is low. a, in malignant tissue, an increase in tumor cell number increases oxygen consumption and demand. As oxygen tension falls, angiogenesis is induced by cytokines to compensate for decreased oxygen tension. However, the newly formed vessels are torturous and inefficient, which may further compromise perfusion. Production of VEGF increases tumor IFP. b, after irradiation, oxygenated cells are destroyed, leaving the radioresistant hypoxic cells. The hypoxic cells can reoxygenate during radiation therapy and improve radiation response and tumor control. c, in cases where tumor control is not achieved, it has been proposed that in response to irradiation, protective cytokines are expressed by the tumor that reinitiate angiogenesis. Angiogenesis and vascular leakage induced by VEGF will compromise perfusion. d, in theory, pretreatment with antiangiogenic agents reduces the number of inefficient vessels and increases perfusion. More oxygenated tumor cells are killed by irradiation, which reduces oxygen consumption, and induction of angiogenesis. No regrowth is observed, and the oxygenated steady state is maintained. The tumor response to fractionated radiotherapy is then greatly improved.
oxygen tension to fall, and angiogenesis is induced by cytokines (103). The newly formed vessels are torturous and inefficient, further compromising perfusion and resulting in hypoxia. Expression of VEGF increases endothelial cell permeability, which can also result in high tumor IFP (21, 104), which has been correlated with tumor hypoxia (101).

Although many biological factors can contribute to tumor radiation response, tumor oxygen tension is one of the most important parameters, and it will be considered here. As shown in Fig. 3b, oxygenated cells are more sensitive to radiation and are destroyed by irradiation, leaving the radioresistant hypoxic cells. During a course of radiotherapy, the hypoxic cells in the tumor may reoxygenate (105), and continued fractions of radiation can lead to tumor control. Studies in patient tumors and tumor models indicate that reoxygenation occurs because of tumor shrinkage (106, 107), decreased oxygen consumption (108), and increased perfusion (105, 108, 109).

In cases where radiation therapy is ineffective, it has been proposed that angiogenesis induced by radiation may compromise tumor response (Fig. 3c). After a radiation insult, the surviving hypoxic cells can produce proteolytic cytokines such as VEGF (10). Angiogenesis can reinitiate, and the tumor cells can reoxygenate and proliferate (110). This scenario could theoretically be avoided if treatment with antiangiogenic agents was used before radiotherapy (Fig. 3d). A reduction in the number of inefficient vessels and in the number of oxygen-consuming tumor cells and endothelial cells by pretreatment with antiangiogenic agents would increase perfusion and reduce consumption, giving rise to more oxygenated tumor cells that are sensitive to radiation. This would prevent tumor regrowth and maintain the oxygenated state. This scenario would be ideal for fractionated radiotherapy because it prevents a reversion back to the hypoxic state during the course of therapy.

In a number of studies, hypoxia was reduced by angiogenesis inhibitors (13–16). In particular, Lee et al. (14) noted that vessel regression induced by an anti-VEGF antibody was associated with a decrease in hypoxia in a U87 xenograft. They suggested that the observed increase in oxygenation, despite a decrease in vascular density, was related to vascular reorganization after inhibition of VEGF. One possible mechanism by which VEGF affects vascular organization is its effect on vascular permeability and IFP within a tumor. Its inhibition decreases vascular permeability (111) and IFP, thereby reducing compression of vessels and allowing for normalization of vascular architecture and perfusion and consequent normalization of pO2. Improvement in tumor oxygenation may also be explained by decreased oxygen consumption resulting from increased killing of tumor and endothelial cells (14). Lee et al. (14) concluded that the oxygenation status of a tumor at time of radiation is probably not the major cause of the increased radiosensitivity to anti-VEGF therapy because they observed similar enhanced responses under both normoxic and hypoxic conditions. They concluded that the critical factor in producing enhanced tumor response to radiation is more likely related to the reduced vessel density induced by anti-VEGF treatment rather than the state of oxygenation of the tumor at the time of radiation.

**Effect of Radiation and Antiangiogenic Agents on Tumor Cell Apoptosis**

Enhancement of tumor response to radiation plus antiangiogenic agents has also been explained by increases in tumor cell apoptosis relative to tumor cell proliferation (87, 112–114). It has been observed that, on the tumor cell level, the proliferation index is unaffected, but the apoptotic index is increased after treatment with antiangiogenic agents such as angiostatin and TNP-470 (87, 114). Tumor cell apoptosis may occur directly by radiation-induced DNA damage to tumor cells or indirectly by antiangiogenic and radiation-induced damage to endothelial cells, upon which tumor cells ultimately depend. The molecular mechanism by which damage to endothelial cells results in increased rates of tumor cell apoptosis is not clear. It has been speculated that angiogenesis inhibition of tumor cell-expressed autocrine growth factors and receptors (115) or loss of endothelial-derived paracrine factors needed for tumor growth contributes to tumor cell cytotoxicity via apoptotic mechanisms (87, 113).

**Radiation and Vascular Targeting Agents**

Vascular targeting agents differ from antiangiogenic agents in their mechanism of action. Vascular targeting agents take advantage of the unique properties of tumor endothelium to selectively target the tumor vasculature (116). They are directed at the immature, rapidly proliferating tumor endothelial cells in existing tumor vasculature rather than the multistep processes involved in neovascularization. Vascular targeting strategies include biological agents, such as targeted gene therapy and antibodies to neovascular antigens, and drug-based agents (117). The drug-based strategies have progressed the furthest in development (117). These agents include drugs related to flavone acetic acid (118–121) and tubulin-binding compounds (122–127). Flavone acetic acid and its derivatives are believed to work by inducing tumor necrosis factor α (128–130), a cytokine known to induce hemorrhagic necrosis in experimental tumor models (131). The tubulin-binding compounds are believed to work by affecting the immature endothelial microtubular cytoskeleton found in tumors, leading to abnormalities in endothelial cell shape, thrombus formation, rapid vasculature shutdown, reduction in tumor blood flow, and secondary induction of tumor necrosis (132). However, after treatment with vascular targeting agents, viable tumor cells have been found at the tumor periphery (122, 123, 125, 132). It has been suggested that increased blood flow in the adjacent normal tissue, together with probable rapid up-regulation of angiogenic factors such as VEGF, directly facilitates rapid growth and expansion of the remaining rim of viable tissue because of the extensive ischemic insult (125, 133). Because the cells surviving the vascular targeting treatment are believed to be well oxygenated and consequently present an excellent target for radiation therapy, vascular targeting has been studied in combination with ionizing radiation. A number of studies have reported an increased antitumor effect with this combined complementary approach. Combretastatin A-4-disodium phosphate and ZD6126, potent and selective tubulin-binding agents against tumor vasculature, enhanced the radiation response of murine mammary carcinoma and sarcoma (123–125). Scheduling was found to be an impor-
tant factor in the efficacy of ZD6126 when combined with radiation (125). ZD6126 increased tumor cell killing in KHT murine sarcoma when administered 24 h before radiation or ≥1 h after radiation, but it was found not to be as effective if administered 1 h before radiation. It was suggested that blood flow needs to be reestablished in the remaining viable tissue to obtain maximum radiosensitization of the tumor.

Overall Tissue Response to Radiotherapy

The respective roles of endothelial cells and tumor cells in the overall tissue response to radiotherapy still remain controversial. In mouse models, Paris et al. (134) showed that a single large dose of radiation administered to mouse gastrointestinal mucosa preferentially damaged endothelial cells of the gut microvasculature that lie in close apposition to the epithelial cells lining the gut. Previous work showed that irradiation of microvascular endothelial cells resulted in the generation of ceramide, a compound that facilitates endothelial cell apoptosis (135). In more recent experiments, Paris et al. (134) showed that systemic administration of bFGF, an endothelial cell survival factor, or deletion of the acid sphingomyelinase gene (the gene upon which ceramide generation depends) can override the apoptotic signal from ceramide, thus protecting gut endothelial cells and epithelial stem cells from the effects of whole body irradiation. They concluded that the endothelial cells may represent the principal targets for radiation and that the death of epithelial stem cells may be a secondary event dependent on endothelial cell death. In an analogous manner, it has been speculated that tumor cell death in response to radiotherapy may represent a secondary event after death of endothelial cells, on which tumor cells ultimately depend (85, 135). However, it should be pointed out that the concept of what is the radiation target in normal tissue may well depend on the radiation dose per fraction. In this regard, it is well established that the effects of a single large dose, i.e., 15 Gy, are not equivalent in terms of normal tissue side effects to a more clinically relevant low-dose fractionated radiotherapy regimen of 2 Gy times 8 (136).

Clinical Trials with Antiangiogenic Agents and Radiation Therapy

To date, only one clinical trial has been completed evaluating an antiangiogenic drug (angiostatin) with radiation therapy. rhAngiostatin is a protein consisting of the first 3 kringles (amino acids 97–357) of human proplasminogen with a single amino acid substitution (Asn308 to Glu308) to prevent N-glycosylation. Previous studies using angiostatin in combination with ionizing radiation have indicated that the antitumor activity of human angiostatin is potentiated in a number of preclinical tumor models, resulting in a significant reduction of tumor volume without increase in toxicity.

A single-center, open-label, dose-escalation, Phase I clinical study at Thomas Jefferson University evaluated the safety and pharmacodynamics of three dose levels (15, 60, and 240 mg/m²/day) of rhAngiostatin i.v. protein in combination with EBRT for the treatment of cancer patients with solid tumors (137). Patients received rhAngiostatin i.v. 5 times/week, 30 min before EBRT (head and neck, thoracic, or pelvic regions), for a minimum of 25 EBRT fractions. This study had a unique design in that it was carried out concurrently with a Phase I drug dose clinical trial at Thomas Jefferson University (137). As safe doses were achieved in the drug alone study, these were used for the radiation study. Twenty-three patients were enrolled and evaluated for safety. Three patients were not evaluable, and three who did not complete the minimum EBRT were excluded from response analysis. The 17 remaining patients with evaluable tumors had advanced head and neck, thoracic, or pelvic cancers. No added toxicity was observed in normal tissue contained within the radiation portal. Mild rash was noted in three patients. No clinical thrombotic or bleeding events occurred in any patient. Tumor responses were demonstrated in 90% of patients who entered the trial with measurable disease in the radiation field. The conclusions from this Phase I trial are that concomitant administration of daily 10-min infusions of rhAngiostatin and EBRT is safe and does not increase radiation-induced toxicity. Local durable tumor responses (National Cancer Institute Response Evaluation Criteria in Solid Tumors) have been observed in this Phase I study, although this would be expected with the use of radiation alone (137).

Summary and Conclusions

Despite the expectation that agents causing tumor vessel regression should increase hypoxia and thus limit the effectiveness of radiation, there is accumulating experimental evidence to refute this expectation. Preclinical evidence suggests that antiangiogenic agents reduce tumor hypoxia and provides a rationale for combining these agents with ionizing radiation. The combination of antiangiogenic/vascular targeting agents and radiation therapy has led to additive or greater than additive tumor responses. The basis for the enhanced tumor responses lies in the complex interactions between the tumor microenvironment (tumor stroma and vasculature) and the tumor cells themselves. Although many factors such as VEGF expression, survivin down-regulation, endothelial sensitization to radiation, and many other complex interactions may be affected by antiangiogenic/vascular targeting agents, these molecules and interactions ultimately affect tumor size and \( pO_2 \), which are primary factors affecting tumor response to radiation (67).

Most tumors exist in a hypoxic and low pH environment by virtue of abnormal tumor vasculature giving rise to poor tumor blood perfusion and high IFP. Hypoxic conditions stimulate up-regulation of angiogenic and tumor cell survival factors by tumor and tumor stromal cells that promote tumor and endothelial cell proliferation and high IFP, all of which give rise to radioresistance. In response to radiation alone, oxic cells are killed, leaving behind a hypoxic, radioresistant fraction that can produce protective cytokines giving rise to a second wave of angiogenesis and tumor cell proliferation. Antiangiogenic agents, by targeting both tumor cells and endothelial cells, can reduce tumor proliferation and improve tumor oxygenation by inhibiting angiogenesis and high IFP through VEGF blockade. Thus, antiangiogenic agents can decrease overall tumor resistance to radiation by targeting both the tumor stroma and the tumor cell compartments, thereby eliminating the codependent cycle of tumor growth and angiogenesis (see Fig. 4 for summary of possible mechanisms of interaction between radiation and antiangiogenic agents).
A close examination of this area of research indicates there is great promise for future clinical treatment protocols. Because angiogenesis is one of the most fundamental processes of development, the complexity and redundancy of the process are not surprising. Whereas complexity and redundancy may make treatment and interpretation of the results difficult, they also provide for many opportunities to interfere with the angiogenic process. Exploring which compounds and combinations of compounds will work best with radiation and how to optimize their use will be a long and difficult task. The potential to significantly benefit patients, however, warrants further investigations.

In conclusion:
(a) Antiangiogenic agents appear to target tumor cells and tumor vasculature by both indirect and direct mechanisms.
(b) Antiangiogenic agents can decrease overall tumor resistance to radiation by targeting both tumor cells and tumor vasculature.
(c) Tumor growth and angiogenesis are part of a codependent cycle. Antivascular treatments can break the cycle and prevent revascularization after radiation.
(d) The differential effects observed with combined radiation and antivascular treatments on tumor growth among various tumor models may arise from differences in the intrinsic radiosensitivities of endothelial cells and tumor cells. The intrinsic radiosensitivity of the endothelial cell compartment in a tumor may be the result of differences in expression of survival factors such as VEGF, survivin, and BCL2 that come about in response to changes in the tumor microenvironment. The intrinsic radiosensitivity of the tumor cell compartment likewise depends on microenvironmental factors influencing expression of growth factors and receptors controlling tumor cell growth, proliferation, and capacity to repair DNA damage.
(e) Many factors are involved in the response to radiation and antiangiogenic/vascular targeting agents, but ultimately these factors affect tumor size and pO₂, which are primary factors affecting tumor response to radiation.
(f) Hypoxia not only participates in resistance to radiotherapy but can also affect the efficacy of antivascular therapy by up-regulating angiogenic and survival pathways. Understanding how tumor oxygenation changes during tumor regression and regrowth during exposure to antivascular agents and radiation is critical for the optimal scheduling of combined treatments.

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References


Tumor Response to Ionizing Radiation Combined with Antiangiogenesis or Vascular Targeting Agents: Exploring Mechanisms of Interaction

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