Drug-Radiation Interactions in Tumor Blood Vessels

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Obliteration of the tumor vasculature is an effective means of achieving tumor regression. Antiangiogenic agents have begun to enter cancer clinical trials. Ionizing radiation activates the inflammatory cascade and increases the

Introduction

Therapeutic irradiation has the potential to enhance the destruction of tumor blood vessels and should be considered when designing clinical trials of antiangiogenic agents. These agents include biological response modifiers, antibodies, enzyme inhibitors, therapeutic genes, analogs of bacterial and fungal products, and cytotoxic drugs. The importance of this approach is that angiogenesis is an essential component of neoplasia.[1,2]

Blood vessel growth involves several processes, each of which represents a therapeutic target to combat neoplasia. Endothelial proliferation is required for the development of a vascular sprout into the tumor. Endothelial growth can be regulated by a number of growth factors, including vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor, and angiopoietins. Endothelial cells migrate into the neoplastic tissue in a manner that maintains the structure of blood vessels. Endothelial cell motility is required for movement into the tissue. The structural and functional integrity of the blood vessel must be maintained to provide circulation into the tumor. Inhibition of any of these processes is a strategy to treat most forms of cancer.

Antiangiogenic Agents

The classic antiangiogenic biological response modifier is tumor necrosis factor-a (TNF).[3] TNF is produced during inflammation and functions at many levels to prevent angiogenesis, including inhibition of endothelial cell proliferation and induction of thrombosis and inflammation. Other more recently identified biological response modifiers that function as antiangiogenic agents include angiostatin, endostatin, and antagonists that bind to angiopoietin receptors.[4,5] Cytokines that are antiangiogenic include TNF, interferon, platelet factor 4, and interleukin-12.[6-8]

In addition to these naturally occurring proteins, a number of macromolecules produced by bacteria and fungi have been identified as antiangiogenic compounds, such as CM101 and TNP470.[9,10]

Other levels of activity of antiangiogenic compounds include antibodies to integrins or growth factors and cytotoxic agents.[11] Recently, it has been shown that antiangiogenic agents can be combined with cytotoxic drugs to enhance tumor control.[12]

Ionizing Radiation

Ionizing radiation is also cytotoxic to the vascular endothelium.[13,14] Growth factors and other biologic response modifiers can influence radiation-induced cytotoxicity in the endothelium. For example, elimination of growth factors enhances endothelial cell killing by ionizing radiation, and administration of growth factors minimizes endothelial cytotoxicity.[15] In addition to the direct cytotoxic effects of radiation on endothelial cells, radiation-induced oxidative injury to the endothelium activates homeostatic responses.[16] In this regard, platelets are also activated within irradiated tissues, resulting in platelet aggregation.[17,18] Ionizing radiation also activates inflammation through the induction of cell-adhesion molecules and cytokines.[19-24] These proteins and proteoglycans activate circulating leukocytes and thereby mediate the inflammatory response to irradiation.[16]

As described below, each of these radiation-induced processes can be enhanced within the tumor vasculature to achieve obliteration of blood vessels. The advantage of ionizing radiation over other
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whether radiation produces exocytosis of Weibel-Palade bodies, we irradiated the vasculature within inflammation. These include P-selectin, von Willebrand factor, interleukin-8, and CD63. To determine Weibel-Palade bodies contain several proteins and proteoglycans that initiate thrombosis and prothrombotic response of the endothelium to radiation.

Radiation Induces a Procoagulative State in the Endothelium

P-selectin staining on platelets. P-selectin exocytosis, therefore, contributes to the proinflammatory localization of P-selectin to the vascular lumen was associated with platelet aggregation and bleeding times compared to those of wild-type mice.[33] Moreover, the slowing of platelet flow activation is inhibited by anti–P-selectin antibodies.[34] P-selectin–deficient mice have prolonged platelet aggregation. These findings indicate that radiation-induced P-selectin expression in irradiated tumor blood vessels, and observed that P-selectin was localized within the endothelium of tumor vessels prior to treatment. At 1 to 6 hours following irradiation, P-selectin was localized to the lumen of blood vessels.

To determine whether radiation-induced vascular lumen localization of P-selectin was tumor type-specific or species-specific, we studied tumors in rats, C3H mice, C57BL6 mice, and nude mice. P-selectin localization to the vascular lumen was present in all tumors and all species studied. Irradiated intracranial gliomas showed P-selectin localization to the vascular lumen within 1 hour, whereas blood vessels in normal brain showed no P-selectin staining in the endothelium and no localization to the irradiated vascular lumen. Radiation-induced P-selectin localization to the vascular lumen increased in a time-dependent manner until 24 hours after irradiation (Figure 1). Since P-selectin in platelets may account for this time-dependent increase in staining, we employed immunohistochemistry for platelet antigen GP-IIIa to differentiate between endothelial and platelet localization of P-selectin.

We found that GP-IIIa staining was not present 1 hour after irradiation, but increased at 6 hours and 24 hours. P-selectin localization to the vascular lumen at 6 to 24 hours was, in part, associated with platelet aggregation. These findings indicate that radiation-induced P-selectin staining in the vascular lumen of neoplasms is associated with platelet aggregation. Radiation-induced localization of P-selectin to the vascular lumen is specific to the microvasculature of malignant gliomas and is not present in the blood vessels of the irradiated normal brain.

The presence of P-selectin in membranous organelles, such as a-granules of platelets and Weibel-Palade bodies in endothelial cells, enables it to be readily available for hemostasis. P-selectin initiates the slowing of circulating platelets, implicating it in hemostasis and thrombosis.[32-34] Platelets roll on stimulated endothelium by interacting with endothelial P-selectin.[32] Platelet activation is inhibited by anti–P-selectin antibodies.[34] P-selectin–deficient mice have prolonged bleeding times compared to those of wild-type mice.[33] Moreover, the slowing of platelet flow through blood vessels is markedly impaired in P-selectin–deficient mice. We observed that localization of P-selectin to the vascular lumen was associated with platelet aggregation and P-selectin staining on platelets. P-selectin exocytosis, therefore, contributes to the proinflammatory and prothrombotic response of the endothelium to radiation.

Radiation Induces a Procoagulative State in the Endothelium

Weibel-Palade bodies contain several proteins and proteoglycans that initiate thrombosis and inflammation. These include P-selectin, von Willebrand factor, interleukin-8, and CD63. To determine whether radiation produces exocytosis of Weibel-Palade bodies, we irradiated the vasculature within
the mouse thorax and performed immunohistochemistry for P-selectin. This experiment demonstrated that rapid exocytosis of Weibel-Palade bodies occurs within 30 minutes of irradiation.[18] We used human umbilical vein endothelial cells to study the mechanisms of radiation-mediated Weibel-Palade bodies exocytosis in vitro. We found that exocytosis was most efficient at 2 to 5 Gy, whereas higher radiation doses cause apoptosis in endothelial cells, which interferes with exocytosis.

The Shwartzman reaction shows the close interrelation between the inflammatory and hemostatic systems.[35] During this reaction, a hemorrhagic vasculitis is provoked by local injection of lipopolysaccharide, followed by injection of TNF-α into the same site.[36] The predominant feature of this vasculitis is platelet and neutrophil sequestration along the vasculature endothelium. Microthrombi composed of platelets, neutrophils, and fibrin occlude the capillaries and venules. The thrombotic component of this vasculitis is markedly attenuated in mice that are deficient in the P-selectin gene.[33] Depletion of neutrophils or platelets attenuates this vasculitis during the Shwartzman reaction.[37]

The mechanism of the increased procoagulative state is transcriptional induction of tissue factor, which is a transmembrane glycoprotein on monocytes. Tissue factor is a high-affinity receptor for coagulation factors VII and VIIa.[38] The resulting tissue factor-VIIa complex rapidly catalyzes the conversion of factor X to factor Xa and factor IX to factor IXa, leading to the formation of thrombin.[39] Monocyte-derived tissue factor can activate both the intrinsic and extrinsic coagulation cascades.

Irradiation of blood vessels produces a procoagulative state. The mechanisms are related to the release of von Willebrand factor[40,41] and interaction with leukocytes.[42] E-selectin and P-selectin bind to leukocytes and thereby activate expression of a number of genes including tissue factor and TNF.[28,29] Tissue factor expression promotes thrombosis.[38,39] TNF has also been shown to increase monocyte tissue factor generation.[38,39] Therefore, there are several potential mechanisms by which radiation creates a procoagulative state in the vasculature.

GP-IIIa is a platelet antigen that is not found in the vascular endothelium. We utilized anti–GP-IIIa antibodies to determine whether the time-dependent increase in P-selectin staining is due to platelet aggregation. Lewis lung carcinoma tumors in C57BL6 mice were irradiated and stained with anti–GP-IIIa antibody. Little GP-IIIa staining was observed in blood vessels at 1 hour following irradiation. However, GP-IIIa staining increased at 6 and 24 hours following irradiation (Figure 2). These findings indicate that the increased P-selectin staining within the vascular lumen of irradiated tumors was partially due to platelet aggregation.

To verify that platelet aggregation was present in these irradiated blood vessels, tissue sections were stained with anti–GP-IIIa antibodies that stained the platelets. We found no P-selectin or GP-IIIa staining in the brain or kidney, but both P-selectin and GP-IIIa staining were present in the irradiated lung, intestine, and tumor vessels. The P-selectin knockout mouse was used to study the correlation between platelet aggregation (ie, GP-IIIa accumulation) and P-selectin staining in the vascular endothelium. The GP-IIIa staining was not localized to the lumen of irradiated blood vessels in the knockout mouse, but extravasated into the irradiated lung, intestine, and tumors. Red blood cells also extravasated from irradiated tissues. Therefore, P-selectin accumulated in irradiated blood vessels correlated with maintenance of the barrier function of the endothelium. Knockout of the P-selectin gene leads to extravasation of platelets and red blood cells.

Enhancing the Thrombotic and Inflammatory Properties of Radiation

TNF acts in part through obliteration of the tumor vasculature.[43] This cytokine has been shown to be the principle component in the Shwartzman reaction in which thrombotic vasculitis occurs following the local administration of lipopolysaccharide followed 24 hours later by systemic lipopolysaccharide.[36] This hemorrhagic vasculitis is provoked by local lipopolysaccharide followed by local injection of TNF into the same site.[44] After lipopolysaccharide injection in the skin, neutrophils and monocytes infiltrate, and local TNF injection at the same site 24 hours later results in sequestration of platelets and neutrophils. Microthrombi, composed of platelets, neutrophils, and fibrin, occlude the capillaries and venules. Consequently, oblitative vasculitis and necrosis are observed at the site of the original TNF injection site.[35,45] We propose that the Shwartzman reaction can be used to induce thrombotic vasculitis in tumor vessels.

Localized ionizing radiation combined with localized TNF gene therapy can mimic the Shwartzman reaction in that a thrombotic vasculitis occurs in the tumor microvasculature.[46,47] Ionizing radiation activates the vascular endothelium to translocate Weibel-Palade bodies to the vascular lumen.[17,18] The importance of this finding is that two factors known to participate in platelet activation, P-selectin and von Willebrand factor, are components of Weibel-Palade bodies, which
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translocate to the vascular lumen by exocytosis in response to irradiation.[17,18] Von Willebrand factor in turn binds to the GP-Ib complex on platelets to initiate their aggregation. We propose that radiation-mediated local inflammation and procoagulative response within the vascular endothelium substitute for the inflammatory stimulus of lipopolysaccharide in the Shwartzman reaction. The first example of such an approach was observed when TNF was combined with radiation therapy. TNF given concurrently with radiation therapy produced excellent response rates.[48] However, the systemic toxicities prevented those trials from developing. An alternative approach was to localize TNF expression in tumors by using gene therapy. TNF gene therapy was administered by intratumoral injection into animal models.[47] This combined approach of localized TNF and localized radiation induced tumor regression without regrowth. The mechanisms of interaction between TNF and radiation included thrombosis of tumor vessels and inflammatory cell infiltration into necrotic tumors. It is therefore proposed that biological agents can enhance the prothrombotic and inflammatory effects of radiation.

The mechanisms by which TNF contributes to thrombotic vasculitis include the induction of inflammation, procoagulation, and apoptosis in the vascular endothelium. TNF stimulates endothelial cell procoagulant activity, and release of platelet-activating factor and von Willebrand factor.[43] TNF stimulates inflammation by inducing expression of ICAM-1, E-selectin, and P-selectin on the luminal surface of blood vessels. TNF also upregulates the integrin CD-llb on neutrophils. Endothelial cells also release factors such as colony-stimulating factor and interleukin-8. TNF stimulates inflammation by inducing expression of ICAM-1, E-selectin, and P-selectin on the luminal surface of blood vessels. TNF also upregulates the integrin CD-llb on neutrophils. Endothelial cells also release factors such as colony-stimulating factor and interleukin-8. TNF activates endo-thelial cells to express CAM, which in turn activates circulating leukocytes.[49] This is the probable mechanism of interaction, because ionizing radiation activates inflammatory cells within irradiated blood vessels.[17,18] Moreover, we have found that platelet aggregation occurs within irradiated blood vessels.[17] We propose that radiation activation of the endothelium elicits intercellular signaling from the vascular endothelium to circulating leukocytes and platelets to produce a procoagulant state and inflammation.

Ionizing radiation induces apoptosis in a small percentage of vascular endothelial cells.[14,50] TNF enhances radiation-mediated apoptosis in the vascular endothelium.[50] Apoptotic vascular endothelial cells become procoagulant,[51] whereas unperturbed endothelial cells provide anticoagulant properties. Endothelial cells undergoing apoptosis showed increased tissue factor and decreased thrombomodulin, heparin, and tissue-factor pathway inhibitor.[51] Radiation thereby complements or amplifies the response to TNF, resulting in microvascular obliteration. The significance of the proposed studies is that determining the mechanism of the tumor-specific thrombotic vasculitis by radiation and TNF will allow us to enhance this reaction. For example, desmopressin induces P-selectin expression and increases leukocyte adhesion.[52]

Histology of Xenografts Treated With TNF Expression Vector and Radiation

It has long been recognized that a primary component of radiation-mediated tissue damage is injury to the vascular endothelium.[15,53] We and others have shown that vascular endothelial injury is enhanced by the presence of TNF.[46,47,50] Vascular thrombosis occurs in tumors treated with a combination of TNF and radiation, whereas tumors treated with either agent alone showed no vascular thrombosis (Figure 3).

Tumors treated with combined TNF and radiation demonstrated marked necrosis on tissue sections, whereas tumors treated with either TNF alone or radiation alone showed no necrosis.[46,47] We have proposed that TNF produced within irradiated tissue augments the injury to the vascular endothelium.

TNF interacts with the endothelium of small vessels to induce microvascular obliteration and subsequent hemorrhagic necrosis.[3] TNF also regulates leukocyte extravasation from the vasculature and subsequent infiltration of inflammatory cells into the tissue. To determine whether necrosis and inflammation were components leading to tumor control by the Egr-TNF genetic construct and radiation, we analyzed histologic sections of xenografts. Tumors treated with TNF expression vector and radiation (5 Gy/day × 4 days) or Ad5 (null virus) and radiation were excised on day 7 and fixed in 10% neutral buffered formalin. Tumors were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, followed by examination for necrosis by light microscopy. Xenografts treated with both TNF expression vector and radiation had necrosis over a mean of 32% of the low power fields (10 fields counted) within 7 days, while tumors treated with null virus and radiation had necrosis over a mean of 1.8% of the tumor volumes (P = .007). This is supported by recent findings that direct tumor cell killing by TNF is associated with apoptosis as well as...
necrosis. To determine whether inflammatory cell infiltration of tumor xenografts was a component of tumor control, we graded the leukocyte infiltration within tumor sections. Leukocyte infiltration was found in tumors treated with TNF expression vector. The amount of inflammatory cell infiltration was greater in tumors treated with a combination of TNF expression vector and radiation compared to tumors treated with null virus and radiation or null virus alone (P = .005). Histologic sections of tumors treated with the Egr-TNF genetic construct and radiation therefore show the classic characteristics of TNF-induced changes (ie, necrosis and inflammatory cell infiltration). The inflammatory component of TNF is consistent with findings observed with recombinant TNF combined with radiation in vivo.

**Improving the Biodistribution of Antiangiogenic Gene Therapy Delivery**

The biodistribution of gene therapy is dependent upon the route of administration. When administered by intratumoral injection, therapeutic gene expression is limited to the track of the needle insertion site (Figure 4). Intravascular administration is an alternative approach and delivers the therapeutic gene directly to the target cells (ie, vascular endothelium). Moreover, the circulation distributes gene therapy vectors throughout the tumor vasculature. We therefore studied the feasibility of achieving gene expression in tumor blood vessels following vascular administration of adenovirus vectors. The replication-deficient adenovirus type 5 containing the beta galactosidase (β-gal) gene was administered by intraarterial injection. Tumors were resected and reporter gene expression was analyzed by immunofluorescence microscopy. Figure 4 shows β-gal immunofluorescence staining in the tumor microvasculature. This demonstrates that it is feasible to deliver gene therapy to tumor blood vessels through intraarterial injection. Strategies to improve gene expression in tumor blood vessels involve modification of the fiber on viral vectors that are designed to bind to heparan sulfate and polyanioanic cellular receptors on the endothelium.

**Direct Interaction Between Radiation and Drug in the Vascular Endothelium**

Antiangiogenic agents potentiate cytotoxic cancer therapy. Radiation-induced cytotoxicity might also be enhanced by antiangiogenics. The first example of an interaction between ionizing radiation and antiangiogenic agents showed that Lewis lung carcinoma tumors in mice had increased growth delays when drugs were combined with radiation, compared to either agent alone. In contrast, mouse mammary carcinomas treated with TNP470 during radiotherapy showed reduced tumor control, compared to radiation followed by TNP470. The authors propose that the lack of interaction between TNP470 and radiation is due to hypoxia in tumor tissue, which reduces radiation sensitivity. We propose that enhancement of the therapeutic effects of radiation by antiangiogenic agents might be specific and dependent upon the mechanism of action. The mechanisms by which ionizing radiation interact with antiangiogenic agents may include enhancement of thrombosis, vasculitis, or the cytotoxic effects of radiation on the endothelium. The interaction between ionizing radiation and angiostatin has been shown to be enhancement of the direct cytotoxic effects on endothelial cells. The combination of these agents achieved no thrombosis or vasculitis but did reduce the quantity of blood vessels in neoplastic tissues. Therefore, a direct interaction between radiation and cytotoxic agents may be one strategy to obliterate tumor blood vessels.

Other agents that enhance radiation-induced cytotoxicity in endothelial cells have recently been identified. It is therefore likely that ionizing radiation combined with cytotoxic agents may interact at the level of the vascular endothelium. The importance of these observations is that the endothelium is unlikely to develop drug resistance. Concomitant chemotherapy and radiation therapy is an established treatment regimen for many solid neoplasms. Interactive killing of endothelial cells by cytotoxic agents must be considered. For example, paclitaxel (Taxol) has been shown to be antiangiogenic and also enhances the therapeutic effects of ionizing radiation in clinical trials. In addition, the combined effects of cytotoxic agents and radiation on normal vascular endothelium must also be considered.

**Conclusions**

The therapeutic effects of ionizing radiation on tumors may be enhanced by antiangiogenic factors.
Ionizing radiation is directly cytotoxic to endothelial cells, and relatively innocuous compounds might increase this effect. In addition, radiation induces a prothrombotic and vasculitic effect in tumor vessels. These effects will be enhanced in tumors by the use of biological response modifiers such as TNF.

References:


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