

Tumor angiogenesis as a strategy for radiosensitization

Review Article

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Abbreviations: tyrosine kinase inhibitors, (TKIs); receptor tyrosine kinases, (RTKs); vascular endothelial growth factor, (VEGF); platelet-derived growth factor, (PDGF); fibroblast growth factor, (FGF); circulating endothelial progenitor cells, (CEPs); high dose rate, (HDR); phosphatidylinositol-3,4,5-triphosphate, (PIP3); Phosphatidylinositol 3-kinases, (PI3Ks); 3-phosphoinositide kinase-1, (PDK1); VE Cadeherin, (VEC); von Willebrand factor, (vWF); PECAM, (CD31)

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Summary

Tumor angiogenesis is crucial for the proliferation, survival and metastases of all malignancies. The response of the tumor microvasculature to ionizing radiation can be modified to improve tumor control in preclinical mouse models of cancer. Recent studies have shown that a variety of anti-angiogenic drugs can enhance radiotherapy. Protein tyrosine kinase inhibitors (TKIs) have been shown to enhance radiation-induced destruction of tumor blood vessels. Among these compounds are inhibitors of a broad spectrum of receptor tyrosine kinases (RTKs). Inhibition of RTKs attenuates downstream signaling from various angiogenic growth factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). RTK inhibitors with various specificities against the receptors for VEGF, PDGF and FGF manifest significant antiangiogenic activities as well. We have shown using tumor vascular window model and tumor growth delay assays that these compounds can enhance tumor radiation response by attacking tumor microvasculature. Furthermore, we have shown that radiation and RTK inhibitors exert their antiangiogenic effect through inhibition of the PI3K/Akt signaling pathway, which results in induction of apoptosis. Inhibition of these signaling pathways may block vascular repair or neoangiogenesis through suppression of endothelial progenitor cells. Our studies have provided a basis for future clinical investigations of combining radiotherapy and RTK inhibitors.

I. Introduction

Tumor Angiogenesis

Tumor angiogenesis plays an important role in the growth, invasion and metastases of solid tumors. In its absence, tumors are limited to a growth of 1-2mm³. Tumor growth is dependent on an adequate supply of oxygen and nutrients as well as removal of toxic metabolites. Angiogenesis occurs when the tumor mass contains 100-300 cells and is driven by endothelial cell proliferation. Recruitment of endothelial progenitor cells also plays an active role (Griffioen, 2000). Folkman et al hypothesized that anti-angiogenic therapy would keep tumors in a dormant state and would enhance effectiveness of other cancer therapies (Folkman, 1971). Since anti-angiogenesis

targets non-malignant endothelial cells, drug resistance that is common for cancer cells, may not develop in anti-angiogenic therapy (Kerbel, 2000). Several pro-angiogenic factors have been identified. Vascular endothelial growth factor (VEGF) plays an essential role in tumor angiogenesis (Ferrara, 2000). Inhibition of VEGF signaling pathway via VEGF receptor (VEGFR) blocking antibodies or VEGFR kinase inhibitors resulted in tumor growth inhibition in animal models (Angelov, 1999) (Fong, 1999). However, resistance to this therapeutic approach can occur by up-regulation of other angiogenic factors such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (Kerbel, 2001). Compounds that inhibit multiple angiogenic pathways may circumvent this problem. However, the potential side effects of these

compounds are uncertain and are under investigation.

II. Radiotherapy and tumor angiogenesis

Ionizing radiation interacts with cells by the Compton effect, which produces electrons and causes DNA breaks in the tissue. Vascular endothelium is highly resistant to the effects of radiation. The presence of VEGF may cause the increased resistance to radiation-induced damage. Preclinical studies suggest that antiangiogenic agents enhance tumor control in response to fractionated irradiation. The response of tumor microvasculature to radiation is time- and dose-dependent (Johnson, 1976). Blood flow studies using irradiated mouse sarcoma showed that blood flow increased within 3 to 7 days (Kallman, 1972). Tumor blood flow increases if low doses are administered and does not decrease unless high doses are used (Kallman, 1972) (Johnson, 1976). This delayed increase in tumor blood flow may be due in part to radiation-induced VEGF expression (Gorski, 1999). Previous studies of tumor blood vessel response to radiation have relied upon the clearance of Xe and blood volume within tumors following treatment with radiation (Kallman, 1972) (Johnson, 1976). Current technology allows for the direct, longitudinal observation of tumor blood volume and blood flow (Fleischer, 2000) (Lin, 1998). Available experimental models include the tumor vascular window (Lin, 1998); Power Doppler to measure blood flow (Fleischer, 2000); and histologic evaluation of tumor blood vessel (Hallahan, 1998). Using these models, we found that blood flow increased in all tumors receiving 2 to 3 Gy, which is the fractionation scheme used during conventional radiotherapy. Doses used for stereotactic radiosurgery, intraoperative radiotherapy and high dose rate (HDR) brachytherapy (6 to 10 Gy) resulted in a reduction in blood flow in all tumor types (Figure 1).

III. Effects of anti-angiogenic agents on tumor vessels and radiation response

A. Angiogenic growth factors and receptor tyrosine kinases (RTKs)

Split-kinase domain RTKs, including the PDGF, Flk-1/KDR and FGF receptors and their angiogenic ligands play important roles in tumor angiogenesis. The inhibition of VEGF by antibodies (Angelov, 1999) and the receptor antagonists enhanced tumor control when combined with cytotoxic therapy (Fong, 1999). Other RTK ligands, including FGF and PDGF, also contribute to angiogenesis and tumor growth (George, 2001). bFGF has been shown to inhibit apoptosis in the microvasculature of mouse lungs and intestines exposed to irradiation (Paris, 2001). FGF may contribute indirectly to angiogenesis through up-regulation of VEGF (Seghezzi, 1998). PDGF also increases VEGF secretion in tumor cell lines (Tsai, 1995). In addition, VEGF, FGF, and PDGF are all up-regulated in response to radiation (Witte, 1989). PDGF is produced by various cancer cells and contributes to both autocrine and paracrine growth and viability (Eshel, 2002). Biopsies

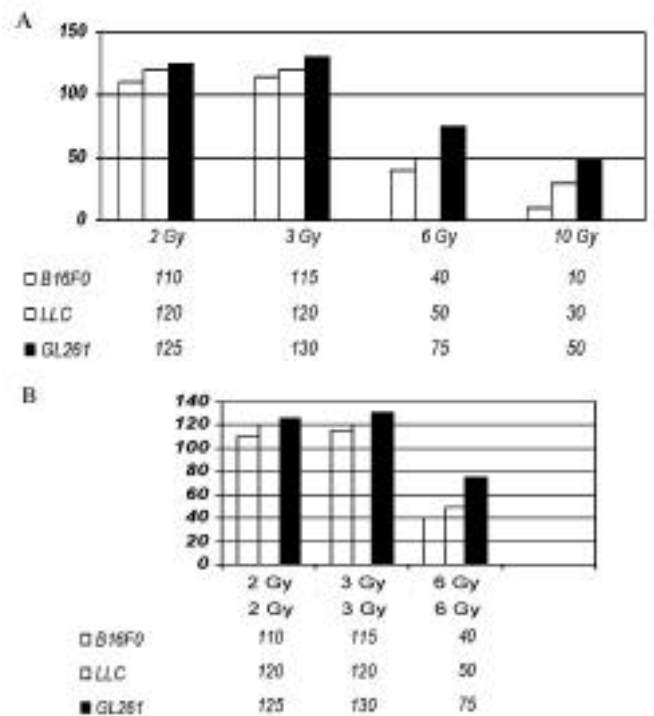


Figure 1. A). Dose-dependent Radiation-response of tumor blood vessels in the vascular window model. Tumor blood vessels were treated with the indicated dosage of radiation. Tumor blood vessels were photographed using light microscopy and vascular density was quantified by use of line morphometry. The vascular density is compared to untreated controls (100%). Shown are the mean and standard error of the mean of 3 experiments, using models of B16F0 melanoma; Lewis lung carcinoma (LLC) and GL261 glioma. **B).** Bar graph of Power Weighted Pixel Density (PWPDP) showing dose-dependent changes in tumor blood flow. Doppler blood flow analysis was performed after irradiation. Shown are dose-dependent changes in tumor blood flow relative to untreated control tumors (100%).

from 95 head and neck cancers showed increased PDGF-B in 54% of cases, which was associated with increased risk of systemic recurrences (Aebersold, 2002). These studies establish potential therapeutic benefits through the inhibition of RTKs and their ligands. RTKs are key elements of signaling pathways (Schlessinger, 2000). Platelet-derived growth factor receptor (PDGFR), a prototypical RTK, contains a core domain flanked by unfolded regions. Specifically, the intracellular portion of PDGFR contains a juxtamembrane region, two kinase domains separated by an unfolded kinase insert, and an unfolded C-terminal tail (Claesson-Welsh, 1994). Posttranslational modification of PDGFR creates a binding site that recruits regulatory proteins. The phosphotyrosines in the RTK form binding sites that are recognized by the SH2 domains of several signaling proteins, including Grb2, and Shc, and the p85 component of PI3K (Claesson-Welsh, 1994). This localized activity increases phosphatidylinositol-3,4,5-triphosphate (PIP3) concentrations at the cell membrane, thus locally activating Akt. Akt is a common target that is activated by various signaling pathways stimulated by RTKs. Although studies suggest that redundancy is an underlying feature of RTK signaling networks, there are also examples in which

specific downstream pathways are required for appropriate cellular response to an RTK-mediated signal (Madhani, 2001).

B. RTK inhibitors (TKIs)

Several TKIs with various specificities against the receptors of VEGF, PDGF and FGF have been developed (Table 1). SU5416 is a quinolone derivative that inhibits VEGFR-2 (Flk-1) tyrosine kinase. SU6668 is an oral tyrosine kinase inhibitor with multiple receptor targets, including VEGFR, PDGFR and bFGFR. We have found that both SU5416 and SU6668 resulted in radiation sensitization in several mouse models of solid tumors (Geng, 2001) (Lu, 2004). SU11248 is an orally available, indolinone-based synthetic molecule, which is a low nM selective inhibitor of the angiogenic receptor tyrosine kinases Flk-1/KDR and PDGFR. It also inhibits cellular signaling via Kit and FLT3. SU11248 exhibited broad and potent anti-tumor activity in

mice, causing regression of A431 human epidermoid and Colo205 human colon tumors, growth arrest of H460 human lung, and substantial growth delay of C6 rat and SF763T human glioma xenografts (Mendel, 2003). SU11248 treatment induced a dose- and time-dependent decrease in tumor microvessel density and tumor cell proliferation and an associated increase in tumor cell apoptosis, culminating in tumor regression. SU11248 is currently in Phase I clinical trials in patients with advanced cancer. PK/PD studies in mice have shown that SU11248 inhibited PDGFR and Flk-1/KDR phosphorylation in a time- and dose-dependent fashion with target plasma concentrations of 50-100 ng/ml. The selectivity of SU11248 is demonstrated by the fact that it does not inhibit EGFR phosphorylation, even at high plasma concentrations. Using the tumor vascular window model, we have shown that SU11248 enhances vascular injury following radiation (Figure 2) (Schueneman, 2003).

Table 1: Known Spectrum of RTK inhibition

	<u>VEGFR2</u>	<u>Flt</u>	<u>PDGFR</u>	<u>c-Kit</u>	<u>EGFR</u>	<u>ErbB</u>
SU5416	+	+	-	-	-	-
SU6668	+	+	+	+	+	-
SU11248	+	+	+	+	-	-

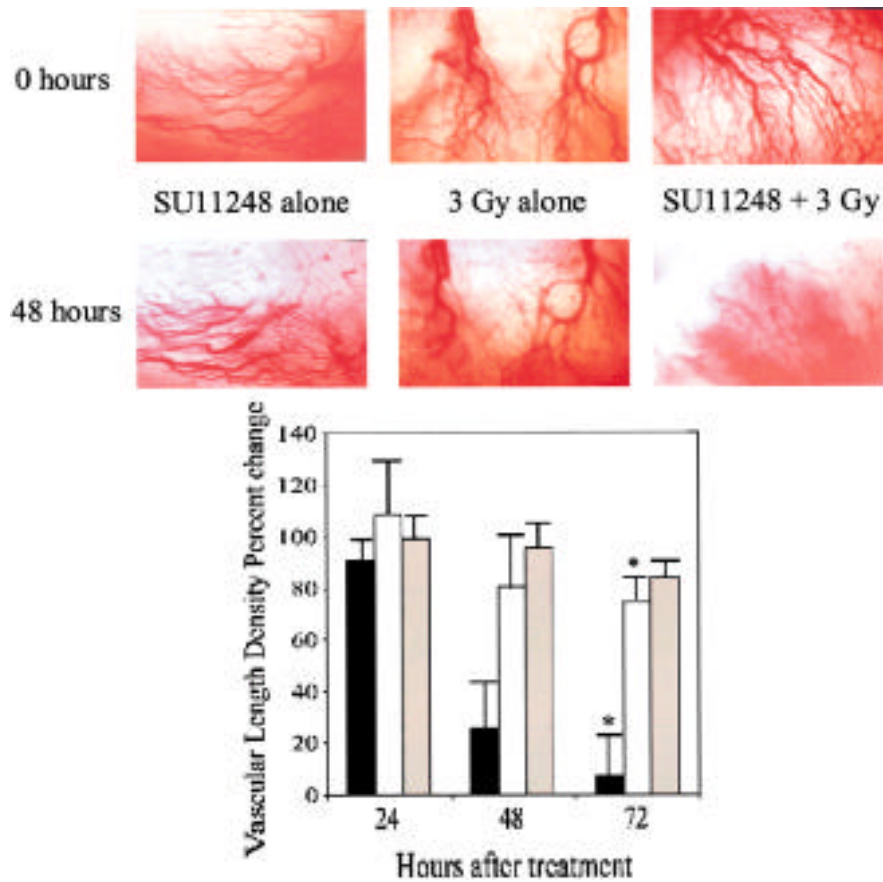


Figure 2. Tumor vascular window model and vascular length density analysis. LLC cells were implanted into the dorsal skin-fold window in C57BL6 mice. Shown are representative photographs of tumor vasculature before and 48 h after treatment with SU11248 (left column), 3 Gy (center), and SU11248 + 3 Gy (right column). Five mice were treated in each of the treatment groups. The vascular length density at 48 h after treatment was quantified. The bar graph shows the means of vascular length densities for each treatment group over 4 days and SE.

SU11248 administered with irradiation achieved significantly greater reduction in tumor vasculature than either agent alone. Furthermore, we found that both LLC and GL261 tumors showed a significant growth delay when SU11248 was added before daily 3 Gy fractions as compared with either agent alone (**Figure 3**). The PDGFR β is one of the molecular targets for SU11248. Phosphorylation of PDGFR β within tumor tissue therefore serves as a biomarker for response to SU11248 in tumor models. PDGFR β phosphorylation was studied through immunohistochemical analysis of tumor sections with phospho-specific antibody. **Figure 3 (C and D)** shows that PDGFR β phosphorylation was detected in the stroma and endothelium of tumors before treatment. 3 hours after SU11248 administration, PDGFR β phosphorylation was undetectable by immunohistochemistry (**Figure 3D**). This indicates that SU11248 is biologically active within mouse tumor models after systemic administration.

C. PI3K cell survival pathway

Phosphatidylinositol 3-kinases (PI3Ks) are activated by RTKs (Wymann, 1998) (**Figure 4**). The ability of RTK-binding growth factors to promote cell survival has been attributed, at least in part, to PI3K. It has been shown that PI3K activity is required for the growth factor-dependent survival of a wide range of cultured cell types. PI3K activity promotes cellular survival even in the absence of trophic support. Active PI3K can block toxin-induced apoptosis (Datta, 1999). PI3K also plays an important role in the response of tumors to radiation. We have shown that PI3K inhibitors such as wortmannin and LY294002 enhance the cytotoxic effects of radiation through inducing apoptosis of tumor vasculature (**Figure 5**) (Edwards, 2002). One important downstream target of D3 phosphorylated phosphoinositides is the serine/threonine kinase Akt-1 (Burgering, 1995). Recruitment to the plasma membrane by 3' phosphorylated phosphoinositides brings Akt-1 in close proximity to the regulatory kinase 3-phosphoinositide kinase-1 (PDK1). PDK1 phosphorylates Akt at Thr308, thus activating it (Alessi, 1997). Once activated, Akt-1 targets a number of downstream substrates that are involved in apoptotic and anti-apoptotic signaling (Datta, 1999). One of the targets is the Bcl-2 family member Bad. Bad promotes cell death through heterodimerization with the survival protein Bcl-XL (Yang, 1995). Formation of this heterodimer leads to release of cytochrome c from the mitochondria, causing cleavage of procaspase-9 and a subsequent cascade that culminates in apoptosis. Active Akt-1 phosphorylates Bad at Ser-136 (Datta, 1997), which blocks Bad/Bcl-XL heterodimerization (Cardone, 1998). This action is sufficient to block Bad-induced apoptosis (Datta, 1997). However, Akt-1 was shown to block apoptosis even after cytochrome c release, which led investigators to search for other downstream targets of Akt-1. Cardone et. al. showed that Akt-1 phosphorylates caspase-9 at Ser196, which prevents cytochrome c induced activation of pro-caspase 9 (Cardone, 1998). Our laboratory recently showed that combination of radiation and SU11248 induced apoptosis in tumor vasculature (**Figure 6A**) (Schueneman, 2003), and we found that cells transfected with adenovirus expressing a dominant

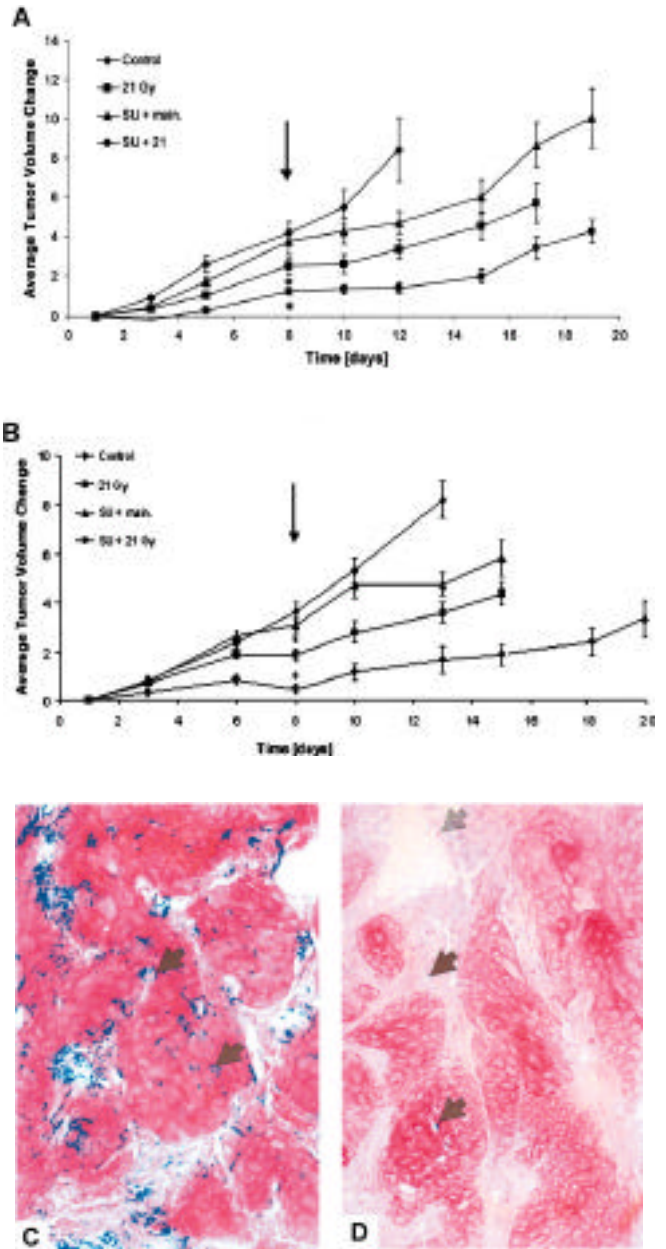


Figure 3. Tumor growth delay analysis. Mice with (A) LLC and (B) GL261 hind limb tumors were treated with SU11248 or vehicle before irradiation. Therapy was halted after day 8 (arrows). Shown are the means of changes in tumor volumes in five mice in each of the treatment groups (vehicle, SU11248 maintenance main, vehicle + 21 Gy, and SU11248 + 21 Gy). Bars indicate SE. PDGFR β phosphorylation was shown by immunohistochemical analysis of tumor sections. C and D show microscopic (x40) photographs of immunohistochemical staining of LLC tumors after i.p. administration of (C) SU11248 or (D) vehicle. Sections were stained for phospho-PDGFR β using alkaline phosphatase (blue) stain and counterstained with eosin. Arrows indicate microvasculature that stains positive for phospho-PDGFR β .

negative mutant of p85 enhanced radiation-induced apoptotic activity, i.e., release of cytochrome c and activation of caspases 3 and 9 in HUVEC cells, a model for tumor angiogenesis (**Figure 6B**) (Tan, 2003).

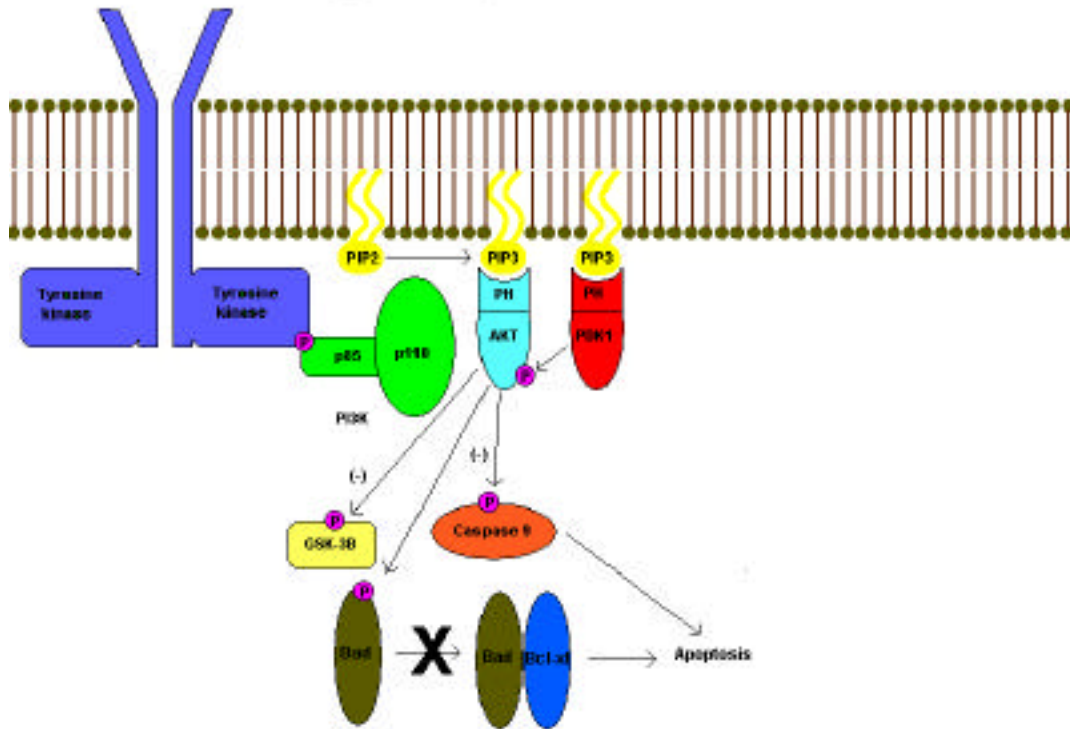


Figure 4. PI3K signaling through Akt. PI3K, when recruited to the plasma membrane by an activated RTK, converts PIP2 to PIP3. PIP3 recruits Akt, which is activated by PDK1. Akt phosphorylates several downstream targets that are important players in apoptotic signaling, cell cycle regulation, and metabolism, including GSK-3 β , Bad, and Caspase-9.

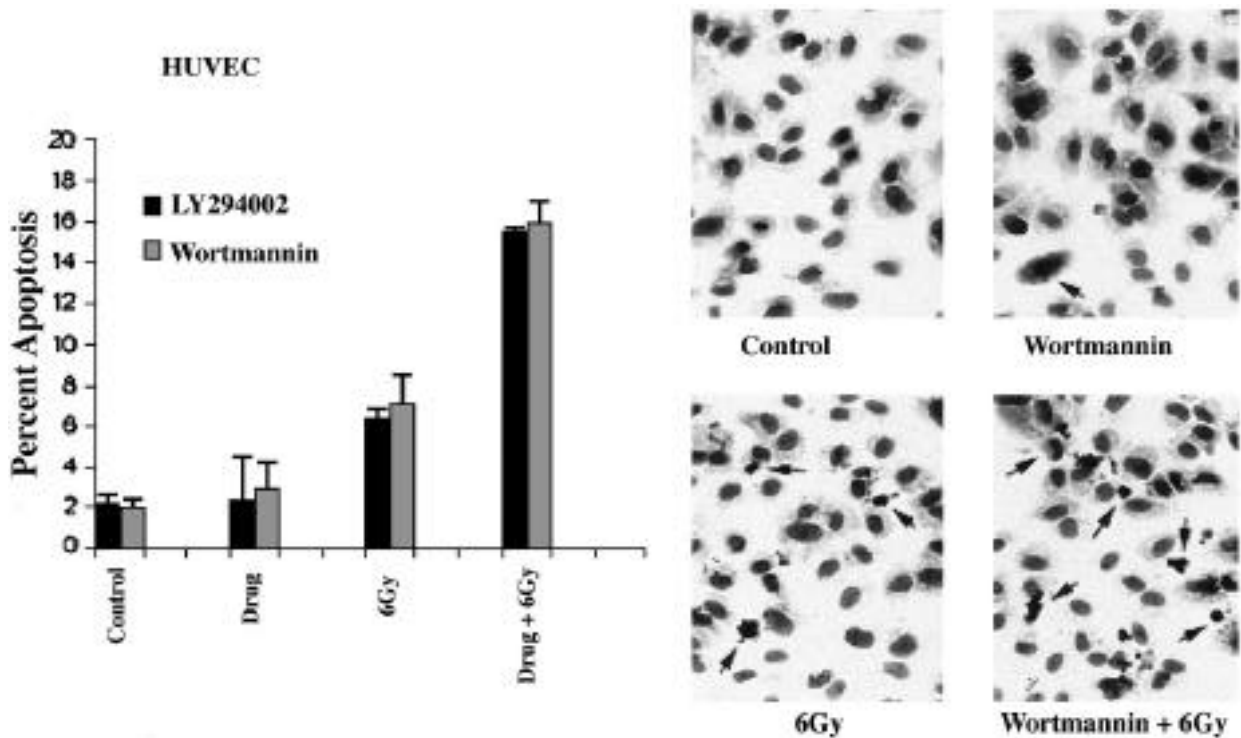


Figure 5. Induction of apoptosis in endothelial cells by PI3K inhibitors. HUVECs were treated with either 2 μ M LY 294002 or 4 nM Wortmannin, incubated for 30 min, and treated with radiation (6 Gy). After a 24-h incubation period, cells were fixed and stained. Four high-powered fields (x400) were observed and counted for each experimental group. Shown is the percentage of apoptotic cells for each experimental group. Photographs show representative HUVECs treated with radiation, LY294002, or LY294002 before irradiation. Arrows, apoptotic nuclei. Bars, SD.

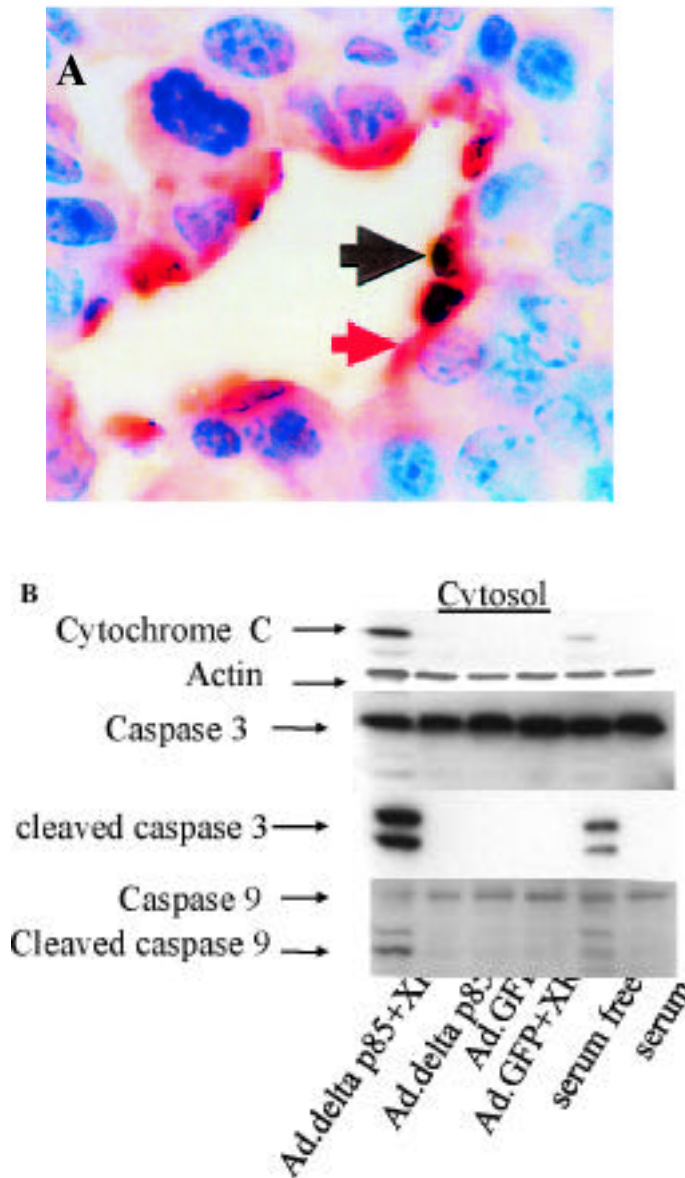


Figure 6. Induction of apoptosis by SU11248 and mutant Akt. A). Sections of tumors were stained for apoptosis in endothelial cells using brown TUNEL (*brown arrows*) and endothelial cell marker using VWF red immunostaining (*red arrows*). B). Cytochrome C released from mitochondria induced by PI3K inhibitor and radiation. cells were transduced with Ad_p83 and radiation alone or together. The adenovirus containing GFP gene insert was using as control.

IV. Endothelial cell precursors in tumor angiogenesis and therapy response

VEGF released by tumors promotes mobilization of circulating endothelial progenitor cells (CEPs) and hematopoietic cells to the vascular bed where they contribute to neovascular formation (Rafii, 2002). CEPs express the VEGFR-2 or FLK1 whereas subsets of hemotopoietic cells express VEGFR1 or FLT1(Rafii, 2002). Co-recruitment of CEPs and hemotopoietic cells facilitates the differentiation and integration of CEPs into rapidly expanding tumor vasculature. These cells express several endothelial protein markers: VE Cadeherin (VEC), von Willebrand factor (vWF), PECAM (CD31), and PIH12. CD34+ endothelial progenitor cells isolated from human peripheral blood differentiate into endothelial cells

(Asahara, 1997). These progenitor cells develop foci of neovascularization in ischemic limbs at 4 weeks after injection (Asahara, 1997). Signal transduction through VEGF participates in differentiation of pluripotent stem cells into endothelial cells (Choi, 1998). Flk-1 receptors also participate in migration of these cells into ischemic tissue (Shalaby, 1995). To determine the origin of these CEPs, bone marrow stem cells containing the Lac Z reporter gene were transplanted into tumor bearing mice (Asahara, 1999). This study showed that CEPs from bone marrow origin were incorporated into tumor neovascularization. VEGF increases the percentage of pluripotent hematopoietic stem cells that stain positive for CD 34 and Flk-1 (Ziegler, 1999) including circulating stem cells.

To study the mechanisms of CEPs' incorporation into tissue, antigen expression was studied. Flk-1 + EPCs with no other initially expressed endothelial cell markers differentiated into VE Cadherin+ PECAM-1+ and CD 34+ cells (Hirashima, 1999). Following activation of the Flk-1 receptor tyrosine kinase, expression of VE Cadherin was induced, which is required for EPC adhesion (Hirashima, 1999) (Vittet, 1997). Monoclonal antibodies to VE Cadherin inhibited vessel formation (Vittet, 1997). VEGF and Flk-1 are also required for proliferation and migration of the EPCs following adhesion.

In summary, tumor microvasculature is novel target for radiation sensitization. Inhibitors of angiogenic RTKs have shown their efficacy in enhancing radiotherapy in several animal models of solid tumors. SU11248, a recently developed RTK inhibitor, is currently planned for clinical trials to be combined with radiotherapy. Inhibition of downstream molecules of RTKs such as PI3K and Akt resulted in similar radiation sensitization. Further investigation of RTK signaling may identify new molecular targets and lead to novel drug development for cancer therapy.

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