Angiogenesis (2013) 16:443–454
DOI 10.1007/s10456-012-9329-2

ORIGINAL PAPER

Dose-dependent response of tumor vasculature to radiation therapy in combination with Sunitinib depicted by three-dimensional high-frequency power Doppler ultrasound

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Received: 27 February 2012 / Accepted: 23 November 2012 / Published online: 12 January 2013

Abstract
Purpose Large doses of radiation (8–20 Gy) preferentially target tumor vasculature. This vascular response is suggested to regulate tumor response to radiotherapy. Here, we investigate the relative contributions of direct cell killing by radiation versus tumor cell death due to radiation effects on the vasculature. We also examine Sunitinib's mechanism of action as a tumor radiosensitizer.
Experimental Design MDA-MB-231 xenografts were treated with radiation doses of 2–16 Gy alone, or in combination with bFGF (endothelial radio-protector) or Sunitinib as pharmacological modulators of the vasculature. Sunitinib was orally administered for 2 weeks at 30 mg/kg before radiotherapy; bFGF was intravenously injected 1 h prior to irradiation. Three-dimensional high-frequency power Doppler ultrasound was used to assess relative changes in tumor vasculature. Immunohistochemistry, clonogenic and tumor growth assays were used to quantify tumor response.
Results Significant reductions in power Doppler signal of up to 50 % were observed for 8 and 16 Gy treatments, along with a dose-dependent increase in cell death. No significant change in power Doppler signal and minimal tumor cell death were noted for tumors treated with radiation and bFGF. Treatments where Sunitinib was combined with radiation demonstrated a significant increase in flow signal at doses equal or greater than 8 Gy. This was accompanied with a significant increase in cell death when compared to radiation or Sunitinib alone.
Conclusion We confirm that tumor response to high doses of radiation is regulated by its vasculature. We also posit that the response observed when radiation is combined with Sunitinib is linked to a vascular "normalization" effect.

Keywords Three-dimensional high-frequency power Doppler ultrasound · SU11248 · bFGF · Vascular normalization

Introduction

Radiation therapy is a primary therapeutic modality in cancer treatment. Although it is well recognized that radiation acts by damaging DNA in tumor cells, recent evidence suggests that host-derived endothelial cells present in tumors are linked to overall tumor response to radiotherapy. Studies have suggested that single large doses of radiation (>8 Gy) primarily damage the microvasculature within hours of treatment via an ASMase-dependent pathway [1–4]. This is followed by vascular destruction and subsequent secondary tumor cell death, and is postulated to be an important mechanism of radiation-induced tumor kill in vivo. Standard 2 Gy fractionated

Electronic supplementary material The online version of this article (doi:10.1007/s10456-012-9329-2) contains supplementary material, which is available to authorized users.

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radiation may however elicit other effects due to hypoxia, reperfusion and reactive oxygen species formation [5–7]; these often include an increased tumor microenvironment heterogeneity associated with poor prognosis and treatment response [8, 9]. Rapid endothelial apoptosis due to high radiation doses has been linked to a 20-fold enrichment of the ASMase enzyme in endothelial membranes compared to epithelial and tumor cells. High doses of radiotherapy (>8–10 Gy) are suggested to cause substantial damage to the endothelial cell's membrane, leading to extensive hydrolyzation by the ASMase enzyme, in turn releasing ceramide to signal for apoptosis. At high radiation doses, endothelial cells can undergo apoptosis through two pathways: by DNA damage, or via a ceramide-signalling apoptosis pathway [3, 10–15]. At lower radiation doses however (<6–8 Gy), it is speculated that insufficient ceramide is released to induce rapid endothelial cell death. Conventional radiation doses are believed not to activate the ceramide-dependent apoptosis pathway. Basic fibroblast growth factor (bFGF) has been shown to inhibit ceramide-dependent messaging for apoptosis [10, 15–19]. In comparison, epithelial and tumor cells are predominantly damaged through direct or indirect DNA damage, and undergo cell death or senescence only when they have accumulated adequate damage [9, 20, 21]. Clinically approved anti-angiogenic agents, such as Sunitinib, have recently been demonstrated to yield a synergistic tumor response when combined with radiation therapy [22, 23]. This observation is potentially due to enhanced vascular radiosensitization following anti-angiogenic therapy. It is possible that such agents could be used to lower a required radiation dose to cause vascular destruction effects similar to that observed at high radiation doses.

Sunitinib is a small molecule tyrosine-kinase inhibitor, which prevents the activation of a wide-spectrum of receptors including VEGF receptors (PDGFRα, PDGFRβ, VEGFR1, VEGFR2, VEGFR3, FLT3, CSF-1R, RET). It acts by inhibiting downstream signalling pathways rather than binding directly to VEGF [24–27], and is currently one of the few FDA approved anti-angiogenic agents for the treatment of renal cell carcinoma and imatinib-resistant gastro-intestinal stromal tumors. Nonetheless, there are still questions in regards to how this agent should be administered, and whether it should be used alone or in combination with other forms of therapies (i.e. chemotherapy, radiation therapy) in order to cause additive or synergistic tumor responses. Furthermore, Sunitinib has been reported to induce a multitude of effects on tumor blood vessels and tumor cells, including in vivo and in vitro radiosensitization in addition to vascular 'normalization' effects [22, 25, 27–34]. Studies by Cuneo et al. [22] have shown that tumor cells can be sensitized to ionizing radiation at low doses given as single fractions when pre-treated with Sunitinib. Their work further demonstrated that combining fractionated radiation therapy with Sunitinib can yield a synergistic response. Recently, Yoon et al. [29] demonstrated that treating with Sunitinib before and after two doses of 10 Gy radiation yielded a synergistic tumor response demonstrated histologically, and a synergistic tumor growth delay. Nevertheless, the mechanism of synergy with radiotherapy remains unknown. Speculations indicate that it may be caused by pre-irradiation vascular normalization resulting in increased radiosensitivity of the whole tumor due to enhanced oxygenation. Alternative models include Sunitinib-induced pre-irradiation endothelial cell radiosensitization leading to complete vascular collapse or destruction at the time of radiotherapy, and subsequent secondary enhanced cell death in the tumor.

In this work, we have used three-dimension high-frequency power Doppler ultrasound to investigate tumor vascular response to therapy, and complementary immunohistochemistry to assess cell death. High-frequency ultrasound is an ideal and inexpensive imaging modality for pre-clinical assessment of tumor vasculature in animal models [35–37]. At clinical frequencies (<15 MHz), power Doppler ultrasound yields greater flow detection and resolution than most other Doppler based techniques [35, 37–39]. At high frequencies (>20 MHz), ultrasound yields enhanced structural resolution and enhanced power Doppler signal detection capable of detecting blood flow down to 1–2 mm/s and vessel sizes of 50–150 μm. It is ideal for longitudinal studies involving large numbers of animal-subjects [40–42]. When used in three-dimensions, power Doppler ultrasound measures flow signal from the whole tumor volume as opposed to single two-dimensional planes, a limiting factor often encountered in most imaging modalities and histopathology. High-frequency power Doppler ultrasound's main limitation is that it is unable to detect the smaller blood vessels of the tumor microcirculation (which can be as small as 5 μm in diameter). On the other hand, changes in smaller capillaries are often reflected in detectable blood flow signal in the tumor microcirculation. A careful observation of the power Doppler flow signal allows detecting such subtle changes in response to anti-angiogenic agents and vascular targeting strategies [39–41, 43–46].

Here, we investigate the relative contributions of direct cell killing by radiation versus tumor cell death due to radiation effects on the vasculature 24 h after irradiation. We also examine the mechanism of action when Sunitinib is combined with radiation. Experiments were conducted in severe combined immune-deficient (SCID) mice bearing xenografts of an aggressive breast cancer cell line, MDA-MB-231. Tumors were treated with radiation doses of 2–16 Gy alone, in combination with basic fibroblast growth factor (bFGF), or Sunitinib. Basic fibroblast growth

factor is a polypeptide produced by several cells, fibroblasts and endothelial cells and is one of the putative factors inducing angiogenic and stromal response in hosts. It is involved in mitogenesis, angiogenesis, cellular differentiation and tissue repair, and has been demonstrated to protect endothelial cells from lethal effects after exposure to clinically relevant radiation doses [10, 15, 16, 18, 19]. Our rational in using bFGF as an endothelial radio-protector is to reduce rapid endothelial cell death, which occurs predominantly via a ceramide signalling pathway after high-radiation doses. This would then allow us to confirm the role of blood vessels in regulating tumor response to radiotherapy and to better understand the relative contribution of direct cell killing by radiation versus tumor cell death due to radiation effects on the endothelium.

Experiments conducted indicate a decrease in power Doppler flow signal of up to 50 % when tumors are treated with 8 or 16 Gy of radiation. A reciprocal dose-dependent increase of tumor cell death is also observed. Animals pre-treated with bFGF demonstrated minimal change in power Doppler flow signal and minimal amounts of tumor cell death at high doses of radiation therapy. This suggests that tumor response is directly linked to the tumor vascular response at such doses. Treatments where Sunitinib is combined with radiation demonstrate no significant change in flow signal at doses lower than 8 Gy. However, in contrast to radiation doses equal to or greater than 8 Gy administered alone, where we observed a decrease in power Doppler signal, combining such doses with Sunitinib yielded a significant power Doppler signal increase. An overall increase in dose-dependent cell death is also observed when compared to Sunitinib alone or radiation alone. We suspect that Sunitinib may be inducing vascular 'normalization', leading to enhanced oxygenation and enhanced overall tumor response.

Methods

Animal preparation

All animal experiments presented in this work were conducted in compliance with internationally recognized guidelines specified in protocols approved by the Sunnybrook Health Science Centre Institutional Animal Care and Use Committee. MDA-MB-231 breast cancer cells were cultured in RPMI 1600 culture medium (ATCC, Manassas, VA, USA), 5 % fetal bovine serum (FBS) with antibiotics (penicillin and streptomycin: Life Technologies, Grand Island, NY, USA) to full confluence and 1×10^6 cells were injected subcutaneously into the hind leg of SCID mice (Charles River Laboratories International, Wilmington, MA, USA). Tumors were grown for a period of 3 weeks, reaching an average of 200 mm^3 before experimentation. An average of five animals per experimental condition were used.

Acute treatment response

Each experimental condition was comprised of a single dose of ionizing radiation therapy alone, or combined with Sunitinib (Pfizer, New York, NY, USA) or bFGF (R&D Systems, Minneapolis, MN, USA). Animals bearing MDA-MB-231 breast cancer xenografts were treated with single radiation doses of 0, 2, 4, 8 or 16 Gy alone, or in combination with bFGF 1 h before irradiation. Another group was given Sunitinib daily for 14 days prior to radiation delivery at a dose of 30 mg/kg prior to irradiation. A separate cohort of tumors was left untreated during Sunitinib administration in order to assess clonogenicity and tumor growth during that period. Similar Sunitinib doses have been demonstrated to cause minimal tumor damage while inducing a synergistic effect when combined with radiation therapy [23, 29, 47]. Mice treated with the vascular protecting agent bFGF received an intravenous injection of 0.45 μg/ml 1 h before irradiation. Radiation therapy was administered with a Faxitron cabinet irradiator (Faxitron Bioptics, Lincolnshire, IL, USA) using X-rays at an energy of 160 kVp, an SSD of 35 cm, and a dose rate of 200 cGy/min. Animals were anesthetised with ketamine (100 mg/kg), Xylazine (5 mg/kg) and acepromazine (1 mg/kg) before imaging and irradiation. For radiation treatment, the body of each animal was covered with a 3 mm thick lead shielding, exposing only the tumor.

Tumor growth assessment

Tumor growth assessment was performed with a different cohort of animals. An additional subset of animals, pre-treated with Sunitinib followed by radiation, continued receiving Sunitinib administration 3 times weekly for up to 30 days after irradiation as a 'maintenance' therapy. Tumors were measured twice weekly up to 30 days after single dose radiation therapy, or until they reached a diameter of 17 mm (end point).

Ultrasound imaging

All animals were imaged with three-dimensional high-frequency ultrasound immediately before treatment and 24 h after treatment. Data was acquired using a Vevo 770 high-frequency ultrasound imaging device (Visualsonics, Toronto, ON, Canada) with a 25 MHz center frequency transducer (RMV-710B: 70 μm axial resolution, 140 μm lateral resolution, focal length of 15 mm). A motorized

scan stage (Visualsonics, Toronto, ON, Canada) was used to acquire 3D B-mode images and power Doppler data, at a step size of 0.1 mm. Power Doppler data was collected with the following optimized settings: a clutter-filter cut-off of 2 mm/s, a scan speed of 2 mm/s, a pulse repetition frequency of 5 kHz, a power Doppler gain of 20 dB and a frame rate of 10 fps). Power Doppler image analysis was conducted using in-house developed software in MATLAB (The MathWorks, Natick, MA). The vascularity index (VI) was computed from power Doppler images by obtaining the volume of all colored objects (colored pixels) over the volume of the selected ROIs. A relative vascularity index was used to assess overall vascular response to therapy, computed as follows: $(VI_{24Hours}/VI_{0Hours}) \times 100$.

Histological analysis

Mice were sacrificed 24 h after irradiation for immunohistochemistry and clonogenic assays. Cross sections of tumor xenografts were stained for DNA breaks using in situ end labelling (ISEL) stain as a cell death marker and cluster of differentiation (CD31) (Santa Cruz Biotechnology, Santa Cruz, CA) to evaluate vascular density. A subset of animals treated with Sunitinib only ($n = 5$) was stained for α-smooth muscle actin (α-SMA) and carbonic anhydrase 9 (CA9). A percentage of cell death was evaluated for each of the tumor cross-sections stained with ISEL. Similarly, a percentage of hypoxia was evaluated for each of the tumor cross-sections stained with CA9. The vascular density was computed from images of CD31 stained tumor cross sections. Three cross-sections per tumor were stained. For each of the cross sections, 4 fields of view (FOV) were analyzed at 10× magnification to compute the density of vasculature for a total of 12 FOVs per tumor, using automated software. To do so, we have developed a toolbox in MATLAB which distinguishes the brown stained regions (CD31 staining of endothelial cells) in the tumor from the background stain (other cells) within a user selected ROI and computes the vascular density per chosen ROI. For clonogenic assays, cells from 30 tumors ($n = 3$ per treatment condition) were disaggregated, suspended in culture medium and plated into petri dishes for colony formation. Cells were fixed, stained and counted 2 weeks after plating. All plating efficiencies were normalized to the 0 Gy condition.

Statistical analysis

Data is presented as mean ± standard error of the mean (SED). Quantified VI, ISEL, CA9 and CD31 staining, colony counts and tumor volume changes were evaluated for statistical significance using a Mann–Whitney test (two-tailed, assuming unequal variances; $\alpha = 0.05$* or $\alpha = 0.01$**). Each treatment condition was compared directly to the 0 Gy control condition. For clonogenic assays, radiation and bFGF or Sunitinib combination treatment conditions were tested against the equivalent irradiation dose in radiation only conditions. Statistical tests were conducted using Prism (GraphPad Software version 5, La Jolla, CA, USA).

Results

Power Doppler ultrasound and CD31 immunohistochemistry analysis

Power Doppler data of in vivo tumors before and after treatment indicated that single large doses of radiation equal to or greater than 8 Gy caused a significant decrease in the flow signal. Figure 1A presents the maximum intensity projection of representative tumor volumes captured with 3D high-frequency power Doppler ultrasound. In Fig. 1B, the relative change in VI at 24 h for a range of radiation doses is exhibited for all conditions. The data demonstrates no significant changes detected with power Doppler ultrasound for the 0, 2 and 4 Gy conditions, while a decrease of approximately 50 % in relative detectable flow for the 8 Gy and 16 Gy ($p < 0.01$) conditions was observed. Experimental conditions where radiation was used in combination with bFGF or Sunitinib resulted in different effects from those using radiation alone. As anticipated, bFGF acted as a vascular protecting agent, and caused only minimal deviations in the relative VI (no drop in the power Doppler flow signal) when combined with 0, 2, 4, 8 or 16 Gy radiation therapy. Sunitinib treatments caused a slight increase in the VI at 2 and 4 Gy, while a significant increase in VI at radiation doses equal or greater than 8 Gy was observed. A summary of statistical test p values is presented in Table S1. Power Doppler results were supported with CD31 micro-vascular density analysis, which demonstrated an acute significant decrease in the micro-vascular density at doses greater than 8 Gy (Fig. 2, Fig. S1 and Table S2) for radiation only conditions. Similarly, no vascular decrease was observed for 8 Gy when bFGF was administered before irradiation. However, Sunitinib treatments caused an overall decrease in the micro-vascular density in tumor cross-sections, which was minimally affected by the administration of radiation therapy.

Cell death assessment using ISEL staining

Representative images of ISEL stained tumor cross-sections and their quantification are presented in Fig. 3. A dose dependent increase in staining was observed for

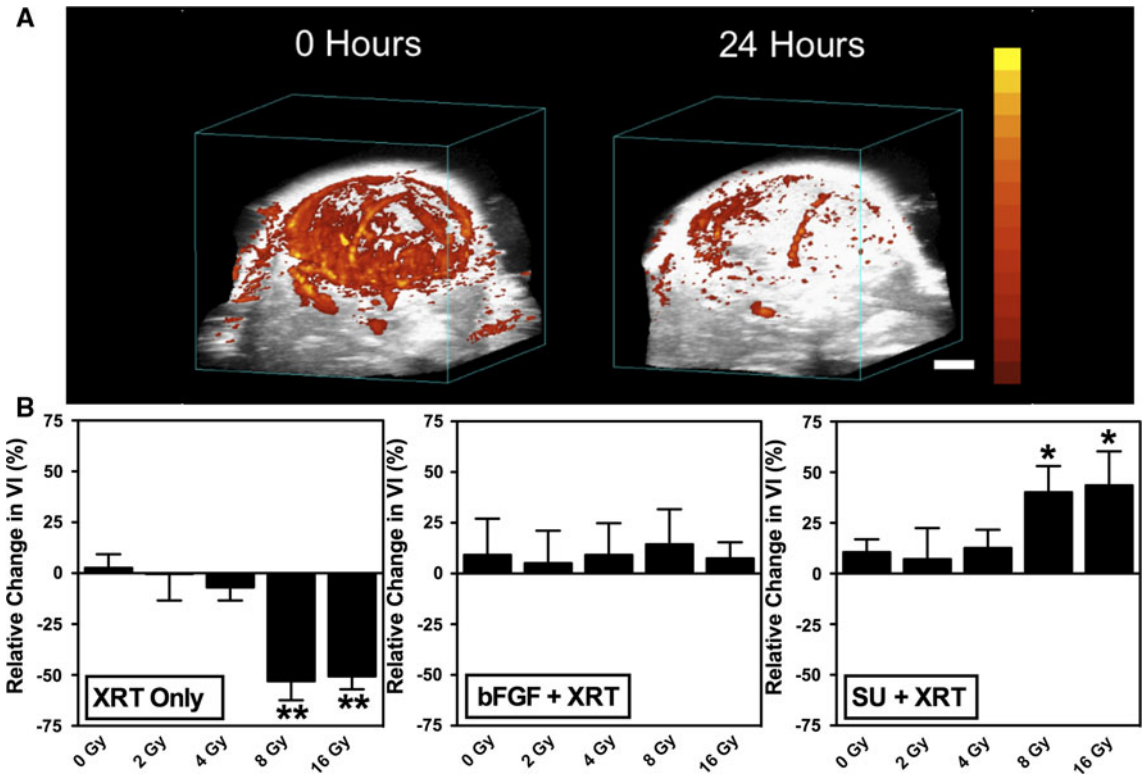

Fig. 1 **A** Representative maximum intensity projections of a tumor volume at 0 and 24 h after treatment with 16 Gy radiation. Data was obtained with 3D high-frequency ultrasound. A decrease in total power Doppler flow signal (blood volume detected) can be observed 24 h after treatment. *Red color* represents the lowest power Doppler intensity (15 dB) while *yellow* represents the highest power Doppler intensity (40 dB). The *scale bar* represents 2 mm. **B** Relative change in VI at 24 h for a range of radiation doses (0, 2, 4, 8 and 16 Gy) alone, in combination with bFGF or Sunitinib. Results indicate that there is a significant decrease in power Doppler flow signal only when tumors are treated with single doses of 8 and 16 Gy. This signal decrease is of almost 50 %. No other statistically significant data (compared to 0 Gy control) is observed. Tumors pre-treated with bFGF appeared to be completely unaffected by these radiation doses. Furthermore, pre-treating with Sunitinib also appeared to cause no significant change in VI at radiation doses lower than 8 Gy, although a significant increase in flow signal was observed in animals treated with 8 or 16 Gy ($p < 0.05$). Each experimental condition is representative of n = 7–9 animals. *Error bars* represent standard error. Statistically significant is indicated with $*p \leq 0.05$ or $**p \leq 0.01$

radiation-only conditions. The observed increase was determined to be significant past the 8 Gy treatment condition ($p < 0.05$). Quantified ISEL staining in animals pre-treated with bFGF was consistent across all radiation conditions, indicating minimal tumor cell death. Sunitinib on its own appeared to cause cell death ($p < 0.05$), which was significantly enhanced when combined with radiation therapy compared to radiation or Sunitinib alone ($p < 0.01$). Despite the non-significant VI changes for Sunitinib treated conditions at low doses, a statistically significant increase in cell death was observed for all radiation doses combined with Sunitinib versus radiation alone. A summary of statistical test p values is presented in Table S3.

Clonogenic assay and tumor growth assessment

Figure 4 shows colony counts for radiation alone, radiation with bFGF, and radiation with Sunitinib, at 0, 2, 4, 8 and 16 Gy. For radiation alone a dose dependent decrease in colony counts was observed. A similar effect was observed when radiation was combined with Sunitinib. Treatments combining radiation and bFGF had lesser effects on colony formations for all radiation doses when compared to radiation alone or radiation combined with Sunitinib. We further tested clonogenic assay results for each combination treatment radiation dose against the same treatment dose in radiation only conditions. Our results indicate that bFGF's endothelial radio-protecting effects caused a significantly ($p < 0.05$) greater number of tumor cells to survive at the 16 Gy dose. Animals treated with Sunitinib alone had a non-significant decrease in the average colony count in comparison to 0 Gy control animals. In contrast, a cohort of tumors receiving no treatment and left to grow in parallel to those treated with Sunitinib had a significant decrease in the average colony count in comparison to control animals ($p < 0.05$). These tumors were also noted to be larger than those receiving Sunitinib after 14 days of treatment. Finally,

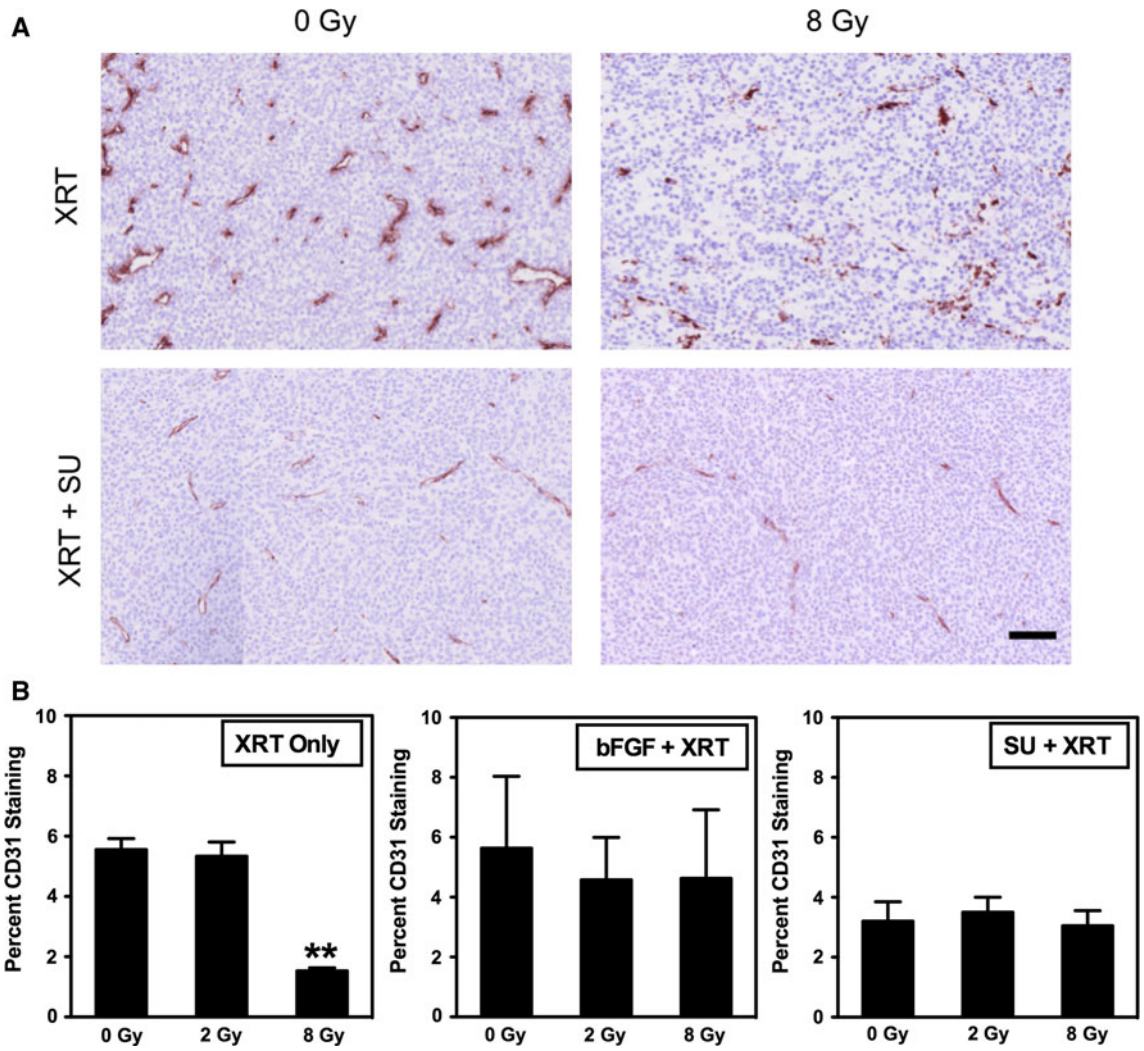

Fig. 2 **A** Representative images of tumor sections (treated with 0, 2 or 8 Gy alone, or in combination with Sunitinib) stained with CD31 for cell death. We observe diminishing amounts of vascular staining with single large doses of 8 Gy, in agreement with our power Doppler data. Sunitinib changes the appearance of the blood vessels causing them to be thinner and more similar to normal vasculature. A decrease in vascular density is also observed. Combining Sunitinib and 8 Gy radiation does not cause an apparent qualitative effect. The scale bar represents 200 μm. **B** Quantified vascular density from CD31 staining of tumors treated with 0, 2 or 8 Gy alone, or in combination with bFGF or Sunitinib. CD31 results confirm that a single large dose of radiation (8 Gy) does impact the tumor vasculature by decreasing the vascular density. Sunitinib alone also decreases the vascular density. However, this decrease is not augmented with the addition of radiation. This is consistent with power Doppler results. Furthermore, bFGF seemed to also protect the vasculature of the tumor by minimally deviating the vascular density measurements for all radiation doses. *Error bars* represent standard error. Statistically significant are indicated with $*p \leq 0.05$ or $**p \leq 0.01$

we found a significantly lower number of colony formations in tumors treated with radiation in combination with Sunitinib than radiation alone (2 Gy—$p < 0.05$; 4 Gy—$p < 0.01$; 8 Gy—$p < 0.05$). A summary of statistical test p values is presented in Table S4.

Figure 5 displays tumor growth curve of treated tumors for up to 30 days. The fold change in tumor volume 15 days after radiation therapy is plotted in Fig. 6. Data indicate that tumor growth is significantly slowed down when treated with a single large dose of 8 Gy ($p < 0.05$). Pre-treating animals with bFGF however seemed to protect tumors from the effect of radiation therapy, causing no significant slow-down in tumor growth. Administering Sunitinib for 2 weeks prior to irradiation caused a slow-down in tumor growth (see curve before arrow in bottom left graph). The average tumor volume was 276 mm^3 following 14 days of Sunitinib administration. Non-treated control tumors left to grow in parallel with those receiving Sunitinib reached an average tumor volume of 630 mm^3. We have also noted that discontinuation of Sunitinib caused tumors to immediately begin growing with a similar trend to control tumors. Vascular rebounds have recently

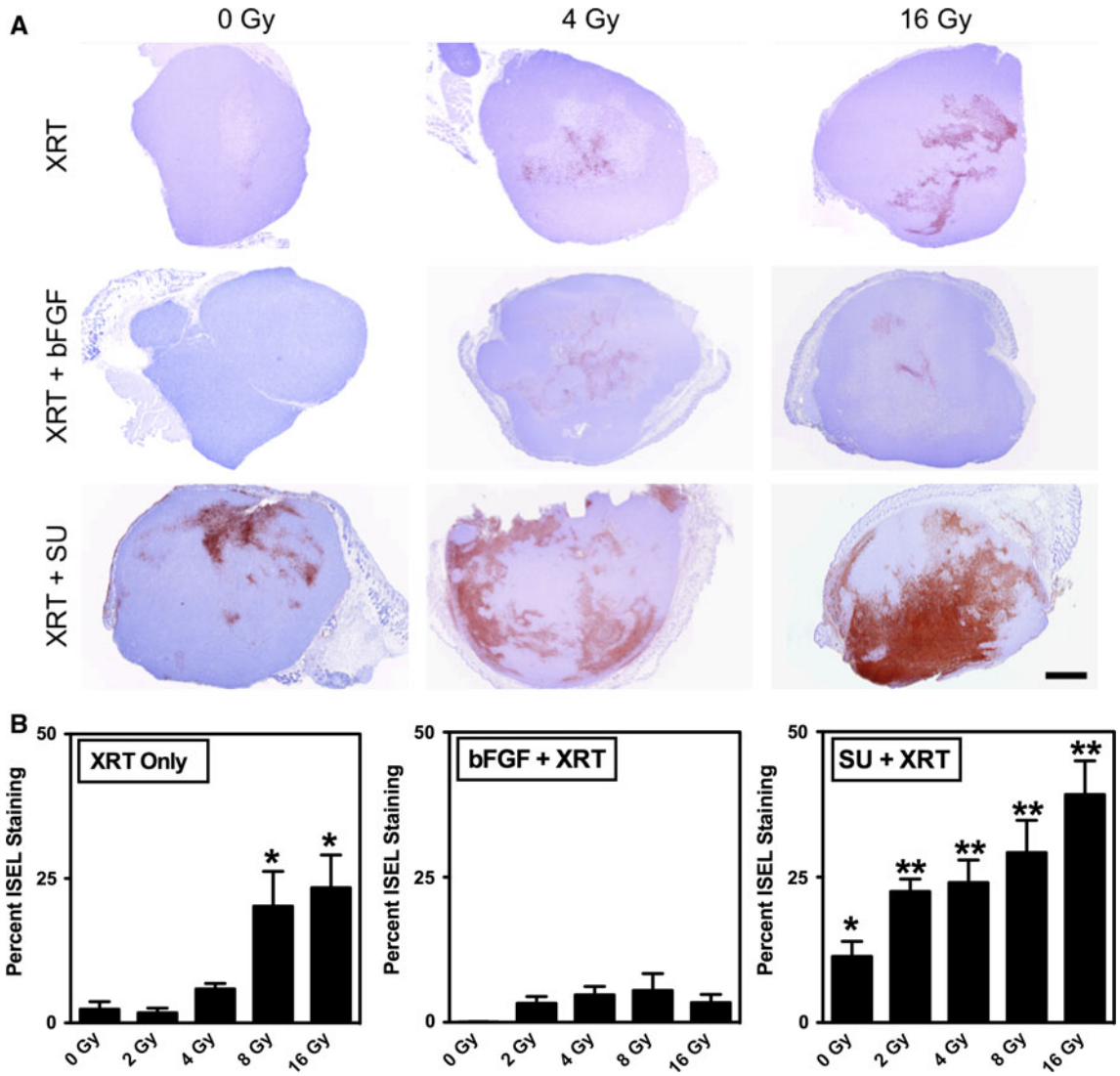

Fig. 3 **A** Representative images of tumor sections (treated with 0, 4 or 16 Gy alone, or in combination with Sunitinib or bFGF) stained with in situ end labelling (ISEL) for cell death. We observed increasing amounts of cell death with increasing radiation doses. There appears to be lesser amounts of ISEL staining when 16 Gy radiation is combined with bFGF than 16 Gy alone. Treatments with Sunitinib alone demonstrate cell death staining equivalent to 16 Gy alone. However, combining Sunitinib with radiation appears to enhance cell death significantly. The *scale bar* represents 1 mm. **B** Quantified ISEL staining data for all 15 treatment conditions. A dose dependent increase in cell death was observed for radiation only conditions. This was significant at doses greater than 8 Gy ($p < 0.05$). This effect did not occur in bFGF treated animals. Sunitinib treatment caused the most significant amount of cell death when combined with radiation therapy, covering at times more than 40–50 % of the tumor cross section at doses greater than 8 Gy. *Error bars* represent standard error. Statistically significant are indicated with $*p \leq 0.05$ or $**p \leq 0.01$

been reported in literature [48]. A growth delay was observed when animals were treated with 2 or 8 Gy radiation therapy following Sunitinib treatment; this was significant only for the 8 Gy condition ($p < 0.05$) at 15 days (Fig. 6). Continuing the administration of Sunitinib post-irradiation had the most drastic effect. Here, a significant slow-down was achieved at 2 Gy ($p < 0.05$) 15 days after radiotherapy (Fig. 6), while an eventual regrowth was observed (Fig. 5). From Fig. 6, no tumor growth was observed 15 days after irradiation when 8 Gy was administered in conjunction with Sunitinib; similar observations were made when Sunitinib was continued after 8 Gy. However, in Fig. 5, comparing the Sunitinib and 8 Gy condition to the same with continued Sunitinib administration, we have found that there is a greater anti-growth effect when Sunitinib is continued following radiotherapy. Here, tumor growth was observed following Sunitinib and 8 Gy (at approximately day 30), while no tumor growth was observed when Sunitinib was continued as maintenance therapy for the duration of the tumor

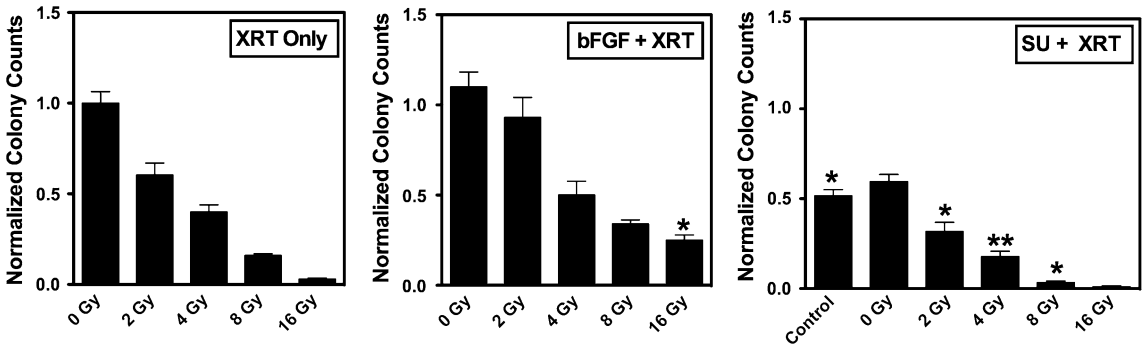

Fig. 4 Clonogenic survival for tumor cells exposed to radiation alone, radiation with bFGF, and radiation with Sunitinib (0, 2, 4, 8 and 16 Gy). All plating efficiencies were normalized to the 0 Gy condition. Data is representative of 3 mice tumors per condition. For radiation alone a dose dependent decrease in colony counts was observed. A similar trend, but greater effect was observed when radiation was combined with Sunitinib. On the other hand, treatments combining radiation and bFGF had lesser effects on colony formations for all radiation doses when compared to radiation alone or radiation combined with Sunitinib. We tested clonogenic assay results for significance at each combination treatment irradiation dose against the same treatment dose in radiation only conditions. Our results indicate that bFGF's endothelial radio-protecting effects caused a significantly ($p < 0.05$) greater number of tumor cells to survive the 16 Gy dose. We further found that non-treated tumors left to grow during Sunitinib administration had a significantly ($p < 0.05$) lower colony count than the 0 Gy condition, while those treated with Sunitinib alone had a non-significant lower colony count. Finally, we found a significantly lower number of colony formations in tumors treated with radiation in combination with Sunitinib than radiation alone (2 Gy—$p < 0.05$; 4 Gy—$p < 0.01$; 8 Gy—$p < 0.05$). *Stars* in figure represent statistical significance for combination treatment doses compared to radiation alone doses. *Error bars* represent standard error. Statistically significant are indicated with $^{*}p \leq 0.05$ or $^{**}p \leq 0.01$

growth experiment. Results for statistical analysis are presented in supplementary data Table S5.

Pericyte and tumor oxygenation assessment

Figure 7 displays representative images of CD31, α-SMA and CA9 staining (A) and average quantification of CA9 staining in control and Sunitinib treated animals (B). Images are of the exact same blood vessel stained with CD31 and α-SMA in control and Sunitinib conditions. Qualitative observations of the α-SMA in control animals indicates abnormalities in perivascular cell morphology in our MDA-MB-231 tumor xenografts, this includes irregular arrangement and loose coverage of blood vessels. In Sunitinib treated animals, pericyte coverage appears to be more 'normal'—a more circumferential and tighter coupling to tumor blood vessels (as marked by CD31). Quantified CA9 staining of Sunitinib and control tumor cross-sections results in a decrease in the average staining in the Sunitinib treated animals. This then suggests that oxygenation levels may be higher in Sunitinib treated animals than control animals, as would be expected if normalization does take place.

Discussion

This study aimed to investigate the acute response of tumors in relation to tumor blood vessel responses to a single radiation dose. We modulated the tumor vascular response to ionizing radiation by administering or inhibiting growth factors involved in endothelial cell survival. This permitted the assessment of the relative contribution of direct tumor cell killing by radiation versus secondary death due to radiation effects on tumor vasculature. We further aimed to investigate whether Sunitinib, an anti-angiogenic agent, can induce tumor radiosensitization, and the mechanism by which it may do so. Tumor vascular response was assessed using previously established high-frequency 3D power Doppler ultrasound methods, while tumor response was assessed using DNA break immunohistochemistry staining (ISEL), standard clonogenic assays, and assessment of tumor growth. Vascular density measurements obtained from CD31 stained tumor cross-sections were used to complement power Doppler measurements. CA9 and α-SMA staining were used to assess Sunitinib's potential mechanism of tumor radiosensitization.

Power Doppler results for tumors treated with radiation alone were in agreement with findings by Garcia-Barros et al. [3], where single large doses of radiation had a vascular effect causing a significant decrease in detectable power Doppler flow signal (Fig. 1). This led to a dose dependent increase in cell death as reflected in ISEL staining (Fig. 3). As anticipated, the administration of bFGF one hour before irradiation prevented a decrease in power Doppler signal and a subsequent increase in tumor cell death 24 h post-therapy. Studies have shown that bFGF can inhibit effects of radiation on endothelial cells if administered into the blood circulation of animals up to an

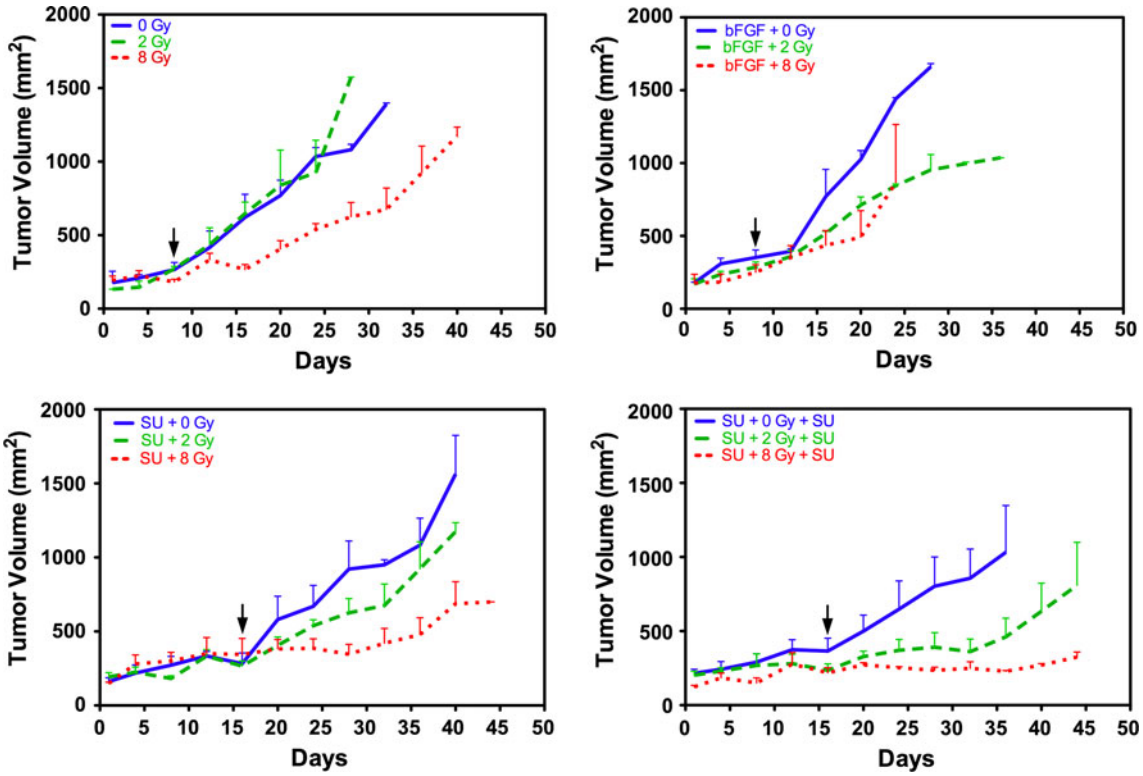

Fig. 5 Long-term tumor growth response to radiation alone, or in combination with bFGF or Sunitinib. Conditions include radiation at 0, 2 or 8 Gy alone, or in combination with bFGF or Sunitinib. A supplementary condition, where Sunitinib is continued following irradiation as maintenance therapy, is also presented. Sunitinib maintenance therapy was administered three times weekly at 30 mg/kg following the initial 14 day dose and radiation treatment delivery. We find a growth delay when animals are treated with 8 Gy radiation therapy alone (*top left* graph). However, this growth delay was not observed for animals pre-treated with bFGF (*top right* graph). Treatments where bFGF is administered alone demonstrate an accelerated tumor growth compared to control animals. We noted that Sunitinib alone caused a slow-down in tumor growth (see curve before *arrow* in *bottom left* graph), and that the discontinuation of Sunitinib caused tumors to immediately begin growing with a similar trend to control tumors. Unlike radiation alone however, administering 2 Gy following Sunitinib therapy caused a significant slow-down of tumor growth. Administering a single 8 Gy dose following radiotherapy blocked tumor growth for nearly 15 days. The use of Sunitinib as a maintenance therapy greatly enhanced tumor growth delay for all three radiation conditions. Furthermore, we observed no tumor growth for the 8 Gy condition. *Black arrow* indicates day of radiation administration. Radiation and Radiation + bFGF conditions have a baseline of 8 days before radiation treatment. Sunitinib conditions have a baseline of 16 days (which include 2 days of baseline followed by 14 days of Sunitinib administration) followed by irradiation on the 16th day

hour before radiation delivery [4, 16]. This in turn hinders tumor cell death associated with vascular destruction. Patches of cell death were noted in ISEL stained tumor cross-sections when bFGF was combined with radiation. However, a dose-dependent increase, such as the one observed for radiation alone, was not observed. These results, supported by clonogenic assays and tumor growth assays, suggest that the blood vessel response to radiation may be essential for increased acute tumor response to radiation, as assessed 24 h after therapy. Taken together, our data is in general agreement with the paradigm described by Fuks et al. [1], which emphasizes the importance of rapid vascular destruction immediately after a single large doses of radiation (>8 Gy) for enhanced tumor cell kill.

Our results suggest that Sunitinib, at the administered dose, may be normalizing tumor blood vessels. We found an increased acute tumor response (percent cell death) to radiation therapy, while tumor blood vessels appeared to be more resistant to single doses of radiation after 2 weeks of Sunitinib treatment. In fact, we noted an increase in power Doppler signal at doses equal to or greater than 8 Gy when these were delivered in conjunction with radiation therapy. These results are potentially linked to a vascular normalization process, as described by Jain [49, 50], which leads to enhanced tumor oxygenation and a potential pruning of the more abnormal and immature tumor blood vessels. Whereas single large doses of radiation destroy the abnormal vasculature of tumors as observed in Figs. 1 and 2, treating with Sunitinib for up to 2 weeks may have

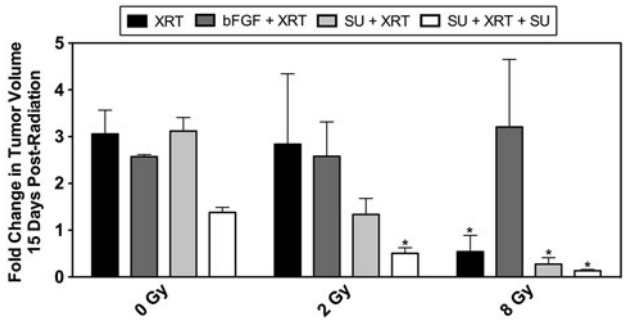

Fig. 6 Fold change of tumor growth 15 days after radiation therapy administration. Conditions include radiation at 0, 2 or 8 Gy alone, or in combination with bFGF or Sunitinib. An extra condition is added to this growth assay, where Sunitinib administration is continued (three times weekly) after the initial 14 day dose and radiation therapy. Results indicate that Sunitinib maintenance therapy seemed to have the most effect on tumor growth for all three radiation doses. Tumors treated with Sunitinib and 8 Gy alone, or through maintenance therapy, caused a near complete cessation of tumor growth over the 15 day period. On the other hand, combining Sunitinib with 2 Gy radiotherapy seemed to slow down tumor growth by half the time. Finally, bFGF seemed to protect tumors from radiotherapy, allowing for continued tumor growth. Error bars represent standard error. Tumors treated with Sunitinib for 2 weeks had their tumor volumes remain at an average of 250 mm^3, while non-treated control tumors grew by a factor of 2. Statistically significant are indicated with $^*p \leq 0.05$ or $^{**}p \leq 0.01$

permitted the tumor vessels to become more "normal-like", yielding better-oxygenated and radiosensitized tumor cells. Since Sunitinib exhibits little effects on established blood vessels in vivo, as suggested by Osusky et al. [25], it may also be that it radiosensitizes immature endothelial cells, leading to capillary destruction after radiation therapy, thereby enhancing flow in larger, more radio-resistant, tumor blood vessels as observed in quantified power Doppler results. Normal micro-vasculature has been shown to be more resistant to radiation therapy, while smaller (and often immature/proliferating) capillaries have been shown to be more sensitive to radiation than arterioles and venules [51–54]. Pericyte staining (α-SMA) suggests that vascular normalization may be taking place while quantification of CA9 staining supports a potential increase in oxygenation levels in the tumor. We also noted increased colony formation when tumors were treated with Sunitinib alone in comparison to control tumors left to grow in parallel to Sunitinib administration.

The enhanced cell death observed in ISEL stained tumor cross-sections when radiation is combined with Sunitinib is potentially related to enhanced oxygenation conditions throughout the tumor. Tumor growth (Figs. 5, 6) was also delayed for these treatment conditions and was further enhanced if Sunitinib treatment was maintained after radiation therapy. However, further investigations will be necessary to better understand the long-term response of tumors after Sunitinib and radiation combined. Such results indicate that a relatively small dose of Sunitinib, such as the one used in this study, administered before irradiation, will likely enhance tumor response to therapy. However, the process by which tumor response is enhanced is not directly linked to a vascular radiosensitization effect. Our current data suggests that the enhanced tumor response may involve a vascular normalization process caused by Sunitinib. Further experimentation will be required to confirm this.

Although the stroma of SCID mice has been described in the past to be more sensitive to radiation effects [55, 56], recent studies suggest that SCID mice also undergo a

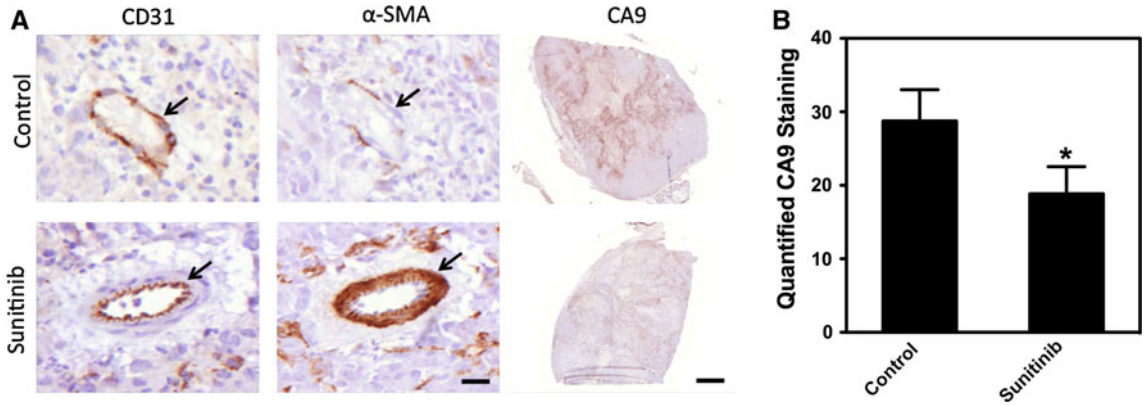

Fig. 7 **A** From *left* to *right*: CD31, α-SMA and CA9 staining of Sunitinib treated and control tumors. *Arrows* indicate the same blood vessel as delineated with CD31 staining in treated versus control conditions in order to assess α-SMA coverage. We observe an abnormal arrangement of the pericyte coverage in control animals, which appears loose and irregular. Contrary to this, Sunitinib treated animals appear to have a more 'normal'-like pericyte coverage, and there is an increase in the amount of micro-vascular coverage. **B** CA9 quantification indicates an increase in oxygenation (decrease in CA9 hypoxia staining; $p < 0.05$) in Sunitinib treated animals. These findings indicate that our treatments may be causing an effect reminiscent of vascular normalization, in at least some of our animals. *Scale bar* is 15 μm in CD31 & α-SMA and 1 mm in CA9 image

similar vascular response to radiation at doses greater than 8 Gy. This vascular response was subsequently linked to overall tumor response [2]. Experiments we have conducted with nude mice indicate a similar VI decrease in response to single large doses of radiation treatment.

Potential designs for clinical trials of radiotherapy combined with anti-angiogenic agents make mention of the potential use of Sunitinib specifically [13], and suggest that there is a growing interest in bringing this type of combination into clinical use [57]. As blood vessels are common to different tumor types and play an important role in the growth and survival of tumor cells, it makes them an ideal target for cancer therapies. The work here forms a basis for such experimentation in concert with radiation.

Acknowledgments This work was supported by the CBCF and the Terry Fox Foundation through a program project grant "Ultrasound for Cancer Therapy". Aspects of the work were also supported by a cancer care Ontario (CCO) research grant in experimental therapeutics and imaging. We would like to thank Clinton Hupple for his help in the technical development of the power Doppler imaging set-up. I would also like to thank Dr. Kolios for scientific insight. Finally, the authors thank Dr. Kerbel for his generous donation of MDA-MB-231 cells. Dr. Gregory Czarnota is supported by a CCO Research Chair in Experimental Therapeutics and Imaging. All animal experiments presented in this paper were conducted in compliance with protocols approved by the Sunnybrook Health Science Centre Institutional Animal Care and Use Committee.

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