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Contrast-Enhanced Ultrasound to Detect Early Microvascular Changes in Skeletal Muscle after High-Dose Radiation Treatment

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Abstract

The biological response of normal tissue to high-dose radiation treatment remains poorly understood. Alterations to the microenvironment, specifically the microvasculature, have been implicated as a significant contributor to tumoral cytotoxicity. We used contrast-enhanced ultrasound (CEU) perfusion imaging, which is uniquely suited to assess functional status of the microcirculation, to measure microvascular blood flow after high-dose irradiation to normal skeletal muscle tissue in a murine model. Proximal hindlimbs of wild-type C57Bl/6 mice were irradiated with a single fraction using 6 MV photons, 1 cm bolus and a dynamic wedge. Quantitative perfusion CEU imaging of the skeletal muscle was performed at days 1 and 8 postirradiation in three different regions of interest (ROIs): 1. 15 Gy external-beam irradiated leg; 2. 12 Gy irradiated 5 mm proximal area; 3. single ROI in the nonirradiated contralateral (CL) hindlimb. Perfusion imaging was also performed in the hindlimb of nonirradiated mice. CEU time-intensity data were analyzed to measure microvascular blood flow (MBF, also referred to as perfusion), and its parametric components of microvascular flux rate and functional microvascular blood volume (MBV). Plasma measurements of two potent vasoconstrictors, endothelin-1 and angiotensin II, were also performed to assess systemic response. CEU perfusion imaging values for the 12 and 15 Gy irradiated limb regions were pooled. At day 1, MBF in the irradiated limb was significantly lower than in the CL limb ($P = 0.016$) but quite similar to the nonirradiated mice. At day 8, both limbs of irradiated mice exhibited a trend towards lower MBF than the limbs of

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SUPPLEMENTARY INFORMATION

Table S1. Comparison of 12 and 15 Gy regions of interest using contrast-enhanced ultrasound.

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nonirradiated mice (28% decrease in mean MBF, $P = 0.149$ for CL; 39% decrease, $P = 0.065$ for irradiated limb). Compared to nonirradiated animals, the reduction in perfusion in irradiated limbs at day 8 may have been more influenced by the microvascular flux rate (25% decrease in the mean, $P = 0.079$) than the MBV (12% decrease in the mean, $P = 0.328$). Examination of vasoactive compounds revealed that the average plasma concentration for endothelin-1 at day 8 postirradiation was significantly higher in 14 irradiated animals than in 4 nonirradiated animals (3.07 pg/ml vs. 2.51 pg/ml; $P = 0.011$). Up to day 8 after high-dose irradiation, flow deficits in irradiated muscle appear to be a consequence of increased vascular resistance more so than loss or functional de-recruitment of microvascular units.

INTRODUCTION

The ability to deliver more precise conformal radiation fields using image guidance has resulted in the development of hypofractionated radiation treatment regimens consisting of high doses of radiation per fraction. These high-dose radiation protocols can induce rapid apoptosis of endothelial cells by promoting acidic sphingomyelinase-mediated generation of ceramide, a pro-apoptotic protein (1, 2). The ability to characterize microvascular responses to irradiation is important for pre-clinical and possibly clinical evaluation of approaches that maximize tumor kill through ischemic sequelae while minimizing off-target effects on adjacent normal tissues. However, there is currently no reliable method to assess these microvascular responses in real time.

Perfusion imaging with contrast-enhanced ultrasound (CEU) relies on the detection of contrast agents consisting of encapsulated gas-filled microbubbles (MB) that are 1–5 μm in diameter and remain entirely within the vascular compartment. This imaging is unique in that it can assess blood flow at a capillary level (3). Signal enhancement from MBs originates from unique acoustic emissions that occur during MB cavitation in the ultrasound field, which allows for differentiation from tissue signal (4). Evaluating microvascular blood flow with CEU relies on kinetic models that assess the rate and extent of MB reappearance after their destruction in an acoustic field with high-power ultrasound (4–6). This approach is particularly advantageous for assessing effects from exposure to radiation since it is not influenced by changes in permeability, but instead evaluates microvascular properties via the parametric estimation of microvascular flux rate and microvascular integrity (blood volume). We employed CEU perfusion imaging to test the hypothesis that differences in microvascular blood flow in normal tissues can be detected days after high-dose radiation exposure.

MATERIALS AND METHODS

Irradiation Protocol

All animal studies were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University (Portland, OR). Wild-type C57B1/6 mice (Jackson Laboratory, Bar Harbor, ME) of both sexes were studied at 8–14 weeks of age. Mice ($n = 24$) were anesthetized with intraperitoneal injection (10–15 $\mu\text{l/g}$ IP) of a solution containing ketamine hydrochloride (10 mg/ml) and xylazine (1 mg/ml), and kept euthermic. As a model

for radiation exposure of normal skeletal muscle tissue, a single proximal hindlimb was irradiated using a linear accelerator (Novalis™ TX, Varian Medical Systems Inc., Palo Alto, CA) in a single fraction with 6 MV photons and a 1 cm tissue-equivalent bolus. Dose was prescribed to the bolus/skin interface. Using a CT simulation of a representative anesthetized mouse, dose was modeled in a treatment planning system (Varian Eclipse, Palo Alto, CA) and prescribed to the bolus/skin interface. Exposed murine hindlimbs were positioned within the linear accelerator light-field while the contralateral hindlimbs were secured outside of the field. A proximal hindlimb on one side was irradiated with a dynamic wedge to create a radiation dose gradient across the entire leg for further testing of the spatial resolution of CEU imaging.

Spatial Registration between Radiation and Imaging

To determine if CEU could detect differences in blood flow, one hindlimb of the mouse was irradiated with a dynamic wedge to create a radiation dose gradient across the entire leg. Dose was prescribed to the bolus/skin interface. Based on spatial patterns of radiation, the irradiated limb was characterized by a higher dose (~15 Gy) region in the more distal portion of the proximal hindlimb with a gradient involving a lower dose (~12 Gy) in the more proximal portion (Fig. 1). Radiation doses were verified with phantom film dosimetry underneath a 1 cm bolus, and the contralateral limb scatter radiation dose was confirmed to be <15 cGy using optically stimulated luminescent dosimetry (OSLD).

CEU Perfusion Imaging

CEU perfusion imaging was performed in the short-axis imaging plane of: 1. both hindlimbs (irradiated and contralateral) of 24 irradiated mice at days 1 and 8 postirradiation; and 2. a hindlimb of 21 nonirradiated, control mice. Lipid-shelled decafluorobutane microbubbles were prepared by sonication of an aqueous lipid dispersion of polyoxyethylene-40-stearate and distearoyl phosphatidylcholine saturated with decafluorobutane gas as described elsewhere (7). Microbubble concentration was measured by electrozone sensing (Multisizer™ 3, Beckman Coulter® Inc., Brea, CA). During imaging, animals were anesthetized with inhaled isoflurane (1.0–1.2%) mixed with room air and were kept euthermic with an external warmer. An internal jugular vein was cannulated for administration of microbubble contrast agents, and microbubbles were infused intravenously at a rate of $1 \times 10^7 \text{ min}^{-1}$. CEU perfusion imaging was performed with a linear-array transducer (15L8) interfaced with an ultrasound system (Sequoia; Siemens Medical Systems, Mountain View, CA). A multipulse algorithm using phase-inversion and amplitude-modulation was used to detect the nonlinear component of the microbubble signal at a transmission frequency of 7 MHz, a mechanical index of 0.18 and a dynamic range of 55 dB. Images in each transaxial plane of the proximal hindlimb described above were acquired using a frame rate of 5 Hz for 12 s after a high-power (mechanical index, 1.0) 5-frame destructive pulse sequence. Data were analyzed for region of interest (ROI) drawn to include the proximal hindlimb adductor muscle groups (adductor magnus and semimembranosus). Time vs. video intensity (VI) data were fit to the function, Eq. (1).

$$y = A(1 - e^{-\beta t}), \quad (1)$$

where y is the intensity at time interval t , A is the plateau intensity of the curve and reflects relative microvascular blood volume, and β is the mean microvascular red blood cell flux rate. Muscle microvascular blood flow was calculated as the product of A and β (6, 8).

Plasma Assay Analyses

Whole blood samples from separate cohorts of mice were collected in tubes containing EDTA by cardiac venipuncture under isoflurane anesthesia prior to sacrifice. Samples were subsequently centrifuged to isolate platelet-rich plasma. ELISA was performed and compared to standard curves for measurement of endothelin-1 (Quantakine® DET100; R&D Systems™, Minneapolis, MN) and angiotensin II (RAB0010; Sigma-Aldrich® LLC, St. Louis, MO) according to manufacture specifications. Only one measurement of plasma endothelin-1 or angiotensin II was technically feasible for each mouse; thus, unlike mice subjected to postirradiation CEU, each irradiated mouse analyzed by ELISA has either a day 1 or 8 postirradiation measurement.

Statistical Analyses

Microvascular outcomes (i.e., CEU image parameters) and plasma concentrations were modeled as continuous variables. Independent two-sample t tests were used to compare separate cohorts of mice and a generalized least squares model with unstructured, correlated errors was applied to the group of irradiated mice who had days 1 and 8 imaging on irradiated and contralateral limbs. Hochberg's step-up Bonferroni method was applied to outcome- and time-specific P values (i.e., one P value adjustment procedure per figure) to control the type I error rate in the presence of multiple comparisons. Group differences in outcomes were considered significant at Hochberg-adjusted $P < 0.05$. Data analysis and graphics were performed with R version 3.6.1.

RESULTS

Spatial Resolution of CEU Perfusion Imaging

Two separate CEU perfusion ROIs on the irradiated limb were acquired corresponding to regions of 15 Gy and 12 Gy (Fig. 1). Because these doses represent clinically-similar ranges, data for the two regions were averaged within each mouse (at a specific time point) to produce the irradiated limb values that were analyzed. Comparisons of these two ROIs for each CEU acquisition time and outcome are shown in Supplementary Table S1 (<https://doi.org/10.1667/RR15471.1.S1>).

Microvascular Imaging Parameters

On day 1 postirradiation, skeletal muscle microvascular blood flow (MBF) in irradiated limbs was significantly lower than in CL limbs of the same irradiated mice ($P = 0.016$; Fig. 2A) but very similar to the MBF in limbs of nonirradiated mice ($P = 0.992$; Fig. 2A). Thus, we observed a trend towards hyperemia in the CL of irradiated mice compared to nonirradiated controls ($P = 0.095$; Fig. 2A). On day 8 postirradiation, the average blood flow in both imaged limbs of irradiated mice trended lower than in limbs of nonirradiated animals (CL: 28% decrease in mean MBF, $P = 0.149$; RT: 39% decrease, $P = 0.065$; Fig. 2B).

Day-specific comparisons between the three groups of mouse limbs differed by parametric component of perfusion. Compared to nonirradiated control mice, the microvascular flux rate in irradiated limbs was nearly identical at day 1 ($P = 0.923$; Fig. 3A) but 25% lower, in terms of group average, at day 8 postirradiation ($P = 0.079$; Fig. 3B). When evaluating the functional microvascular blood volume of irradiated limbs versus nonirradiated mouse limbs, there were minor differences at day 1 (7% increase in mean MBV in the irradiated limbs, $P = 0.485$; Fig. 4A) and at day 8 (12% decrease in mean in irradiated limbs, $P = 0.328$; Fig. 4B). Interestingly, the day 8 relationship between CL limbs and irradiated limbs differed by imaging parameter in the time versus VI function [Eq. (1)]. Specifically, when compared to CL limbs at day 8, irradiated limbs had, on average, lower flux rates (mean irradiated-contralateral difference = -0.08 s^{-1} , $P = 0.171$; Fig. 3B) but unexpectedly higher blood volumes (mean irradiated-contralateral difference = 8.7 VIU, $P = 0.328$; Fig. 4B).

Plasma Concentration of Vasoconstrictors

To further elucidate the mechanism for short-term radiation-induced differences in microvascular blood flow and flux rate, we applied ELISA to quantify plasma concentrations of potent vasoconstrictors (endothelin-1 and angiotensin II) in nonirradiated mice and in irradiated mice at either day 1 or 8 postirradiation. Plasma endothelin-1 levels were significantly higher in irradiated mice imaged at day 8 than in nonirradiated mice ($P = 0.011$; Fig. 5). No significant difference was observed for plasma levels of angiotensin II ($P = 0.774$; Fig. 6).

DISCUSSION

To our knowledge, this study is the first to evaluate *in vivo* microvascular response to irradiation in normal skeletal muscle utilizing non-invasive contrast-enhanced ultrasound perfusion imaging. We observed that CEU perfusion imaging is a sensitive and useful tool to assess microvascular changes after irradiation. We were surprised to observe that perfusion in irradiated limbs at day 1 was similar to perfusion in nonirradiated animal limbs since we expected radiation would lead to a perfusion deficit (Fig. 2A). However, our observation of higher perfusion levels in the CL limbs of irradiated mice compared to nonirradiated animals at day 1 is suggestive of an inflammatory and hyper-perfused state immediately after high-dose irradiation (Fig. 2A). Although we did not observe this hyper-perfusion in irradiated limbs, we believe this was mainly a consequence of the lower flux rate measured in irradiated limbs in contrast to CL limbs (Fig. 3A). Combined with the finding that, by day 8, microvascular blood volumes were greater in irradiated limbs than in CL limbs, this suggests that radiation increases microvascular resistance in the absence of microvascular unit loss when applied to healthy murine skeletal muscle.

Lin *et al.* previously used partial irradiation techniques to minimize inter-subject variability for spatial assessment of microvascular imaging modalities (9). Rather than employing a dynamic wedge as we have here, this group partially treated hindlimb tumors with a stereotactic radiosurgery cone to effectively create a steep dose gradient across the studied limb and then assessed microvascular changes utilizing dynamic contrast-enhanced and blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI) within 1 week

of exposure. Lin *et al.* and others found a significant increase in K_{trans} , a reduced BOLD response to carbogen and a reduced microvascular density within the exposed regions. Similarly, when comparing irradiated mice to nonirradiated mice using CEU imaging, we found microvascular changes exemplified by a markedly lower blood flow (~30–40% decrease in mean MBF) in both the irradiated and remote tissues of the irradiated cohort as early as 8 days after single-fraction high-dose irradiation.

However, unlike Lin *et al.*, based on our findings, we were able to determine that the larger contributor to the observed decline in microvascular blood flow in the radiation-exposed limb was flux rate rather than blood volume. This was unexpected, since we had anticipated that radiation-induced endothelial apoptosis and subsequent microvascular rarefaction would reduce microvascular blood volume (1). Importantly, since the percentage of actively perfused capillaries under resting conditions is so low, a radiation-reduced morphologic capillary density can still be consistent with a higher functional MBV because of acute metabolic needs. A lower microvascular flux rate is usually attributable to increases in tone in resistance arterioles, although changes in rheology at the capillary level (such as those caused by tissue edema or by changes in the relative apparent viscosity of blood,) can also contribute (10, 11). In regards to vascular tone, we sought to identify increased expression of potent vasoconstrictors after irradiation that could explain the decline in flux rate. Although systemic plasma concentrations of endothelin-1 in irradiated animals at both days 1 and 8 postirradiation were higher than in nonirradiated animals, the differences between group means (0.2 and 0.6 pg/ml, respectively) are probably not biologically meaningful. However, it is possible that the radiation-induced elevation in endothelin-1 is even larger at the irradiated site than what we observed in the plasma upon cardiac venipuncture.

There were several limitations in our study. Only two jugular vein cannulations for infusion of microbubbles were possible per animal, given the technical inability to re-cannulate a jugular vein at a later time point. This meant that only days 1 and 8 postirradiation CEU perfusion imaging could be performed, preventing us from acquiring preirradiation measurements from irradiated animals. We understand this is a relatively short time period postirradiation and that vascular modeling changes could certainly occur later than this. We plan to obtain measurements at later time points to assess long-term alterations of microvascular blood flow after high-dose irradiation. Furthermore, although we observed a decreased RBC velocity in radiation-exposed tissue, the mechanism by which this occurs remains unclear, as plasma endothelin-1 and angiotensin II are global vasoconstrictors and thus may not capture localized changes. We were hopeful that the gradient radiation methodology utilized in this study would allow for intra-animal comparison of imaging parameters and correlation with radiation dose. However, given the overall small size of mouse hindlimbs and technical inability for creating vastly disparate radiation doses across 5 mm, imaging parameters in the irradiated limb were averaged. Lastly, irradiations were performed only in skeletal muscle of healthy mice. It is unclear whether these results would be comparable in other tissue types or disease states.

In conclusion, our non-invasive CEU imaging method was effectively employed to detect radiation-induced changes in microvascular blood flow and RBC velocity. Further studies using other radiation doses and tissue types are warranted to corroborate our findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Diagram of hindlimb radiation experiments and subsequent imaging planes studied. Using a dynamic wedge, a radiation dose gradient was created across the mouse hindlimb with cross-sectional imaging planes 5 mm apart.

FIG. 2.

Microvascular blood flow (MBF) individual measures, quartiles (delineated by boxes) and means (denoted by diamonds) for contralateral and irradiated legs of irradiated mice at day 1 (panel A) and day 8 (panel B) postirradiation vs. the legs of nonirradiated mice. Some random variation has been applied to the individual measures (i.e., dots) so that all values are visible. *P* values involving comparisons with nonirradiated mice are from two sample *t* tests, while the *P* value for the contralateral vs. irradiated limb comparison is from a linear model accounting for correlation between measures from the same mouse. Hochberg multiplicity

correction was applied to produce the final displayed P values. Contralateral (CL) leg, region of interest (ROI); irradiated leg = irradiated ROIs (12 and 15 Gy average); video intensity unit = VIU.

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FIG. 3.

Microvascular flux rate individual measures, quartiles (boxes) and means (diamonds) for contralateral and irradiated legs of irradiated mice at day 1 (panel A) and day 8 (panel B) postirradiation vs. the legs of nonirradiated mice. Some random variation has been applied to the individual measures so that all values are visible. P values involving comparisons with nonirradiated mice are from two sample t tests, while the P value for the contralateral vs. irradiated limb comparison is from a linear model accounting for correlation between measures from the same mouse. Hochberg multiplicity correction was applied to produce the

final displayed P values. Contralateral (CL) leg, region of interest (ROI); irradiated leg = irradiated ROIs (12 and 15 Gy average).

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FIG. 4.

Microvascular blood volume (MBV) individual measures, quartiles (boxes) and means (diamonds) for contralateral and irradiated legs of irradiated mice at day 1 (panel A) and day 8 (panel B) postirradiation vs. the legs of nonirradiated mice. Some random variation has been applied to the individual measures so that all values are visible. P values involving comparisons with nonirradiated mice are from two sample t tests, while the P value for the contralateral vs. irradiated limb comparison is from a linear model accounting for correlation between measures from the same mouse. Hochberg multiplicity correction was applied to

produce the final displayed P values. Contralateral (CL) leg, region of interest (ROI); irradiated leg = irradiated ROIs (12 and 15 Gy average); video intensity unit = VIU.

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FIG. 5.

Endothelin-1 plasma concentration individual measures, quartiles (boxes) and means (diamonds) in separate cohorts of irradiated mice imaged at either day 1 or 8 postirradiation vs. nonirradiated mice. Group sample sizes are displayed above the x-axis.

FIG. 6.

Angiotensin II plasma concentration individual measures, quartiles (boxes) and means (diamonds) in separate cohorts of irradiated mice imaged at either day 1 or 8 postirradiation vs. nonirradiated mice. Group sample sizes are shown above the x-axis.