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Application of a Novel Murine Ear Vein Model to Evaluate the Effects of a Vascular Radioprotectant on Radiation-Induced Vascular Permeability and Leukocyte Adhesion

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Abstract

Vascular injury after radiation exposure contributes to multiple types of tissue injury through a cascade of events. Some of the earliest consequences of radiation damage include increased vascular permeability and promotion of inflammation, which is partially manifested by increased leukocyte-endothelial (L/E) interactions. We describe herein a novel intravital imaging method to evaluate L/E interactions, as a function of shear stress, and vascular permeability at multiple time points after local irradiation to the ear. This model permitted analysis of quiescent vasculature that was not perturbed by any surgical manipulation prior to imaging. To evaluate the effects of radiation on vascular integrity, fluorescent dextran was injected intravenously and its extravasation in the extravascular space surrounding the ear vasculature was measured at days 3 and 7 after 6 Gy irradiation. The vascular permeability rate increased approximately twofold at both days 3 and 7 postirradiation ($P < 0.05$). Leukocyte rolling, which is indicative of L/E interactions, was significantly increased in mice at 24 h postirradiation compared to that of nonirradiated mice. To assess our model, as a means for assessing vascular radioprotectants, we treated additional cohorts of mice with a thrombopoietin mimetic, TPOM (RWJ-800088). In addition to stimulating platelet formation, thrombopoietin can protect vasculature after several forms of injury. Thus, we hypothesized that TPOM would reduce vascular permeability and L/E adhesion after localized irradiation to the ear vasculature of mice. If TPOM reduced these consequences of radiation, it would validate the utility of our intravital imaging method. TPOM reduced radiation-induced vascular leakage to control levels at day 7. Furthermore, L/E cell interactions were also reduced in irradiated mice treated with TPOM, compared with mice receiving irradiation alone, particularly at high shear stress ($P = 0.03$, Kruskal-Wallis). We conclude that the ear model is useful for monitoring quiescent normal tissue vascular injury after radiation exposure. Furthermore, the

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application of TPOm, for preventing early inflammatory response created by damage to vascular endothelium, suggests that this drug may prove useful in reducing toxicities from radiotherapy, which damage microvasculature that critically important to tissue function.

INTRODUCTION

Normal tissue injury is a negative consequence of radiotherapy, as well as accidental total-body irradiation (TBI). Although organs have a range of cellular targets of radiation, vascular damage contributes to tissue damage through several mechanisms. First, the vasculature serves as an avenue for delivery of nutrients and removal of waste. Second, the vasculature prevents hemorrhage and inflammation by acting as a relatively impermeable barrier to fluid extravasation. Finally, quiescent vasculature downregulates adhesion molecules to limit interactions with circulating immune cells, thereby inhibiting their aggregation and extravasation. At high TBI doses, vascular damage may contribute to a cascade of events that culminates in organ failure, sepsis or death (1). In contrast, localized radiation doses that are used therapeutically interfere with microvascular function, cause local hypoxia and further downstream organ damage (2). Therefore, preventing radiation-mediated injury to the vasculature may reduce tissue-specific injury and mortality. For example, Jacobson *et al.* reported that treatment with pentoxifylline and vitamin E reduced tissue fibrosis after radiotherapy for breast cancer. The mechanism of this dual treatment was putatively related to improved microvascular blood flow, although this effect was not confirmed in the patients with reduced fibrosis scores (3). Mitigating late injury by preventing early vascular damage is an important and overlooked area of research.

Within 24 h postirradiation, vascular endothelial cells express adhesion molecules (e.g., L-selectin), which promotes leukocyte adhesion and extravasation, and can lead to an inflammatory response (4, 5). These early vascular effects may be accompanied by exposure of the basement membrane and hemorrhage, which then leads to microclot formation (6). The formation of microclots could damage downstream organ function as a result of depletion of oxygen and nutrient supply.

We had the opportunity to examine a thrombopoietin mimetic (TPOm or RWJ-800088) as a new class of vascular protectant for local irradiation (7). The drug was originally examined for its protective effects after TBI. In TBI, the extent of microclot formation may lead to platelet and fibrinolytic factor depletion. Because the platelet population cannot be efficiently replenished due to radiation exposure, thrombocytopenia ensues, leading to further vascular thinning and a progression to disseminated intravascular coagulopathy. Therefore, protection of the vascular endothelium may mitigate radiation-induced injury and mortality after TBI (8).

TPOm is a novel, potent, synthetic TPO-mimetic peptide, comprised of 29 amino acids and two 20 kDa moles of linear polyethylene glycol and has a mean half-life of 36 h in humans (9). TPOm has no homology with endogenous TPO, which is important because human TPO is not a viable therapy in humans, due to induction of cross-reactive antibodies to endogenous TPO, resulting in chronic thrombocytopenia. TPO may act directly on vasculature by binding to c-mpl receptors located on vascular endothelial cells (10). There

have been several studies demonstrating direct vascular protective effects of thrombopoietin in animal models of doxorubicin mediated cardiovascular injury (11), cardiovascular ischemia reperfusion injury (12) and stroke (13). Our group has demonstrated that TPOm preserves penile artery function nine weeks after prostate radiation therapy in rats (Ashcraft *et al.*, unpublished data).

Previous studies examining radiation effects on normal tissue vasculature have utilized window chambers (14, 15). A drawback of window chambers is that the surgical technique inevitably induces some degree of inflammation to the tissue being observed. Thus, the results may not be reflective of response of a quiescent vasculature that would be more typical of normal tissues. We sought to develop an *in vivo* model of a nonperturbed normal vasculature that would be amenable to serial measurements of: 1. vascular permeability and 2. leukocyte-endothelial (L/E) cell interactions. These two end points were chosen because changes in these parameters typically occur within hours of exposure to radiation, but can persist for many days or even weeks (6). As proof of principle, we examined the effects of TPOm on these assessments of vascular damage after 6 Gy irradiation. The mouse ear is a perfect choice for a nonperturbed microvasculature because it is amenable to repeated measures, using confocal microscopy through the intact overlying skin.

Ears were assessed 24 h postirradiation (6 Gy) for L/E cell interactions, and at days 3 and 7 postirradiation for changes in vascular permeability.

METHODS

Animals

Flk1-mCherry mice (16) were bred within the Duke University Breeding Core facility (Durham, NC). Male and female mice of at least 8 weeks of age were transferred to a nonbarrier facility and group housed throughout the experiments. The vascular endothelial cells in Flk1-mCherry mice constitutively express mCherry, which facilitates intravital microscopic imaging and analysis. All animal work was approved by the Duke University School of Medicine Institutional Animal Care and Use Committee.

Ear Irradiation

Mice were anesthetized with 80/8 mg/kg ketamine/xylazine intraperitoneal (i.p.) injection. Mice were placed in cylindrical lead jigs that shielded the entire body with the exception of the left ear pinna and were irradiated using an Orthovoltage X-ray 320 irradiator (Precision X-ray Inc., North Branford, CT) at a dose rate of 203 cGy/ min (320 kVp/10 mA with a 2-mm aluminum filter). All dosimetry was confirmed by Duke Radiation Physics prior to initiation of experiments.

TPOm Synthesis and Administration

TPOm was synthesized by Janssen Pharmaceutica (Beerse, Belgium) as described elsewhere (17), and shipped to Duke University in dried form. TPOm was reconstituted in sterile saline, sterile filtered, aliquoted and stored at -20°C until use. Immediately prior to

injection, aliquots were thawed and diluted. Mice received 300 µg/kg subcutaneously 6 h postirradiation.

Intravital Microscopy

Mice were anesthetized via isoflurane (2%, mixed with oxygen) and the ear was chemically depilated (Nair™) and cleaned with 70% ethanol. The mouse was placed on a heated stage underneath a Zeiss Observer.Z1 (Carl Zeiss AG, Oberkochen, Germany) inverted microscope. The irradiated ear was affixed to a Plexiglas® slide using double-sided tape. Images were acquired using Zen Imaging software (Carl Zeiss). The vascular endothelial cells in Flk1-mCherry mice express mCherry, which was visualized using a Zeiss Texas Red filter (Ex. 560/40; Em. 630/75). Acridine orange (Sigma-Aldrich® LLC, St. Louis, MO) and FITC-dextran were visualized using a Zeiss eGFP/ FITC/Alexa Fluor® 488 filter (Ex. 470/40; Em. 525/50).

Leukocyte-Endothelial Cell Interactions

After orienting the mouse on the microscope, 0.5 mg of acridine orange, dissolved in sterile saline, was intravenously (i.v.) injected. Acridine orange labels all nucleated cells in the blood (i.e., leukocytes). Vasculature was imaged for 60 s, with images taken every 200 ms. Rolling leukocytes were defined as cells that marginated along the vessel wall and were clearly dissociated from blood flow. Four venules per mouse were identified, based on their diameter, and the number of leukocytes that rolled past three preselected points in each venule were counted. The vessel diameter (D) and free-flowing leukocyte velocities (V) were also measured, and used to calculate pseudo shear rate (18), defined as $(8 \times V)/D$. All videos were analyzed by researchers blinded to the treatment groups. Groups were compared using Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) followed by Dunn's multiple comparisons test using GraphPad Prism software (LaJolla, CA).

Vascular Permeability Measurement and Assessment

After orienting the mouse on the microscope, a 30g needle attached to a syringe with Tygon® Microbore tubing catheter (Harvard Apparatus, Holliston, MA) was inserted into the tail vein. Z-stack images of both the FITC and mCherry channels were acquired every 10 s, and 0.5 mg of FITC-conjugated 70 kDa dextran injected through the catheter after the third set image of images was collected. Image acquisition was done every 10 s for 25 min.

We assumed a one-compartment model of permeability using the equation for $C_T(t)$. The tissue concentration of dextran in extravascular tissue at time t [known as a time activity curve (TAC)] is given by:

$$\frac{dC_T(t)}{dt} = k_1 C_B(t - \Delta). \quad (1)$$

Equation (1) postulates that the change in tissue concentration at time t is proportional to $C_B(t - \Delta)$, concentration of dextran in the vasculature at a delay Δ . For simplicity, we excluded the possibility of dextran returning from extravascular tissue back into the vascular

compartment. In this setting, the quantity of interest for us is k_1 , the permeability rate. Assuming the initial condition that $C_B(0) = 0$ (there is no dextran in the tissue at time 0), we can solve the differential Eq. (1) as:

$$C_T(t) = k_1 \int_0^t C_B(s - \Delta) ds. \quad (2)$$

Estimating k_1 from Eq. (2) requires knowledge of both the intra- and extravascular concentration of dextran at time t , $C_B(t)$ and $C_T(t)$. Due the lack of a reliable vascular marker, it was difficult to accurately separate the vascular and extravascular components in the images. However, we were able to identify part of the vasculature because the earliest dextran signals are seen only in the vasculature. By drawing a region of interest (ROI) over this region (Fig. 1), we could estimate the vascular TAC $C_B(t)$. We made the assumption that the TAC is uniform through other parts of the vasculature. Instead of attempting to identify the extravascular region, we computed the mean activity over the entire field of view $C_F(t)$, which is composed of both the vascular and tissue components. Without loss of generality, we can express this overall activity as:

$$C_F(t) = v_b C_B(t) + (1 - v_b) C_T(t), \quad (3)$$

where v_b is the (unknown) fraction of area in the field of view composed of the vasculature. We could then replace the unknown tissue TAC $C_T(t)$ in Eq. (3) using Eq. (2), as follows:

$$C_F(t) = v_b C_B(t) + (1 - v_b) k_1 \int_0^t C_B(s - \Delta) ds. \quad (4)$$

Assuming the vascular TAC, $C_B(t)$ to be strictly positive (>0), we could divide both sides of Eq. (4) by it to obtain a transformed equation:

$$\frac{C_F(t)}{C_B(t)} = v_b + (1 - v_b) k_1 \frac{\int_0^t C_B(s - \Delta) ds}{C_B(t)}, \quad (5)$$

i.e., $y(t) = v_b + mx(t)$, where $y(t) = \frac{C_F(t)}{C_B(t)}$, $x(t) = \frac{\int_0^t C_B(s - \Delta) ds}{C_B(t)}$ and $m = (1 - v_b)k_1$. Equation

(5) can be seen to be in the form of a straight line, which can be fit using linear regression. The unknown constants v_b and m are estimated as the slope and intercept of the fitted regression line. This estimation technique, usually known as Patlak graphical analysis, is used to estimate rates of tissue transport of radiotracer in dynamic PET studies (19). In practice, it takes time for equilibrium to be reached before the linear relationship described in Eq. (5) results in the slopes shown in Fig. 2. For this reason, we fit the linear relationship

by excluding the first 10 min of the study. For our purposes, we could estimate the permeability constant as: $\hat{k}_1 = \hat{m}/(1 - \hat{v}_b)$.

Circulating Blood Cell Analysis

Mice were euthanized with 50 μ l Euthasol® (Virbac Animal Health Inc., Fort Worth, TX) injected i.p. After mice became nonresponsive to toe pinch, but prior to heartbeat cessation, 500 μ l of blood was collected by cardiac puncture via heparinized syringe and immediately vortexed with 20 μ l heparin. CBC analysis was performed by the Duke University Veterinary Diagnostic Laboratory, using a ProCyte Dx Hematology Analyzer (IDEXX Laboratories Inc. Westbrook, ME).

RESULTS

Effects of Radiation on Vascular Permeability

One of the consequences of radiation-mediated vascular endothelial cell apoptosis is reduced vascular integrity, resulting in vascular leakage (20). We sought to assess vascular integrity by quantifying dextran extravasation from the vasculature after i.v. injection of 70 kDa FITC-dextran. This size of dextran was selected because it is near the size of albumin; therefore, its extravasation is physiologically relevant (21). Dextran was injected 30 s after imaging was initiated, to establish the background signal. After injection, a strong fluorescent signal was observed within the vasculature, which spread throughout the vascular network. Leakage was observed as “spreading” of FITC signal that originated within the vessels. (Supplementary Videos S1 and S2; <http://dx.doi.org/10.1667/RR14896.1.S1> and <http://dx.doi.org/10.1667/RR14896.1.S12>, respectively)

Permeability measurement method

Although we considered a mathematical model that included reverse transport from the tissue to the vasculature, the standard model with transport in both directions assumes that the blood TAC has a negative exponential shape, suggesting that the blood TAC monotonically decreases over time. Based on the observed blood TAC presented herein, this is violated in a number of cases, with some individuals increasing, then decreasing, at later times during the imaging. This suggests that the standard model would be a poor fit for some data. The model we have fitted is stronger because it uses the empirically observed data without presupposing any particular form for the blood TAC.

Vascular Radioprotectant Therapy Preserved Vascular Integrity by Reducing Leakage after Irradiation

Nonirradiated control mice showed strong vascular integrity; the dextran signal remained within the vessels and did not appreciably change over a 25-min imaging session (Supplementary Video S1; <http://dx.doi.org/10.1667/RR14896.1.S1>). The average permeability rate for nonirradiated control mice was 0.0012. In mice where vascular integrity had been compromised by radiation, FITC signal clearly extended beyond the vascular mask (Supplementary Video S2; <http://dx.doi.org/10.1667/RR14896.1.S2>). Permeability rates were calculated for each mouse and are shown in Fig. 3. Radiation

significantly increased permeability rates at days 3 and 7 postirradiation to 0.0027 and 0.0025, respectively ($P < 0.05$, compared to controls) (Table 1). Permeability rates from mice treated with TPOM after irradiation were not significantly different from nonirradiated TPOM controls (0.001) at either time point (0.0021 and 0.0011 for 3 and 7 days postirradiation, respectively). There was no correlation between permeability rates at days 3 and 7 in individual mice, suggesting that injury may not peak at the same time among animals.

Effects of Radiation on Leukocyte-Endothelial Cell Interactions

Previous studies have utilized intravital microscopy to quantify radiation-mediated inflammatory responses resulting in increased rolling leukocytes in the terminal venules of irradiated small bowel (22), skin (15) and sublingual mucosa (23). To assess L/E interactions, we obtained real-time images of ear vasculature at 24 h postirradiation (6 Gy). Leukocyte nuclei were labeled by intravascular acridine orange injection, which permitted visualization by fluorescence microscopy. Rolling leukocytes were clearly observed marginating along the vessel walls, at a rate that was distinctly separated from the free flow (Fig. 4A) in both sham (Supplementary Video S3; <http://dx.doi.org/10.1667/RR14896.1.S3>) and irradiated (Supplementary Video S4; <http://dx.doi.org/10.1667/RR14896.1.S4>) mice. Post-capillary venules were used for analysis and were distinguished from other vessels based on diameters (approximately 15–60 μm in diameter) and evidence of convergent blood flow. Neither radiation nor TPOM had significant effects on average blood flow velocity, diameter or shear rates among the vessels selected. (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR14896.1.S5>).

Shear rate, the force of blood flow parallel to vessel walls, has a direct effect on L/E interactions. Higher shear rates tend to reduce leukocyte interactions with adhesion molecules. This translates to fewer L/E interactions at high shear rates and a negative slope when plotting shear rate vs. L/E interactions. To account for this relationship while investigating the role of radiation-induced leukocyte adhesion, shear rates and L/E interactions were determined for at least four vessels per mouse, and the values for each vessel were graphed as scatter plots (Fig. 4B). Slopes were calculated for the vessels studied in each individual mouse. As expected, L/E interactions decreased with increasing shear rates, yielding a slope of $-14.4 \pm 5.1 \text{ mm}^{-1}$ (Fig. 4C). However, at 24 h postirradiation, sufficient endothelial activation had occurred such that L/E interactions persisted, even at higher shear rates, resulting in a positive average slope of $5.6 \pm 3.6 \text{ mm}^{-1}$.

In mice treated with TPOM prior to either sham or 6 Gy irradiation to the ear, the sham-irradiated group showed an average slope of $-14.4 \pm 10.9 \text{ mm}^{-1}$, whereas the irradiated + TPOM-treated group had an average slope of $-9.6 \pm 6.4 \text{ mm}^{-1}$. The slopes were significantly different between groups ($P = 0.03$, Kruskal-Wallis). These findings support the hypothesis that TPOM reduces vascular endothelial leukocyte interactions after irradiation, thereby promoting maintenance of vascular integrity after irradiation.

To rule out the possibility that our observations resulted from systemic changes in leukocyte counts, complete blood counts were performed (Supplementary Table S1; <http://dx.doi.org/10.1667/RR14896.1.S5>). By 24 h postirradiation, there was a slight decrease in platelets in

the irradiated mice, but this was not significant. There were no significant differences in total leukocytes, neutrophils or lymphocytes across groups, or as an effect of radiation, TPOm or the interaction. Therefore, the observed increase in L/E interactions was not merely a byproduct of altered blood leukocyte composition.

DISCUSSION

Acute changes in the vascular endothelium develop after radiation exposure. These include both cellular and tissue dysfunction followed by increased vascular permeability, edema formation, secretion of various cytokines and growth factors leading to the activation of fibroblasts and endothelial cells, as well as the recruitment of macrophages and other white cells, all of which results in radiation-induced inflammation (24). The role of vascular endothelial cell dysfunction in this cascade of events remains controversial; nevertheless, changes in this tissue clearly occur. Initially, key adhesion molecules become upregulated, including P-selectin, E-Selectin, ICAM-1, VCAM-1 and E-cadherin (25–27). This has been consistently observed across a wide variety of cell types and tissues (28). There is precedent for targeting adhesion molecule upregulation as a means to reduce radiation-induced changes. We previously reported that a platelet activating factor antagonist can prevent radiation-induced leukocyte rolling using intravital microscopy to evaluate microvessels in the nontumor-bearing skin-fold window chamber of rats (15).

A key role of adhesion molecules is to mediate cellular interactions. These interactions are crucial for maintenance of normal tissue after injury. Coinciding with early upregulation of adhesion molecules, a host of inflammatory and immune response markers are affected after radiation exposure. As leukocytes accumulate in the affected area, vascular dysfunction permits their extravasation to surrounding tissue. The presence of neutrophils is a characteristic of acute inflammation. Other cells, including macrophages and monocytes, release pro-inflammatory and pro-fibrotic mediators including IL-1 β , IL-6, IL-8, CXCL1 (Gro α , KC), CXCL2 (MIP2 α), TNF α and TGF β (29–31). In addition, during this early phase, keratinocytes can produce large amounts of cytokines in response to radiation, specifically IL-1 β and TNF α (32). The upregulation of these key cytokines has been observed across a variety of settings, including IL-6 and CXCL1 (Gro α , KC) are upregulated in lung models (27) and CXCL10, IL-6, IL-8, CCL2 (MCP-1), CCL4 (MIP1 α) in skin models (26). In more complex models investigating combined radiation injuries, Fukumoto *et al.* observed that IL-6, CXCL1 (Gro α , KC), G-CSF, IL-14, IL-1 β , IL-9, IL-10, eotaxin, MIP1 α / β were all dysregulated after irradiation followed by wound trauma in mice (33).

Increases in vascular permeability may result from endothelial cell apoptosis. Although some groups have suggested that endothelial apoptosis can occur within 24 h postirradiation, we did not detect cleaved caspase 3 or TUNEL staining 24 h after 6 Gy irradiation (data not shown) which is consistent with other reports in the literature (34). An alternative hypothesis is that radiation-induced vasodilation increases endothelial gap sizes, which is supported by studies showing radiation-induced increases in eNOS activation (34).

The studies described herein demonstrate that ear vasculature provides a suitable site for assessing vascular changes after irradiation via intravital microscopy. This model is complementary to the dorsal skin-fold window chambers as a means of imaging aspects of normal (15, 18, 35, 36) and tumor (37–39) vasculature such as vascular density, hemodynamics and angiogenesis. The benefits of the window chamber are that it is easier to keep the tissue in place during imaging, and it is far easier to return to the same area of the vessel bed for serial imaging. However, there is a low level of inflammation remaining in window chamber tissue as a result of the surgical implantation and the chamber viability is typically limited to one week after implantation. The ear model may be optimal for observing vascular changes in normal tissue after irradiation, because it is not surgically manipulated. Thus, any vascular and inflammatory effects would reflect that of native previously unperturbed tissue. As the ear has been shown to be useful for engraftment of multiple tissue types (40), these approaches could be combined for intravital imaging of tissue-specific vascular responses to radiation.

The ear model does exhibit some limitations. We observed variability among mice within treatment groups, particularly with the permeability measurements. The effect of radiation on increased vascular permeability was driven by a few mice at each time point. There was no correlation between permeability at day 3 vs. day 7, suggesting that the timing of injury is variable. A focus of future studies may be to examine vessels at multiple time points to catch each mouse at its time of peak leakage. This model of vascular injury could be improved by expanding the number of days the mice are imaged to encompass all days between 3 and 7 as well, and then scoring injury using a binary leakage/no leakage rubric. We speculate that this may be a less noisy means of assessing injury, because it would eliminate “false negatives” of mice having a permeability index that is similar to controls for some days, but elevated on others. The acridine orange L/E analysis is also inherently variable, as are the shear rates, which were determined by vessel diameter and blood flow velocity. We used a narrow window of vessel diameters when selecting venules for analysis, but blood velocity could not be controlled. Thus, shear rates were subject to inter-mouse differences in two variables, as highlighted in Supplementary Fig. S1 (<http://dx.doi.org/10.1667/RR14896.1.S5>). We were able to take advantage of the inherent variation by examining slopes of shear rate vs. L/E interaction. Here, there were obvious differences in slope, with irradiated vessels consistently showing higher L/E interactions persisting at higher shear rates. Of the two models presented herein, the L/E interaction model showed a clearer distinction between irradiated mice treated with saline compared to the vascular radioprotectant, whereas the dextran vascular permeability was complicated by temporal differences in peak dextran extravasation among mice.

In previously published studies, earlier time points after exposure have been considered, and L/E interactions were examined as early as 1–2 h after 19–20 Gy irradiation (41, 42). However, earlier time points were meaningless for our assessment of TPOM, because TPOM was not administered until 6 h postirradiation. For preclinical analysis of radioprotectants (i.e., therapeutics given prior to radiation treatment), earlier time points may be worth considering. For example, administration of a platelet activating factor inhibitor 5 min prior to 6 Gy prevented upregulation of L/E cell rolling 30–60 min postirradiation (15). We initially intended to assess L/E interactions at both 24 and 48 h postirradiation; however, we

found that the acridine orange signal did not clear well from the ear tissue between these two time points, making serial imaging difficult. Because preliminary evidence indicated that there was no difference in L/E interactions between groups at 48 h, we did not pursue additional studies at this time point. Genetically engineered mouse models could certainly be beneficial to this type of research, if dual reporter genes could be used to identify leukocytes and vasculature endothelium, in the absence of extrinsic labeling.

CONCLUSIONS

We have described here a novel ear vein model for quantifying radiation-induced changes to the vasculature at different time points. We have presented two imaging techniques that can be used to monitor vascular changes at 24 h, and 3 and 7 days postirradiation in the same mouse. These procedures may be adaptable to examine chronic vascular injuries and arteritis. We propose that this model can be used for future studies on vascular injury after irradiation, as well as to evaluate potential vascular radioprotectants at a variety of time points and radiation doses. In this scenario, TPOM may be useful in reducing localized microvascular dysfunction and subsequent hypoxic microenvironments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Scheme for extraction of time activity curves (TACs), demonstrated using a mouse in the irradiation + TPOM arm. Panel A: A rectangular region of interest (ROI) is selected around vasculature showing expression of dextran at an early time point in the study. Panel B: A subregion of the vasculature is identified by thresholding on the 80th percentile of the intensity within the ROI in panel A. Panel C: The same field as in panel A, seen at the last time point of the study. Notice the enhanced extravascular expression relative to panel A. Panel D: TACs for both the entire field of view (black lines) and the vascular ROI in panel B, measured at 150 time points, spaced 10 s apart. Each observation is the average expression over all pixels in the relevant region at that time point. There are three lines for each type of ROI, representing three successive slices of tissue. Note that the vascular TAC is higher than the overall TAC at all times.

FIG. 2.

Slice-wise estimation of permeability rate using Patlak analysis, based on the extracted TACs for the study shown in Fig. 1. The black dots show the observed Patlak plot of the quantities $x(t)$ vs. $y(t)$, as given in Eq. (5). The red line shows the least squares fit to the data. The equation of the fitted line is given in red. The permeability rate is estimated from the coefficients of the fitted line. Note the low variability of estimated coefficients across slices.

FIG. 3. Dextran permeability rates before treatment, days 3 and 7 postirradiation; saline and TPOm (300 $\mu\text{g/kg}$) were administered at 6 h postirradiation. N = 9–17/group and each symbol represents a mouse. Error bars represent the standard error.

FIG. 4. Leukocyte-endothelial cell interactions, assessed using acridine orange intravital microscopy. Panel A: Leukocytes that rolled along the vascular endothelium were easily distinguished due to their slowed velocity compared to surrounding blood flow. A representative image series showing three marginating leukocytes traveling over the course of 1 s is shown. Panel B: Shear rates vs. L/E interactions were plotted, for each mouse, and the slope was calculated. Panel C: Radiation significantly altered the relationship between shear rate and L/E interactions, whereas TPOM prevented L/E interaction at higher shear

rates. A Kruskal-Wallis nonparametric one-way ANOVA showed significant ($P=0.03$) differences among all groups. *Dunn's post hoc multiple comparisons identified a significant pair-wise difference between saline 0 Gy and saline 6 Gy irradiated groups. There was no significant difference between 0 Gy TPOm and 6 Gy TPOm (n.s.).

TABLE 1

ANOVA Results of Dextran Permeability Rate Comparison

Term	Effect	Std. Error	T value	P value
Baseline	0.0012	0.0003	3.7025	0.0003
Day 3 postirradiation	0.0013	0.0006	2.0089	0.0483 ^a
Day 7 postirradiation	0.0015	0.0006	2.2727	0.0260 ^a
Day 3 postirradiation + TPO	0.0009	0.0007	1.2689	0.2086
Day 7 postirradiation + TPO	-0.0002	0.0007	-0.2346	0.8152

^aSignificant at $P < 0.05$, compared to controls.