Radiation therapy causes loss of dermal lymphatic vessels and interferes with lymphatic function by TGF-β1-mediated tissue fibrosis

Tomer Avraham,1 Alan Yan,1 Jamie C. Zampell,1 Sanjay V. Daluvoy,1 Adriana Haimovitz-Friedman,2 Andrew P. Cordeiro,1 and Babak J. Mehrara1

1Division of Plastic and Reconstructive Surgery, Department of Surgery, and 2Department of Radiation Oncology, Memorial Sloan-Kettering Cancer Center, New York, New York

Submitted 8 December 2009; accepted in final form 1 June 2010

LYMPHEDEMA is a debilitating disorder that affects more than 3 million Americans (2, 48). In the United States the most common cause of lymphedema is lymph node dissection for the treatment of a variety of malignancies (44, 60). Because of the relative prevalence of breast cancer and the fact that lymphatic metastasis is the primary source of metastasis in these patients, axillary lymph node dissection (ALND) is the most commonly encountered cause of lymphedema clinically in Western countries (23). Patients with lymphedema are easily recognizable by their dependence on tight-fitting garments needed to prevent the inevitable progression of this disorder. Because of the lack of effective treatment options, these patients have significantly decreased quality of life with frequent infections, decreased function, and disfigurement (3, 60, 61).

A large number of clinical studies have identified risk factors for the development of lymphedema (22, 35, 42, 59, 61). The vast majority of these studies have identified radiation therapy as an independent risk factor for the development of lymphedema (23). In fact, it is estimated that postoperative radiation therapy increases the risk of developing lymphedema by as much as 10-fold (44). In addition, it appears that the combination of surgery and radiation is necessary for the development of lymphedema since radiation alone is infrequently associated with clinically apparent lymphedema. Nevertheless, the potential mechanisms by which radiation therapy increases the risk of lymphedema remain essentially unknown.

One previous study demonstrated that intestinal lymphatic endothelial cells (LECs) are relatively radioresistant compared with microvascular endothelial cells (57). This finding was not reproduced by a more recent study demonstrating intestinal lymphatic endothelial apoptosis shortly after total body irradiation (1). The clinical relevance of studies on intestinal LECs is somewhat difficult to interpret since lymphatic dysfunction and subsequent extremity lymphedema are related to cutaneous lymphatic channels, which may be structurally or functionally different from intestinal lymphatics. In fact, Jackowski et al. (27) have shown that decreased numbers of cutaneous lymphatics after radiation therapy in breast cancer patients are associated with an increased rate of lymphedema. In addition, Mortimer et al. (40) have shown in limited studies that radiation therapy is associated with cutaneous lymphatic dysfunction in pigs. Therefore, it is possible that radiation therapy contributes to the development of lymphedema by causing depletion or dysfunction of cutaneous lymphatic channels.

Radiation therapy may also increase the risk of lymphedema after ALND by promoting tissue fibrosis. Radiation therapy is well known to cause tissue fibrosis as a result of transforming growth factor (TGF)-β1-dependent mechanisms (36, 37). We recently showed (8) that fibrosis is a critical regulator of lymphatic regeneration and that this effect is largely dependent on TGF-β1 expression. In addition, we (8) and others (41) have shown that TGF-β1 is a direct inhibitor of LEC proliferation and function. Therefore, it is feasible that at least some of the negative consequences of radiation therapy on the lymphatic system may be secondary to tissue fibrosis. This hypothesis is supported by the fact that clinical factors that increase the risk of fibrosis (e.g., extensive surgery, infections, or obesity) also significantly increase the risk of lymphedema (34, 35).

The purpose of these studies was to determine the effects of ionizing radiation therapy on cutaneous lymphatic vessels. Using a mouse tail model, we show that radiation causes a...
dose-dependent, long-term decrease in lymphatic function. Impaired lymphatic function resulting from radiation is associated with a combination of LEC apoptosis, long-term decrease in the number of cutaneous lymphatic vessels, and soft tissue fibrosis. We show that LECs in acid sphingomyelinase (ASM)-deficient animals, similar to microvascular endothelial cells, are significantly protected from radiation-induced apoptosis. However, this protective effect does not inhibit development of tissue fibrosis or improve lymphatic function at later time points. Short-term inhibition of TGF-β1 significantly decreased tissue fibrosis and markedly improved lymphatic function but did not provide a protective effect for LEC apoptosis or lymphatic depletion. Finally, radiated lymphatics demonstrated evidence of endothelial-mesenchymal transition (EMT) in vivo. In vitro exposure of LECs to recombinant TGF-β1 increased expression of collagen and markers of fibroblast differentiation while simultaneously decreasing the expression of lymphatic markers [lymphatic vessel endothelial receptor-1 (LYVE-1)]. Together, these results indicate that lymphatic dysfunction resulting from radiation therapy does not cause clinically apparent lymphedema but may predispose to the development of lymphedema when combined with other insults by promoting tissue fibrosis (e.g., surgery). In addition, our results indicate that prevention of tissue fibrosis resulting from radiation may be a means of protecting against this potentially devastating complication.

**METHODS**

**Mouse tail irradiation.** All animal experiments were approved by the Research Animal Resource Center Institutional Animal Care and Use Committee (IAUC) at Memorial Sloan-Kettering Cancer Center. C57BL/6 mice were anesthetized with ketamine-xylazine and then carefully shielded, and their tails were then irradiated with the X-RAD 320 (Precision X-Ray, North Branford, CT) with doses of 15 or 30 Gy. We chose the mouse tail for irradiation because it is an appendage that has been previously used extensively in studies of lymphatic function and regeneration and is also easily shielded from the rest of the animal. Control mice received anesthetic and were placed in the irradiator but received no radiation. Mice were then euthanized for tail harvest after 4 h, 10 h, 1 wk, 4 wk, 12 wk, and 24 wk following irradiation (n = 5 for each dose at each time point evaluated).

**Irradiation of acid sphingomyelinase-deficient mice.** Mice deficient in the ceramide ASM have been previously shown to be protected from radiation-induced apoptosis in a variety of cell types including microvascular endothelial cells (43). As such, we evaluated the impact of this deficiency on LEC apoptosis following irradiation. ASM−/− mice established on the SV129/C57BL/6 background along with wild-type (WT) littermates underwent tail irradiation as described above. Tissues were harvested at 10 h and 4 wk after radiation (n = 3 for each time point). The 4 wk time point was chosen as the longest follow-up since this represented our longest follow-up in other groups for assessment of apoptosis.

**Short-term TGF-β1 blockade with LY-364947.** To determine the effectiveness of TGF-β1 blockade in alleviating the effects of radiation therapy on lymphatic function, we treated animals with LY-364947 (Sigma, St. Louis, MO), a well-described small-molecule inhibitor. This molecule is an ATP-competitive inhibitor of TGF-β receptor (TGF-βRI) and blocks association with TGF-βRII and subsequent Smad3 phosphorylation (52). LY-364947 was dissolved at a concentration of 5 mg/ml in DMSO. Experimental animals (n = 5) were treated with LY-364947 (1 mg/kg diluted in PBS) delivered intraperitoneally starting 1 day before tail irradiation with 15 Gy. Animals were subsequently treated every other day for three additional doses. Control mice were irradiated in an identical manner but treated with DMSO diluted in PBS. While long-term inhibition of TGF-β1 may be associated with immune dysfunction, short-term inhibition as performed in our study is well tolerated (71). No adverse side effects were noted in our animals treated with LY-364947.

**Tail volume measurements.** To evaluate the degree of acute lymphedema following irradiation, tail volumes were determined with the truncated cone formula \[ V = \frac{1}{2} \pi C (r_1^2 + r_2^2 + r_1 r_2) \] (C is circumference) as previously described (53). Briefly, tail circumference was measured by blinded reviewers at four points at 10-mm intervals starting 20 mm from the tail base with a digital caliper. Tail volume changes from baseline were calculated for each animal in order to minimize the potential effects of interanimal baseline differences in tail volumes.

**Lymphoscintigraphy.** To quantify lymphatic transport after irradiation, [99mTc]-sulfur colloid (100-nm particle size; 400–800 μCi in ~50 μl) was injected intradermally ~20 mm from the tail tip with our previously reported methods (5). This colloid is taken up by the lymphatics and transported to the lymph nodes at the base of the tail and is a sensitive, easily quantifiable measure of lymphatic transport. Briefly, dynamic planar gamma camera images were acquired in 15- to 600-s frames for up to 130 min after injection with an X-SPECT (Gamma Medica, Northridge, CA) fitted with low-energy parallelogram collimators. Resulting dynamic images were analyzed with ASIPro (CTI Molecular Imaging, Knoxville, TN), and region of interest (ROI) analysis was performed to derive the decay-adjusted activity at the lymph nodes at the base of the mouse tail. Time-activity data were then fit to a function exponentially decreasing to an asymptotic value for the injection site or a function exponentially increasing to an asymptotic value for the individual lymph nodes. Maximal [99mTc] uptake was calculated and plotted.

**Specimen preparation, histology, and immunostaining.** After euthanasia, mouse tails were fixed in 4% parafomaldehyde at 4°C for 24 h and then decalcified in Immunocal (Decal Chemical, Tallman, NY). A 1-cm portion of tail was harvested 2 cm from the base of tail and sectioned longitudinally. Additionally, 2-mm cross sections of tail were obtained both immediately distal and proximal to this piece. All three pieces of tail were embedded in paraffin and sectioned at 5 μm.

Sections were histologically stained with standard protocols and evaluated with a Leica microscope (Wetzlar, Germany). Histochemical staining for scar tissue was performed with picro-Sirius red (Direct Red 80, Sigma) as previously described (16). Sirius red-stained specimens were examined by polarized light microscopy (Leica TCS AOBSSP2), and the scar index was calculated as previously described with Metamorph Offline software (Molecular Devices, Sunnyvale, CA) in a minimum of three 70,000-μm sections per animal (16). A minimum of three animals were evaluated per group/ per time point. Sirius red staining quantification of scarring and fibrosis is based on the fact that normal skin is characterized by thin, randomly oriented collagen fibers demonstrating yellow-green birefringence while scarring is associated with deposition of thick parallel collagen bundles with orange-red birefringence (15, 16). The scar index is the ratio of orange-red to yellow-green pixels, with higher values representing increased scarring.

Immunohistochemical and immunofluorescent staining were performed as described previously to localize LECs, growth factors, and myofibroblast markers (39). Primary antibodies used were against LYVE-1 (rabbit polyclonal, Abcam, Cambridge, MA), podoplanin (rabbit monoclonal, Abcam), phosphorylated Smad3 (pSmad3, rabbit polyclonal, Abcam), and α-smooth muscle actin (α-SMA, Abcam). Immunofluorescent secondary antibodies used fluorescein (R&D Systems, Minneapolis, MN) or Cy3 Zymed (Invitrogen Molecular Probes, Carlsbad, CA). For immunohistochemistry, secondary antibody was from the VECTASTAIN ABC Kit (Vector, Burlingame, CA) and developed with 3,3’-diaminobenzidine (DAB). For all immunohistochemical or immunofluorescent studies, negative control included tissue incubated with secondary antibody but not primary antibody.
Images were obtained by bright-field microscopy (Leica TCS) for immunohistochemistry and with a confocal microscope for immunofluorescence (Leica). Lymphatic vessel and lymphatic endothelial (LEC) cell counts were performed by two blinded observers in three to five random high-power fields (HPFs) in a minimum of three animals/group fields. There was minimal interobserver variation in the analysis. For colocalization of podoplanin and α-SMA, slides were scanned with the Mirax Slide Scanner (Zeiss). Captured bright-field and fluorescent images taken at the same location on the slide were overlaid with Adobe Photoshop (Adobe, San Jose, CA).

In vivo detection of LEC apoptosis was performed on paraffin-embedded tissue sections. Apoptotic cells were detected with the Apoptag Red In Situ Apoptosis Detection Kit per the manufacturer’s protocol (Millipore, Billerica, MA). This assay labels apoptotic cells by using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining. To localize apoptotic LECs, sections were double stained with anti-LYVE-1 antibody as described above, labeled with a fluorescein secondary antibody, and imaged with a confocal microscope (Leica). For quantification of LEC apoptosis the total number of LYVE-1+ cells was counted by two blinded reviewers in three to five random HPFs (>400 magnification) in a minimum of three animals/time point/group. We identified cells that expressed both TUNEL and LYVE-1, and these were considered apoptotic LECs; their proportion was expressed as a percentage of the total number of LECs.

Western blot analysis. Total cellular protein was isolated from tail sections inclusive of the skin and subcutaneous tissues 1 wk after irradiation from animals treated with LY-364947 or vehicle control. Protein was harvested with the Qiagen DNA/RNA/Protein Mini Kit according to the manufacturer’s protocol (Qiagen, Valencia, CA) and quantified with the Bradford method. Western blotting was performed as previously described (8). Western blot analysis for TGF-β-induced (TGFβi), a protein that is upregulated by and represents a surrogate marker of TGF-β1 activity, was performed with a rabbit polyclonal antibody (Abcam) and normalized to actin levels (24, 62). Antibodies for N-cadherin, E-cadherin, LYVE-1, fibroblast activation protein (FAP), and collagen I were all purchased from Abcam. Immunoreactivity was determined with the ECL chemiluminescence detection system (Amersham, Arlington Heights, IL).

Cell culture and reagents. Human dermal lymphatic endothelial cells (HLECs) were obtained from PromoCell (Heidelberg, Germany), cultured in ECGM-MV containing 0.4% ECGS/H, 5% FCS, EGF (10 ng/ml), hydrocortisone (1 μg/ml), and penicillin-streptomycin (50 U/ml) (Invitrogen), and passaged every 48 h. LEC morphology was confirmed with immunofluorescent staining for LYVE-1 and prox-1 (not shown). Early-passage cells (<10) were used for all experiments.

For in vitro assay of LEC apoptosis, cells were grown to near-confluence in six-well plates or chamber slides. They were then irradiated with varying doses of radiation and evaluated at various time points. Flow cytometric analysis for apoptosis by TUNEL assay was carried out with the Apo-Direct TUNEL kit per the manufacturer’s protocol (Millipore). Flow cytometry was performed on the FACS-caliber flow cytometer (BD Biosciences, San Jose, CA) and analyzed with Flowjo software (Tree Star, Ashland, OR). Immunofluorescent confirmation of LEC apoptosis was carried out with the Dual Apoptosis Kit per the manufacturer’s protocol (Biotium, Hayward, CA). This kit allows concomitant detection of annexin V and caspase-3 as dual markers of apoptotic cell death.

To assess LEC senescence after radiation therapy in vitro, cells were grown to ~60% confluence in six-well plates and then exposed to various doses of irradiation and grown for an additional 4 days. After 4 days, the medium was removed and the cells were briefly fixed in 4% paraformaldehyde followed by staining with the Biovision Senescence Detection Kit (Biovision, Mountain View, CA). This assay is based on the fact that senescent cells stain for senescence-associated (SA)-β-galactosidase at a pH of 6.0 with X-gal and has been used in a large number of studies to identify cellular senescence in vitro (25). The number of stained cells as a function of total number of cells present was quantified by blinded reviewers in a minimum of four plates per group.

To evaluate the direct effects of TGF-β1 on LECs, LECs were grown in vitro in fibronectin-coated flasks (Sigma). At the initiation of experiments medium was changed to DMEM with 5% FCS with or without 10 ng/ml recombinant human (rh)TGF-β1 (R&D Systems). Cells were then evaluated at specified time points.

Statistical analysis. Multigroup comparison was performed by one-way ANOVA with the Tukey-Kramer post hoc test. Student’s *t*-test was used for analyzing differences between two groups. Data are presented as means ± SD or SE as noted, with *P* < 0.05 considered significant.

**RESULTS**

Radiation is associated with severe long-term lymphatic dysfunction. To assess the impact of radiation therapy on tail edema, mouse tails were radiated with a single dose of 0, 15, or 30 Gy and tail volume changes compared with baseline were evaluated with the truncated cone formula (Fig. 1A). Four weeks after radiation therapy with either 15 or 30 Gy, a modest although significant and dose-dependent increase in tail volumes was noted. Animals treated with a dose of 15 Gy had a >15% increase, while those treated with 30 Gy demonstrated an ~25% increase in baseline tail volume (*P* < 0.005 and 0.002, respectively, compared with 0 Gy). These acute increases in tail edema were nearly resolved by 12 wk after radiation therapy, demonstrating no significant differences among the various groups. In addition, no statistical changes in tail volumes were noted in animals followed for as long as 24 wk after irradiation. Thus, similar to the clinical scenario, radiation therapy alone does not appear to cause chronic swelling or lymphedema in our mouse tail model.

In an effort to evaluate the effect of radiation on dermal lymphatic function, we performed lymphoscintigraphy with 99mTc-labeled sulfur colloid and quantified the maximal uptake of this material in the lymph node basin at the base of the tail. This analysis demonstrated that radiation therapy caused significant and dose-dependent decreases in lymphatic function 4 wk after treatment compared with controls (*P* < 0.0001 and *P* < 0.003 for 30 Gy and 15 Gy, respectively, compared with controls; Fig. 1B). Interestingly, lymphatic dysfunction persisted at the later time points examined (12 and 24 wk), demonstrating severe lymphatic dysfunction despite resolution of measurable tail lymphedema (Fig. 1B). In addition, both radiation doses appeared to cause significant lymphatic dysfunction, with an apparent progression of this phenomenon in animals treated with 15 Gy such that lymph node uptake was equivalent in severity with the 30-Gy-treated animals (Fig. 1B). Decay-adjusted lymph node uptake of radiolabeled sulfur colloid was nearly fivefold lower in the treated animals compared with control (*P* < 0.001). Image analysis of lymphoscintigraphic data demonstrated gross impairment in nodal uptake of radiolabeled colloid in irradiated animals compared with non-irradiated control animals (Fig. 1C).

Radiation therapy decreases number of dermal capillary lymphatic vessels and lymphatic endothelial cells. To determine the impact of radiation therapy on the number of cutaneous lymphatic vessels present tail sections were stained with the lymphatic-specific marker podoplanin by immunohistochemistry and positively stained luminal structures were
The number of lymphatic vessels in nonirradiated skin did not vary significantly over time (not shown), and on average 8.4 ± 1.3 lymphatic vessels were found per HPF. In contrast, tails irradiated with 15 Gy had only 3.6 ± 1.2 lymphatic vessels/HPF 4 wk after irradiation (P < 0.001). There was no statistical difference in the lower and higher doses of radiation therapy used in our study (15 and 30 Gy, respectively), implying that a single 15-Gy dose is sufficient to cause lymphatic vessel loss. The decrease in the number of lymphatic vessels in irradiated animals was persistent even after 12 and 24 wk following irradiation, with the number of lymphatic vessels per HPF ranging from 3.4 ± 25 to 6 ± 1.7 (P < 0.004 for all).

As most of the dermal lymphatics are collapsed with no obvious lumen, lymphatic vessel counts may underestimate the degree of lymphatic injury and depletion resulting from radiation therapy. Therefore, in order to confirm our vessel counts we stained sections with LYVE-1, another lymphatic specific marker, and identified individually stained cells by colocalization with DAPI by confocal microscopy (Fig. 2, C and D). These findings corroborated our vessel counts, demonstrating that radiation therapy caused a significant and persistent decrease in the number of LECs present in the dermis and subcutaneous tissues. We noted a modest but dose-dependent decrease in LEC number 1 wk after radiation. This effect became more pronounced at the later time points, resulting in a nearly fourfold decrease in the number of LECs at the latest time point (24 wk; P < 0.01). In addition, similar to our findings with vessel counts other than the 1 wk time point, we found no statistically significant differences between the two doses of radiation administered, further implying that a dose of 15 Gy is sufficient to cause significant injury to dermal lymphatics.

Radiation is associated with LEC apoptosis and promotes cellular senescence in vitro. In light of the persistent loss of dermal LECs associated with radiation therapy and the known effects of radiation on microvascular endothelial cells, we hypothesized that radiation therapy causes LEC apoptotic cell death in vivo. To test this hypothesis we performed immunofluorescent colocalization of the LEC marker LYVE-1 with TUNEL detection of apoptosis, and the ratio of apoptotic LECs as a function of total number of LECs was calculated. Counts of TUNEL-positive LECs demonstrated a dose-dependent increase in LEC apoptosis that peaked at 10 h after irradiation.

Fig. 1. Radiation therapy causes severe, long-term lymphatic dysfunction. A: tail volume changes from baseline after irradiation with 0, 15, or 30 Gy. Although there was a significant, dose-dependent increase in tail volume 4 wk after radiation therapy (P < 0.008 and 0.002, respectively), these differences resolved in long-term follow-up (12, 24 wk). B: analysis of lymphatic function with lymphoscintigraphy 4, 12, and 24 wk after irradiation with 0, 15, or 30 Gy. Decay-adjusted maximal uptake of 99Tc-labeled sulfur colloid in the lymph nodes at the base of the tail after injection at the tip of the tail is presented. Note significant, dose-dependent impairment in lymph node uptake at the 4 wk time point (*P < 0.001 for both doses compared with nonirradiated control, #P < 0.05 for 15 Gy compared with 30 Gy at 4 wk after radiation therapy). Also note sustained and progressive lymphatic dysfunction in animals treated with 15 or 30 Gy and analyzed after 12 and 24 wk (*P < 0.001 for both doses). C: representative heat maps of 99Tc radioisotope uptake in a nonirradiated animal (top) and an animal 24 wk after exposure to 30 Gy (middle). Red color indicates areas of most intense radioactivity, and yellow, green, and blue represent progressively lower activities. Arrowheads indicate the site of radio-labeled colloid near the tip of the tail, and arrows indicate uptake in the lymph node basin at the base of the tail. Note far greater uptake in the lymph nodes of the nonirradiated mouse at the conclusion of the study, indicating more efficient lymphatic transport. A picture of a mouse tail is presented for orientation at bottom.
We identified very few apoptotic LECs in the nonirradiated animals (1.1%). In contrast, 10 h after irradiation, animals treated with 15 Gy demonstrated apoptosis in 8.0% of LECs ($P < 0.005$ compared with 0 Gy), while 30 Gy of radiation resulted in apoptosis in nearly 20% of LECs ($P < 0.001$; Fig. 3A and B).

To confirm our in vivo findings, we irradiated isolated human dermal LECs in vitro with 0, 15, or 30 Gy of gamma irradiation and identified apoptotic cells with flow cytometry and TUNEL assays 4 and 10 h later (Fig. 3C). LECs demonstrated a statistically significant increase in apoptosis compared with controls with a 30-Gy dose 10 h after irradiation (20.4% vs. 6.8%, $P < 0.001$), but not with a 15-Gy dose. This could be a function of a higher background rate of apoptosis in this in vitro study. It should be noted that the baseline rate of LEC apoptosis in vivo was 1.1%, while in vitro it was 6.8%. This
phenomena is well recognized in endothelial cells, as these often have a significant rate of background apoptosis in vitro even with meticulously maintained culture conditions (32). LEC apoptosis following exposure to radiation therapy was confirmed by immunofluorescence for annexin V and caspase-3 (Fig. 3D). This finding is interesting as it suggests that in vivo tissue injury following radiation may sensitize LECs to apoptosis. Indeed, we recognize that a limitation of in vitro irradiation studies is that these conditions fail to mimic changes in the specific niche of individual cells and may therefore underestimate the effect of irradiation on isolated cells. However, in vitro radiation experiments have been used in multiple previous studies with the dose range described as a means of validating and supporting in vivo observations (13, 51, 58, 63, 70).

Irradiation has been previously shown to cause cellular senescence by upregulating cell cycle regulators such as p21 and p16 (63, 74). Senescent cells are metabolically active but demonstrate significantly reduced potential for proliferation and differentiation (10). To determine the effects of sublethal doses of irradiation on cellular senescence in isolated dermal LECs, cells were treated with 0, 4, 8, or 12 Gy. LECs were then grown for an additional 4 days and evaluated for senescence by β-galactosidase staining at pH 6 (Fig. 3). Interestingly, this analysis demonstrated that radiation therapy, even at low doses, causes a statistically significant and dose-dependent increase in LEC senescence compared with controls at all doses tested. This finding implies that radiation therapy may impair lymphatic function by causing premature senescence of these cells.

Protection of LECs from radiation-induced apoptosis does not prevent long-term lymphatic dysfunction. To study the relative impact of fibrosis and LEC apoptosis on the pathogenesis of radiation-associated lymphatic dysfunction we irradiated the tails of ASM-deficient (ASM−/−) mice and WT littermates. Evaluation of LEC apoptosis 10 h after treatment with 15 Gy demonstrated that ASM−/− mice had significantly less apoptosis compared with WT control mice (4.5% vs. 8.1%, P < 0.03; Fig. 4, A and B). Interestingly, however, this protection from apoptosis did not translate to significantly improved lymphatic function when assessed by lymphoscintigraphy 4 wk after radiation (Fig. 4C). In addition, ASM deficiency did not significantly decrease radiation-induced fibrosis as assessed by Sirius red staining (Fig. 4, D and E).

Radiation causes soft tissue fibrosis and increased TGF-β1 activity. Radiation is known to cause soft tissue fibrosis (4, 68). In fact, clinically, many of the long-term wound healing and surgical complications associated with radiation are directly related to soft tissue fibrosis. Previous studies have shown that postradiation skin fibrosis is in large measure caused by activation of TGF-β1 and its downstream mediator Smad3 (17). Furthermore, we (5, 8) and others (41) have previously demonstrated that TGF-β1 is an antilymphangiogenic cytokine by inhibiting LEC proliferation and function and that fibrosis is a significant inhibitor of lymphatic function during wound healing. Therefore, to determine the effects of fibrosis in general, and TGF-β1 function in particular, we evaluated fibrosis mouse tails treated with 0, 15, or 30 Gy of irradiation.

As expected, radiation therapy resulted in loss of hair follicles, epidermal thinning, dermal thickening, loss of fat, fibrosis, and increased inflammation at all times evaluated after radiation (not shown). Using Sirius red staining and birefringence microscopy to calculate the scar index, we found that radiation caused progressive fibrosis with a scar index that is significantly higher than control starting at 4 wk after irradiation (P < 0.03 for all doses at time points compared with control) (Fig. 5, A and B). In addition, immunohistochemical localization of pSmad3, an important downstream mediator of TGF-β1 signaling, demonstrated a significant increase in the number of pSmad3-positive cells as early as 4 wk after radiation therapy. Similar to previous reports, this increase in TGF-β1 activity persisted even 24 wk after radiation therapy in animals treated with 30 Gy (P < 0.03) (Fig. 5, C and D).

Treatment with LY-364947, a small-molecule inhibitor of TGF-BRI, effectively blocks TGF-β1 signaling. Roberts and colleagues (69) have previously shown that treatment of mice with small-molecule inhibitors of TGF-β1 for 3–5 wk can decrease the fibrosis associated with radiation therapy. However, the effects of short-term TGF-β1 blockade on tissue fibrosis and lymphatic function remain unknown. This is important since long-term inhibition of TGF-β1 function can cause significant morbidity due to the protean effects of this growth factor. Therefore, we inhibited TGF-β1 function with a small-molecule inhibitor of TGF-BRI (LY-364947) for just 1 wk after radiation and evaluated the effects of this intervention on fibrosis, lymphatic function, and lymphatic vessel depletion. Western blot analysis of tissues harvested 1 wk after radiation (15 Gy) from animals treated with or without LY-364947...
Fig. 4. Protection of LECs from radiation-induced apoptosis does not prevent induction of lymphatic dysfunction. A: identification of apoptotic LECs by colocalization of LYVE-1 (red) and TUNEL (green) in acid sphingomyelinase (ASM)/−/− and wild-type (WT) mice (means ± SD). Note significant decrease in LEC apoptosis 10 h after radiation in ASM−/− mice (*P < 0.034) B: representative immunofluorescent micrographs (×200) of colocalization of the LEC marker (red) with TUNEL (green) and DAPI nuclear stain (blue) 10 h after irradiation with 15 Gy. C: lymphoscintigraphy of ASM−/− and WT mice 4 wk after 15 Gy of irradiation. Data represent mean maximal decay-adjusted uptake of a minimum of 3 animals per time point/group (not significant). D: scar index of tissue sections from ASM−/− and WT animals that received 15 Gy of irradiation 4 wk after radiation (not significant). E: representative ×200 micrographs of tissue sections from ASM−/− (top) or WT (bottom) mice 4 wk after radiation with 15 Gy. No differences are observed.
demonstrated a nearly fivefold reduction in the expression of TGFβi in the LY-364947-treated animals (Fig. 6, A and B). TGFβi protein expression is directly related to TGF-β1 activity; therefore, reductions in TGFβi in LY-364947-treated animals confirm reduction in TGF-β1 signaling (24). This conclusion is further supported by immunohistochemical localization of pSmad3, the downstream mediator of the TGF-β receptor, demonstrating a significant reduction in the number of pSmad3-positive cells/HPF in LY-364947-treated animals both 4 and 12 wk after radiation (Fig. 6, C and D; \( P < 0.03 \)). This finding is consistent with previous reports demonstrating that TGF-β1 expression is regulated, at least in part, by positive feedback mechanisms (50).

Short-term inhibition of TGF-β1 signaling reduces radiation-induced soft tissue fibrosis and lymphatic dysfunction. Interestingly, even very short-term blockade of TGF-β1 func-
tion with LY-364947 after irradiation with 15 Gy significantly decreased soft tissue fibrosis compared with control animals. The scar index of irradiated/LY-364947-treated animals was more than twofold less than vehicle treated/irradiated animals even as long as 12 wk after radiation (Fig. 7, A and B; $P < 0.01$). Short-term blockade of TGF-$\beta_1$ function was associated with decreased tail volume 4 and 12 wk after irradiation with 15 Gy (mean $\pm$ SD). Note significant and persistent decrease in the number of pSmad3-positive cells in animals treated with LY-364947 for 1 wk ($P < 0.03$). D: representative $\times$200 micrographs of tissue sections from animals treated with vehicle (top) or LY-364947 (bottom) for 1 wk after irradiation with 15 Gy. Tissue sections were harvested 12 wk after radiation therapy. Note marked decrease in the number of pSmad3-stained cells in the LY-364947-treated animals.

Fig. 6. Treatment with LY-364947, a small-molecule inhibitor of TGF-$\beta_1$ receptor (TGF-BR)I, effectively blocks TGF-$\beta_1$ signaling. A and B: Western blot (A) and quantification (B) for TGF-$\beta_1$-induced (TGFBi) in vehicle- and LY-364947-treated animals 1 wk after irradiation with 15 Gy. Each lane represents protein isolated from an individual animal. Note marked reduction (nearly 5-fold decrease) in TGFBi protein expression in LY-364947-treated animals, indicating inhibition of TGF-$\beta_1$ signaling ($^*P < 0.02$). C: calculation of the number of pSmad3-stained cells in 3–5 sections/animal in a minimum of 3 animals per group 4 and 12 wk after irradiation with 15 Gy (mean $\pm$ SD). Note significant and persistent decrease in the number of pSmad3-positive cells in animals treated with LY-364947 for 1 wk ($^*P < 0.03$). D: representative $\times$200 micrographs of tissue sections from animals treated with vehicle (top) or LY-364947 (bottom) for 1 wk after irradiation with 15 Gy. Tissue sections were harvested 12 wk after radiation therapy. Note marked decrease in the number of pSmad3-stained cells in the LY-364947-treated animals.

Improved lymphatic function in LY-364947-treated animals was not due to a protective effect on LECs since quantification of dermal LECs did not show significant differences in the LY-364947- or vehicle-treated animals (Fig. 7F). In addition, TGF-$\beta_1$ blockade did not significantly alter LEC apoptosis when evaluated 10 h after treatment with 15 Gy (Fig. 7, G and H). Similarly, treatment of isolated LECs with recombinant TGF-$\beta_1$ (10 ng/ml) did not result in increased rates of apoptosis compared with controls.
Fig. 7. Short-term inhibition of TGF-β1 signaling reduces radiation-induced soft tissue fibrosis and lymphatic dysfunction. A: scar index of tissue sections from animals that received 15 Gy of irradiation and were treated with vehicle or LY-364947 for 1 wk after irradiation (mean ± SD scar index). Tissues were harvested 4 or 12 wk after radiation. Note marked decrease in scar index in LY-364947-treated animals compared with control animals at both time points evaluated (*P < 0.002, #P < 0.02). B: representative ×200 micrographs of tissue sections from animals treated with vehicle (top) or LY-364947 (bottom) 12 wk after radiation with 15 Gy. Note decreased scarring as represented by decreased red-orange and increased yellow-green birefringence in the LY-364947-treated section. C: tail volume measurements in animals treated with vehicle or LY-364947 4 or 12 wk after irradiation with 15 Gy (mean ± SD). Treatment with LY-364947 resulted in mild but nonsignificant reduction in tail edema after irradiation. D: lymphoscintigraphy of animals treated with vehicle or LY-364947 4 or 12 wk after 15 Gy of irradiation. Data represent mean maximal decay-adjusted uptake of a minimum of 3 animals per time point/group. Note statistically significant increased lymph node uptake in LY-364947-treated animals, particularly at the 4 wk time point (*P < 0.0001). Statistically significant improved lymphatic function was also noted at the 12 wk time point; however, this difference was less marked than the 4 wk analysis (#P < 0.03). E: representative heat maps of 99Tc radioisotope uptake in mice irradiated with 15 Gy and treated with either vehicle (left) or LY-364947 (right) at 4 and 12 wk after irradiation. Red color indicates areas of most intense radioactivity, and yellow, green, and blue represent progressively lower activities. Arrowheads indicate the site of radiolabeled colloid near the tip of the tail, and arrows indicate uptake in the lymph node basin at the base of the tail. Note far greater uptake in the lymph nodes of animals treated with LY-364947, indicating more efficient lymphatic transport.
Short-term inhibition of TGF-β1 signaling may reduce radiation-induced LEC EMT and lymphatic fibrosis. TGF-β1 is a known inducer of endothelial-mesenchymal cell transdifferentiation (EMT) resulting in vascular fibrosis (45–47). In addition, we have more recently shown (8) that LECs in animals treated with TGF-β1 or in an environment containing high endogenous expression of TGF-β1 coexpress lymphatic and smooth muscle cell markers (LYVE-1 and α-SMA, respectively) in dermal lymphatic capillaries. This is an abnormal phenotype, as dermal lymphatic capillaries are not associated with pericytes. In addition, lymphatic vessel fibrosis is a known clinical hallmark of lymphedema resulting in lymphatic vessel obliteration and dysfunction (56). Therefore, to determine whether radiation therapy caused expression of fibroblast markers by lymphatic capillaries, we colocalized podoplanin and α-SMA in nonirradiated animals and in animals irradiated with 15 Gy and treated with or without LY-364947 (Fig. 8A). As expected we found no LECs that expressed α-SMA in nonirradiated animals. Similarly, we found no lymphatic vessels that expressed α-SMA in irradiated animals treated with LY-364947. In contrast, we noted that in animals treated with vehicle and 15 Gy of irradiation, lymphatic capillaries that coexpressed α-SMA could be seen in some sections, albeit these represented a minority of lymphatic capillaries present.

To more directly study the effects of TGF-β1 on LEC dedifferentiation and EMT, we treated isolated LECs with recombinant TGF-β1 (10 ng/ml) and analyzed the expression of a panel of fibroblast and LEC markers at various time points.
Interestingly, stimulation of LECs with recombinant TGF-β1 markedly increased the expression of putative EMT markers collagen I, N-cadherin, and FAP (73). Conversely, expression of the LEC marker LYVE-1 and the endothelial marker E-cadherin was markedly reduced by rhTGF-β1 treatment, providing further evidence for dedifferentiation and cellular transformation.

**DISCUSSION**

Although radiation therapy following lymphadenectomy is a significant risk factor for the development of chronic lymphedema, the mechanisms that contribute to this risk remain unknown (23, 49, 54). This gap in our knowledge is an important barrier to the development of targeted therapeutic or preven-
tative strategies for lymphedema. In the present study we demonstrate that radiation therapy causes lymphatic dysfunction due to depletion of LECs and soft tissue fibrosis. Similar to the clinical scenario in which lymphedema is rare following radiation therapy without surgery, we found that radiation in our model resulted in only subtle increases in tail volume that resolved over time. Interestingly, however, we found that lymphatic function was markedly decreased after radiation therapy, implying that radiation decreases the baseline clearance of lymphatic fluid without overwhelming the transport capacity of existing vessels. This finding is supported by the findings of Mortimer and colleagues (40) demonstrating impaired dermal lymphatic function as measured by lymphoscintigraphy in a porcine model after 18 Gy of radiation. Our study expands the findings of Mortimer et al. by demonstrating that lymphatic transport capacity is decreased for prolonged periods of time (even 6 mo after radiation). These findings support the hypothesis that the long-term reduction in lymphatic function resulting from radiation therapy is not usually sufficient to overwhelm the transport capacity of extremity but when combined with additional injury from surgery goes on to cause chronic lymphedema.

We hypothesized that one mechanism by which radiation therapy may decrease lymphatic function is by directly injuring or depleting LECs. This hypothesis is supported by the fact that microvascular endothelial cells are highly sensitive to radiation injury and undergo apoptosis (43). In fact, depletion of these cells is thought to be the underlying pathogenic mechanism for gastrointestinal toxicity resulting from radiation therapy (43). In the present study we show that dermal lymphatic vessels are also susceptible to apoptosis after radiation injury and that this response is, at least in part, due to LEC apoptosis in a process that is mediated, at least in part, by ASM. Interestingly, however, we found that prevention of LEC apoptosis did not translate to improved lymphatic function as assessed by lymphoscintigraphy, suggesting that other processes such as tissue fibrosis play an overall larger role in the regulation of lymphatic function after radiation injury.

This proapoptotic effect of radiation was maximal 10 h after radiation but was apparent even at the earliest time point we selected (4 h). Our findings are also supported by the work of Abtahian et al. (1), who demonstrated intestinal LEC apoptosis 4 h after 12 Gy of total body irradiation. In contrast, however, Sung et al. (57) have previously shown that intestinal and peritumoral lymphatics are resistant to radiation-induced apoptosis. This difference from our findings may be related to a number of factors. Most importantly, Sung and colleagues used a dose of 15 Gy and evaluated intestinal LECs. In the present study, while we found a modest increase in LEC apoptosis after 10 h with 15 Gy (~7% of LECs), 30 Gy caused a more significant increase in the number of apoptotic cells. The relative doses of radiation to the skin are also likely to be higher since they are superficial compared with the intestinal villi exposed to total body irradiation. It is also possible that dermal lymphatics are phenotypically different than intestinal lymphatics and therefore more susceptible to apoptosis after ionizing radiation injury. This idea is supported by the fact that intestinal lymphatics, for example, are important in lipid transport, whereas dermal lymphatics are not. Furthermore, cellular responses to radiation injury are known to be tissue dependent and influenced by the surrounding cellular niche. For example, previous studies have shown that growth factors such as heparanase that are found in the extracellular matrix may provide a radioprotective effect for local endothelial cells (19). Thus dermal LECs may be exposed to a microenvironment that is more inductive of apoptosis than that of intestinal LECs.

Interestingly, we demonstrated that LECs in culture are relatively radioresistant and undergo apoptosis only when exposed to high doses such as 30 Gy. The observed differences in in vivo and in vitro radiation susceptibility may be due to differences in the microenvironment of the cell in vivo. This hypothesis is supported by the fact that impairment of lymphatic vessel function in response to ultraviolet B radiation in vivo is mediated by the release of VEGF-A, implying that radiation therapy not only affects individual cells but also changes the cellular microenvironment (28). Furthermore, in vivo radiation may cause generation of free oxygen radicals or other toxic substances that may increase the susceptibility to injury. Kajiya and colleagues (29) have also demonstrated that ultraviolet B radiation therapy markedly downregulates the expression of VEGF-C, leading to lymphatic dysfunction. Similar changes may contribute to decreased LEC and lymphatic vessel numbers in our study since activation of VEGF receptor (VEGFR)-3 is required for LEC survival and proliferation (18).

Our in vitro studies also demonstrated that human dermal LECs are sensitive to radiation therapy in terms of cellular senescence. We found that even relatively low doses of radiation caused an increase in the number of senescent cells. This is an interesting finding as it suggests that chronic reduction in LEC and lymphatic vessel numbers may be related to premature senescence of LECs. Similar findings have been demonstrated in vitro in a variety of cell types including fibroblasts, hematopoietic stem cells, and mesenchymal stem cells (7, 14, 26, 64).

An important finding in our study that supports the concept that LECs are susceptible to radiation injury is the fact that radiation therapy caused a significant depletion of LECs and lymphatic vessels in tissues even up to 6 mo after exposure. This is important as it demonstrates a direct mechanism by which radiation therapy decreases the transport capacity of the lymphatic system. Jackowski and colleagues (27) have evaluated the effects of radiation therapy on lymphatic vessels, using biopsies obtained at various times after treatment obtained from women treated for breast cancer. Similar to our study, the authors demonstrated that the number of small lymphatic vessels (<10 μm) was decreased in the early periods after radiation (0–5 mo), although this difference was not statistically significant. However, in the patients evaluated between 11 and 14 mo the authors found a significant increase in the number of small lymphatic vessels in the skin that they termed radiogenic lymphangiogenesis. These differences were no longer apparent 17+ mo after radiation therapy. These findings imply that the timing of evaluation after radiation therapy is important and may be a reason for the differences observed in our study. Furthermore, the differences in the number of lymphatic vessels after radiation between our study and that of Jackowski et al. may be related to the radiation dose or fractionation protocols. Clinically patients were treated with a total of 40 Gy of radiation delivered in a fractionated manner, whereas animals treated in our study were treated with a single dose. Thus fractionation may have a protective effect for
lymphatic vessels. This may be reflected in the finding that the subset of patients that developed lymphedema failed to mount a lymphangiogenic effect in response to radiation, implying that these patients had a more severe injury. Therefore, similar to our findings, patients with a reduction in the number of cutaneous lymphatic vessels appeared to have decreased lymphatic function and subsequent lymphedema.

The differences in the observations of Jackowski et al. from our study may also stem from significant variability in the number of lymphatic vessels between patients. In fact, these differences forced the authors to use a treated-to-control ratio for their analysis that may have altered the findings in some way. This idea is further supported by studies demonstrating that genetic polymorphisms play a role in an individual’s tissue response to radiation (6, 31). Interestingly, polymorphisms in the gene coding for TGF-β1 have been directly implicated as modulators of tissue response to radiation (72). The use of genetically inbred animals for the study of lymphatic function obviates these differences and may simplify analysis. The inherent variability in baseline lymphatic vessel numbers observed in clinical studies is interesting as it suggests that genetic or other differences may influence the baseline lymphatic transport capacity and therefore contribute to the risk of developing lymphedema. This concept is supported by the fact that lymphatic imaging studies have demonstrated that the rate of lymphatic transport in patients is highly variable both after surgery and at baseline, and that patients with low baseline and postsurgical clearance rates have a higher risk of developing lymphedema (55).

Another potential mechanism by which radiation therapy can decrease lymphatic function is soft tissue fibrosis. Fibrosed soft tissues lose compliance and are characterized by loss of normal structures, which are replaced by scar tissue. In fact, the clinical hallmarks of lymphedema are soft tissue and lymphatic vessel fibrosis (56). Anatomic and cadaver studies have shown that lymphatic vessels undergo fibrosis after lymphadenectomy, losing compliance and in many instances becoming completely stenosed and nonfunctional (56). We have previously shown (5, 8) that lymphatic fluid stasis is associated with soft tissue fibrosis and lymphatic dysfunction and that this effect is due, at least in part, to increased expression of TGF-β1. In addition, we (8) and others (41) have shown that TGF-β1 has antilymphangiogenic effects by inhibiting LEC proliferation and tubule formation. These findings are important since radiation therapy is known to cause tissue fibrosis as a result of chronically increased TGF-β1 expression and may therefore be additive to the effects of lymphatic stasis in promoting soft tissue fibrosis and lymphatic dysfunction. This hypothesis is supported by our findings in ASM−/− mice, in which LEC apoptosis was significantly decreased but lymphatic dysfunction and tissue fibrosis persisted at later time points.

Several studies have shown that inhibition of TGF-β1 function either in knockout animals or with small-molecule inhibitors of TGF-β1 can decrease skin fibrosis resulting from radiation (17, 36, 69). These findings have been difficult to translate clinically, however, since long-term inhibition of TGF-β1 function was required. In fact, the shortest effective time for treatment reported was 3–5 wk with the small-molecule inhibitor halofuginone (69). Prolonged TGF-β1 inhibition can have significant negative side effects due to immunosuppression and the protean effects of TGF-β1 in a variety of organ systems (33, 68). To circumvent these potential difficulties, we evaluated the effects of short-term (1 wk) TGF-β1 inhibition on radiation fibrosis and lymphatic function. We hypothesized that if the autoregulatory upregulation of TGF-β1 is avoided then long-term sequelae of radiation can be minimized. Indeed, we found that short-term blockade of TGF-β1 with LY-364947 resulted in marked reduction of the progressive fibrosis that is associated with radiation injury. This finding is consistent with the study by Wormstone and colleagues (66), who showed that even a 2-day exposure to TGF-β results in long-term signaling changes in matrix contraction, transdifferentiation, and fibrosis. In our study, TGF-β1 activity as measured by Smad3 phosphorylation was attenuated far beyond the duration of therapy, suggesting that we had at least partially succeeded in breaking the positive feedback loop of TGF-β1 activity that is seen with progressive fibrosis (68). These findings directly correlated with better lymphatic function in the LY-364947-treated animals, consistent with the hypothesis that fibrosis is a mediator of lymphatic dysfunction. Interestingly, treatment with LY-364947 did not appear to confer any direct radioprotection to LECs in the present study, as evidenced by quantification of LEC depletion. While TGF-β1 has been shown to be inhibit LEC tubule formation and proliferation, there are no data implicating this growth factor in LEC death. Thus the present study indicates that radiation-induced LEC apoptosis is likely independent of TGF-β1.

Previous studies have shown that TGF-β1 can cause EMT and microvascular vessel fibrosis (45, 46, 68). In addition, we have previously shown (8) that increased TGF-β1 expression is associated with coexpression of lymphatic and fibroblast markers in capillary lymphatics. In the present study we found that radiation therapy also induced the fibroblast marker α-SMA by capillary LECs and that this effect could be abrogated by short-term blockade of TGF-β1 function. These findings, together with our in vitro experiments demonstrating that TGF-β1 increases expression of EMT-associated markers and simultaneous downregulation of lymphatic and endothelial markers, support the hypothesis that radiation therapy may directly contribute to lymphatic dysfunction by promoting lymphatic vessel fibrosis as a result of increased TGF-β1 expression.

Radioprotective strategies are employed in a variety of clinical scenarios to decrease the morbidity of radiation (11, 20–21). For instance, these approaches have been used to decrease the early morbidity of head and neck cancers such as intraoral blistering and swelling. In addition, radioprotection has been effectively used to decrease the incidence of late radiation-induced sequelae such as osteoradionecrosis and growth disturbances in children (12). These strategies are not commonly used for breast cancer treatment, however, since radiation in this setting is thought to be relatively well tolerated, causing little more than skin blistering and swelling. Our findings, however, suggest that radioprotective approaches may have a role in the prevention of lymphedema by preventing lymphatic dysfunction. This is critical since lymphedema remains the most dreaded complication of breast cancer treatment and is a major source of morbidity for more than 500,000 breast cancer survivors (22, 38). Future studies from our laboratory will explore these approaches in an effort to identify...
optimal methods of limiting radiation-induced injury to lymphatic channels.

ACKNOWLEDGMENTS

The authors thank Dr. Zvi Fuks for his invaluable advice and guidance in the planning and analysis of the presented studies.

GRANTS

This work was funded in part by grants from the Plastic Surgery Educational Foundation to B. J. Mehrara and T. Avraham and the Breast Cancer Alliance.

Technical services provided by the Memorial Sloan-Kettering Cancer Center Small-Animal Imaging Core Facility, supported in part by National Institutes of Health (NIH) Small-Animal Imaging Research Program (SAIRP) Grant R24-CA-83084 and NIH Center Grant P30-CA-08748 are gratefully acknowledged.

DISCLOSURES

None of the authors has any commercial associations or financial relationships that would create a conflict of interest with the work presented in this article.

REFERENCES


