Fibrosis Worsens Chronic Lymphedema in Rodent Tissues

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Running Title: Fibrosis Worsens Acute and Chronic Lymphedema
Secondary lymphedema in humans is a common consequence of lymph node dissection (LND) to treat breast cancer. A peculiar characteristic of the disease is that life-long swelling often precipitously appears several years following the surgical treatment, often due to an inflammatory stimulus. Although the incidence of secondary lymphedema dramatically increases following radiation therapy, the relationship between fibrotic scarring and the eventual appearance of lymphedema remains unclear. In order to clarify the role of fibrosis in secondary lymphedema initiation, we chemically increased fibrosis in rodent tissues with bleomycin and assessed the ability of the local lymphatic system to prevent lymphedema, either acutely or in a chronic state induced by inflammation. We found that bleomycin injections exacerbated fibrotic matrix deposition in an acute mouse tail lymphedema model (p<0.005), reduced wound closure (p<0.005), and impaired the ability of the tail lymphatics to regenerate (p<0.005) and reduce the swelling (p<0.05). When fibrosis was worsened with bleomycin following axillary LND in the rat foreleg, the ability of the foreleg lymphatic system to reduce the chronic state swelling induced by stimulated inflammation was severely impaired (p<0.005). Indocyanine Green (ICG) lymphography in the ALND-recovered rat forelegs revealed a worsened lymphatic drainage due to the inflammation and bleomycin pre-treatment. Although inflammation reduced the drainage of dextran fluid tracer from the control forelegs (p<0.05), the reduction in fluid drainage was more severe post-ALND when fibrosis was first increased (p<0.005). The findings demonstrate that fibrosis reduces the lymphatic capacity to functionally regenerate and prevent the chronic appearance of lymphedema.

Key words: lymphangiogenesis; lymphatic drainage; axillary lymph node dissection; chronic secondary lymphedema; inflammation;
Introduction

Secondary lymphedema in humans is a common consequence of axillary lymph node dissection (ALND) to treat breast cancer, which disconnects lymphatic vessels and excises extracellular matrix from the axilla (7). The intervening surgical space forms into scar tissue, which has been demonstrated to hinder lymphatic regeneration and interstitial flow (1, 23, 27, 31, 32). We have hypothesized that an inability of the lymphatic system to adequately regenerate during normal wound repair due to fibrotic scarring may predispose the tissue to develop secondary lymphedema (23, 31). A number of studies have attempted to clarify the regulation of interstitial flow and lymphatic regeneration across the surgical obstruction and within edematous tissues by molecules and matrix proteins expressed during wound repair. This has been the case in particular for proteins that regulate the extent of fibrotic scarring (1, 2, 8, 11, 23, 31, 34, 35). Importantly, it has been shown that a simple collagen gel implanted at the site of surgery, which reduces wound site fibrosis, can dramatically increase interstitial flow and lymphatic regeneration across the obstruction without the need for exogenous lymphatic growth factors (1, 6, 11, 23, 31).

An important characteristic of secondary lymphedema is that ~25% of patients experience the chronic life-long swelling after an often years-long delay during which the initial lymphatic related injury has healed, whereas the other ~75% never experience the swelling despite undergoing the same treatment (5, 7, 15, 19, 20). Notably, the incidence of secondary lymphedema dramatically worsens when radiation therapy is involved (12, 29), potentially due to the worsened fibrotic scarring incurred at the site of surgery (3, 9). Thus, the clinical features of the disease and accumulating experimental evidence in acute animal models suggest a
relationship between fibrotic scarring at early times and the years-later appearance of lymphedema. However, the role of fibrosis as an underlying cause of chronic lymphedema as well as the mechanism by which fibrosis mediates its inhibitory effects on lymphatic adaptation remains unclear.

We recently demonstrated that the lymphatic system in the rodent foreleg remains chronically and latently impaired following ALND, even though there is no evidence of edema or lymphatic deficiency following the acute recovery (17, 18). The latent deficiency is made manifest and measurable in the form of an increased tissue swelling and decreased lymphatic drainage by an exogenous inflammatory stimulation (18). The etiology of swelling onset in the experimental model is similar to the induction of chronic swelling often seen in humans (5). Here, we hypothesized that fibrotic scarring may regulate the chronic and latent lymphatic impairment that follows ALND in the rat foreleg. To test this hypothesis, we compared the ability of the post-ALND regenerated foreleg lymphatic system to drain fluid and prevent edema during an acute inflammatory challenge in the presence or absence of a worsened wound site fibrosis. Because the complexity of the rat foreleg model can hinder mechanistic clarifications, a mouse tail model of lymphedema was used to gain insight into the mechanism by which fibrosis-stimulation may impair lymphatic regeneration. Such an investigation is necessary to reveal whether fibrosis may regulate the chronic and latent post-ALND lymphatic function deficiency and to clarify the mechanisms of secondary lymphedema initiation in humans.
Methods

Rat Foreleg Lymphedema Model

Female Sprague Dawley rats at 250-300 grams were used for all foreleg experiments. Rats were anesthetized with 2.5% isoflurane mixed with oxygen gas for surgical procedures and were euthanized at experimental endpoints under 5% isoflurane and oxygen gas by diaphragm puncture followed by cardiac excision. Axillary lymph nodes were removed from female rats, similar to what has been described previously for mice (17, 28) and rats (18). Briefly, a 10 mm long surgical incision through the dermis was placed across the axilla on the right side and the axillary lymph nodes were identified and excised along with visible portions of pre and post-nodal collecting vessels under an Olympus SZX7 stereo microscope. Two groups of twenty rats were each divided into four groups, those receiving surgery or not (ALND + or -), both with and without bleomycin injections (Bleomycin + or -). One group was used to confirm that the bleomycin injections promoted wound site fibrosis. The second group was used to assess the effects of acute inflammation on lymphatic foreleg drainage in the bleomycin treated rat axillas, with or without ALND. All protocols for the rats were approved by the Animal Care and Use Committee of Michigan Technological University.

Mouse Tail Lymphedema Model

Tail skin edema was created in 6-8 week old balb/c mice by excising a 1-mm circular band of dermis (which contained the lymphatic capillary network) 2 cm from the base of the tail, leaving the underlying bone, muscle, tendons, and major blood vessels intact, similar to previous studies (23). Two groups of forty mice were each divided into four groups, those receiving surgery or not (Surgery + or -), with or without injections of bleomycin (Bleomycin + or -). One group was used to investigate the effects of bleomycin treatment on fibrosis and lymphatic
regeneration. The second group was used to assess the effects of the worsened fibrosis on the evolution of the tail edema. All protocols for the mice were approved by the Animal Care and Use Committee of Michigan Technological University.

**Bleomycin Treatment**

Bleomycin has recently been used to induce fibrosis in rodent models of lymphedema (4). We injected bleomycin (Sigma-Aldrich) directly into rat forelegs and mouse tails to induce fibrosis. Repeated injections of 0.45U of bleomycin into the rat axillas was found in our preliminary study to induce fibrosis, yet created overt discomfort for the rat, used here as a general gauge of tissue injury. Because a major concern when using bleomycin is the induction of cell death, in addition to fibrosis (14), we alternated the bleomycin dose between a high and a medium level. This dose induced fibrosis without overt animal discomfort. For the present study, right foreleg rat axillas were injected with .45U of bleomycin in 45 μl of phosphate-buffered saline (PBS) at day 0. Injections then alternated between 0.3U in 30 μl of PBS and .45U in 45 μl of PBS every 3 days until day 12, for 5 total injections. For mouse tails, injections were made immediately distal to the wound site every 5 days, from day 5 to day 25, for 5 total injections. Each tail injection consisted of 0.025U of bleomycin in 2.5 μl PBS. Control animals received the same volume of PBS at the same times and anatomical locations.

**Oxazolone Exposure**

Oxazolone (Sigma, catalog number E0753) is a potent pro-inflammatory agent. The rats were allowed to fully recover from axillary lymph node dissection (ALND). At 45 days post-ALND surgery, at which time there was no edema (data not shown), oxazolone was applied as recently described (18). Briefly, the rats in all groups were sensitized by applying 300 μl of 2% oxazolone in ethanol to the abdomen. To initiate dermatitis, 300 μl of a 1.6% oxazolone solution
of acetone and olive oil (4:1) was applied to the right forelegs of all rats starting at day 7 post-sensitization (day 52 post-ALND surgery) and proceeding every three days thereafter until day 61 post-surgery, for a total of 4 applications. Optical images of the right and left rat forelegs were collected before each application of oxazolone. Finally, rats were imaged via ICG lymphography, injected with lymphatic fluid tracer, and euthanized for cryosectioning of the foreleg on day 64 post-ALND.

Quantifying Interstitial Transport and Lymphatic Function

Tetramethylrhodamine-conjugated lysine fixable dextran 70,000 MW (Invitrogen, Carlsbad, CA, catalog number D1818) at 1mg/mL in PBS was used as a fluorescent lymph tracer to quantify fluid drainage in the rat foreleg, as described recently (18). At the specified day post surgery, 15 μL of fluorescent tracer solution was injected intradermally into the posterior of both foreleg paws. We allowed all rats to recover for 6 hours following dextran injections to quantify lymph drainage post-surgery. Because the presence and distribution of the tracer across the foreleg depends upon lymphatic drainage, the coverage of fluorescent tracer that is measured later in foreleg cross sections can serve to quantify interstitial transport and lymphatic function. The extent of fluid marker coverage in cross sections made at the wrist is a direct measurement of the ability of fluid marker to spread interstitially from the paw injection site towards the lymphatics proximal to the wrist. Collected forelegs were cryo-sectioned at the wrist to produce 100 μm cross-sections. Sections were counterstained for cell nuclei with 4’, 6-diamino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, catalog number H-1200) and imaged under an Olympus BX51 Fluorescent Microscope. Fluorescent tracer area of coverage was quantified using Metamorph Offline 6.3r7 software and expressed as a percentage of total cross-sectional area of the foreleg tissue section.
Physiological measurements

Digital images were collected of mouse tails and rat foreleg every 5 days, in addition to other times specifically indicated, with a DP71 camera mounted to a stereomicroscope (for imaging mouse tails) and a Nikon D5000 camera mounted to a tripod (for imaging rat forelegs). All swelling measurements for the mouse tail and rat foreleg were made from these digital images by using Metamorph as well as Image J imaging software (Molecular Devices, PA). Mouse tail diameter was measured at the point of greatest diameter distal to the wound site. Mouse tail area was measured by outlining the tail area beginning immediately distal to the wound site and proceeding down the tail for 10 mm. Wound closure was measured as the average distance between healthy tissue on both sides of the wound. Foreleg wrist thickness was measured from the digital images of the rat foreleg. Foreleg area was measured by outlining the paw, wrist, and leg 2.5 cm proximally from the wrist. Foreleg measurements were normalized to their respective pre-oxazolone, day 52, condition.

Imaging of functional lymphatic vessels via ICG fluorescence lymphography

We employed indocyanine green (ICG) fluorescence lymphography to identify functional lymphatic vessels following ALND, bleomycin treatment, and oxazolone exposure (n=5 per group). An imaging system recently developed by Drs. N. Unno, F. Ogata, and Eva M. Sevick-Muraca (21, 22, 24, 25, 30) was used to detect functional lymphatic vessels in the rat foreleg. 7.5 μl of a 5 mg/ml solution of the fluorescent near-infrared dye Indocyanine Green (ICG; Akorn, catalog number 17478-701-02) was injected into the rat paw. Detection of ICG was performed with an electron multiplying charge-coupled device (CCD) (Hamamatsu, C9100-13) using Hamamatsu recommendations as described previously (30). ICG was illuminated with an array of 760 nm LEDs (Epitex Inc) placed before a 760 nm band pass filter (model 760FS10-50,
Andover Corporation) to provide the excitation light for activating ICG. A 785 nm and 763 nm custom made holographic notch band rejection filter (model HNPF-785.0-2.0 and HNPF-763.0-2.0, Kaiser Optical Systems) and a 830 nm image quality bandpass filter (model 830FS20-25, Andover Corporation) were placed before the camera lens to selectively reject the excitation light and pass the emitted 830 nm wavelength. Following microdose paw injections of ICG dye, images were collected at 10 minutes to visualize the path of ICG drainage through the rat foreleg.

Detection and Measurement of Fibrosis and Lymphatic Regeneration

A Mason’s Trichrome stain was used on cross sections of mouse tails and rat axillas to detect fibrotic changes in these tissues, by following the manufacturers directions (Sigma Aldrich, catalog number HT15-1KT). The chemical treatment stains cell cytoplasm and muscle fibers red and connective tissue blue/purple, thereby allowing fibrotic material to be distinguished. Fibrosis was quantified in the mouse tail skin by measuring the thickness of the blue/purple matrix dense region in the stained cross sections. Antibodies against LYVE-1 (abcam: ab14917) were used to fluorescently label lymphatics in the mouse tail skin cross sections. For comparison purposes, the natural gap in lymphatic coverage seen in thin tail skin cross sections due to the hexagonal architecture of the tail skin lymphatics was quantified along the longitudinal axis by measuring the distance between regularly repeating clusters of lymphatic structures. For the bleomycin + surgery treated tails, the distance was quantified by measuring the large lymphatic coverage gap that was clearly evident in the cross sections.

Statistical Methods
Five rats and ten mice were used for each experimental group, respectively. Data are presented as means with standard deviations (SD). P values were calculated using ANOVA or repeated measures ANOVA.
Results

Fibrosis Promotes a Sustained Acute Lymphedema

We have previously shown that reducing fibrosis at the wound site of the mouse tail lymphedema model with a collagen gel implant significantly reduces tissue swelling (23). Here, in order to determine the effects of increased wound site fibrosis in the regulation of fluid drainage, we compared the ability of the mouse tail lymphatic system to drain accumulating fluid following lymphatic injury in the presence or absence of bleomycin treatment at the site of obstruction. We found that the injections of bleomycin near the wound site produced visible fibrotic tissue in the context of tissue injury and swelling, whereas fibrosis was not externally visible on the non-injured control tissue despite the same course of bleomycin injections (Figure 1). Tissue swelling, as measured by the tail diameter and tail area, was strongly increased following surgery relative to both control groups, irrespective of bleomycin treatment (p<0.005, by repeated measures ANOVA). Bleomycin treatment without surgery did not produce swelling (p>0.5, by repeated measures ANOVA). Bleomycin treatment following surgery (Surgery +, Bleomycin +) caused a significant increase in tail area relative to the untreated surgical control (Surgery +, Bleomycin -, p<0.05, by repeated measures ANOVA). Thus, wound site fibrosis caused a worsened tail swelling. Both surgical groups experienced a similar initial tail diameter and tail area trend, consisting of a swelling that peaked at the same time and to the same degree, but which then proceeded to diverge over time as the swelling was sustained in the bleomycin (+) group and declined in the bleomycin (-) group. Thus, in the acute lymphedema model, worsened fibrosis with bleomycin treatments was found to impair fluid drainage across the site of obstruction and to sustain tissue swelling, rather than worsen the degree of swelling. The ability of the wound site to heal naturally was strongly inhibited by the bleomycin treatments, as...
shown by the large disparities in wound closure over time (p<0.005, by repeated measures ANOVA).

As noted above, we observed the formation of fibrotic material during wound repair in the mouse tails that had received bleomycin injections following the surgery. The fibrosis in these tails became apparent over time at the wound site and was generally absent by day 60 (Figure 1), consistent with the timing of bleomycin injections. A Mason’s Trichrome stain was employed in histological cross sections of the tails collected at day 30 to confirm the presence of fibrosis and compare its distribution in deeper levels of the tissue. We found minimal signs of fibrosis in control tails (either with or without bleomycin). In contrast, extensive fibrosis consisting of increased connective tissue density was evident in the tails that had experienced tissue swelling due to the surgery in combination with bleomycin treatment (p<0.005 by ANOVA; Figure 2). The dense fibrotic biomatrix extended well into the subcutaneous space in the tails that had received both bleomycin and surgery. The findings demonstrate that the bleomycin treatment had promoted an extensive synthesis of fibrotic tissue in the swollen tail skin.

Fluorescent staining for lymphatics in cross sections from the same mouse tails demonstrated dramatic lymphatic hyperplasia in the swollen control skin and a potent suppression of lymphatic growth at the site of bleomycin injections (p<0.005 by ANOVA; Figure 3). The sustained tissue swelling from the bleomycin in the acute model may therefore be causally related to the reduced wound closure and suppressed lymphangiogenesis due to the fibrosis. Indeed, regenerating lymphatics and accumulating fluid both require a suitable matrix bridge across the obstruction and within the swollen tissue in order to spread towards proximal lymphatics. Furthermore, the
fibrosis detected in the deeper connective layer of the skin may have compromised the functional regeneration of the deep collecting lymphatics, thereby contributing further to the worsened tissue swelling. Surprisingly, treatment of the first group of mouse tails with oxazolone at day 70 to induce inflammation did not result in significant tissue swelling in any group, despite four oxazolone applications over the subsequent twelve days (data not shown). Our inability to manifest inflammation-swelling in the mouse tail stands in stark contrast to the significant swelling that we have reported following oxazolone treatment in the rat foreleg (18) and may be explained by the stiffer tissue of the tail relative to the axilla and foreleg. Therefore, in order to detect chronic post-surgical lymphatic deficiencies due to fibrosis, we employed the rat foreleg lymphedema model.

Fibrosis Worsens the Post-ALND Swelling Induced by Inflammation

In order to reveal the presence of any worsened chronic lymphatic deficiencies due to the fibrotic scarring, we compared the ability of the regenerated foreleg lymphatic system following ALND and bleomycin treatment to prevent edema during an acute inflammatory challenge with that of the PBS treated post-ALND foreleg lymphatic system (Figure 4). Following the ALND surgery and bleomycin treatments and at all times up to day 52 (measurements made every five days until day 50) there was no detectable swelling in any of the forelegs (data not shown). Beginning at 52 days post-ALND, long after quiescence of the acute wound healing phase, all the right forelegs of sensitized rats were exposed to the pro-inflammatory agent oxazolone to challenge the foreleg lymphatic system and reveal the presence of any worsened latent lymphatic deficiencies due to the bleomycin treatments. We detected a significant increase in the wrist diameter between oxazolone-treated ALND (-) Bleomycin (-) control forelegs and both ALND (+) Bleomycin (-) and ALND (+) Bleomycin (+) forelegs (p<0.05, by repeated measures
ANOVA). Measurements of the arm area revealed a significant increase in foreleg swelling between ALND (-) Bleomycin (-) control forelegs and all other groups, with a highly significant difference found relative to the ALND (+) Bleomycin (+) forelegs (p<0.005, by repeated measures ANOVA). The results show that fibrosis, even in the absence of ALND, causes a chronic lymphatic deficiency and that worsened wound site fibrosis in conjunction with ALND creates the most severe chronic lymphatic deficiency. The presence of fibrosis at the wound site due to the bleomycin injections was confirmed in histological cross sections of the rat axilla, at day 15 post-surgery (Figure 5).

Fibrosis Worsens the Post-ALND Inflammation-Induced Lymphatic Drainage Impairment

In order to clarify the mechanism by which fibrosis causes an increased post-ALND swelling, we visualized lymphatic drainage with indocyanine green (ICG) fluorescent lymphography at day 64, prior to euthanization. Indocyanine Green (ICG) dye in ALND (-) Bleomycin (-) Oxazolone (-) forelegs was seen to drain from the injection site by large lymphatic vessels leading towards the axillary lymph nodes (Figure 6A), indicative of normally functioning lymphatic vessels in the healthy foreleg. In the presence of oxazolone, lymphatic drainage was reduced for all conditions, including for forelegs that did not undergo ALND or pre-treatment with bleomycin (Figure 5B-5E). However, ALND (-) Bleomycin (-) forelegs appeared to have incurred the least reduction in lymph drainage (Figure 6B). In contrast, ALND (+) Bleomycin (+) forelegs appeared to have incurred the largest reduction in lymph drainage (Figure 6E), as ICG dye in these forelegs was mainly restricted to the paw, near the injection site, indicative of a more serious lymphatic transport impairment.

Fibrosis Reduces Post-ALND Interstitial Transport and Lymphatic Function

A second method was employed to quantitatively assess the degree of lymph drainage
impairment in the rat forelegs. Fluorescent rhodamine-dextran was injected into rat paws to trace the path of lymph drainage through the foreleg and quantitatively assess the ability of the lymphatic system to drain macromolecules from the paw. Quantification was made by measuring fluorescent fluid tracer area coverage in histological foreleg cross sections made at the wrist, near the injection site. Fluid marker was found to be strongly present interstitially and subcutaneously at the wrist location of all left forelegs, which were not treated with oxazolone (Figure 7). Fluid marker was weakly present at the wrist location of all right forelegs, which were treated with oxazolone. A statistical analysis showed a significant reduction in tracer coverage in the right vs. left foreleg cross sections for all groups (p<0.05 by ANOVA), but identified a highly significant reduction in the ALND (+) Bleomycin (+) group (p<0.005 by ANOVA). The data indicates a worsened interstitial transport of fluid marker from the injection site in the paw to more proximal lymphatic vessels due to the ALND in conjunction with bleomycin treatment. Thus, fluid drainage was the most severely suppressed by the inflammatory stimulus following ALND surgery when fibrosis was first exacerbated with bleomycin. Our cumulative findings of a worsened foreleg swelling, worsened lymph drainage as seen with the ICG imaging, and reduced fluid transport as measured using rhodamine-dextran tracer demonstrates that increased fibrosis worsens the chronic lymphatic deficiency that follows ALND and precedes lymphedema.
Discussion

Although fibrosis is a natural consequence of wound repair following ALND to treat breast cancer and inadvertently stimulating fibrosis at the wound site with radiation therapy dramatically increases susceptibility for secondary lymphedema, it remains unclear how fibrotic scarring contributes to the years-later appearance of chronic lymphedema in the arm. Here, we have used two rodent models of lymphedema to demonstrate that fibrosis is a key regulator of both short and long term lymphatic function. Using an acute mouse tail lymphedema model, we first showed that fibrosis stimulation with bleomycin significantly impairs lymphangiogenesis and lymphatic drainage relative to controls. The sustained swelling and suppressed lymphatic regrowth that we found in the mouse tail when fibrosis was increased stands in sharp contrast to the increased functional lymphangiogenesis and rapidly ameliorated lymphedema that we recently reported when fibrosis was reduced with collagen gel implants (23). Secondly, using a rat foreleg model with a similar period of latency that follows ALND and fibrotic stimulation as is found in the human, we showed that the stimulated fibrosis worsens the latent and chronic lymphatic deficiency relative to what follows a physiological regeneration of the dissected axilla. We found that this worsened chronic lymphatic deficiency reduces the ability of the foreleg lymphatic system to prevent edema during acute inflammation, possibly by weakening lymphatic pump function due to the increased regional lymphatic outflow resistance. Our findings therefore demonstrate a clear link between fibrotic scarring and a worsened lymphedema, and demonstrate a more chronically impaired lymphatic drainage and suppressed lymphangiogenesis as contributing mechanisms.
The mechanism by which fibrosis regulates lymphatic function and regeneration remains to be clarified. The components of fibrotic matrix differ from that of natural matrix, being comprised mainly of thick bundles of collagen that reduce matrix compliance (33). Because lymphatics are often anchored to the surrounding matrix (26) and depend upon a compliant tissue to become functional (16), a reduced tissue compliance may directly impair the function of lymphatics that traverse a fibrotic region. In addition, it has been shown that the fibrotic scarring produced from physiological wound healing impairs lymphangiogenesis and interstitial flows (1-4, 8, 11, 23, 31, 34, 35), most likely through molecular mediated signaling mechanisms and changes to matrix resistance to interstitial flow. Through both molecular inhibition of lymphangiogenesis signaling pathways and inhibition of interstitial flows shown to stimulate lymphangiogenesis (6), fibrosis at a wound site may inhibit the regeneration of lymphatics that become damaged during ALND and SLND. The present data supports the notion that fibrosis may mediate its pro-edema effects by suppressing lymphatic regeneration and drainage. Clinical data on human subjects is strongly supportive of a critical role for fibrosis in impairing lymphatic regeneration during wound repair. For instance, radiation therapy without lymph node dissection (which induces fibrotic changes that reduce tissue compliance) or lymph node injury without radiation are both far less likely to result in secondary lymphedema relative to radiation therapy in conjunction with lymph node dissection (which creates an additional fibrotic barrier to lymphatic regeneration) (10, 13). We have recently found that a relatively scar-free regeneration of extracellular matrix at the wound site (starting from a collagen implant) provides an interstitial bridge for lymph fluid to spread across the wound site to functional proximal lymphatics and serve as a scaffold for functional lymphangiogenesis (23, 31). These previous studies, when taken together with the current study,
highlight the critical role of fibrosis in regulating lymphangiogenesis and interstitial flows during wound repair.

Unlike the time-delayed life-long swelling experienced by humans, swelling appears immediately following surgery and completely resolves in acute animal models of lymphedema. Thus it has often been difficult to apply experimental findings with acute models to clarify mechanisms in the human disease. Here we have demonstrated a causal relationship between fibrotic scarring during wound repair and the latter appearance of lymphedema. We showed that fibrotic scarring suppresses lymphatic regeneration and contributes to a chronic lymphatic deficiency that is able to remain latent and become manifest during periods of inflammation. We have shown recently that the chronic and latent lymphatic functional deficiency produced by ALND in the rat foreleg may consist of a reduced maximum lymph flow rate caused by an insufficient wound healing lymphangiogenesis (18). Worsening the fibrotic scarring with bleomycin, as we have done here, may further reduce the maximum lymph flow rate by more strongly impairing interstitial flow and lymphangiogenesis. In conditions of acute inflammation, the reduced lymphatic reserve results in a greater lymphatic flow reduction and an increased fluid accumulation relative to what occurs post-ALND following physiological wound repair. A similar mechanism of reduced capacity to drain an inflammation-induced swelling due to pre-existant fibrosis and deficient lymphangiogenesis may play a role in the often times sudden appearance of lymphedema in humans and in the increased incidence of lymphedema in humans who receive radiation therapy.

Several aspects of our results warrant additional comment. We found that bleomycin injections
into the tail had no real consequence in the absence of surgery and tissue swelling, while the ALND (-) Bleomycin (+) control was found to have elevated arm area in the rats. This may be accounted for by the softer tissues present in the foreleg relative to the tail, which may allow the foreleg to swell under lower interstitial fluid pressures. Indeed, although we saw an increased arm area, the wrist diameter (which lacks spaces containing soft connective tissue) was not increased despite a particularly severe response as visualized by the ICG in figure 5. We speculate that fluid preferentially accumulates in the arm relative to the wrist due to the presence of softer fatty tissues in the arm. The accumulation of fluid simultaneously in both the arm and the wrist is therefore generally indicative of worsened lymphatic drainage relative to fluid accumulation in the arm alone. Our finding that tails did not swell in the Surgery (-) Bleomycin (+) mouse group contrasts to an earlier report where bleomycin injections resulted in increased tail thickness even without surgery (4). This may have occurred because we injected 2.5 μls into the mouse tails to avoid inducing an injection-related injury. In contrast, 10 μls were injected in the earlier report, which may have induced a stretch related injury in the tail. Indeed, our results have shown that bleomycin may induce a more extensive fibrosis in the context of tissue swelling, which may facilitate the spread of bleomycin through the matrix. We also found a non-significant jump in wound closure at day 45 in the Surgery (+) Bleomycin (+) group. This may have been due to long-term changes in cell synthesis of fibrotic matrix induced by the bleomycin.

Presently, therapies for treating secondary lymphedema have largely been directed at increasing lymphatic drainage with excess lymphatic growth factor to clear accumulated fluid after disease onset. The appearance of a chronically worsened lymphatic drainage and tissue swelling due to
the increased fibrosis, as we have found in the present study, supports the hypothesis that fibrosis
is a key regulator of interstitial flow and lymphangiogenesis across the site of obstruction and
within the swollen limb. They also suggest that reducing fibrosis during wound repair and
radiation therapy, in place of or in addition to stimulating lymphangiogenesis, may be a viable
therapeutic strategy to reduce the incidence of secondary lymphedema in humans.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).
**Figure Legends**

**Figure 1. Fibrosis Promotes a Sustained Acute Lymphedema.**
Shown are representative images of mouse tails at the indicated conditions at post-surgical days 0, 20, 40, and 60 post-surgery. Proximal to distal direction is shown top to bottom in each image. Black scale bar in lower right tail image = 5 mm. Yellow and green arrows indicate normal wound repair and fibrosis due to bleomycin, respectively, in the two enlarged images. Tail diameter, tail area, and wound closure data are shown for all groups in graphs at the right. * indicates statistical significance (p<0.05) and ** indicates high statistical significance (p<0.005). n=10 per group.

**Figure 2. Bleomycin and Tissue Swelling Promote Fibrosis of the Mouse Tail**
Shown are representative images of Mason’s Trichrome-stained mouse tail cross-sections at day 30 post-surgery for tails receiving (A) PBS injections, (B) Bleomycin injections, (C) PBS injections + Surgery, and (D) Bleomycin injections + Surgery. Epidermis is shown at the top of all images. Distal to proximal direction is shown left to right in each image. Yellow scale bar at bottom right = 0.5 mm. Data is graphically depicted in E for treatment groups shown in A-D, with ** indicating high statistical significance (p<0.005). n=10 per group.

**Figure 3. Fibrosis Inhibits Lymphatic Regeneration**
Shown are representative low magnification images of LYVE-1-stained mouse tail thick cross-sections (150 μm) at day 30 post-surgery for tails receiving (A) PBS injections, (B) Bleomycin injections, (C) PBS injections + Surgery, and (D) Bleomycin injections + Surgery. Also shown are representative high magnification LYVE-1-stained images from thin cross-sections (10 μm) for the same treatment groups (E-H), respectively. Epidermis is shown at the top of all images. Distal to proximal direction is shown right to left in each image. White scale bar is 0.5mm and
0.1mm at bottom right of images D and H, respectively. Data related to the distance between clusters of lymphatic structures is graphically depicted in panel I, with ** indicating high statistical significance (p<0.005). n=10 per group.

**Figure 4. Fibrosis Worsens the Post-ALND Swelling Induced by Inflammation.**

Shown are representative images of right rat forelegs at the indicated conditions at days 52 and 64. Black scale bar in lower right foreleg image = 10 mm. Wrist diameter and arm area were measured and graphed for all groups, shown below. * indicates statistical significance (p<0.05) and ** indicates high statistical significance (p<0.005) between groups. n=5 per group.

**Figure 5. Bleomycin Stimulates Fibrosis in the Rat Axilla**

Shown are representative images of Mason’s Trichrome-stained rat axillas at day 15 post-surgery for those receiving (A) PBS injections, (B) PBS injections + Surgery, (C) Bleomycin injections, and (D) Bleomycin injections + Surgery. Epidermis is shown at the top of all images. Green scale bar at bottom right = 0.5 mm. n=5 per group.

**Figure 6. Fibrosis Worsens the Post-ALND Inflammation-Induced Lymphatic Drainage Impairment.**

Shown are ICG fluorescence lymphography images captured from ALND (-) Bleomycin (-) Oxazolone (-) (A), ALND (-) Bleomycin (-) Oxazolone (+) (B), ALND (-) Bleomycin (+) Oxazolone (-) (C), ALND (+) Bleomycin (-) Oxazolone (+) (D), and ALND (+) Bleomycin (+) Oxazolone (+) (E) forelegs at 64 days post-surgery. Images were captured at 10 minutes following the ICG paw injection. The paw injection site is shown to the left and the axilla to the right of each image. n=5 per group.

**Figure 7. Fibrosis Reduces Post-ALND Interstitial Transport and Lymphatic Function.**
Dextran fluid marker was imaged in left and right foreleg wrist cross-sections from each condition, as shown. Red color in each image is fluorescent dextran lymph fluid tracer. Blue color in each image is DAPI labeled cell nuclei. Yellow arrows identify visible fluid marker. Rhodamine fluorescence was equally augmented in all images of right foreleg cross sections to facilitate visualization. White scale bar in low right image = 0.5 mm. Fluid marker coverage is graphically depicted below. * indicates statistical significance (p<0.05) and ** indicates high statistical significance (p<0.005) between right and left forelegs. n=5 per group.
References


Day 0  |  Day 20  |  Day 40  |  Day 60
---     |         |         |         
Bleomycin - Surgery - 

Bleomycin + Surgery - 

Bleomycin - Surgery + 

Bleomycin + Surgery + 

Day 40: Bleomycin - Surgery + 

Day 40: Bleomycin + Surgery + 

**Statistics**

- Tail Diameter (cm)
- Tail Area (cm²)
- Wound Closure (cm)

*Days Post-Surgery*