Interstitial Flow as a Guide for Lymphangiogenesis

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Abstract—The lymphatic system is important in tissue fluid balance regulation, immune cell trafficking, edema, and cancer metastasis, yet very little is known about the sequence of events that initiate and coordinate lymphangiogenesis. Here, we characterize the process of lymphatic regeneration by uniquely correlating interstitial fluid flow and lymphatic endothelial cell migration with lymphatic function. A new model of skin regeneration using a collagen implant in a mouse tail has been developed, and it shows that (1) interstitial fluid channels form before lymphatic endothelial cell organization and (2) lymphatic cell migration, vascular endothelial growth factor-C expression, and lymphatic capillary network organization are initiated primarily in the direction of lymph flow. These data suggest that interstitial fluid channeling precedes and may even direct lymphangiogenesis (in contrast to blood angiogenesis, in which fluid flow proceeds only after the vessel develops); thus, a novel and robust model is introduced for correlating molecular events with functionality in lymphangiogenesis. (Circ. Res. 2003;92:801-808.)

Key Words: lymphatic capillary • interstitial fluid flow • vascular endothelial growth factor-C • lymphatic vessel hyaluronan receptor 1 • matrix metalloproteinases

The lymphatic system is an important component of the circulation; however, our understanding of lymphatic biology lags far behind that of the blood vasculature, even though the lymphatics are the primary route of metastasis of many common cancers, including breast, colon, and skin cancer. Several important molecular regulators have been implicated in lymphangiogenesis, including vascular endothelial growth factor (VEGF)-C and VEGF-D1–4 and their receptor Flt-4,5 as well as Prox16 and basic fibroblast growth factor 7; furthermore, a CD44-like hyaluronan receptor specific for lymphatics (lymphatic vessel hyaluronan receptor 1; LYVE-1) has been identified.8–10 However, the morphological, spatial, and temporal aspects of lymphangiogenesis remain poorly understood. This is due in great part to the lack of an adequate model in which to study the spatial organization of lymphatic endothelial cells (LECs) relative to fluid flow as well as the development of functionality and patterning in an emerging lymphatic capillary network.

The extent of similarity between the processes of lymphangiogenesis and blood angiogenesis is poorly understood, although they share many molecular regulators and serve complementary functions. The latter, which is much more extensively studied and characterized, occurs in embryonic development, wound healing, and tumor growth through a sprouting process managed by a number of polypeptide growth factors such as VEGF-A and angiopoietin-2.11 The primary physiological driving force for blood angiogenesis is oxygen concentration, which is directly correlated with the primary function of the blood vasculature. Indeed, a number of growth factors including VEGF-A and erythropoietin are expressed under the influence of hypoxia inducing factor-1, an oxygen-sensitive transcription factor.12 The primary function of the lymphatic system, in contrast to the blood circulation, is to maintain interstitial fluid balance and provide lymphatic clearance of interstitial fluid and macromolecules, thereby sustaining osmotic and hydrostatic gradients from blood capillaries through the interstitium and stimulating convection for interstitial protein transport.13 Thus, although it is clear that VEGF-C and VEGF-D are important molecular regulating factors, it is plausible to suggest that an important physiological regulating factor of lymphangiogenesis may be the maintenance of interstitial fluid balance and protein convection (analogous to oxygen transport as a physiological regulating factor in blood angiogenesis) and, therefore, that interstitial fluid flow may play a role in lymphangiogenesis.

Previous research into the physiological regulation of lymphangiogenesis has been hindered by the lack of appropriate experimental models. In the present study, we introduce a new model of lymphatic development in a regenerating region of skin. The model is unique and robust in that it allows for the observation of fluid channels and (through the concurrent use of molecular markers) differential identification of functional lymphatic vessels. We developed this model in the skin of the mouse tail, which has been used previously to measure lymph flow velocity,14 to evaluate tissue fluid balance parameters,15 and to observe lymphatic architecture in tissue grafts16 and in transgenic mice.
because the CDE is initially acellular, any cells or structures in the distal portion of the tail is prevented. Furthermore, CDE provides an effective path for fluid transport, and edema inserted into the prepared gap (Figures 1b through 1d). The fluid-bridging mechanism would result in marked edema of the tail.15,16 Instead, to bridge the distal and proximal portions of skin is surgically removed from the tail without disturbing the lymphatic network and the flow of interstitial fluid out of the tail tip are completely interrupted by the procedure. Left under the sleeve and allowed to gel (g).

Figure 1. Lymphangiogenesis: model construction and features. a, Lymphatic capillary network located within the dermis of the normal mouse tail, as seen with FM. Bar=1 mm. b, CDE skin replacement model in mouse tail to investigate morphological patterns of lymphangiogenic growth. Bar=1 cm. c, Close-up photograph of CDE. d, Schematic diagram of CDE. An annulus of skin is surgically removed from the tail without disturbing the tail core (tc, including large blood vessels, tendons, muscle, and bone). Bar=1 mm. The silicone sleeve (s) is placed over the wound and fixed in place with surgical glue. Collagen is injected under the sleeve and allowed to gel (g).

To create this model, a collagen “window” is formed in the tail skin, through which lymph and interstitial fluid necessarily move in a proximal direction. A 2-mm-wide circumferential band of dermal tissue is removed midway up the tail, leaving the underlying bone, muscle, major blood vessels, and tendons intact. Blood flow to the tail remains uninterrupted because of the large subcutaneous blood vessels, but the lymphatic network and the flow of interstitial fluid out of the tail tip are completely interrupted by the procedure. Left alone, this interruption of the lymphatic network without a fluid-bridging mechanism would result in marked edema of the tail.15,16 Instead, to bridge the distal and proximal portions of the tail skin and restore the proximally routed unidirectional flow of lymph, a collagen dermal equivalent (CDE) is inserted into the prepared gap (Figures 1b through 1d). The CDE provides an effective path for fluid transport, and edema in the distal portion of the tail is prevented. Furthermore, because the CDE is initially acellular, any cells or structures (particularly lymphatic vessels) later observed within the CDE are presumably the result of cell migration, proliferation, and organization.

This model has enabled us to observe the development of functional fluid channels and to correlate these functional features with migrating LECs and blood endothelial cells (BECs). In the present study, we report the temporal and spatial relationship between lymphatic development within CDE and interstitial fluid channeling, showing that (1) interstitial fluid channeling precedes LEC organization, and (2) fluid channeling, LEC migration, and functional vessel formation are initiated only in the direction of lymph flow. (Note: here and later, the term migration refers to the net result of cell translocation, which is a function of cell survival and possibly proliferation as well as migration.) We also show that matrix protease activity before fluid channeling is more pronounced at the upstream (distal) rather than downstream (proximal) side of the CDE, further hinting that interstitial flow may play a role in the mechanism of the observed directional fluid channel formation and cell migration. Furthermore, we find that expression of VEGF-C is similarly more pronounced at the distal side of the CDE before LEC infiltration. These observations are in contrast to the events that take place during blood angiogenesis, which occurs equally from all sides of the implanted tissue equivalent and is not preceded by interstitial fluid channeling, consistent with decades of observations of blood angiogenesis in a variety of models.8,11,17 Therefore, the process of lymphangiogenesis seems distinct from that of blood angiogenesis and may involve a new mechanism related to interstitial fluid flow. On the basis of our observations, we suggest that interstitial flow can drive the formation of fluid channels along which LECs migrate, proliferate, and finally reorganize into a functional lymphatic capillary network.

Materials and Methods

Twenty-one Balb/C mice were used in the present study. Animals were anesthetized with a subcutaneous mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Postsurgical analgesic (buprenorphine 2 mg/mL) was administered subcutaneously twice a day for up to 1 week. All work was conducted with the approval of the Northwestern University Animal Care and Use Committee.

Surgical Preparation

Midway up the mouse tail, a 2-mm-wide circumferential annulus of skin was removed from the tail, leaving the underlying bone, muscle, major blood vessels, and tendons intact (Figure 1). Great care was taken to ensure that the deeper draining lymphatics running alongside the major blood vessels were also severed. A diameter-matched silicone sleeve was placed over the wound and fixed in place with surgical glue to the intact skin on the proximal and distal edges. A 0.3% solution of type I rat tail collagen18 was injected into the wound under the sleeve and allowed to gel; this formed the CDE. The tail was then wrapped with self-sticking tape to protect the surgical site from interference by the animal. The CDE is not meant to mimic the granulation tissue of a healing wound (in fact, it never scars because of the silicone sleeve); rather, it is simply meant as a tissue bridge for interstitial fluid flow and as a scaffold for cell migration and growth.

Microlymphangiography

After removing the silicone sleeve for optimal visualization, we evaluated the functional lymphatics of the mouse tail by use of fluorescence microlymphangiography (FM).19 By this technique, a
fluorescently labeled macromolecule (in this case, lysine-fixable FITC-dextran of 2000 kDa; Molecular Probes) was injected intradermally at a constant pressure of 35 mm Hg into the tip of the tail (which was roughly 25 to 30 mm away from the site of the CDE). Because of its large size, the tracer was taken up by the lymphatics but was excluded from the blood vasculature. Because the tracer fills the network downstream from the injection site, the fluorescent tracer is clearly visible within lymphatic capillaries and any connecting fluid channels (Figure 1a). The entire procedure was monitored with a Nikon Eclipse TE 200 inverted microscope; digital images were captured with a Spot RT slider camera (Diagnostic Instruments).

**Specimen Preparation**

Perfusion fixation was used to cross-link the lysine-fixable FITC-dextran tracer in place, marking the lymphatic vessels and fluid channels in thin cryosections. Immediately after FM at various time points (10, 25, and 60 days), the animals were fixed with Zamboni’s fixative20 (2% formaldehyde, 15% picric acid, and 0.1 mol/L phosphate buffer, pH 6.9) by perfusion through the abdominal aorta. After fixation, the animals were then perfused with cryoprotectant (10% sucrose in PBS, pH 7.2), the tails were removed, and a 6-mm region centered around the CDE was embedded in freezing media and flash-frozen in melting isopentane. Longitudinal cryosections (10 μm) were obtained from each specimen.

**Immunofluorescence and Immunohistochemistry**

We identified various endothelial markers by use of standard immunofluorescence techniques. Cryosections were incubated with rat anti-mouse CD31 (PharMingen), rabbit anti-mouse LYVE-1 (kind gift of Dr David Jackson, John Radcliffe Hospital, Headington, Oxford, UK), goat anti-mouse Flt-4 (R&D Systems), and rabbit anti-mouse CD31 (PharMingen), rabbit anti-mouse LYVE-1 (kind gift of Dr Gwendalyn Randolph, Mount Sinai Medical School, New York, NY) antibodies and labeled with the appropriate rhodamine red-X-conjugated IgG (Jackson Immunoresearch Laboratories). The mounting medium contained DAPI (Vectorshield, Vector) for visualizing cell nuclei. We also identified VEGF-C expression within the CDE using standard immunohistochemical techniques. Cryosections were incubated with goat anti-mouse VEGF-C (A-18, Santa Cruz Biotechnology). Sections were further incubated with biotinylated rabbit anti-goat IgG (Vector) and then visualized with the ABC-AP kit by the use of Vector Red substrate (Vector). In this case, cell nuclei were counterstained with hematoxylin.

**In Situ Zymography**

We mapped the location of matrix protease activity using an in situ zymographic technique.21 Briefly, DQ Collagen (Molecular Probes) is heavily loaded with fluorescein such that fluorescence is initially quenched. Digestion by collagenolytic proteases yields a highly fluorescent product whose intensity is proportional to proteolytic activity. We incubated freshly frozen tissue sections overnight at room temperature with DQ type I collagen in reaction buffer (50 mmol/L Tris-HCl, 0.15 mol/L NaCl, 5 mmol/L CaCl2, and 0.2 mmol/L sodium azide, pH 7.6). We examined the sections without washes or fixation.

**Results**

Overall, 3 different types of markers were used in evaluating the lymphangiogenic process: fluid channel markers (via FM), cellular markers (via immunofluorescence), and soluble molecular markers (via immunohistochemistry and in situ zymography); furthermore, each of these could be correlated in space and in time. At each time point, FM was performed on the live animal before perfusion fixation, cryosectioning, and immunostaining. Because FM allowed the lysine-fixable cutaneously injected fluorescent tracer to be tracked from its interstitial deposit into the local lymphatic capillaries and then proximally within the lymphatic network and because the fluid tracer was then fixed in place before sectioning, we could directly correlate functional fluid channels with LECs or other molecular markers via immunostaining.

**Fluid Channeling Precedes Lymphatic Network Organization**

We used FM to track lymph flow pathways (either as fluid channels or lymphatic vessels) within the CDE over a period of 60 days after the procedure. We saw that after an initial phase of at least 10 days (during which lymph pooled and moved interstitially through the CDE), fluid channels eventually formed to bridge the distal and proximal portions of the lymphatic system; finally, those channels gave way to an organized network of lymphatic capillaries. In normal tail skin, FM showed a highly regular hexagonal network of lymphatic vessels (Figure 1a). Ten days after the skin was removed and replaced with the CDE, the tracer appeared to move through the construct diffusely and without discrete channeling, and it pooled uniformly throughout the CDE (Figure 2a). This was also seen at earlier times, eg, at 3 and 7 days (data not shown). In contrast, by 25 days, the tracer was seen concentrated within crude but distinct fluid channels (Figure 2b). Finally, within 60 days, the tracer in the CDE was present within a continuous network of fluid channels between the upstream and downstream intact lymphatics (Figure 2c), restoring lymphatic continuity and lymph flow. Remarkably, the remodeled fluid channels within the CDE had recovered the unique hexagonal patterning of a normal lymphatic network.

**Observation of LECs Versus BECs**

The ability to identify functional lymphatic vessels and differentiate LECs from BECs through combined functional and molecular techniques is a key advantage of our model. Immediately after FM, the animals were perfusion-fixed, and the tails were flash-frozen. Later, the tails were cryosectioned...
and immunostained for various markers to distinguish interstitial fluid channels from endothelium-lined vessels as well as to differentiate blood from lymphatic vessels. Fixation was initiated when lymph, saturated with lysine-fixable FITC-dextran, was present within the CDE as well as the entire lymphatic network; thus, the fixation cross-linked the fluid tracer in place within both those fluid channels and the lymphatic vessels. This allowed us to distinguish between interstitial fluid channels (positive for FITC-dextran but negative for the putative lymphatic markers Flt-4 and LYVE-1), functional lymphatic vessels (positive for FITC-dextran and Flt-4 and/or LYVE-1), nonfunctional lymphatic structures (negative for FITC-dextran but positive for Flt-4 and/or LYVE-1), and nonlymphatic endothelial structures (negative for both FITC-dextran and Flt-4 or LYVE-1 but positive for other endothelial markers such as CD31). Because these markers are not always specific for LECs and BECs under certain conditions, we carefully examined the specificity of these markers in our model before confirming these classifications.

In normal skin, 83±13% (mean±SD) of structures that were LYVE-1+ (Figure 3a) and Flt-4+ (not shown) colocalized with tracer-filled fluid channels, whereas only 18±9% of the CD31+ structures similarly colocalized with the FITC-dextran. Furthermore, the CD31+ structures that did colocalize with the fluid channels had a lower staining intensity relative to the CD31+ vessels that did not colocalize with fluid channels (Figure 3e); this is consistent with the stronger expression of CD31 by BECs relative to LECs. In experimental animals in which normal skin was replaced with the CDE, CD31+ structures were seen distributed equally throughout the CDE as early as 10 days after the procedure (Figure 3f), whereas Flt-4+ and LYVE-1+ structures were absent from the CDE at day 10 (Figure 3b). At 60 days, both CD31+ and Flt-4+/LYVE-1+ structures were present within the CDE (Figures 3d and 3h), but only the Flt-4+/LYVE-1+ structures strongly colocalized with fluid channels (80±15% of Flt-4+/LYVE-1+ structures colocalized with fluid tracer, but only 10±2% of CD31+ structures colocalized with fluid tracer). Thus, these additional observations allowed confirmation of the classifications suggested above.
We observed in the CDE that the development of the lymphatic system lags behind that of the blood vasculature; this finding is concordant with findings in earlier studies involving scar and granulation tissue.\textsuperscript{27–29} In observing the CD31 staining patterns, it can be seen that the epithelialization and vascularization of the CDE occurred more quickly than lymphatic development (Figures 3b and 3f). The entire CDE was completely vascularized (CD31$^+$/H11001$^+$/FITC-dextran$^+$/H11002$^+$) by day 10 after the procedure, although organization and trimming continued through day 60 (Figures 3f through 3h). By contrast, lymphatic development (LYVE-1$^+$/H11001$^+$/Flt-4$^+$/H11001$^+$) within the CDE occurred between 25 and 60 days (Figures 3b through 3d). Furthermore, although LEC migration occurred primarily in the direction of interstitial fluid flow (at 25 days, 79% of Flt-4$^+$/LYVE-1$^+$ cells were in the upstream half of the CDE), there were no discernible differences in BEC migration or organization between the upstream or downstream directions, even at 3 (data not shown) and 10 days, emphasizing the morphological and physiological differences between blood and lymphangiogenesis.

**LEC Migration Follows Direction of Lymph Flow Along Fluid Channels**

By comparing the relative distribution of fluid channels and LECs within the CDE in cryosections at various time points, we observed lymphangiogenesis as a process whereby fluid channel formation and LEC migration along those channels preceded the eventual organization of the fluid channels into endothelium-lined vessels (Figures 3a through 3d) and developed from the upstream edge of the CDE (we assume that the only differences between the upstream and downstream edges of the CDE were in the direction of interstitial fluid flow, inasmuch as reepithelialization and blood vascularization occurred equally from both ends). At 10 days after the procedure, the fluid tracer was diffusely dispersed throughout the CDE interstitium without any LECs present (Figure 3b). By day 25, we observed discrete fluid channels within the CDE that bridged the distal and proximal lymphatic networks of the intact skin, consistent with the microlymphangiography data in Figure 2. However, we also detected LECs (Flt-4$^+$/LYVE-1$^+$ cells) primarily within those fluid channels, but only as single cells and not as a continuous vessel developing from the preexisting vessels of the intact skin. Furthermore, those cells were located mostly on the upstream (distal) edge (Figure 3c), consistent with the direction of lymph flow (again, 79% of LYVE-1$^+$ structures were located in the upstream half of the CDE). Finally, by day 60, the fluid tracer appeared to be present in smaller, more regularly spaced, and more numerous clumps, and the fluid channels were fully colocalized with LECs (Figure 3d) similar to those seen in normal skin (Figure 3a). These observations, together with
those shown in Figure 2, indicate that the fluid channels had been transformed into a dense network of functional lymphatic vessels with a normal hexagonal architecture.

The observation that LECs translocate along fluid channels primarily in the direction of lymph flow (from distal to proximal) is corroborated by comparing the spatial localization of fluid channels with expression patterns of CD31, LYVE-1, Flt-4, and SLC (another putative lymphatic marker also known as 6Ckine, exodus-2, and CCL21) between the upstream versus downstream edges of the CDE (Figure 4). Because lymph flows through the mouse tail skin and the CDE in a single direction (proximally), fluid channels are necessarily initiated at the upstream edge of the CDE. At 10 days, CD31+ cells were seen equally in both the upstream and downstream regions (with no apparent relationship to fluid channels), but we did not observe LYVE-1, Flt-4, or SLC expression within the CDE. At 25 days, CD31+ structures remained distributed uniformly throughout the CDE, but LYVE-1, Flt-4, and SLC were seen only in cells migrating along fluid channels in the upstream region. Finally, at 60 days, upstream and downstream regions showed staining patterns of CD31, LYVE-1, Flt-4, and SLC similar to those of normal tissue, ie, colocalizing with several small fluid channels. Thus, the migrating cells within the CDE express these 3 putative LEC markers, and our observations suggest that they were responsible for creating the organized hexagonal lymphatic network seen in Figures 2c and 3d.

Patterns of Protease and VEGF-C Expression in the CDE

Matrix degradation was examined to investigate the relationship between fluid flow and fluid channeling (as seen in Figures 2b, 3c, and 3g). Because the rate of collagen hydrolysis is much faster for matrix metalloproteinases (MMPs) compared with other collagenolytic proteases and enzymes such as cathepsin K, trypsin, and thermolysin, it was assumed that collagen degradation was due primarily to MMP activity. Therefore, we mapped the location of collagen-degrading proteases using in situ zymography at each of our time points and saw the majority of activity only at early times. After 3 days after implantation, we observed several points of MMP activity present at both ends of the CDE (Figures 5a and 5c), indicative of individual migrating cells. However, by 7 days, 2 patterns of MMP activity emerged (Figure 5b): (1) MMPs from adjacent intact dermis streamed into the CDE in the direction of fluid flow (only at the distal edge, Figure 5e); this stood in contrast to activity at the proximal edge of the CDE, where MMP activity did not infiltrate the CDE against the direction of fluid flow (Figure 5f). (2) A path of MMP activity, independent of that produced by the intact dermis, was present within and through most of the CDE.

Figure 5. a and b, Collagen-degrading MMPs within the CDE, as indicated by green fluorescence using in situ zymography in specimens at 3 days (panel a) and 7 days (panel b) after CDE implantation. Dashed lines demarcate edges of CDE. Flow is from left to right (distal to proximal). c through f, Enlarged distal and proximal views of 3- and 7-day tails. Cell nuclei were counterstained with propidium iodide (red). Bar=100 μm.

Figure 6. VEGF-C protein expression within the CDE using immunohistochemistry. a and d, VEGF-C staining (red) appeared to be strongest within the CDE at 10 days, primarily in the distal edge. b and e, Decreased, but more distributed, VEGF-C staining was also observed at 25 days after procedure. c and f, VEGF-C cannot be detected anywhere in the CDE at 60 days. Flow is from left to right (distal to proximal). Cell nuclei were counterstained with hematoxylin (blue). Bar=200 μm.
the length of the CDE. These observations lead to speculation that there is at least 1 potential mechanism of unidirectional interstitial fluid channel formation: proteases, transported by the interstitial fluid flow, preferentially degrade matrix in a downstream direction, leading to directed cell migration and fluid channel formation in the direction of flow.

Finally, we correlated the spatial and temporal expression of VEGF-C with the lymphangiogenic events occurring within the CDE by immunohistochemistry. At 10 days after the procedure, a large amount of VEGF-C was seen at the distal but not the proximal edge of the CDE (Figures 6a and 6d). The pattern of VEGF-C distribution suggested, in a similar manner to the location of MMPs, that VEGF-C transport and expression were likely directed by interstitial fluid flow. Because VEGF-C is known to be a mitogen for LECs, this could provide a significant chemotactic factor for LEC proliferation and migration, preferentially from the distal, rather than proximal, edge. By 25 days, VEGF-C was only weakly observed and appeared to be distributed throughout the CDE (Figures 6b and 6e), and by 60 days, VEGF-C could not be detected within the CDE (Figures 6c and 6f).

**Discussion**

The data in the present study reveal the spatial and temporal patterns of interstitial fluid flow and cell migration involved in lymphangiogenesis in an implanted skin equivalent. The significance of these data are 2-fold. First, our results introduce a potential new physiological regulator in lymphangiogenesis by implicating interstitial fluid flow in the process and signify a potential new mechanism of vessel formation. In this mechanism, lymphatic vessel formation is initiated along preestablished routes of fluid flow. This is in contrast to the known mechanisms of blood vessel growth and remodeling (eg, angiogenesis, vasculogenesis, arteriogenesis, and intussusception), through which fluid flow occurs only after an intact vascular network has developed. In our proposed mechanism, fluid channels form within the extracellular milieu, and then the LECs migrate along these channels to organize into a functional lymphatic network, contiguous with the upstream and downstream lymphatic networks. Indeed, this is consistent with the function of the lymphatic capillaries of maintaining interstitial fluid balance and convection.

Second, our results present a new and robust model for studying lymphangiogenesis. Because the implanted CDE is circumferential, there is no possibility for fluid shunting around the implant, and lymph (fluid contained in the upstream lymphatics) must necessarily pass through the implant to continue moving in downstream lymphatics. Therefore, the fluid pathways through the CDE can be positively tracked with FM originating from the upstream lymphatics and can later be correlated with LECs by immunohistological examination. This is the first model to our knowledge in which function can be definitively examined, because there is no alternative drainage pathway as in other models; indeed, the question of whether identified LEC-lined structures are functional lymphatics has been an ongoing controversy in recent literature.

The question of the mechanism of fluid channel formation naturally arises from our observations. Although this issue is clearly interesting and important and warrants further study, one potential mechanism can be suggested from the pattern of MMP activity within the CDE. Skewed by interstitial convection, the MMPs preferentially degrade matrix in a downstream direction, leading to directional fluid flow and cell migration. Furthermore, the temporal and spatial patterns of VEGF-C distribution suggest that growth factor distribution and thus LEC mitogenesis may be influenced by interstitial fluid flow. This would potentially lead to the preferential proliferation and migration of LECs from the distal, rather than the proximal, edge. This may help to at least partially explain the translocation of LECs along fluid channels (as evidenced in both FM and immunofluorescent staining) before the eventual organization of a complete and functional lymphatic capillary network.

The phenomenological evidence obtained in the present study for the role of interstitial fluid channeling in functional lymphatic vessel formation is consistent with and complementary to the rapidly growing literature on molecular regulation of lymphangiogenesis, which is particularly focused on VEGF-C and its receptor Flt-4. For example, it has been observed that upregulating the lymphatic growth factor VEGF-C in the dermis of transgenic mice induces lymphatic hyperplasia but does not change lymphatic density or architecture; this is consistent with our findings because the blood vasculature, extracellular matrix, and thus, presumably, interstitial fluid balance were normal in these transgenic mice. When Flt-4 was made inactive through mutation or was blocked, a decrease in lymphangiogenesis was observed, along with marked edema (a state of increased fluid accumulation and decreased interstitial flow). Thus, VEGF-C appears to be required for LEC proliferation, a necessary step in lymphangiogenesis, but additional factors are necessary to alter lymphatic architecture or to induce new vessel growth.

In summary, we introduce a new model of lymphangiogenesis in which physiological function can be incorporated with molecular regulation, and we propose physiological mechanisms for lymphangiogenesis that complement molecular regulation. We show that fluid channeling and LEC migration precedes lymphatic capillary formation and that unlike blood angiogenesis, these events occur primarily in the direction of flow into the implanted dermal equivalent. In exploring one possible mechanism for these events, we observed that in the upstream region of interstitial flow, MMP activity is increased, which could lead to preferential cell migration in the direction of flow. We also showed that increased expression of the LEC mitogen VEGF-C occurs primarily in upstream regions of the CDE, which (with subsequent unidirectional transport) could then enhance cell migration and proliferation even further in the direction of flow. Thus, interstitial flow may represent an important transport mechanism to help guide growth and organization of a developing lymphatic capillary network.

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