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Regulation of lymphatic capillary regeneration by interstitial flow in skin

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Goldman J, Conley KA, Raehl A, Bondy DM, Pytowski B, Swartz MA, Rutkowski JM, Jaroch DB, Ongstad EL. Regulation of lymphatic capillary regeneration by interstitial flow in skin. *Am J Physiol Heart Circ Physiol* 292: H2176–H2183, 2007. First published January 12, 2007; doi:10.1152/ajpheart.01011.2006.—Decreased interstitial flow (IF) in secondary lymphedema is coincident with poor physiological lymphatic regeneration. However, both the existence and direction of causality between IF and lymphangiogenesis remain unclear. This is primarily because the role of IF and its importance relative to the action of the prolymphangiogenic growth factor vascular endothelial growth factor (VEGF)-C (which signals primarily through its receptor VEGFR-3) are poorly understood. To clarify this, we explored the cooperative roles of VEGFR-3 and IF in a mouse model of lymphangiogenesis in regenerating skin. Specifically, a region of lymphangiogenesis was created by substituting a portion of mouse tail skin with a collagen gel within which lymphatic capillaries completely regenerate over a period of 60 days. The relative importance of IF and VEGF-C signaling were evaluated by either inhibiting VEGFR-3 signaling with antagonistic antibodies or by reducing IF. In some cases, VEGF-C signaling was then increased with exogenous protein. To clarify the role of IF, the distribution of endogenous matrix metalloproteinases (MMPs) and VEGF-C within the regenerating region was determined. It was found that inhibition of either VEGFR-3 or IF suppressed endogenous lymphangiogenesis. Reduction of IF was found to decrease lymphatic migration and transport of endogenous MMP and VEGF-C through the regenerating region. Therapeutic VEGF-C administration restored lymphangiogenesis following inhibition of VEGFR-3 but did not increase lymphangiogenesis following inhibition of IF. These results identify IF as an important regulator of the pro-lymphangiogenic action of VEGF-C.

vascular endothelial growth factor receptor-3; endothelial cell

LYMPHATIC VESSELS DRAIN interstitial fluid and thereby guide interstitial flow (IF; see Refs. 3 and 19). Although IF has been identified as an important organizing factor in lymphangiogenesis both in vivo and in vitro, its relationship to vascular endothelial growth factor (VEGF)-C signaling has not been established. In vivo, a model of lymphangiogenesis in regenerating mouse tail skin demonstrated that the direction of IF (formed from lymph that collected from upstream lymphatic capillaries and then transported interstitially through the regenerating region) correlated with the formation of crude prelymphatic fluid channels, transport of matrix metalloproteinases (MMPs) and lymphatic growth factors, and ultimately the direction of lymphatic endothelial cell (LEC) migration and organization (4, 15, 21). In vitro, IF was shown to induce endothelial cell migration and capillary morphogenesis (11) by skewing cell-secreted proteases that liberate matrix-bound growth factors (7). Despite such progress, a causal relationship between IF and lymphangiogenesis has not been demonstrated, primarily because it is difficult to directly and selectively inhibit IF in a clearly defined region of regenerating skin where lymphatic regrowth can be observed over time. Furthermore, the ability of exogenous VEGF-C to rescue lymphangiogenesis in tissues with reduced IF is unclear.

In contrast to the lack of studies on IF in lymphangiogenesis, numerous studies have been conducted to determine the role of vascular growth factors and their receptors in lymphangiogenesis (20). It has recently been shown that excess VEGF-C can augment lymphatic growth and function in adult tissues. For example, lymphatic size, density, and function have been reported to increase in skin when VEGF-C is overexpressed (5, 16–18, 24, 25) or delivered as recombinant protein (22). It is now well accepted that VEGF-C activation of VEGF receptor-3 (VEGFR-3) is necessary for lymphangiogenesis (1, 2, 9, 10, 12, 13, 24), and, in the same tail skin regeneration model, blocking antibodies against VEGFR-3 prevented lymphangiogenesis (13). Because of this, VEGF-C holds potential for lymphangiogenic therapy in diseases of inadequate lymphatic drainage. However, the relationship between VEGF-C/VEGFR-3 signaling with IF during lymphangiogenesis is unknown. For example, in the regenerating skin model, we recently reported that excess VEGF-C induced hyperplasia of regenerating lymphatics but was unable to increase lymphatic migration or density above that of controls (6), possibly because IF is unaltered in both conditions. In another study, VEGF-C-overexpressing transgenic mice were shown to have incurred lymphatic hyperplasia in the tail skin without any apparent changes to the density of the lymphatic network (9). Thus, whereas the importance of VEGF-C in lymphangiogenesis is well established, the extent to which VEGF-C may, by itself, promote new functional lymphatic growth and increase IF in conditions of lymphedema remains debated.

Here we evaluate the ability of exogenous VEGF-C to promote new lymphatic growth in a clearly defined region of lymphatic-deficient skin using a previously characterized model of mouse tail skin regeneration (4). In this model, a circumferential section of tail skin is removed and replaced by a collagen matrix within which skin regenerates in a relatively scar-free manner. Lymphatic capillaries are not present in the region initially, and IF is always directed from the distal toward proximal regions of the tail (formed from lymph collected by "upstream" lymphatics). We can induce lymphatic-

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free skin regeneration in the following two ways: *1*) by employing VEGFR-3 neutralizing antibodies(13) or *2*) by reducing IF. We then investigate the ability of exogenous VEGF-C to promote lymphangiogenesis in both cases: in the first, where IF is normal, vs. in the second, where IF is severely reduced, as would be the case in chronic lymphedema. By testing the ability of VEGF-C to augment lymphangiogenesis under each conditions, we can clarify the extent to which lymphatic growth depends on preexisting IF.

MATERIALS AND METHODS

Lymphangiogenesis model. A previously described model of lymphangiogenesis in regenerating tail skin of adult mice was used (4, 15, 21). For all studies, 6- to 8-wk-old female BALB/c mice (Charles River Laboratories and Harlan) weighing 18–20 g were used; at least four mice were examined for each condition at each time point. Mice were anesthetized with a subcutaneous injection of ketamine (65 mg/kg), xylazine (13 mg/kg), and morphine (2 mg/kg). An analgesic, butorphanol (0.05 mg/kg), was administered subcutaneously two times daily for 3 days following the procedure. Protocols were approved by the Animal Care and Use Committee of Michigan Technological University and the Veterinary Authorities of the Canton Vaud, Switzerland.

Regenerating regions were created in two ways. Either a 2-mm-wide circumferential region (4) or a 2×2 mm square region (15) of dermal tissue (containing the dermal lymphatic and blood capillary networks) was excised midway up the tail, leaving the underlying bone, muscle, major blood vessels, and tendons intact. The area was then covered with a close-fitting, gas-permeable silicone sleeve and filled with type I rat tail collagen (BD Pharmingen). The collagen provides a well-controlled environment in which skin can regenerate; thus, any LECs or lymphatic structures later observed within this region are the result of newly initiated cell migration, proliferation, and organization.

Experimental model 1: VEGFR-3 neutralization. For murine blocking studies, anti-mouse VEGFR-3 (mF4-31C1; see Ref. 13) was used (Imclone Systems). In groups receiving mF4-31C1, a 2-mm circumferential regenerating region was prepared as described above in the lymphangiogenesis model section and 0.625 $\text{mg} \cdot \text{dose}^{-1} \cdot \text{mouse}^{-1}$ of the blocking antibody was administered postsurgery every 2 days by intraperitoneal injection for a duration that varied by experimental group. By employing this model, we can prepare a circumferential region of regenerating skin with normal IF but without functional VEGFR-3.

Experimental model 2: IF inhibition. IF through a regenerating region of skin can be reduced by creating a square injury midway up the tail. It has been recently shown that upstream lymph circumvents this regenerated square region by flowing through the intact surrounding lymphatic network (15). By employing this model, we can reduce IF through a regenerating square of skin.

Experimental model 3: IF inhibition. IF through the regenerating mouse tail skin is formed from lymph that is collected from upstream tissue (4, 6, 13). Therefore, IF through the regenerating region can be reduced by surgical amputation of the upstream tissue. To reduce IF, mice were anesthetized and fitted with a silicone sleeve to identify the location for tail amputation. The tail was then completely severed distal to the fitted sleeve, the sleeve was removed, and the tail end was cauterized to prevent bleeding with special care undertaken to minimize cautery-induced trauma. Mice were allowed to recover for 3 wk, whereupon they were reanesthetized, and a regenerating region was prepared as described above, with the modification that the regenerating region was placed at the edge of the severed tail instead of midway up an intact tail (as was done in *experimental model 1*). By employing this model, we can reduce IF through a regenerating circumferential region of skin.

It should be noted that the surgical amputation process induces injury-associated inflammation and wound healing, which may alter local IF conditions. To monitor the degree of injury, the presence of macrophages was assessed at the tip of the amputated tail 3 wk subsequent to the amputation. Few macrophages, identified by the F4/80 marker, were present at 3 wk at the amputated tail tip (data not shown), suggesting that the 3-wk delay may have been sufficient for natural wound healing processes to diminish in the tail skin.

VEGF-C therapy. To determine the ability of VEGF-C to augment lymphangiogenesis in the absence of prior VEGFR-3 signaling or under reduced IF, mouse tail skin regenerating regions were prepared as described for *experimental models 1* and *2*. Mice with circumferential regenerating skin, as described in *experimental model 1*, received mF4-31C1 for the first 10 days postsurgery. Mice with square regenerating skin were created as described in *experimental model 2*. At *day 60* (at which time the skin and blood vasculature in both models had completely regenerated but neither contained functional lymphatics), mice in both groups (*groups 1* and *2*) received daily injections of carrier-free recombinant human VEGF-C (R&D Systems) at the site of regenerated skin over a period of 15 days at 4 $\mu\text{g} \cdot \text{dose}^{-1} \cdot \text{mouse}^{-1}$ (12 μl total volume/dose) for a total delivery of 60 μg VEGF-C per mouse over this time period. Control mice received saline injections (12 μl total volume/dose) over this period of time.

Detection of functional lymphatic capillaries via microlymphangiography. To visualize lymph flow patterns in situ, a 1% solution of FITC or tetramethylrhodamine-conjugated dextran of 7×10^4 Da dextran (Molecular Probes/Invitrogen, Carlsbad, CA) was injected intradermally in the tail end where it was taken up and transported by the lymphatics in the proximal direction, revealing fluid channels and functional lymphatic capillaries.

Immunofluorescence and immunohistochemistry. Tail specimens were cut into either 10- or 60-μm longitudinal cryosections and immunostained. To detect LECs, a rabbit polyclonal antibody against the lymphatic-specific hyaluronan receptor LYVE-1 (Upstate) was used along with an Alexa Fluor 488 goat anti-rabbit secondary antibody (Molecular Probes). LECs were defined as a cell nuclei surrounded by LYVE-1 staining. To detect blood endothelial cells (BECs), a biotinylated rat antimouse CD31 antibody (BD Pharmingen) was used along with Alexa Fluor 555 streptavidin (Invitrogen). BECs were defined as a cell nuclei surrounded completely by CD31 but not LYVE-1 staining (because LECs weakly express CD31). Cell nuclei were labeled with 4′,6-diamino-2-phenylindole (Vector Laboratories). To determine the cell density, LECs and BECs in the regenerating region were counted, and this number was divided by the surface area of the region.

Biotinylated antibodies (R&D Systems) were used to detect VEGF-C, MMP-2, MMP-3, and MMP-9 in 10-μm longitudinal cryosections. Antibody labeling was visualized with an avidin-biotin complex-alkaline phosphatase kit and vector red substrate (Vector Laboratories). Cell nuclei were counterstained with Gill's hematoxylin. Images were analyzed with Metamorph image analysis software (Molecular Devices) to determine the percent coverage of positive pixels in the regenerating region.

Statistical methods. At least three sections were counted per specimen. Data are presented as means (SD). *P* values were calculated using a two-tailed Student's *t*-test.

RESULTS

Lymphangiogenesis is completely inhibited by transient VEGFR-3 neutralization. Microlymphangiographies of mouse tail skin at 60 days demonstrated regeneration of normal hexagonal capillary architecture around the circumference of regenerated skin in saline-treated control mice. However, neither normal architecture nor any apparent functional lymphatic

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capillaries had regenerated after 60 days in mice treated with VEGFR-3 neutralizing antibodies for the 10, 17, or 25 days following surgery (Fig. 1). In addition, immunostaining of cryosections for LYVE-1 demonstrated that LECs were present at *day 60* throughout the regenerating region in saline-treated controls but had failed to migrate in the regenerating region when VEGFR-3 neutralizing antibodies were administered for the first 25, 17, or 10 days following surgery (Fig. 1). Thus even 10 days of VEGFR-3 blocking appeared to prevent lymphatic regrowth in the regenerated skin. These results confirmed earlier findings where VEGFR-3 blocking over the entire 60-day period of regeneration was found to prevent lymphatic capillary regrowth (13) and suggest that VEGFR-3 is active mainly in the early stages of lymphatic capillary regeneration. These results also show that VEGFR-3 blocking during the early stages of skin regeneration is sufficient to generate chronic lymphatic deficiency in the fully regenerated skin.

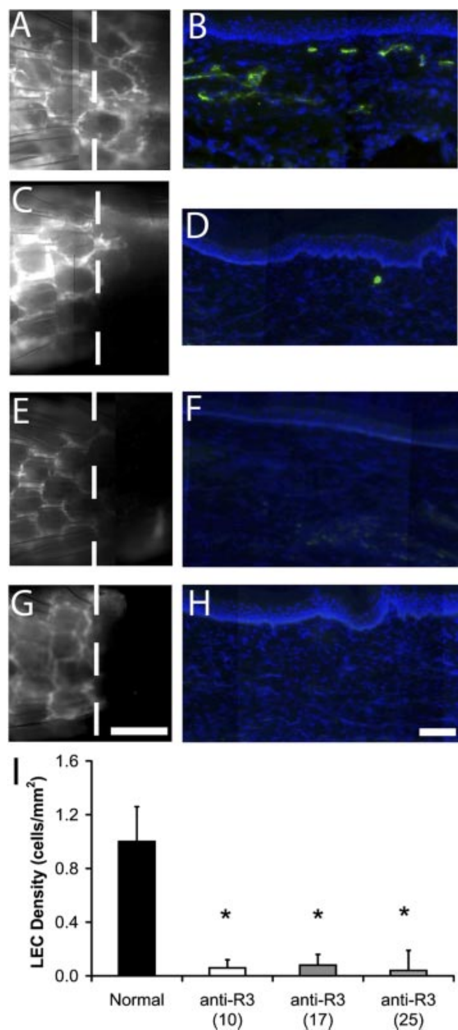

Fig. 1. Lymph fluid transport and lymphangiogenesis in regenerating skin. Lymph fluid flows in the proximal direction, left to right. Dashed white lines in the microlymphangiographies indicate location of the distal regenerating region boundary. After surgery in saline-treated controls (60 days), functional lymphatic capillaries with normal hexagonal lymphatic architecture have reappeared in the regenerating region (*A*). Cryosections (10 μm) were immunostained against the lymphatic endothelial cell (LEC)-specific receptor LYEV-1 (green), and cell nuclei were stained with DAPI (blue). LECs are present throughout the regenerating region at 60 days, demonstrating complete regeneration of lymphatics (*B*). After either 10, 17, or 25 days of anti-vascular endothelial growth factor receptor (VEGFR)-3 administration, regeneration of capillaries with normal hexagonal lymphatic architecture have been completely prevented (*C*, *E*, and *G*, respectively), when evaluated at *day 60*. Very few LECs are present in their corresponding LYEV-1-stained sections (*D*, *F*, and *H*), demonstrating effective inhibition of lymphangiogenesis. Bar in *G* = 1 mm. Bar in *H* = 100 μm. *I*: LEC density was determined from immunostained cryosections of mouse tail skin at *day 60* in normal controls and after 10, 17, or 25 days of VEGFR-3 neutralization. LEC densities and SD values are presented normalized to the mean average control density.

Exogenous VEGF-C augments lymphangiogenesis following VEGFR-3 neutralization. It is well established that VEGF-C can induce lymphatic capillary growth in normal mammalian skin (2). To verify that exogenous VEGF-C could augment lymphangiogenesis in a region of skin with normal IF that was rendered chronically lymphatic-deficient by VEGFR-3 blocking, recombinant VEGF-C protein was administered to the regenerated region of mouse tail skin following systemic VEGFR-3 neutralization. To accomplish this, VEGFR-3 neutralizing antibodies were administered systemically for the first 10 days of skin regeneration, and no antibodies were delivered from *day 10–60*, as before. Next, from *day 60* to *75*, VEGF-C protein was injected in the tail skin at 4 $\mu g \cdot dose^{-1} \cdot mouse^{-1} \cdot day^{-1}$. These VEGF-C injections induced lymphatic growth, resulting in complete rescue of lymphangiogenesis relative to normal regenerating controls (Fig. 2, *D* and *E*), whereas daily injections of saline from *day 60* to *75* did not improve lymphangiogenesis (Fig. 2, *B* and *E*). Thus exogenous VEGF-C can induce lymphangiogenesis in lymphatic-free (regenerated) skin when IF is present. This result allows us to compare the ability of VEGF-C to promote lymphatic capillary growth in skin with normal vs. reduced IF. Blood angiogenesis was unaffected by either VEGFR-3 neutralization or VEGF-C therapy (Fig. 2, *A* and *C*). It should be noted that neither surgical preparation of the 2-mm skin injury nor VEGFR-3 neutralization resulted in tail skin edema at any time during the period of experimental observations.

Reduction of IF inhibits physiological and therapeutic lymphangiogenesis. To clarify the role of IF in physiological and therapeutic adult lymphangiogenesis, IF can be reduced in the mouse tail. This can be accomplished by preparing regenerating square regions midway up the intact tail. Because the lymphatic network that surrounds the regenerating square region remains intact, upstream lymph circumvents the square regenerating region. Thus IF is demonstrably reduced in the regenerating region of this model.

The square model of skin regeneration was employed to investigate the ability of exogenously administered VEGF-C to augment lymphangiogenesis in the presence of reduced IF. Reduced IF and deficient lymphatic function were found in the square region of control mice (Fig. 3*A*), in accordance with previous findings (15). When 60-μm-thick sections were labeled for the LYVE-1 marker, poorly organized and nonfunctional individual LECs were found populating the center of the regenerated square (Fig. 3*B*), confirming the role of IF in physiological lymphatic regeneration. To determine whether VEGF-C could augment lymphatic growth and function in this reduced IF region, recombinant VEGF-C was administered from *day 60* to *75* postsurgery to the regenerated square region.

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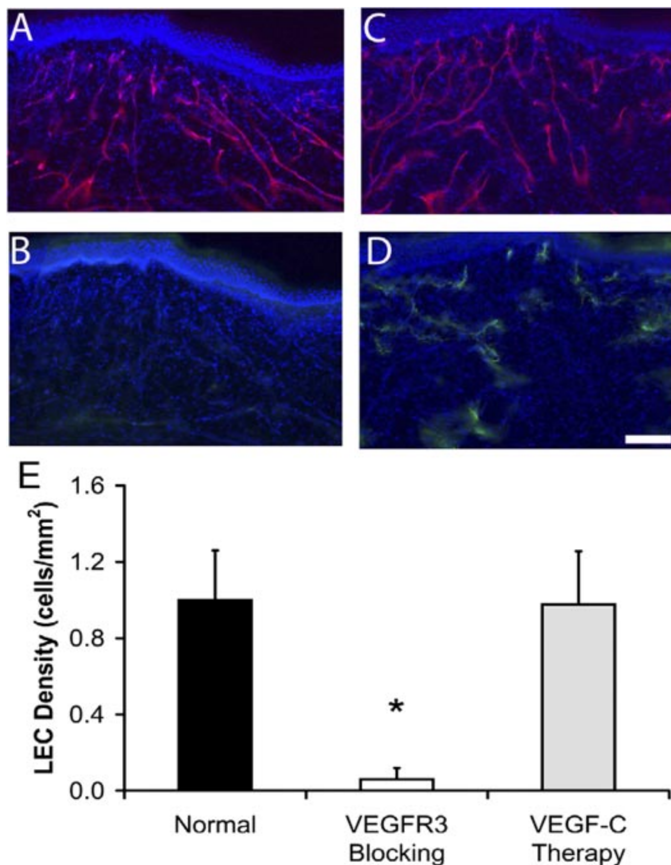

Fig. 2. Lymphangiogenesis following vascular endothelial growth factor (VEGF)-C administration. Cryosections (60 μm) were immunostained against the LEC-specific receptor LYVE-1 (green) and the CD31 blood endothelial cells (BEC) marker (red), and cell nuclei were stained with DAPI (blue). Shown are fluorescence images of the regenerating region, with the proximal direction from right to left. VEGFR-3 was neutralized for the first 10 days postsurgery followed by saline injections from *day 60* to *day 75*. LECs are not present in the regenerating region at 60 days (*B*), although BECs are unaffected by VEGFR-3 neutralization (*A*). Administration of VEGF-C protein from *day 60* to *75* following VEGFR-3 neutralization from *day 0* to *10* resulted in rescue of lymphangiogenesis as shown by the presence of LECs throughout the regenerating region (*D*), whereas BEC distribution is unaffected by this treatment (*C*). *E*: LEC density was determined from immunostained 10-μm cryosections of mouse tail skin at the end point of each experimental condition using an antibody against LYVE-1 and counting LYVE-1-positive cell nuclei in the regenerating region. LEC densities and SDs are presented normalized to the mean average control density; $n = 5$ for normal regeneration and VEGFR-3 neutralization, and $n = 4$ for VEGF-C rescue. Bar = 100 μm.

It was found that the regenerated square region was still devoid of functional lymphatic capillaries, as demonstrated by microlymphangiography (Fig. 3*C*). Following therapeutic VEGF-C treatment, nonfunctional individual (i.e., unorganized) LECs were found populating the center of the regenerated square (Fig. 3*D*), similar to controls. These results suggest that IF may regulate the prolymphangiogenic action of VEGF-C.

Reduction of IF inhibits lymphatic migration. At early times in normal (nonamputated) regenerated tail skin, before lymphatics have regenerated, IF initially collects in the regenerating region from upstream lymphatics and moves through the region interstitially in bulk flow patterns (4, 6). Over time, upstream lymph that collects in the region moves interstitially through discrete fluid channels that become populated with LECs (4, 6). In the mouse tail, IF can be reduced through the regenerating region by amputating tails midway up the tail and preparing circumferential regenerating regions at the tail edge. Because IF is formed from upstream lymph, which is generated from upstream tissue, removal of upstream tissue in the amputated model reduces IF through the circumferential regenerating region.

It has recently been demonstrated that IF can generate gradients of VEGF that direct endothelial cell migration and tube formation in vitro (7). Based on these previous investigations, we hypothesized that reduced IF in skin might reduce LEC migration and capillary formation by diminishing VEGF transport and thereby decreasing VEGF gradients. The regenerating region of the circumferential injury model with reduced IF is similar in geometry to the regenerating region of the normal flow model. The similar geometry allows lymphatic invasion and repopulation of the region to be compared between these models. Therefore, the circumferential reduced IF model was employed to clarify the mechanism of reduced VEGF-C effectiveness in low IF skin. It should be noted that, whereas the square regenerated model allows IF reduction to be directly demonstrated by microlymphangiography (and we have used this model to correlate reduced IF with reduced endogenous and exogenous lymphatic growth), it can be difficult to directly demonstrate IF reduction at the tip of an

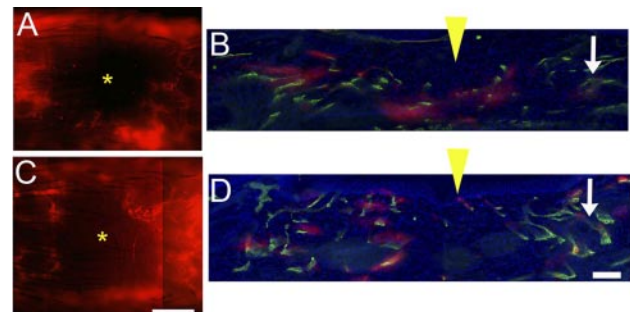

Fig. 3. Lack of increased functional lymphatic growth following VEGF-C therapy in a reduced interstitial flow (IF) model. *A*: microlymphangiography of control skin (proximal direction is from right to left) shows functional lymphatic capillaries transporting lymph (red) around a regenerated square region (yellow star), demonstrating both reduced IF and impaired functional lymphatic vessel regrowth in the square region of regenerated skin. *B*: 60-μm immunostained image from a mouse that received daily saline injections in a regenerated square injury from *day 60* to *75* postsurgery. Shown are fluorescence images with LYVE-1-positive LECs (green), lymph fluid tracer from microlymphangiography (red), and DAPI-stained cell nuclei (blue) in the regenerating square region. The yellow arrowhead indicates the center of the regenerated square injury where multiple individual LECs are present, demonstrating reduced lymphatic regrowth. Some lymph fluid tracer that was below the detection threshold of the microlymphangiography can be seen in the interstitial space in the image. This fluid tracer is not contained within the lymphatics in the regenerating square but is contained within lymphatic capillaries on the outer edge of the square region (indicated by white arrow), confirming reduced lymphatic function in the low IF region. *C*: microlymphangiography from a mouse that received exogenous VEGF-C injections shows flow of lymph (red) mostly around the regenerated square region (yellow star), similar to control microlymphangiography. *D*: 60-μm immunostained image from a mouse that received daily VEGF-C injections in a regenerated square injury from *day 60* to *75* postsurgery. The yellow arrowhead indicates the center of the regenerated square injury, and the white arrow identifies a functional lymphatic vessel at the outer edge of the square region. Multiple individual LECs are present in the square region in a manner similar to the saline-treated controls; $n = 5$ for each group. Bar = 500 μm in *C* and 100 μm in *D*.

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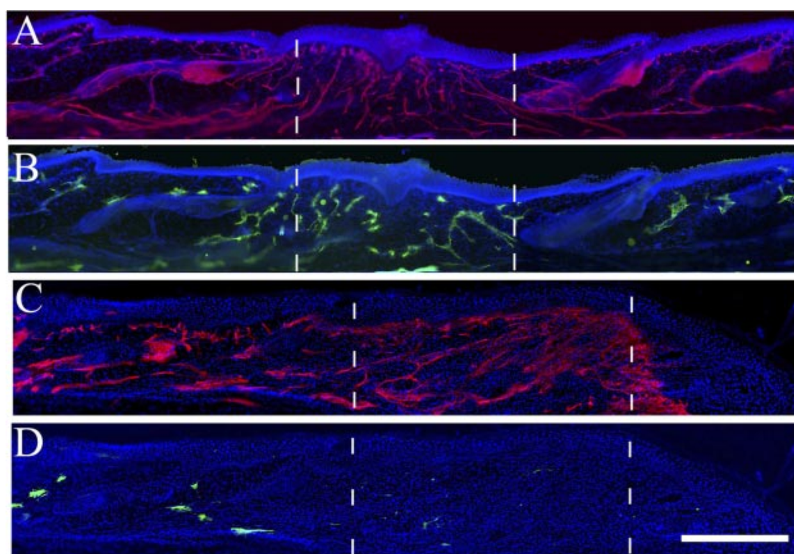
amputated mouse tail. However, the similar regenerating region geometries between the circumferential reduced IF and normal IF models allow for a more direct comparison of lymphatic migration than is possible with the regenerating square model.

It was found that lymphatics filled the entire regenerating region in normal flow controls after 25 days (Fig. 4*B*), whereas few LECs were found in the regenerating region of the reduced circumferential IF model at this time (Fig. 4*D*). The presence of BECs appeared similar in each condition (Fig. 4, *A* and *C*). Quantification of LEC density was compared with normally regenerating control skin (Fig. 4*E*). This quantification confirmed our observations that reduced IF conditions strongly inhibited lymphatic migration at this time ($P < 0.005$). This result provides evidence in support of the hypothesis that IF may be important for directing LEC migration.

Increased presence of VEGF-C and MMP in the absence of IF. Interstitial transport of proteins is directed by lymphatic drainage from the blood capillaries toward the lymphatic capillaries in the form of IF. It has been hypothesized that transport of MMPs by IF may be important for forming prelymphatic fluid channels, along which LECs migrate during the initial stages of lymphangiogenesis (4, 15). It has also been hypothesized that transport of VEGF-C by IF and the generation of VEGF-C gradients may be important for directing the migration of LECs (4, 7, 15). Because IF may generate growth factor and protease gradients in regenerating skin, IF may be an important regulator of the pro-lymphangiogenic activity of MMPs and VEGF-C.

To directly demonstrate the importance of IF for the transport of VEGF-C and MMPs, we examined the transient and spatial distribution of VEGF-C, MMP-2, MMP-3, and MMP-9 via immunostaining of 10-μm cryosections from circumferential models of normal and reduced flow dermis collected at *day 10* (Fig. 5). VEGF-C, MMP-2, and MMP-3 were found to be present in a diffuse pattern and at low levels in the control skin but were concentrated at the distal portion of the deep dermis and hypodermis of the reduced IF skin. MMP-9 was strongly present throughout both normal-flow and reduced-flow regenerating dermis. Increases in the percent coverage in the regenerating region of VEGF-C, MMP-2, and MMP-3 were highly significant relative to controls ($P < 0.005$), whereas the mean increase in MMP-9 coverage was not significantly different from controls (Fig. 6), suggesting that MMP-9 distribution may not be flow dependent. The increased presence of endogenous VEGF-C in the reduced IF skin is particularly striking because lymphangiogenesis was strongly inhibited by reduced IF. These results demonstrate that lymphatic growth factors and proteases collect in the subdermis of regenerating skin in the absence of IF. Thus IF may be important for transporting



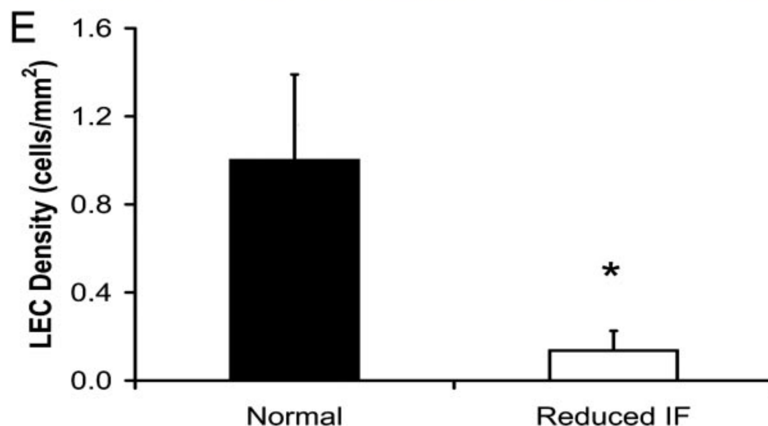


Fig. 4. Reduced lymphatic migration in low interstitial flow model. Cryosections (60 μm) were immunostained against the LEC-specific receptor LYVE-1 (green) and the CD31 BEC marker (red), and cell nuclei were stained with DAPI (blue). Dashed white lines indicate the regenerating region. The proximal direction is from right to left in all images. Blood (*A*) and lymphatic (*B*) capillaries regenerated at *day 25* in skin with normal interstitial flow. Following inhibition of interstitial flow, LECs are not found in the regenerating region at 25 days (*D*), although BECs are unaffected (*C*), demonstrating specific inhibition of lymphatic migration. *E*: LEC density was determined from immunostained cryosections of mouse tail skin at the end point of each experimental condition using an antibody against LYVE-1. LEC densities and SDs are presented normalized to the mean average control density; $n = 5$ for each experimental group. Bar = 1 mm.

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growth factors and proteases during skin regeneration. These results support the hypothesis that interstitial transport of growth factors and proteases may be important for lymphangiogesis.

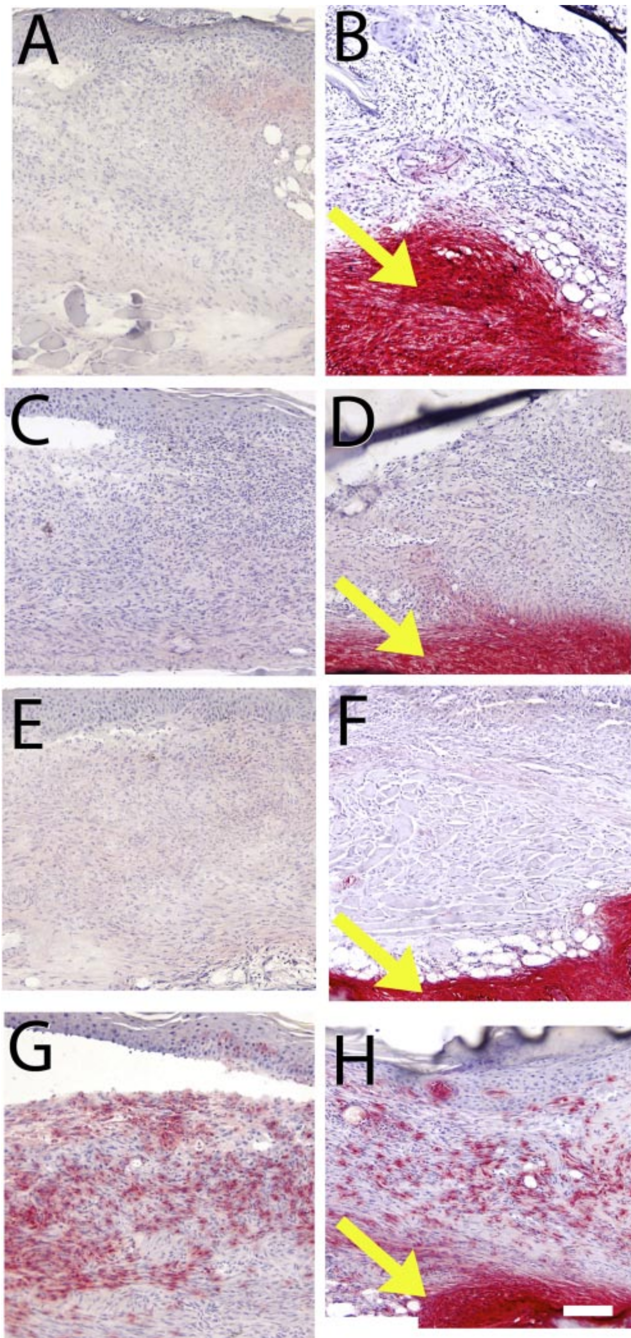

Fig. 5. Increased VEGF-C and matrix metalloproteinase (MMP) presence in low-flow-regenerating skin. Cryosections were immunostained against either VEGF-C (*A* and *B*), MMP-2 (*C* and *D*), MMP-3 (*E* and *F*), or MMP-9 (*G* and *H*) (red in each image) at *day 10* in normally regenerating skin (*column 1*) or reduced interstitial flow skin (*column 2*). The epidermis is located at the *top* of each image. Shown is the distal half of the regenerating region under each condition. VEGF-C, MMP-2, and MMP-3 are present at very low levels in the control skin. However, VEGF-C and MMP-2 and -3 are seen pooling in the deep dermis of the distal regenerating region of the reduced interstitial flow skin (indicated by the yellow arrow). MMP-9 is strongly present throughout both normal flow and reduced flow regenerating skin. Bar = 100 μm; $n = 5$ for controls and for reduced interstitial flow.

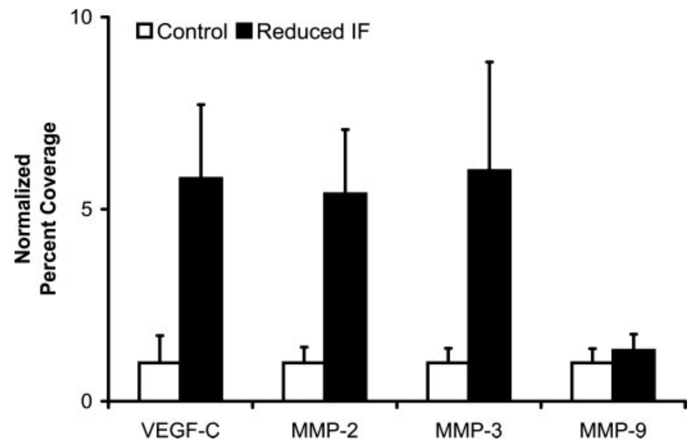

Fig. 6. Quantification of VEGF-C and MMP coverage in low flow and normally regenerating skin. Cryosections (10 μm) obtained from skin following 10 days of regeneration were immunostained against either VEGF-C, MMP-2, MMP-3, or MMP-9. Presented as values normalized to controls, the percent coverage of VEGF-C, MMP-2, and MMP-3 was found to be strongly increased in the low-flow-regenerating skin relative to the normally regenerating control skin. The percent coverage of MMP-9 was not found to be significantly increased in the low-flow-regenerating skin. All differences were highly significant ($P < 0.005$); $n = 4$ for controls and $n = 5$ for reduced interstitial flow.

DISCUSSION

We demonstrate here that VEGF-C is able to rescue lymphangiogenesis in lymphatic-deficient skin when IF is present but has a reduced ability to augment lymphangiogenesis when IF is severely reduced. This may be because of the importance of MMP and VEGF-C transport by IF for lymphangiogenesis. It has been widely reported that excess VEGF-C can induce the growth of new lymphatic capillaries in the adult (5, 16–18, 22, 24, 25). However, our results suggest that VEGF-C may have a reduced ability to promote lymphatic growth in skin with low IF. Our results confirm earlier findings that showed lymphatic capillaries failed to organize in a modified version of the mouse tail skin regeneration model in which a square wound was created instead of a circumferential wound, allowing lymph flow to circumvent the regenerating region (15).

It has been previously hypothesized that IF may promote lymphatic migration by transporting VEGF-C and proteases (4). Indeed, it has been reported in the same model of skin regeneration that crude fluid channels, possibly generated from soluble proteases transported by IF, precede lymphatic migration (4). The present study supports the notion that transport of proteases and VEGF-C by IF may be important for the prolymphangiogenic action of VEGF-C. In further support of this notion, we previously reported that excess VEGF-C does not enhance the rate of LEC migration in regenerating skin or in vitro through a proteolytically sensitive extracellular matrix (6). We also found that VEGFR-3 neutralization prevents lymphatic migration but does not prevent IF through the regenerating region (13). In the present study, we have found that VEGF-C has a significantly reduced ability to induce functional lymphatic capillary organization when IF is reduced. Together, these findings highlight the cooperative roles of IF and VEGF-C/VEGFR-3 signaling in lymphatic migration and functional organization.

In further support of our findings, it was recently found that IF promoted blood and lymphatic capillary morphogenesis

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in vitro (11). When bioavailability of VEGF was dependent upon cell-secreted proteases (7), IF synergized with VEGF in driving structure organization. It was hypothesized (and shown computationally) that the slow IF skewed the pericellular protease gradients, which in turn created amplified gradients of liberated VEGF, leading to directed and enhanced cell-cell communication and thus enhanced structure organization. While these in vitro studies were missing many important components of a wound healing environment, such as infiltrating immune cells and other sources of cytokines and growth factors, they presented a novel mechanism that may help explain how IF can work together with morphogenetic growth factors to direct capillary organization. In the present study, we have found that reduced IF diminishes VEGF-C and protease gradients and causes decreased lymphatic migration and capillary organization in vivo. These results support the hypothesis that normal IF may generate gradients of morphogenetic growth factors that direct LEC migration and capillary organization.

It is believed that excess VEGF-C expression by itself can promote lymphangiogenesis and will be useful for lymphangiogenic therapy. Results from numerous investigations have suggested that overexpression of one of the VEGFR-3 ligands, VEGF-C or VEGF-D, can generate an increased density of hyperplastic lymphatic capillaries that improve lymphatic function (5, 14, 23, 25), whereas other investigations have reported that overexpression of VEGF-C may induce lymphatic hyperplasia without increasing lymphatic density (6, 9, 24) and may also induce irregular lymphatic function (8). These later results support our findings that increased VEGF-C expression by itself may not necessarily lead to improved lymphatic function.

Lymphatic flow in a mouse tail occurs in one direction, allowing for a reduction in lymph volume and IF by amputation of the tail (which removes the source of upstream lymph) or by creating a square injury, allowing lymph to circumvent the region. By surgically preparing a region of regenerating skin in this manner, we were able to create a permanent state of lymphatic deficiency and poor IF. Thus the mouse tail, when prepared in such a manner, may be useful for clarifying the role of IF in lymphangiogenesis and for testing therapeutic strategies to promote lymphatic regrowth in tissues with reduced IF. We have used this model to correlate reduced transport of VEGF-C and MMPs in skin with poor IF with reduced endogenous and therapeutic lymphangiogenesis.

In summary, our results are consistent with the substantial literature demonstrating the necessity of VEGF-C/VEGFR-3 signaling for initiating lymphangiogenesis by inducing LEC migration and proliferation. However, we have found a reduced ability of VEGF-C to increase lymphatic function in tissue with poor IF by demonstrating that transport of VEGF-C by IF may be important for the prolymphangiogenic activity of VEGF-C.

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