Increased Interstitial Protein Because of Impaired Lymph Drainage Does Not Induce Fibrosis and Inflammation in Lymphedema

C.E. Markhus,* T.V. Karlsen,* M. Wagner, Ø.S. Svendsen, O. Tenstad, K. Alitalo, H. Wiig

Objective—The pathophysiology of lymphedema is incompletely understood. We asked how transcapillary fluid balance parameters and lymph flow are affected in a transgenic mouse model of primary lymphedema, which due to an inhibition of vascular endothelial growth factor receptor-3 (VEGFR-3) signaling lacks dermal lymphatics, and whether protein accumulation in the interstitium occurring in lymphedema results in inflammation.

Methods and Results—As estimated using a new optical-imaging technique, we found that this signaling defect resulted in lymph drainage in hind limb skin of K14-VEGFR-3-Ig mice that was 34% of the corresponding value in wild-type. The interstitial fluid pressure and tissue fluid volumes were significantly increased in the areas of visible swelling only, whereas the colloid osmotic pressure in plasma, and thus the colloid osmotic pressure gradient, was reduced compared to wild-type mice. An acute volume load resulted in an exaggerated interstitial fluid pressure response in transgenic mice. There was no accumulation of collagen or lipid in skin, suggesting that chronic edema presented in the K14-VEGFR-3-Ig mouse was not sufficient to induce changes in tissue composition. Proinflammatory cytokines (interleukin-2, interleukin-6, interleukin-12) in subcutaneous interstitial fluid and macrophage infiltration in skin of the paw were lower, whereas the monocyte/macrophage cell fraction in blood and spleen was higher in transgenic compared with wild-type mice.

Conclusion—Our data suggest that a high interstitial protein concentration and longstanding edema is not sufficient to induce fibrosis and inflammation characteristic for the human condition and may have implications for our understanding of the pathophysiology of this condition. (Arterioscler Thromb Vasc Biol. 2013;33:266-274.)

Key Words: cytokines ■ fluid balance ■ inflammation ■ lymphedema ■ mouse model

Any imbalance, where capillary filtration exceeds lymphatic drainage results in edema formation. Lymphedema is a special form of edema, and human primary lymphedema is a congenital pathology of dysfunctional lymphatic drainage characterized by swelling of the limbs, thickening of the dermis, and fluid and lipid accumulation in the underlying tissues.1,2 Several key determinants of lymphatic development have recently been identified, and one of the most studied lymphangiogenic regulators is vascular endothelial growth factor C and its signaling via VEGFR-3 expressed on lymphatic endothelial cells. Defects in this signaling pathway lead to insufficient organization of dermal lymphatic capillaries, resulting in deficient lymph clearance. In humans, VEGFR-3 signaling defects are associated with Milroy disease, a hereditary primary lymphedema condition with swelling of the extremities.3

There are still unresolved questions concerning the pathophysiology of lymphedema. This especially applies to factors concerning fluid transport in the tissue and into initial lymphatics, mechanisms for collagen and lipid accumulation, as well as the role of inflammation in tissue modulation as a response to chronic lymphedema, and furthermore, how the interstitium reacts to perturbations in fluid balance. In a previous study in the Chy model,3 representing a model of Milroy disease, we found an exaggerated response to fluid load, suggesting that the interstitial compliance was reduced as a result of tissue modulation in lymphedema.4 These findings called for studies, where lymph flow was quantified in mouse models with lymphatic system derangements for quantitative evaluation of the phenotype with respect to development of edema.

Here, we investigated pathophysiological consequences of impaired lymph drainage with special focus on tissue microcirculation in a transgenic mouse model of lymphedema, the K14-VEGFR-3-Ig mouse.5 In this model, a soluble VEGFR-3-Ig, expressed by the keratinocytes of the epidermis, is released systemically and leads to a competitive inhibition of the vascular endothelial growth factor C/D–VEGFR-3 signaling pathway. This prevents proper lymphatic capillary maturation and leads to a reduced lymphatic drainage capacity,

Received on: October 10, 2011; final version accepted on: November 27, 2012.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.112.300384/-/DC1.

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.112.300384
resulting in edema formation. We asked how this genotype affected transcapillary fluid balance parameters, particularly focusing on quantification of lymph flow and the response to hydration. Moreover, we asked whether impairment of lymph transport and the associated protein accumulation in the interstitium resulted in inflammation assumed to be central in the pathophysiology of lymphedema, for example. As a consequence of the lymphatic pathology, we found that this model had a lymph flow in hind paw averaging 34% of that in wild-type (WT) mice, limb lymphedema, a significant increase in interstitial fluid hydrostatic, and a decrease in plasma colloid osmotic pressure (COP). In spite of manifest lymphedema, and in contrast to humans, there was no change in interstitial collagen and fat in adult mice, a reduction in some of the macrophage-associated cytokines, and a reduced macrophage infiltration in hind limb skin. Collectively, our data suggest a suppressed immune response in the limbs of mice with this form of lymphedema, and that high interstitial protein concentration and longstanding edema is not sufficient to induce fibrosis and inflammation characteristic for the human condition.

Materials and Methods

Animals

We used the K14-VEGFR-3-Ig transgenic mice on a C57Bl/6 background as model of congenital lymphedema. Mice were anesthetized with a mixture of ketamine (12.2 mg/mL; Ketalar, Pfizer, New York, NY) and medetomidine (24.3 µg/mL; Domitor, Orion Pharma, Espoo, Finland) in saline, 0.2 to 0.3 mL given subcutaneously, or with isoflurane in a combination with O2 and N2O in the optical-imaging experiments (see below). All experiments were performed in accordance with recommendations given by the Norwegian State Commission for Laboratory Animals and were approved by the local ethical committee (Approval # STFDU 1221).

Interstitial Fluid and Tissue Analyses

Interstitial fluid pressure was measured by micropipettes, and interstitial fluid was sampled with implanted wicks or by tissue centrifugation. Tissue fluid volumes were measured with radioactive tracers or by weighing, and COPs on a colloid osmometer designed for submicroliter samples. Cytokines were assayed with multiplex analysis, and collagen and lipids as described in Rutkowski et al. Tissue immune cells were visualized by staining for CD45 and F4/80, and cell distribution in blood, spleen, and bone marrow analyzed using flow cytometry.

Measurement of Lymph Flow

Lymph drainage from the skin was quantified by optically monitoring the depot clearance of near-infrared labeled albumin, and subsequent calculation of removal rate constants (k) in isoflurane anesthetized mice. Skin inflammation was induced by topical application of oxazolone representing delayed-type hypersensitivity inflammatory reaction. For a more detailed description of experimental procedures, including immunohistochemistry, please see the extended Methods section in the online-only Data Supplement.

Results

All K14-VEGFR-3-Ig mice seemed healthy and developed normally except for a modest swelling of the limbs, in agreement with original data from this model. There were no apparent size or weight differences between the K14-VEGFR-3-Ig mice and their WT littermates.

Normal Tissue Morphology in K14-VEGFR-3-Ig Mice Except for a Modest Swelling of the Hypodermis

Chronic lymphedema in humans is associated with irreversible changes in tissue architecture and composition. Common morphological characteristics include interstitial fibrosis with collagen and lipid accumulation. We therefore looked for morphological changes in the edematous skin of K14-VEGFR-3-Ig mice. Sections from hind paw demonstrated normal tissue morphology, except for a slight hypodermal swelling in the lymphedematous mice. Immunohistochemical analysis with lymphatic vessel endothelial hyaluronan receptor (LYVE-1) verified the absence of lymph vessels in dermis (Figure 1), whereas in deeper layers of the skin, that is, the subcutis, lymphatics appeared normal. The distribution of blood vessels was similar in WT and K14-VEGFR-3-Ig mice, as visualized by CD31 staining (Figure 1).

No Accumulation of Collagen and Lipids in K14-VEGFR-3-Ig Mice in Skin Areas Investigated

Lipid and collagen accumulation are hallmarks of longstanding lymphedema in humans. We therefore explored whether similar changes could be found in the K14-VEGFR-3-Ig mouse. Lipid extraction in young adult WT mice (3 months) revealed a high fat fraction in back skin (30.1%) compared with hind paw (13.5%) and thigh muscle (13.4%). No significant differences were observed between the K14-VEGFR-3-Ig (n=7) and WT (n=6) mice in the regions investigated (Figure IA in the online-only Data Supplement), contrasting the reduction of fat content observed in tail skin in this transgenic model. In older mice (aged 12 months), we found a significantly higher fat content in WT hind paw skin (n=6; P=0.01) as compared with the K14-VEGFR-3-Ig (n=6) mice (Figure IB in the online-only Data Supplement). No other regions revealed any significant differences between the strains. Interestingly, old WT mice showed a significantly increased lipid content in all regions investigated, compared with their younger littermates, and fat fraction in old WT mice demonstrated a 110% increase in back skin (P=0.002), 26% increase in hind paw (P=0.043), and 104% increase in thigh muscle (P=0.001). None of these differences were observed in the K14-VEGFR-3-Ig mice.

Quantitative analysis of collagen content revealed no significant differences between the 2 genotypes in any of the tissues or regions investigated neither from young (n=5 for WT;
n=6 for K14-VEGFR-3-Ig) nor old (n=6 and 6) mice (Figure IC and ID in the online-only Data Supplement). In general, collagen content was 10-fold higher in skin than in muscle. Comparing the 2 age groups, there was a significant reduction in collagen content with increasing age in hind paw skin of the K14-VEGFR-3-Ig (P=0.006) as well as WT (P=0.013) mice.

Significant Alterations in Tissue Fluid Exchange Parameters in K14-VEGFR-3-Ig Mice in Areas of Visible Swelling Only

Edema of the K14-VEGFR-3-Ig mouse is only visible in the paws. Estimation of interstitial fluid volume verified the presence of increased tissue hydration in the paws only (Figure 2A). Although average control values in fore and hind paw skin were 1.73 mL/g dry weight (DW; n=6) and 1.43 mL/g DW (n=6), respectively, corresponding mean volume estimates in K14-VEGFR-3-Ig were 2.45 mL/g DW (n=6; P=0.020) and 3.54 mL/g DW (n=6; P<0.001). Muscle samples showed no significant differences in interstitial fluid volume between the 2 strains, WT values averaged 0.52 mL/g DW (n=6).

Measurements of total tissue water content (TTW) in skin and muscle followed the same pattern as for interstitial fluid volume, with an accumulation of tissue fluid only in areas of visible edema. K14-VEGFR-3-Ig mice (n=6) showed a 37% (P=0.013) and 130% (P<0.001) increase in TTW compared with WT (n=6) in fore and hind paw skin, respectively (Figure IIA in the online-only Data Supplement). No differences were observed in back skin, thigh skin, or muscle between the 2 genotypes.

The interstitial fluid pressure (Pif) is a determinant of interstitial fluid volume. In the case of lymphedema, an abnormal accumulation of fluid in the interstitium is expected to result in an increase in Pif, which under normal physiological conditions are slightly subatmospheric in the skin and muscle.13 We found that Pif was significantly higher in fore paw (P=0.001), hind paw (P<0.001), and thigh skin (P=0.013) in K14-VEGFR-3-Ig mice (Figure 2B). The most pronounced difference was found in hind paw skin, where Pif on average was 1.6 mm Hg higher in the K14-VEGFR-3-Ig (n=13) as compared with WT mice (n=12). There were no significant differences in back skin and muscle Pif between the 2 groups (Figure 2B).

To investigate a possible impact of longstanding edema, we measured Pif in 12-month-old K14-VEGFR-3-Ig (n=6) and WT (n=6) mice. As summarized in Figure IIB in the online-only Data Supplement, we found a similar pattern as in the younger mice, the difference in Pif being most pronounced for hind paw skin, averaging 1.9 mm Hg. There is accordingly an increased Pif in overhydrated, dependent, regions in transgenic mice that will counteract further fluid filtration and also represent an increased filling pressure for the initial lymphatics.2

Functional properties of the microcirculation include opposing hydrostatic and colloid osmotic forces working across the vascular wall. Under normal circumstances, the key factor that restrains fluid loss from the capillaries is the intravascular COP. In plasma, the COP was found to be significantly lower (P=0.001) in the K14-VEGFR-3-Ig mice, averaging 16.7 mm Hg (n=7), compared with the corresponding pressure in WT mice of 21.2 mm Hg (n=5). Interstitial fluid from fore paw, hind paw, thigh, and back skin together with thigh muscle of K14-VEGFR-3-Ig mice, had an average COPif ranging from 11.4 to 12.4 mm Hg. In the corresponding areas in WT mice, mean COPif ranged from 10.1 to 12.5 mm Hg (Figure 2C). The COP gradient across the capillary wall (∆COP) is a determinant of the transcapillary fluid flux,14 and we therefore calculated the difference between COPif...
Reduced Level of Proinflammatory Cytokines in K14-VEGFR-3-Ig Mice

We searched for a possible inflammatory response suggested to play a role in the characteristic remodeling of the interstitium1,15 in the transgenic lymphedema model by studying local production of inflammatory mediators in the skin and serum of adult (3 months) and old (12 months) mice. In serum, proinflammatory cytokines interleukin (IL)-1α (P<0.001), monocyte chemotactic protein (MCP)-1 (P<0.05), and macrophage inflammatory protein (MIP)-1α (P=0.001) were significantly lower in adult K14-VEGFR-3-Ig mice (n=6), when compared with WT mice (n=6; Figure 3A–3C). IL-2 level was also lower in both serum (P=0.024) and paw skin interstitial fluid (P=0.045) of the K14-VEGFR-3-Ig mice, when compared with WT mice (n=6 for both; Figure 3D). A significantly reduced cytokine concentration of proinflammatory mediators IL-6 (n=5 for K14-VEGFR-3-Ig and n=6 for WT; P=0.012) and IL-12 (n=6 for both; P<0.01) was found in interstitial fluid from hind paws (Figure 3E and 3F). In contrast, the level of IL-17 (Figure 3G) was elevated in paw interstitial fluid of K14-VEGFR-3-Ig mice, averaging 37.0 pg/mL (n=6), whereas not detectable in WT mice (n=5; P=0.05). The other cytokines analyzed, IL-1β, IL-4, IL-10, granulocyte colony stimulating factor, granulocyte macrophage colony stimulating factor, chemokine (C-X-C motif) ligand 1, interferon-γ, interferon-γ induced protein 10, regulated and normal T cell expressed and secreted (RANTES), and tumor necrosis factor-α, showed no significant differences between the 2 strains, neither in serum nor in interstitial fluid (data not shown). Thus, except for IL-17, there was a general reduction in macrophage-related proinflammatory cytokines (IL-1α, MIP-1α, IL-6, and IL-12) in the transgenic lymphedema mice, suggesting a reduced macrophage infiltration or local cytokine production in the skin.

Altered Distribution of Immune Cells in K14-VEGFR-3-Ig Mice

Our finding that there was a significant reduction of proinflammatory cytokines in the interstitial fluid from hind paws of K14-VEGFR-3-Ig mice led us to determine the number of immune cells in the skin from hind paws. Results from the immunohistochemical analysis (Figure 4) showed a significantly decreased number of macrophages (P<0.05), and a tendency to decreased number of leukocytes (P=0.06) in the dermis and subcutis from hind paws of K14-VEGFR-3-Ig mice compared with the WT (n=6 for both groups).
As a consequence of the reduced number of immune cells in skin, we also investigated relative expression of the monocyte/macrophage lineage marker CD11b, on leukocytes in blood, spleen, and bone marrow (Figure 5). Interestingly, there was an increased fraction of double-positive CD45/CD11b cells in blood (Figure 5A) and spleen (Figure 5B) in K14-VEGFR-3-Ig mice compared with WT, whereas the distribution was similar in the 2 strains in bone marrow (Figure 5C). Together, these observations suggest that the mobilization of immune cells to the skin is reduced in the mutant.

Reduced Lymph Flow in K14-VEGFR-3-Ig

To investigate the role of the missing lymphatics on interstitial protein removal from skin tissue, we used a novel method to quantify the lymphatic function based on washout of fluorescently labeled albumin.12 The intradermal injection of Albumin-Alexa 680 rendered only a small, hardly visible vesicle at the dorsal surface of the hind paw. After an initial distribution phase of 60 minutes, when little solute was cleared, we followed the washout of tracer for 6 hours (Figure 6A). All experiments followed a near-log-linear decrease in the quantity of labeled macromolecule (Figure 6B), indicating stable dye washout. k-values, calculated from the slope of the regression lines for each individual mouse, averaged 0.14±0.02% min–1 in K14-VEGFR-3-Ig mice (n=5), which was significantly lower (P<0.001) than the corresponding k of 0.41±0.03% min–1 found in WT mice (n=5), thus showing that the transgene expression resulted in a lymph flow in skin averaging 34% of that in WT in the lymphedema mice.

K14-VEGFR-3-Ig Mice Have an Exaggerated Pressure Response to Fluid Volume Overload

One of the main functions of the lymphatic vasculature is to remove excessive fluid from the interstitial compartment. To test the lymphatic drainage capacity, we therefore exposed both K14-VEGFR-3-Ig and WT mice to a volume overload of Ringer solution, 15% of body weight during 60 minutes. The relationship between tissue fluid volume and Pif during changes in tissue hydration was evaluated, as previous experiments had suggested an exaggerated response to overhydration in lymphedema.4 Control values were acquired from euvolemic animals. In WT mice, Pif and TTW averaged –0.7±0.1 mm Hg and 1.9±0.1 mL/(g DW), respectively. Corresponding values of Pif and TTW during overhydration were plotted as single observations (Figure 6C). K14-VEGFR-3-Ig mice had a different starting point with control values in a similar area as overhydrated WT mice, Pif and TTW averaging 0.9±0.2 mm Hg and 4.2±0.3 mL/(g DW), respectively. Similar to WT mice, volume expansion in K14-VEGFR-3-Ig mice resulted in marked rise in Pif and TTW (Figure 6C), and both parameters differed from its corresponding value in WT mice (P<0.001 for both comparisons). Whereas the TTW increased to a similar extent in both genotypes, the fluid challenge resulted in a more pronounced increase in Pif in K14-VEGFR-3-Ig (4.7±0.7 mm

Figure 4. Distribution and quantification of macrophages and leukocytes in skin. Sections from wild-type (A and C) and K14-VEGFR-3-Ig (B and D) mice hind paw stained for F4/80 (macrophages) (A and B) and CD45 (leukocytes) (C and D). Arrows indicate positive staining. Bar=20 µm. Image quantifications (E and F) confirm reduction of macrophages and leukocytes in the subcutis of K14-VEGFR-3-Ig mice (white bars). Values are mean±SE; n=6 animals. *P<0.05 compared with WT mice (black bars).
Hg) than in WT (2.5±0.5 mm Hg; \( P<0.05 \)). Of note, 2 of the mutant animals had a very marked increase in volume and pressure on volume loading. Although not as pronounced as in the Chy mice, 4 this response suggests that also the K14-VEGFR-3-Ig mice have an exaggerated \( P_f \) response to volume loading.

**Similar Response in K14-VEGFR-3-Ig and WT Mice to Inflammation**

Because of the exaggerated response in the K14-VEGFR-3-Ig mice to an acute volume load, we decided to test the response to a more chronic perturbation induced by oxazolone application. 16 Challenge by oxazolone resulted in fluid accumulation in WT as well as K14-VEGFR-3-Ig mice, as shown by TTW of 2.9 and 4.7 mL/(g DW), respectively (Figure 6D; \( P<0.001 \)), both values significantly increased from respective control values (Figure IIA in the online-only Data Supplement). Moreover, \( P_f \) increased significantly after oxazolone challenge in WT (0.8±0.2 mm Hg; \( P<0.05 \)) as well as mutant mice (0.6±0.4 mm Hg; \( P<0.01 \); paired \( t \) test; Figure 6E). As in the control situation, \( P_f \) was higher in K14-VEGFR-3-Ig than in WT mice after oxazolone treatment (\( P<0.001 \); 1-way ANOVA), whereas the pressure rise because of treatment was not different. In spite of the increase in TTW and \( P_f \), the lymph flow rate constants were unaffected by the challenge and were similar to their respective controls (Figure III in the online-only Data Supplement). Moreover, the number of leukocytes and macrophages did not differ significantly in WT and K14-VEGFR-3-Ig mice after oxazolone treatment (Figure IV in the online-only Data Supplement), and suggests that this model, when challenged, can respond with a normal T-cell response, 16 in agreement with previous data. 17

**Discussion**

Here, we have evaluated a transgenic mouse model, which because of inhibition of VEGF-3 signaling lacks dermal lymphatics, and studied the pathophysiological consequences
of this phenotype. These mice developed lymphedema in fore paws and hind paws, and had a significantly elevated level of $P_i$, in areas of edema only. Furthermore, a generally increased COP was relative to plasma suggests that the model represents a high-protein lymphedema. Using a novel optical-imaging approach, we could demonstrate that the lymphatic vessel phenotype resulted in a dramatic reduction in washout of injected macromolecules, suggesting that lymph flow is only $\approx$ one third of that in WT mice. In spite of chronic edema, the mice did not develop significant changes in tissue architecture or composition. Much to our surprise, and in contrast to what has been observed in secondary lymphedema in humans,15 this model also had reduced levels of some proinflammatory cytokines in serum and tissue, as well as macrophages and leukocytes in skin. These findings suggest that there is a suppressed rather than increased humoral immune response, and thus reduced immune competence in the lymphedematous tissue, in agreement with a recent study in this model.17

Microcirculatory Consequences of Impaired Lymph Flow

Although the derangement in lymph function in the K14-VEGFR-3-Ig mice is caused by a different mechanism than in the Chy mice, the subsequent changes in interstitial fluid pressure and volume in the control situation were quite similar, although they tended to be somewhat less pronounced in the K14-VEGFR-3-Ig model. Furthermore, the COP was relative to plasma was increased, suggesting that the removal of proteins by lymphatics is impeded, as was actually demonstrated by recording of lymph flow. The reason for the high relative COP is that the COP was significantly reduced because of hypoalbuminemia in the mutant, whereas the COP in the 2 strains. The reason for this finding is not readily evident. It is well known that cytokines affect liver synthesis of albumin (for review see Saini et al18), and as we found that cytokine levels were affected in our model, one possibility is that the low levels of some proinflammatory cytokines affected the liver synthesis of albumin in the K14-VEGFR-3-Ig mice. Interestingly, we could demonstrate that although the K14-VEGFR-3-Ig mice lack initial lymphatics in the dermis, they still possess about one third of the drainage capacity of the WT mice in the lymphedematous paws. Although this reduction is significant, the resulting edema is relatively modest compared with most forms of primary lymphedema in humans, indicating that compensatory mechanisms are activated. These compensatory mechanisms are also active in inflammation, as shown by the modest rise in $P_i$, TTW, and unaltered lymph flow in the induced delayed hypersensitivity reaction.
The lymph flow measurements together with the fact that only the paws present edema indicate that the K14-VEGFR-3-Ig mouse must have an alternative route for removal of filtered interstitial fluid, when dermal lymphatics are missing. Recent data from this mouse model suggest that an increased hydraulic conductivity could counteract the swelling tendency. As observed here, Rutkowski et al. found an increased tissue hydration in the K14-VEGFR-3-Ig versus WT mice, and the tissue composition with respect to collagen was not different in the 2 strains. Actually, in their study, the content of subcutaneous fat was reduced and not similar to WT that may be because of the site studied (tail skin versus paw). The tissue hydraulic conductivity, however, was almost 3-fold higher in the K14-VEGFR-3-Ig mice. Hydraulic conductivity is strongly dependent on hydration, which, in our experiments, was 2- to 3-fold as compared with the modest increase of 10% reported by Rutkowski et al. Thus, even though the lymphatics are missing from the dermis in this model, fluid could more easily be transported to the lymph vessels located in subcutis, which would compensate to keep the edema moderate.

Role of Inflammation in Lymphedema

Tissue collagen and lipid accumulation found in human chronic lymphedema are thought to originate from chronic inflammation induced by a high interstitial protein concentration. The fact that K14-VEGFR-3-Ig mice present with a relative high-protein edema notwithstanding, our results from the cytokine analysis show no signs of inflammation, suggesting that a high protein concentration in the interstitial fluid per se is not sufficient to induce an inflammatory reaction, or the fibrotic and hyperlipemic condition seen in humans. Actually, whereas skin fat increased with age in the WT mice, such a change was not observed in K14-VEGFR-3-Ig mice, showing an opposite effect of what is seen in lymphedema patients. Moreover, several of the proinflammatory cytokines and, in particular, those associated with macrophage function were even reduced in the lymphedema mice. In plasma, levels of IL-1α, associated with transmigration of immune cells, together with MIP-1α, a chemotactic and proinflammatory protein that recruits leukocytes to sites of infection, were reduced. Furthermore, monocyte chemotactic protein-1 that has a similar function as MIP-1α, was found at lower levels, suggesting that macrophage migration may be affected in K14-VEGFR-3-Ig mice. This assumption is supported by flow cytometry data showing accumulation of cells of monocyte/macrophage lineage in blood and spleen, and also of recent data showing reduced dendritic cell trafficking in this model. In interstitial fluid from hind paw, IL-6, a typical proinflammatory cytokine released mainly by monocytes and macrophages, was found at reduced concentrations, as was IL-2, a potent T-cell growth factor produced mainly by activated T-lymphocytes. The most pronounced difference concerned the IL-12 levels in hind paw skin interstitial fluid. IL-12 is a proinflammatory cytokine, produced by dendritic cells and macrophages, that during infection induces the production of interferon-γ, favors the differentiation of T-helper 1 cells and forms a link between innate resistance and adaptive immunity. Our findings of reduced inflammation are contrasting recent data from Avraham et al. In a mouse model of secondary lymphedema in the tail, they found increased fibrosis and a T-helper 2 cell-based inflammatory reaction mediated via TGF-β in lymphedematous tissue. They suggested that these reactions resulted from lymph stasis, whereas our data show that lymph stasis per se does not induce inflammation. An alternative explanation could be that a postoperative reaction to skin resection in the lymphedema tail model, combined with the lymph stasis, could induce the observed changes.

Considering the reduced levels of proinflammatory cytokines discussed above, the finding of an elevated level of IL-17 in paw skin was somewhat surprising, as the differentiation of naive T-cells to IL-17-producing T-helper 17 cells is induced by IL-1 and IL-6 that were found to be downregulated in hind paw skin. This observation may, however, be a consequence of the fact that IL-2, a known inhibitor of IL-17 production, was reduced in lymphedematous skin. Taken together, the cytokine analysis data may suggest that there is an impaired macrophage and dendritic cell function in the K14-VEGFR-3-Ig lymphedema model.

Input of dendritic cell-bound and -unbound antigenic material to lymph nodes from nonlymphoid tissue like skin through afferent lymphatics is a fundamental element in immune surveillance and homeostasis. Such transport is slow in steady state, but increases dramatically during inflammation and is a central element in the initiation of an immune response. Accordingly, the lack of dermal lymphatics and the associated reduction in lymph flow in our lymphedema model may result in a reduced antigen presentation in the lymph nodes and a reduced host immune defense that is even discernible in steady state. This assumption is supported by a markedly diminished skin dendritic cell migration and impaired humoral immunity observed by others in models with reduced lymph flow, and the marked suppression of cytokines observed in our study.

Summary and Implications

The K14-VEGFR-3-Ig lymphedema model may be used to further our understanding of the pathophysiology of this condition. Like the Chy model, the K14-VEGFR-3-Ig model lacks lymphatics in the dermis, resulting in a high hydrostatic pressure and COP in interstitial fluid. For the first time, we have managed to quantify the effect on lymph flow of the engineered lymphatic vessel defect, and found that it decreased lymph flow to one third of that in corresponding tissues of the WT mice. Although it has been assumed that a high protein concentration in interstitial fluid induces inflammation, fibrosis, and lipid accumulation, our finding of normal or reduced levels of proinflammatory cytokines, and normal levels of lipids and collagen suggest that a high protein concentration per se, or longstanding edema, is not sufficient to induce the mentioned changes in tissue architecture. Our findings of low levels of proinflammatory cytokines in interstitial fluid from lymphedematous limbs as well as reduced number of macrophages in the tissue suggest a reduced host defense capability in the affected regions. Although the K14-VEGFR-3-Ig mouse is an interesting experimental model for lymphedema, it fails to mimic the severe symptoms of chronic lymphedema in humans, making it less suitable for studies of pathophysiology of lymphedema in its advanced stages, involving changes in tissue architecture.
Sources of Funding

Financial support from The Research Council of Norway, The Norwegian Cancer Society, and The Western Norway Regional Health Authority is gratefully acknowledged. CEM was a recipient of a PhD-stipend from The Norwegian Council on Cardiovascular Diseases.

Disclosures

None.

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Arterioscler Thromb Vasc Biol. 2013;33:266-274; originally published online January 3, 2013;
doi: 10.1161/ATVBAHA.112.300384

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

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In the article by Markhus et al, which appeared in the February 2013 issue of the journal (*Arterioscler Thromb Vasc Biol*, 2013;33:266–274. DOI: 10.1161/ATVBAHA.112.300384), author “M. Wagner” should have appeared as: Marek Wagner. The online version of the article has been corrected.
Increased interstitial protein due to impaired lymph drainage does not induce fibrosis and inflammation in lymphedema

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Methods

Animals
We used the K14-VEGFR-3-Ig transgenic mouse model of congenital lymphedema as described in detail by Mäkinen et al on a C57Bl/6 background. Offspring were genotyped by PCR analysis, using 5’-GAA AGC CCA AAA CAC TCC AAA CAA TG-3’ as the forward and 5’-TCC TTG TCT CCG GTG GCT GGC G-3’ as the reverse primer. Mice were of both sexes, and wt littermates were used as controls. They were anesthetized with a mixture of Ketamine (12.2 mg/ml; Ketalar, Pfizer, New York, NY) and medetomidine (24.3 µg/ml; Domitor, Orion Pharma, Espoo, Finland) in saline, 0.2–0.3 ml given subcutaneously. The depth of the anesthesia during the experiments was determined by testing the withdrawal reflex of the paws by applying a pinch between the toes. Supplemental doses of anesthesia (0.05 – 0.1 ml) were added when necessary. In the experiments where we sampled blood for flow cytometry or quantified lymph flow, mice were anesthetized with isoflurane in a combination with O₂ and N₂O. Cardiac arrest was induced while under anesthesia with an intravenous or intracardiac injection of an overdose anesthetic or by cervical dislocation. All experiments were performed in accordance with recommendations given by the Norwegian State Commission for Laboratory Animals and were approved by the local ethical committee (Approval # STFDU 1221).

Interstitial Fluid Pressure Measurements
Interstitial fluid pressure was measured by micropuncture technique as described in detail previously. A sharpened glass capillary (diameter; 4-7 µm) connected to an automatic counter-pressure system was inserted in thigh skin and muscle and in the fore paw, hind paw and back skin of anesthetized mice. Measurements in skin were performed through intact skin, while thigh muscle was exposed by a skin incision prior to measurements and covered with a drop of paraffin to prevent evaporation.

Sampling of Interstitial fluid
Interstitial fluid was sampled from thigh muscle by the wick the implantation method and from skin by a tissue centrifugation. All procedures and sample handling were performed in a humidity chamber (100% relative humidity) to avoid evaporation from skin and collected interstitial fluid.

Interstitial Fluid Volume and Total Tissue Water
Interstitial fluid volume (IFV) was measured as the 90 min extravascular distribution volume of ⁵¹Cr-EDTA (MW 341 Da) after neprectomy as detailed in a previous publication, whereas the intravascular volume was determined as the 5 min distribution volume of ¹²⁵I-labeled human serum albumin (¹²⁵I-HSA, Institute for Energy Technology, Kjeller, Norway). Tissue samples were excised from fore paw, hind paw, thigh and back skin and from thigh muscle. Interstitial fluid volume was calculated as the difference between extracellular and intravascular volume. Total tissue water was obtained as the difference between tissue wet and dry weight after drying the samples until constant weight.

Experimentally Induced Overhydration
Surgical procedures were performed as described above. To induce fluid volume expansion, Ringer solution, corresponding to 15% of body weight, was infused intravenously over a period of 60 minutes. 90 minutes after infusion start, Py was
measured, the mice were sacrificed and samples harvested for total tissue water
determination as described above.

**Colloid Osmotic Pressure (COP)**
Interstitial fluid (1-5 µl) and serum (5 µl) were analyzed on a colloid osmometer
designed for submicroliter samples 7 equipped with a PM 30 ultra-filtration membrane
(Millipore; molecular mass cutoff, 30 kDa).

**Analysis of serum albumin concentration**
The distribution of macromolecules in plasma was determined by size exclusion
chromatography as described previously 8. We used two 4.6 mm ID x 30.0 cm
TSKgel Super SW3000 columns coupled in series (Tosoh Biosciences, Stuttgart,
Germany) with an optimal separation range for globular proteins of 10-500 kDa. The
albumin concentration in the elution fluid was measured by UV detection at 220 nm
on a Ettan™ LC System (GE Healthcare) and the buffer/mobile phase was 0.1M
Na$_2$SO$_4$ in 0.1M phosphate buffer pH 6.7 – 7.0. Samples were diluted 1:100 in mobile
phase buffer, and 20 µL were injected on the HPLC system for separation and
analysis.

**Tissue Lipid Analysis**
Determination of tissue lipid content in hind paw and back skin together with thigh
muscle was done by a chloroform-methanol extraction technique 9. Skin and muscle
specimens were freeze-dried for 1 week, weighed and put in glass tubes with a 4 ml
mixture of chloroform and methanol (2:1) for 16 h. After adding 1 ml of chloroform
and 1 ml dH$_2$O, glass tubes were mixed and left standing for 2 h. The solvent fraction,
containing the lipids, was removed and the tissue specimen was freeze-dried again to
obtain fat-free dry weight and fat content. Tissue samples were then further processed
for determination of collagen content.

**Collagen Content**
Tissue collagen was quantified according to the spectrophotometric method described
by Woessner 10 based on determination of hydroxyproline content as described in a
previous publication 11. The absorbance was read at 557 nm on a Spectramax pluss
384 spectrophotometer (Molecular Devices, Sunnyvale, CA). Hydroxyproline
concentration was quantified by comparison with a standard curve of L-4-
hydroxyproline (Fluka Chemie GmbH, Buchs, France) in 1 mM HCl. The collagen
values were calculated assuming a 6.94 to 1 collagen to hydroxyproline ratio 12.

**Multiplex Cytokine Assay Analysis**
In order to investigate a possible inflammatory pathogenic role in lymphedema, a
panel of cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, G-CSF, GM-
CSF, GRO-KC, IFNγ, IP-10, MCP-1, MIP-1α, RANTES and TNFα) was quantified
using a mouse multiplex fluorescent bead immunoassay kit (Linco Research Inc, St.
Charles, MI, USA) according to the manufacturers’ instructions. Total surface
fluorescence was measured with a flow-based dual laser system (Luminex 100,
Luminex Corporation, Austin, TX, USA).
Double immunofluorescence staining for CD31 and LYVE-1 in intact hind paw

Frozen samples of intact hind paw were cut into 10 µm sections using a cryostat. Sections were fixed in ice-cold acetone for 10 min and blocked with 10% chicken serum in PBS for 1 hour in room temperature (RT). To detect both lymphatic endothelial cells and blood endothelial cells the sections were incubated overnight at 4°C in a mixture of rabbit polyclonal LYVE-1 antibody (1:400, RELIATech, Braunschweig, Germany) and rat polyclonal CD31 antibody (1:50, BD Pharmingen, San Diego, CA). Thereafter sections were incubated for 1 hour in RT with a mixture of the corresponding secondary antibodies; Alexa Fluor 488-conjugated chicken anti-rabbit and Alexa Fluor 596-conjugated chicken anti-rat secondary antibody (Both 1:200, Molecular Probes, Carlsbad, CA). Finally, the sections were mounted with DAPI mounting medium (Vector Laboratories, Burlingame, CA, USA).

Staining for immune cells

Intact hind paws were surgically removed and demineralized in ethylenediaminetetraacetic acid (EDTA) for 14 days. All tissue samples were rinsed in 0.1 M phosphate buffer, soaked in 30% sucrose solution overnight for dehydration and then stored at -80°C until sectioning. Sections (8 µm) from intact paws were mounted onto poly-L-lysine-coated glass slides and dried overnight. Next day sections were fixed in acetone for 10 minutes, dried for 15 minutes and treated with 0.3% hydrogen peroxide in 90% methanol for 30 min. Sections were incubated for 1 hour in 2.5% rabbit serum (Vector Vectastain Elite ABC Staining Kit, Vector Laboratories Inc.) in 0.5% PBS-TX to prevent unspecific binding. To visualize immune cells sections were stained with an antibody against common-leukocyte antigen CD45 (Abcam) or macrophage antigen F4/80 (Abcam) with the avidin-biotin complex (ABC) method using commercially available kit (Vector Vectastain Elite ABC Staining Kit, Vector Laboratories Inc.) together with 3,3’-diaminobenzidine (DAB). Sections were incubated overnight at 4 °C with mouse CD45 (1:200 dilution) or F4/80 (1:200 dilution) monoclonal antibodies raised in rat. Following PBS rinses, the sections were incubated with secondary antibody (1:200 dilution, Vector Vectastain Elite ABC Staining Kit, Vector Laboratories Inc.) for 1 hour. After PBS rinses sections were incubated with the avidin-biotin complex for 1 hour. To visualize bound antigen sections were incubated with DAB for 10 minutes. Nickel was added to the solution to intensify DAB staining. Sections were counterstained with Richardson’s Stain. Macrophages and leukocytes were counted in 100 x 100-µm fields of 400x magnification.

Flow cytometry

In animals anesthetized with isofluorane, blood was obtained by orbital puncture and collected into a VACUTAINER® tube with EDTA anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Mice were killed by cervical dislocation and spleens were excised and squeezed through a 70µm mesh cell strainer (BD Bioscience) to obtain single cell suspension. Bone marrow was flushed out of the femur using 25G needle and a syringe. All samples were collected in Dulbecco’s PBS (Gibco) with 2mM EDTA and stored on ice. Red blood cells were lysed in RBC Lysis Buffer according to the manufacturer’s protocol (BD Pharmingen). Cells were counted and re-suspended in 100µl of flow staining buffer (1% BSA v/v, 0.5M EDTA in PBS). Cells were blocked with Fc-blocking reagent (Miltenyi Biotec) to prevent nonspecific binding, labeled with mouse CD45-PE-Cy5™ (1:100 dilution, BD Pharmingen) and
mouse CD11b-FITC (1:50 dilution, BD Pharmingen) antibodies for 30 min at 4°C, washed three times and then acquired on Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA), and analyzed using CFlow Software provided with the cytometer.

Quantification of Lymph Flow
Lymphatic drainage from hindlimb skin was quantified directly by optically monitoring the depot clearance of near-infrared labeled albumin and subsequent calculation of removal rate constants (k), as described previously. Briefly, 0.5 µl Alexa 680-conjugated bovine serum albumin (Invitrogen) was injected intradermally with a graded Hamilton syringe (34 G), and the mice were imaged macroscopically every 60 min for a 6-hour period. Mice were only anesthetized (1% isoflurane) during imaging and remained awake during measurements. For calculations of depot clearance rates (k), the natural logarithm of the fractional amount of counts remaining at the injection site was plotted against time. The resultant monoexponential curves were fitted with linear regression, and k found as the slope of each curve. For imaging we used an Optix® MX system (GE Healthcare, Munich, Germany) with a fixed pulsed laser diode as an illumination source with an excitation wavelength at 670 nm with a 80 MHz repletion rate. A photomultiplier tube detector with a 700 nm long-pass filter collected the fluorescent signal from the specimen. Images were analyzed using eXplore Optix Optiview software (GE Healthcare), and the number of counts calculated for each region of interest.

Induction of skin inflammation
A delayed type hypersensitivity inflammatory reaction was induced by topical application of oxazolone as described by Kunstfeld et al in the hind paw of K14-VEGFR3-Ig and WT mice. In these experiments mice were sensitized by topical application of a 2% oxazolone (4-ethoxymethylene-2 phenyl-2-oxazoline-5-one; Sigma, St Louis, MO) solution in acetone/olive oil (4:1 vol/vol) to the closely clipped abdominal skin (50 µl) and to each forepaw (5 µl) after control measurements of Pif in the hind paw. Five days after sensitization, both hind paws were challenged by topical application of 10 µl of a 1% oxazolone solution. Two days after oxazolone challenge, lymph flow was measured in the hind paw followed by recording of Pif. Thereafter, the experiment was terminated and hind paw skin was harvested for determination of total tissue water serving as an indicator of the degree of inflammation.

Statistical Methods
Results are presented as Means ± SE. Statistical significance was evaluated using paired and unpaired Student’s t-tests or One-way Analysis of Variance (ANOVA) as appropriate. A value of P < 0.05 was considered statistically significant.

References
Supplemental figures

Supplemental Figure I

**Supplemental Figure I.**
Lipid and collagen content in hind paw, back skin and thigh muscle in K14-VEGFR-3-Ig (white bars) and wild type (black bars) mice of adult (3 months) and old (12 months) mice. Except for reduced lipid content in K14-VEGFR3-Ig mice in old mice, lipid content in young (A) and old (B) mice showed no significant differences between the K14-VEGFR-3-Ig and wt mice. Collagen content in skin was similar in the two strains in young (C) as well as old (D) mice. Values are mean ± SE. *P = 0.01.
Supplemental Figure II

A: Total tissue water in fore and hind paw, back and thigh skin and skeletal muscle in K14-VEGFR3-Ig (white bars) and wt mice (black bars). Total tissue water was significantly higher in fore and hind paw of K14-VEGFR-3-Ig mice compared with wt. Values are mean ± SE. *P < 0.05 and ***P < 0.001. B: Interstitial fluid pressures (Pif) in skin from fore paw, hind paw, back and thigh, and skeletal muscle in old (12 months) K14-VEGFR3-Ig (open symbols) and wt (filled symbols) mice. Pif was significantly higher in fore paw and hind paw skin of old K14-VEGFR-3-Ig mice (B). Values are mean ± SE. *P < 0.05 and ***P < 0.001
Supplemental Figure III

Supplemental Figure III.
Washout of an intradermal injection depot of fluorescent Albumin-Alexa® 680 (0.5 µl) in paw skin of K14-VEGFR-3-Ig (K14) and wild type (WT) mice after induction of delayed hypersensitivity reaction by oxazolone application. Average Log<sub>e</sub> fraction of counts remaining in intradermal injection depot of fluorescent albumin in paw skin of K14-VEGFR-3-Ig (open symbols) and WT (closed symbols) in control (circles) and oxazolone treated (triangles) mice recorded at various time points after injection. Values are mean ± SE, n = 6. Treatment did not result in any statistical difference in the removal rate constant k for any of the genotypes, but k was significantly different between the genotypes in control (p<0.001) (*) and after oxazolone treatment (p<0.001) (†) (One-way ANOVA).
Supplemental Figure IV

Quantification of leukocytes (CD45 positive cells) (panel A) and macrophages (F4/80 positive cells) (panel B) in skin after inflammatory challenge using oxazolone in sensitized wild type and K14-VEGFR-3-Ig mice. Values are mean ± SE, n = 3 animals/group.