Growth Factor Therapy and Autologous Lymph Node Transfer in Lymphedema

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Background—Lymphedema after surgery, infection, or radiation therapy is a common and often incurable problem. Application of lymphangiogenic growth factors has been shown to induce lymphangiogenesis and to reduce tissue edema. The therapeutic effect of autologous lymph node transfer combined with adenoviral growth factor expression was evaluated in a newly established porcine model of limb lymphedema.

Methods and Results—The lymphatic vasculature was destroyed within a 3-cm radius around an inguinal lymph node. Lymph node grafts and adenovirally (Ad) delivered vascular endothelial growth factor (VEGF)-C (n=5) or VEGF-D (n=9) were used to reconnect the lymphatic network in the inguinal area; AdLacZ (β-galactosidase; n=5) served as a control. Both growth factors induced robust growth of new lymphatic vessels in the defect area, and postoperative lymphatic drainage was significantly improved in the VEGF-C/D–treated pigs compared with controls. The structure of the transferred lymph nodes was best preserved in the VEGF-C–treated pigs. Interestingly, VEGF-D transiently increased accumulation of seroma fluid in the operated inguinal region postoperatively, whereas VEGF-C did not have this side effect.

Conclusions—These results show that growth factor gene therapy coupled with lymph node transfer can be used to repair damaged lymphatic networks in a large animal model and provide a basis for future clinical trials of the treatment of lymphedema.

Key Words: lymphangiogenesis ■ lymphatic vessels ■ vascular endothelial growth factor C ■ vascular endothelial growth factor D

The lymphatic vasculature plays a key role in the maintenance of tissue fluid homeostasis by collecting extravasated fluid and macromolecules back to the blood circulation.1 The lymphatic system also has a major role in immune defense. Therefore, lymphatic vessels and lymph nodes are involved in several human diseases such as lymphedema, inflammation, and tumor metastasis.2 The lymphatic capillaries in the peripheral tissues merge with larger collecting lymphatic vessels, specialized for the transport of large volumes of lymph, which in turn connect with chains of lymph nodes. Chronic lymphedema, caused commonly by infection and surgical or radiation therapy of metastatic cancer, remains a common clinical problem that lacks curative treatment options. The effective treatment of cancer often requires removal of regional lymph nodes and the associated collecting lymphatic vessels to eradicate metastases. This leads to a disruption in the lymphatic flow at the operated area, which frequently leads to lymphedema of the affected limb. The conventional treatment for chronic lymphedema aims at alleviating the symptoms and is based mainly on physiotherapy and/or controlled compression therapy, whereas current surgical treatment options are limited.3–5 This is due chiefly to difficulties in identifying and preserving the lymphatic vessels even by modern microsurgical methods. Recently, microvascular lymph node transfer into axillas of patients who had undergone axillary lymph node dissection in response to disseminated breast cancer was shown to improve lymphatic drainage in some patients.6

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Understanding of the molecular mechanisms of lymphangiogenesis has increased considerably in recent years.2,7 Vascular endothelial growth factors (VEGFs) are important regulators of angiogenesis and lymphangiogenesis.2,8 VEGFs

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stimulate cellular responses by binding to tyrosine kinase receptors (VEGFRs) that are specifically expressed in blood and lymphatic endothelial cells that line the luminal surface of vessels. VEGF-A binds to VEGFR-1 and VEGFR-2 and induces mainly angiogenesis. VEGF-C and VEGF-D signaling induces lymphangiogenesis via VEGFR-3.9,10 However, the proteolytically processed short forms of both VEGF-C and VEGF-D also bind to VEGFR-2 and have blood vascular effects in some tissues.9,11–14

Growth factor therapies with VEGF-C or VEGF-D have shown promising effects in small animal models of lymphedema.12,15–19 Results from a mouse model demonstrated that a damaged lymphatic network can be repaired with growth factor therapy and lymph node transfer.19 Although lymph node transfer without growth factor therapy has been shown to provide at least some benefit in human lymphedema patients,6 autologous lymph nodes incorporate into existing lymphatic vasculature at a low frequency (22% to 31%).6,19 This poor efficiency may compromise the outcome of the operation because connection with lymphatic vessels is required for maintenance and function of the lymph nodes.20

The aim of the present study was to evaluate the potential of combined lymph node transfer and viral growth factor expression for the treatment of lymphedema in a large animal model. Combination therapy was chosen because, in the clinical situation, patients with a history of malignant disease and recurrent infections could benefit from both functional lymphatic vessels and lymph nodes. The efficacy of the 2 adenovirally overexpressed lymphangiogenic growth factors, VEGF-C and VEGF-D, was evaluated with a newly established porcine model.

**Methods**

**Porcine Lymphedema Model**

All porcine experiments were performed with domestic pigs (National Laboratory Animal Center, Kuopio, Finland) weighing 28 to 32 kg and were approved by the Finnish Experimental Animal Committee. Unlike the chains of lymph nodes found in the inguinal region of human patients, the pig has 1 superficial lymph node located in the inguinal ring area of each hind limb. The collecting lymphatic vessels in the thigh are connected to this lymph node, and lymphatic vessels from this node drain proximally into the paraaortic plexus (Figure 1). To block lymphatic drainage from the lower limb, pigs were sedated with an intramuscular injection of 1.5 mL atropine (1 mg/mL; Leiras, Helsinki, Finland) and 6 mL azaperone (Stresnil 40 mg/mL; Janssen-Cilag, Wien, Austria) and operated on under general propofol (Propofol-Lipuro 20 mg/mL; B. Braun, Frankfurt, Germany) anesthesia introduced intravenously at 15 mg · kg⁻¹ · h⁻¹, combined with analgetic phentanyl 10 mg · kg⁻¹ · h⁻¹ (Fentanyl; Janssen-Cilag) infusion, as follows. We injected 0.3 mL Patent Blue tracer dye (Guerbet, Villepinte, France) intradermally into the right distal hind limb to visualize the lymphatic vessels. A small collecting lymph vessel in the calf region was then cannulated, and Lipiodol radiography tracer (Guerbet) was injected to the lymph vessel. A 10-cm skin incision was made into the inguinal ring area, and the superficial lymph node was located. The vascular pedicle (artery and vein) coming from the femoral vessels to the lymph node was identified, and the inguinal lymphatic network was then destroyed by excising a 3-cm piece of the collecting lymphatic vessels draining both distally and proximally from the node (Figure 1). Perioperative Lipiodol x-ray lymphangiography was used to visualize the lymphatic vessel network in the inguinal area to ensure that the defect of the lymphatic network in the inguinal area was complete and of standard width (3 ± 3 cm). The blood vascular pedicle was left intact to simulate microsurgical Anastomosis of blood vessels to the lymph node. The contralateral (left) lymph node was left intact.

After the lymphatic vessels were destroyed, 1 × 10¹⁰ viral particles of adenoviral vectors (Ad) encoding full-length VEGF-C (5 pigs), VEGF-D (6 pigs), or control AdLacZ gene (5 pigs) were injected subcapsularly into the exposed lymph nodes.21,22 Because of pronounced seroma fluid accumulation at this dose of AdVEGF-D, a viral dose of 1 × 10¹⁰ viral particles (3 animals) was also evaluated. The pedicular lymph node was then attached into the remaining tissue 4 cm lateral from its original position. This procedure mimics lymph node transfer in human lymphedema patients, in which the lymph node graft is vascularized but lacks all lymphatic vessel connections. Wounds were closed with 4–0 Polysorb sutures (Co-
vidien, Dublin, Ireland). Postoperative analgesia (Rimadyl, Pfizer, New York, NY; and Temgesic, Merck/Schering-Plough, Haar, Germany) and prophylactic antibiotics (Zinacef, GlaxoSmithKline, Brentford, Middlesex, UK) were used.

Postoperative Follow-Up

The animals were observed for 2 weeks postoperatively by an observer blinded to the experimental groups to evaluate accumulation of seroma fluid in the operation area. All animals were inspected at the same time, and the amount of fluid was visually estimated in deciliters. If the estimated amount exceeded 2 L, a value deemed harmful by the animal facility veterinarian, the fluid collections were drained with a sterile needle. Samples of the seroma fluid were collected from all animals at day 11, and the total amount of seroma was measured.

The lymphatic vessel function in the limb and inguinal area was analyzed at 2 months postoperatively by following the transport of Evans Blue/Patent Blue dye mix (method described below) and by Lipiodol x-ray lymphangiography in deep anesthesia. Afterward, the animals were euthanized and tissue samples were collected for histology and Western blot analysis.

Viral Transduction of Cultured Cells

To compare expression levels of the viral vectors, human umbilical vein endothelial cells were plated into 6-well plates (150 000 cells per well) and transduced in triplicate with adenoviruses encoding VEGF-C, VEGF-D, or β-galactosidase (LacZ) with a multiplicity of infection (MOI) of 10 and 50. The cells were washed with Hank balanced salt solution (Invitrogen, Carlsbad, CA) 12 hours after transduction and fed with fresh growth medium. Cell culture mediums were harvested 72 hours after transduction. The same viral lots as in the in vivo animal studies were used.

Enzyme-Linked Immunosorbent Assay

VEGF-C and VEGF-D protein levels were measured from the harvested cell culture medium by use of the appropriate ELISA kits (catalog No. DVECO0 and DVED00, respectively; R&D Systems, Minneapolis, MN). Results were plotted on standard curves generated by use of samples supplied with the kits, and the values were converted to nanograms per milliliter.

Western Blot

Snap-frozen seroma fluid samples from the 1×10^{11} viral particles dose group animals were thawed and diluted to electrophoresis sample buffer, separated in a 12% polyacrylamide gel, transferred to nitrocellulose membrane, immunblotted, and detected with commercially available VEGF-D and VEGF-C antibodies (AF-286 and AF-752, respectively; R&D Systems) plus horseradish peroxides–commercially available VEGF-D and VEGF-C antibodies (AF-286 and AF-752, respectively; R&D Systems) plus horseradish peroxides–commercially available VEGF-D and VEGF-C antibodies (AF-286 and AF-752, respectively; R&D Systems) plus horseradish peroxides–commercially available VEGF-D and VEGF-C antibodies (AF-286 and AF-752, respectively; R&D Systems) plus horseradish peroxides–

Lymphatic Vessel Imaging and Evaluation for Lymphatic Function in the Porcine Model

X-ray lymphangiographies were recorded with a Siemens Siremobil 3000 C-arm (Siemens, Berlin, Germany) x-ray machine during Lipiodol injection before the operation, after the operation, and at time of euthanasia. The total number of the Lipiodol-positive lymph vessels in the hip region (anatomic size, 40 cm²) was counted from x-ray images taken at the time of death.

To quantify lymphatic vessel flow postoperatively, a mix of 0.5 mL of Patent Blue (Guerbet) and 0.5 mL of 3% Evans Blue was injected distally into the hind limb, and serum samples were collected from the tail vein 13 minutes after the injection.15 Formamide (4 mL) was added to the samples, and the samples were incubated for 48 hours at 60°C. The amount of the marker dyes absorbed into the formamide was then measured from the serum with a spectrophotometer at 670 nm.

Tissue Samples

The sizes of the operated (right side) and nonoperated (left side) porcine inguinal lymph nodes were measured in 3 dimensions. Tissue samples from the lymph nodes and the surrounding tissues of the thigh were collected. The structure of the lymph nodes and the lymphatic vasculature of the lymph nodes and surrounding soft tissues were assessed by a pathologist blinded to the study.

Histochemistry and Immunohistochemistry

Porcine tissue samples were fixed and embedded in paraffin, and 7- and 2-μm sections were cut and stained with hematoxylin-eosin or the chromogen diaminobenzidine (Vector Laboratories, Burlingame, CA) after platter endothelial cell adhesion molecule-1 (PECAM-1; R&D Systems), lymphatic vessel endothelial HA receptor (LYVE-1; R&D Systems), Prospero homeobox protein 1 (PROX-1; R&D Systems), dendritic cell (CD23; DakoCytomation, Denmark, Glostrup, Denmark), B-cell (CD20; DakoCytomation, Denmark), or T-cell (CD3; DakoCytomation, Denmark) immunostaining. PECAM-1 (with the Vector Blue; Vector Laboratories) and α-smooth muscle actin (α-SMA; Sigma-Aldrich, St Louis, MO) double staining was used to identify blood vessels. PROX-1/α-SMA double staining was used to evaluate the amount of collecting lymphatic vessels. Vessel areas were calculated in the lymph nodes and surrounding tissue from the microscopic images of PECAM-1/α-SMA and PROX-1/α-SMA double-immunostained sections with AnalysSIS (Olympus Soft Imaging Solutions, Münster, Germany).

Statistics

Results are expressed as mean±SD. Data from individual experiments were plotted to a quantile (Q-Q) plot and residuals of data to a histogram to determine whether data approximately follow normal (approximate gaussian) distribution. Data in Figure 2A were determined not to follow normal distribution. Normally distributed data were analyzed with PASW statistics 18.0 (IBM Corp, Somers, NY) and tested with 1-way ANOVA followed by a Dunnett posthoc test and P value correction for multiple comparisons, except for Figure 2E, in which only 2 groups were tested against each other with an independent-samples Student t test and no correction for multiple comparisons was made, and Figure 3C, in which data were tested with 1-way ANOVA followed by a pairwise comparison and Bonferroni correction for multiple comparisons. For the data in Figure 2A, statistical analyses were performed with R Project for Statistical Computing version 2.12 and nonlinear mixed-effects R-package version 3.1–97. Linear mixed-effect models were used to analyze group differences at baseline and during follow-up. Volume was used as a quantitative dependent variable, and volume data were transformed to a logarithmic scale to account for nonnormal distribution. Sample identifiers were included as a grouping random effect. An interaction term between group and follow-up time point (1 to 10 days after treatment) was used to examine group-related changes during follow-up. The AdLacZ group was used as a reference group in comparisons of group differences. Data were analyzed only at 1 to 10 days so that fluid drainage at day 11 would

Vascular Permeability Assay

To explain the effects of VEGF-C and VEGF-D on postoperative seroma formation, a vascular permeability assay in mice was performed. The mouse experiments were approved by the Finnish Experimental Animal Committee. The right hind limbs (m. gastrocnemius) of C57/Bl mice were injected with AdVEGF-C, VEGF-D, or β-galactosidase (LacZ) with a multiplicity of infection (MOI) of 10 and 50. The cells were washed with Hank balanced salt solution (Invitrogen, Carlsbad, CA) 12 hours after transduction and fed with fresh growth medium. Cell culture mediums were harvested 72 hours after transduction. The same viral lots as in the in vivo animal studies were used.

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Results

AdVEGF-D Gene Transfer Causes Increased Seroma Formation Through Increasing Vascular Permeability

The porcine lymphedema model was used to compare the effects of AdVEGF-C or AdVEGF-D gene transfer in combination with lymph node transfer (Figure 1A). Variation in preoperative lymph node size between the groups was not significant (data not shown). Total excision of the proximal and distal lymph vessels from an area of 3-cm radius surrounding the lymph node was achieved, leaving the lymph node without distal-to-proximal lymphatic circulation as visualized by Lipiodol lymphangiography (Figure 1B). The surgical wound and excision of the lymphatic vasculature resulted in seroma fluid formation in the inguinal region. The accumulation of fluid started immediately after the operation, and differences between the groups were observed 7 days later (Figure 2A). The amount of fluid in the VEGF-D–treated animals continued to rise for 2 weeks after the operation. Fluid volumes in VEGF-C–treated and control pigs were at comparable levels, and seroma collections had dissipated in these groups by 2 weeks (P<0.05 for VEGF-C versus LacZ; n=5 for both). There was a statistically significant increase in fluid formation in the VEGF-D–treated pigs compared with LacZ control (P=0.00005 for VEGF-D; n=6 for VEGF-D, n=5 for LacZ; Figure 2A). The VEGF-D–treated pigs also required fluid drainage, whereas in the other groups, the fluid dissipated spontaneously.

To explain the differences in seroma fluid accumulation, the viral vector constructs were tested for differences in protein expression levels in human umbilical vein endothelial cell culture. ELISA analysis of the culture mediums indicated that with a low MOI (MOI 10), protein levels in both AdVEGF-C and AdVEGF-D were very similar, whereas with a higher MOI (MOI 50), AdVEGF-C induced slightly higher (1.5-fold) levels of expressed protein compared with AdVEGF-D (Figure 2B). A new series of pigs was operated on, transduced with a 10-fold-lower dose of AdVEGF-D (1×10^10 viral particles), and followed up for seroma fluid drainage and required fluid drainage 2 weeks after treatment (P=0.00004 for VEGF-D low dose versus LacZ; n=5 for LacZ, n=3 for VEGF-D low dose; Figure 2A).

Because differences in viral dose levels did not account for the differences in seroma buildup, the proteolytic processing of the secreted growth factors was studied. The fully processed short forms of VEGF-C and VEGF-D can induce angiogenesis and increase blood vessel permeability.9,11,13,14 To explain the effect of VEGF-D on fluid accumulation, Western blot analysis of the seroma fluid was carried out. The top band represents the full-length form; the lower band, the mature form. P indicates positive control; L, LacZ; C, VEGF-C; and D, VEGF-D. E, Densitometric analysis of the Western blots to compare the amount of mature (short-form) protein relative to the full-length form (3 samples per group in triplicate). F, Mean blood vessel area in the operated lymph nodes and surrounding tissue in sections immunostained for PECAM-1/aSMA (VEGF-D, n=6; others, n=5). G, Vascular permeability assay in indicated study groups (n=5). *P<0.05.
not in the AdVEGF-C– or AdLacZ-transduced pigs (Figure 2D). The amounts of processed short forms were compared with the amounts of full-length forms in Western blot densitometry. The ratios of short processed versus full-length forms were 0.53±0.11 for VEGF-D and 0.30±0.025 for VEGF-C, indicating that VEGF-D was more prominently processed into its shorter isoform (P=0.03; n=3 in triplicate).

To account for the angiogenic effects of the proteolytically processed forms of AdVEGF-C and AdVEGF-D, mean blood vessel areas were calculated from the PECAM-1/α-SMA–immunostained sections. A 2-fold increase in mean blood vessel area was observed in the AdVEGF-D group compared with the AdLacZ group (P=0.017), whereas AdVEGF-C did not stimulate angiogenesis (P=0.814; Figure 2F). To further determine the effect of AdVEGF-D and AdVEGF-C on vessel permeability, a modified Miles assay was performed. AdVEGF-D, AdVEGF-C, or AdLacZ viruses were injected into the right hind limb (m. gastrocnemius) of C57Bl/6 mice, and extravasation of the Evans Blue dye was measured at day 6. On average, AdVEGF-D resulted an 8.9-fold increase in extravasation compared with control AdLacZ (P<0.001; n=5), whereas the leakage of the Evans Blue in the AdVEGF-C–treated hind limbs was on average 3.5-fold greater than in the AdLacZ–transduced control hind limbs, although this difference failed to reach statistical significance (P=0.135; n=5; Figure 2G).

**Both VEGF-C and VEGF-D Gene Transfers Induce Growth of Functional Lymphatic Vasculature**

Lymphatic vessel function in the inguinal area was studied by Lipiodol lymphangiography 2 months after the operation and gene transfer. Both VEGF-C and VEGF-D induced a statistically significant increase in the number of lymphatic vessels compared with control pigs (16.4±2.8 vessels, P<0.001 for VEGF-C; 13.6±3.6 vessels, P=0.002 for VEGF-D; n=6 for VEGF-D, n=5 for VEGF-C) as calculated from the lymphangiographies (Figure 3A and 3B and Figure I in the online-only Data Supplement). Some spontaneous lymphatic vessel growth was detected in the control pigs (5.8±1.6 vessels; n=5; Figure 3A and 3B). Many of the vessels found in the VEGF-C– and VEGF-D–treated pigs had integrated into the lymph node graft, although vessels bypassing the graft were also observed in both groups. To assess the improvement in lymphatic vessel function, the accumulation of the distally injected lymphatic tracers, Patent Blue and Evans Blue, was followed in the systemic blood. Both AdVEGF-C and AdVEGF-D increased dye transport into the blood, indicating increased lymphatic vessel flow from the distal part of the lower limb to the central circulation (Figure 3C). AdVEGF-C and AdVEGF-D gene transfers led to 50% and 40% increases in lymph flow, respectively (P<0.001 for VEGF-C versus LacZ, P=0.003 for VEGF-D versus LacZ; n=5 for VEGF-C and LacZ, n=3 for VEGF-D), 2 months after the operation compared with the AdLacZ controls (Figure 3C).

Immunohistochemical staining of the lymph nodes revealed a dense LYEVE-1–positive lymphatic vessel network in both AdVEGF-C– and AdVEGF-D–treated tissues (Figure 4A through 4F). In the AdVEGF-C–treated pigs, lymphatic vessels were detected in the pericapsular area and near the pedicle of the transferred lymph node (Figure 4A and 4D). In the AdVEGF-D–treated samples, lymphatic vessels were more concentrated in the connective tissue surrounding the lymph nodes (Figure 4B and 4E). Interestingly, double immunostaining for PROX-1 and α-SMA showed that, although the collecting lymphatic vessels in VEGF-C–treated samples were larger than in the LacZ–treated pigs, the vessels in the VEGF-D–treated pigs were actually smaller than in the control, although this difference did not quite reach statistical significance (P=0.005 and P=0.052, respectively; n=6 for VEGF-D, n=5 for LacZ and VEGF-C; Figure 4G through 4J). The number of PROX-1/α-SMA double-positive vessels, however, was increased in both the AdVEGF-C and AdVEGF-D groups compared with the AdLacZ group (P=0.008 for VEGF-C, P=0.029 for VEGF-D; n=6 for VEGF-D, n=5 for VEGF-C and LacZ; Figure 4K).

**VEGF-C–Treated Lymph Nodes Retain Their Structure**

Hematoxylin–eosin staining of the nodal tissues indicated that the majority of lymph nodes in both AdVEGF-C– and AdVEGF-D–treated pigs had a normal follicular structure (Figure 4L and 4M). However, in a few AdVEGF-D–treated pigs, areas of connective and adipose tissue were observed (asterisk in Figure 4M). In contrast, the normal follicular lymph node structure was replaced by adipose and connective tissue in many areas in the AdLacZ–treated pigs (P=0.004 for AdVEGF-C versus AdLacZ, P=0.005 for VEGF-D versus...
AdLacZ; n=6 for VEGF-D, n=5 for VEGF-C and LacZ; Figure 4N and 4O). Furthermore, groups of lymphocytes and siderophages were detected around the vessels in the AdLacZ samples (asterisk in Figure 4N). Interestingly, the lymph nodes in the AdLacZ- and AdVEGF-D–transduced pigs were also significantly smaller than the normal lymph nodes (P=0.014 for AdLacZ, P=0.049 for AdVEGF-D; Figure 4P). AdVEGF-C–transduced lymph nodes had retained their original size or were even slightly bigger than the control nodes (P=0.245; Figure 4P). No differences in the numbers of B cells, T cells, or dendritic cells were observed between the groups (Figure II in the online-only Data Supplement). Furthermore, there were no pathological changes in any of the internal organs (heart, lung, liver, kidney, spleen, gonads) in any of the groups when examined by autopsy, including analysis of hematoxylin-eosin–stained sections by a pathologist (data not shown).

**Discussion**

We show here that AdVEGF-C or AdVEGF-D therapy greatly increases the survival and functionality of transferred lymph nodes. AdVEGF-C and AdVEGF-D gene transfer proved effective in restoring lymphatic vasculature to the site of surgical damage. Both growth factors increased lymphatic vessel number and promoted lymphatic drainage when they were analyzed 2 months postoperatively. However, AdVEGF-C gene transfer resulted in somewhat better lymphatic vessel function, collecting vessel formation, and lymph node histology than AdVEGF-D treatment. Control-treated lymph nodes regressed, and their follicular structure was replaced by adipose and fibrotic tissue. This is in concordance with previous data indicating that the lymph nodes need lymphatic vasculature and exposure to lymph flow to remain intact.19,20 These findings clearly favor the use of growth factors in conjunction with lymph node transfer to augment incorporation of the grafted lymph node into the resident lymphatic vascular tree.

Although lymphatic vessel function at 2 months was significantly improved in both growth factor groups, AdVEGF-D gene transfer led to increased accumulation of seroma fluid after surgery. Some seroma fluid, however, was accumulated after lymphatic vessel excision in all study groups. Most of the fluid likely originated from the dissected lymphatic vessels. Newly forming lymphatic vessels are also known to be leaky.19 However, hyperpermeability of the blood vessels was provoked in particular by VEGF-D, and this could account in part for the increase in the accumulation of seroma fluid. The blood vascular effects of VEGF-D could also be due to more complete proteolytic processing of the protein into its short isoform VEGF-D\(_{\text{A\text{N}A\text{C}}}\) by endogenous

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Lymph node structure is best preserved in the VEGF-C–treated pigs. A through F, Immunohistochemical staining for the lymphatic vessel marker LYVE-1 at 2 months after gene transfer. Note the strong lymphangiogenic effect characterized by dilated sinuses in the AdVEGF-C–treated lymph nodes (A and D) and less prominent lymphangiogenesis in the AdVEGF-D–treated lymph nodes (B and E). G through I, PROX-1(blue)/α-SMA(brown) immunostainings of VEGF-C–, VEGF-D–, and LacZ–treated lymph nodes. J and K, Quantification of the PROX-1/α-SMA–positive vessels (VEGF-D, n=6; others, n=5). L through N, Hematoxylin-eosin staining of the lymph nodes. Preserved lymph node architecture featuring several follicles in an AdVEGF-C–treated pig (L). Note the mainly preserved nodal structure in AdVEGF-D–treated lymph node (M) but also slight deterioration in some areas (asterisk). In the control AdLacZ–transduced pigs (N), lymphatic follicles were often replaced by fibrous and adipose tissue. Note also the grouped lymphocytes and siderophages (asterisks). O, Quantification of the amount of fibrosis or fat in the lymph node sections (VEGF-D, n=6; others, n=5). P, Lymph node volumes in the different study groups (VEGF-D, n=6; others, n=5). Magnifications: A through C, ×12.5; D through F, L, and M, ×40; G through I, ×200; N, ×100. Scale bar=500 μm. NS indicates P>0.05. *P<0.05, **P<0.01.
proteases compared with VEGF-C. Proteolytic processing of VEGF-C and VEGF-D has been shown to regulate their activity toward the blood vessel endothelium, leading to angiogenesis and increased vascular permeability via VEGFR-2 activation.9,13,14 A recent study has identified a processed form of VEGF-D that stimulates blood vessel growth but not lymphangiogenesis.24 Interestingly, AdVEGF-C did not increase the amount of seroma fluid compared with the AdLacZ control, which could be explained by the lower levels of the fully processed VEGF-C form observed in the seroma fluid. Taken together, these findings favor the use of VEGF-C over VEGF-D in future lymphedema trials.

Most previous reports of growth factor therapy in lymphedema are based on mouse or rabbit models, which have several limitations when considering their clinical translation to the therapy of human patients.12,15,16,19,25,26,27 First, the hydrostatic conditions are dramatically different in mice because humans are considerably taller. This also means that the absolute lymphatic area damaged in humans is greater, and the regenerating lymphatic vessels must span a longer distance to form anastomoses with both the distal and proximal ends of the lymphatic vascular tree. The lack of large animal models that better recapitulate conditions in human lymphedema has limited experimental research of lymphedema.28 Previously used canine models were complex and would be considered unethical today. We present here a novel large animal model of lymphatic vessel damage and lymph node transfer. However, this model also has limitations. The inguinal lymphatic anatomy is different between pigs and humans. In addition, unlike in chronic lymphedema, our experimental model is based on acute lymphatic damage. Despite these limitations, our findings indicate for the first time that lymphangiogenic growth factor therapy could be feasible in humans. It is important to note that VEGF-C and VEGF-D have been shown to promote lymphatic metastasis of tumor cells, to increase blood vascular permeability, and to stimulate extravasation of lymph from the lymphatic vessels. On the other hand, VEGF-C stimulates less angiogenesis and primary tumor growth than VEGF-A.12,19,29–32 In light of these studies, safety is an important consideration in the identification of patients for future clinical trials.

Conclusions

Combining autologous lymph node transfer and therapeutic growth factors provides a promising tool for the curative treatment of lymphatic vessel dysfunction after infection, surgery, or radiation therapy (Figure 5). Brief AdVEGF-C gene expression seems to be sufficient for the development of stable collecting lymphatic vessels in a large animal model. Once the lymphatic neovascularization becomes functional, it remains stable and mature in the target tissue even without growth factor support.

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Disclosures

Drs Saaristo and Seppo Yli-Herttuala are members of the Board of Directors of OY Lx Therapies Ltd. The other authors report no conflicts.

References

10. Achen MG, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a...
The lymphatic vascular system is crucial for the regulation of tissue fluid homeostasis, fat metabolism, and elicitation of immune responses. Lymphatic dysfunction, either resulting from gene alterations or secondary to damage to the lymph vessels, may lead to lymphedema, a debilitating condition characterized by chronic tissue edema, impaired immunity, and accumulation of subcutaneous fat. Lymphedema is a common and incurable problem. Interestingly, during recent years, application of lymphangiogenic growth factors has been shown to induce lymphangiogenesis and to reduce tissue edema in small animal models. The aim of the present study was to evaluate the potential of growth factor therapy in a clinically relevant novel large animal model. Lymphedema patients have recurrent infections and thus would benefit from both functional lymphatic vessels and lymph nodes. In an attempt to restore the lymphatic anatomy, we combined growth factor therapy with lymph node transfer. Interestingly the lymph nodes transduced with vascular endothelial growth factors (C and D) survived and formed both afferent and efferent connections with the preexisting lymphatic vessel network, whereas the control vector–treated nodes regressed. We compared the effect of 2 different endothelial growth factors to select the best candidate for the clinical trials. Combining autologous lymph node transfer and therapeutic growth factors provides a promising tool for the curative treatment of lymphatic vessel dysfunction after infection, surgery, or radiation therapy.
Supplemental figure 1: Inflammatory cell counts from treated lymph noduses. (a) CD23cy positive cell counts. (b) Dendrite cell counts. (c) CD3 positive cell counts. Ns = p > 0.05.

Supplemental figure 2: High resolution angioraphies. (a) AdVEGF-C treated nodus. (b) AdLacZ control nodus. Scale bar 10 centimeters.