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Strategies in Modulating Lymphedema

Jin-Hong Chang*, Joshua H. Hou, Sandeep Jain and Dimitri T. Azar*
Department of Ophthalmology and Visual Sciences, University of Illinois Chicago
United States of America

1. Introduction

Lymphedema is the accumulation of interstitial fluid within tissues due to the impairment of lymphatic function. Dysfunction can result from direct obstruction of lymphatic vessels, absence of lymphatic vessels, or inadequate lymphatic function. From congenital forms of lymphedema, such as Milroy disease, to acquired forms of lymphedema, such as filiarisis lymphedema or post-surgical lymphedema, lymphatic dysfunction contributes significantly to the world's human disease burden.

Due to the inherent difficulties in visualizing lymphatic channels, research in the pathogenesis and treatment of lymphedema has lagged behind similar investigations in vascular pathology. Over the past two decades, aggressive research efforts have vastly improved our understanding of the lymphatic system, but significant advances in medical therapies for lymphedema and lymphatic regeneration are still lacking. To date, the majority of treatment strategies for lymphedema (compression stockings, massage, and exercise) do not address the underlying molecular pathophysiology (Nakamura & Rockson, 2008).

Several nonspecific pharmacological agents, such as selenium and benzo-pyrones, have been studied with limited success. A review of ten different trials of pharmacological therapies for lymphedema by Kligman *et al*. concluded that insufficient evidence exists to support the use of these nonspecific medical therapies at the moment (Kligman et al., 2004).

With improvements in our understanding of the molecular mechanisms of lymphedema and lymphangiogenesis, however, an increasing number of potential pharmacological targets for specific therapies are being identified. Due to ongoing efforts by numerous researchers in the study of therapeutic lymphatic regeneration, significant progress is being made towards targeted pharmacological therapies for lymphedema. Specific molecules that have shown the most promise as therapeutic targets include the vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs), cyclooxygenase 2 (COX-2) selective inhibitors, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β.

*Corresponding author

2. Nonspecific treatments

To date, studies have been unable to confirm the effectiveness of nonspecific treatment strategies such as selenium and benzo-pyrones on lymphedema, despite their common use in clinical practice. Selenium is a drug that is used to prevent or minimize the adverse effects of radiotherapy, chemotherapy, or surgery in oncology patients; however, after rigorous testing of this therapy, Dennert and Horneber concluded that inadequate evidence exists to advocate for or against the use of selenium for lymphedema (Dennert & Horneber, 2006). Similarly, benzo-pyrones have been considered a plausible treatment strategy for lymphedema, as these molecules reduce vascular permeability and thereby, reduce subcutaneous fluid. Furthermore, benzo-pyrones also increase macrophage activity and encourage protein degradation, which, in turn, reduces the formation of fibrotic tissue in the lymphedematous limb. A recent review of 15 trials of benzo-pyrones in the treatment lymphedema, however, failed to uncover any conclusions due to the poor quality of the analyzed trials (Badger et al., 2004). The limited and questionable efficacy of current nonspecific treatments has led many researchers toward using molecular strategies for the development of newer targeted pharmacological therapies. Specific attention has been paid to factors responsible for lymphangiogenesis, such as VEGF-C. By stimulating lymphangiogenesis and regeneration of lost or damaged lymphatics, VEGF-C offers significant promise as a treatment for all-cause lymphedema.

3. Targeted therapies

Currently, a number of molecular strategies for the treatment of lymphedema are being studied in animal models. Promising results have been obtained in the treatment of mouse models of lymphedema via methods of promoting lymphangiogenesis; however, significant work is still required before clinical application of these therapies becomes a reality.

3.1 Treatment with VEGF-C

In particular, VEGF-C has been found to be a potent regulator of lymphangiogenesis through its actions on two receptor tyrosine kinases, VEGFR-2 and VEGFR-3 (Haiko et al., 2008; Tammela et al., 2011). Multiple studies have evaluated the efficacy of VEGF-C gene therapy and plasmid transfection and revealed surprising success in a variety of animal lymphedema models.

The first study to document improvement in the clinical and pathologic features of lymphedema by therapeutic enhancement of lymphatic drainage with human VEGF-C gene therapy was performed in 2003 by Yoon *et al*. In two animal models, a rabbit ear model and a mouse tail model, these authors treated lymphedema with naked plasmid DNA that encoded human VEGF-C (phVEGF-C) injected subcutaneously. Following treatment, improvements in both lymphedema and lymphatic function were noted (Yoon et al., 2003).

Cheung *et al*. similarly found improvement in surgically-induced lymphedema in a mouse tail model using recombinant human VEGF-C. Mice were treated with cautery ablation of the large collecting lymphatics of the tail after identification of the vessels with injected methylene blue. A lymphedematous state was then documented by evidence of dilated cutaneous lymphatics, acute inflammation and hypercellularity, and impairment of immune

trafficking via *in vivo* bioluminescent imaging. Three days post-surgery, the animals were then treated with parenteral recombinant human VEGF-C generated from engineered DNA encoding the human VEGF homology domain (amino acid residues Thr103-Arg227) fused to a human CD33 signal peptide at the N-terminus and a 10x-histidine tag at the C-terminus. Upon examination, treated animals were found to have reversal of the lymphedematous state to the normal state with resolution of edema, hypercellularity, inflammatory changes, and microlymphatic dilation. Both lymphatic vessel number and cross-sectional area were reduced following exogenous administration of the recombinant VEGF-C (Cheung et al., 2006).

In another mouse model of chronic obstructive lymphedema, treatment with VEGF-C also improved lymphedema in the studied animals. The authors injected a pcDNA3.1-VEGF-C plasmid into the tail of these mice. Subsequent overexpression of VEGF-C enhanced lymphangiogenesis *in vivo* and improved lymphedema (Hu et al., 2008).

Using a rat hind limb model of lymphedema, Liu *et al*. also observed significant improvement in lymphedema following focal transfection with VEGF-C DNA. Rats were treated with a plasmid DNA encoding human VEGF-C (pcDNA3.1-VEGF-C) and monitored for resolution of their surgically-induced lymphedema in comparison to controls. Lymphedema was quantitatively reduced at 2 and 4 weeks in the therapy group as documented by magnetic resonance imaging (MRI), B-scan ultrasound, and water displacement volumetry measurements. Furthermore, numerous newly formed lymphatic vessels were observed in treated mice on both histological and immunofluorescence analysis (Liu et al., 2008).

Finally, Tammela *et al*. found a significant role for adenovirally-delivered VEGF-C in improving outcomes of lymph node dissection and transplantation in mice. In their study, lymph node dissection and transplantation in combination with adenovirally-delivered VEGF-C induced the formation of functional collecting lymphatic vessels and the reconstitution of a functional immunological barrier (Tammela et al., 2007).

3.2 Caveats to VEGF-C Therapies

Despite the growing body of evidence in support of the efficacy of VEGF-C gene therapy, the exact mechanism by which VEGF-C-induced lymphangiogenesis facilitates resolution of lymphedema remains controversial. In an effort to elucidate the mechanisms by which VEGF-C induces lymphatic microvascular remodeling, Jin *et al*. examined the effects of anti-VEGFR-3 neutralizing antibodies in a mouse tail model of post-surgical lymphedema. This study demonstrated that VEGFR-3 plays a central mechanistic role in lymphedema remodeling. In the presence of the neutralizing antibody, lymphatic remodeling was greatly attenuated due to blockage of VEGF-C-induced signaling (Jin da et al., 2009).

In contrast, Uzarski *et al*. failed to observe inhibition of edema resolution across surgically-induced wounds in a mouse tail lymphedema model upon blockage of VEGFR-3. In their study, two mouse models were compared. In the first mouse model, scar-free lymphatic obstruction was simulated with dissection and removal of the superficial lymphatics from a mouse tail. Distal lymphedema was noted, and resolution was stimulated by VEGF-C. Edema resolution was not, however, inhibited by VEGFR-3 neutralizing antibodies, although lymphangiogenesis was reduced. In the second mouse model, scar-containing

lymphatic obstruction was simulated with dissection and removal of the superficial lymphatics with cautery. Subsequent treatment with either VEGF-C or VEGFR-3 neutralizing antibodies resulted in no improvement in lymphedema. The authors concluded that interstitial flow dynamics and lymphedema may actually be more dependent on the extracellular matrix that reforms at the site of the injury than on lymphangiogenesis and that this effect may be impeded by the formation of scar tissue (Uzarski et al., 2008).

Following the finding that resolution of lymphedema may be more dependent on interstitial flow than on VEGFR-3 or VEGF-C, Ongstad *et al*. set out to clarify the role of VEGFR signaling during edema resolution and to probe the mechanism by which VEGF-C hastens resolution of edema. In their study, inhibition of VEGFR-3 or VEGFR-2 alone in mouse models did not significantly change the evolution of lymphedema relative to controls; however, inhibition of both VEGFR-2 and VEGFR-3 led to reduced tissue repair and reduced resolution of tail swelling at 40 and 50 days post surgery. Thus, tissue repair was crucial to the resolution of edema as this process provides a matrix bridge for fluid drainage. These authors then hypothesized that edema resolution in the mouse may be VEGFR signaling dependent, but lymphangiogenesis independent (Ongstad et al.).

Careful analysis by Jin *et al*. identified 120 mouse genes, many of which share homology with human genes, that are upregulated in the presence of lymphedema and normalized following therapeutic VEGF-C administration. Many of these genes were found to be involved in processes unrelated to lymphangiogenesis, suggesting an underlying, but incompletely understood, complexity to VEGF-C-induced lymphedema resolution. It is likely that numerous processes, including inflammation, immune response, wound healing, angiogenesis, oxidative stress response, and adipogenesis, play important roles in the pathogenesis and therapeutic resolution of the disease (Jin da et al., 2009). Additional research in this area is certainly warranted.

3.3 VEGF-C and stem cell combined therapies

Several recent studies further complicated our understanding of the role of VEGF-C and the optimal application of VEGF-C therapy in the treatment of lymphedema. In two papers, augmentation strategies using synthetic extracellular matrix material, such as gelatin and/or stem cells, co-administered with VEGF-C resulted in greater resolution of lymphedema compared to VEGF-C therapy alone. This further highlights the complexity of lymphedema and VEGF-C therapy.

Gelatin is a natural and abundant polymer used for tissue engineering. Gelatin-based hydrogels are biodegradable, non-immunogenic, and non-toxic and are able to mimic the properties of the extracellular matrix. This polymer can be used to distribute growth factors in a localized, sustained, controlled manner to obtain an effective dose response. Due to these properties and the success of VEGF-C treatment in lymphedema mouse models, Hwang *et al*. created a mouse hind limb model of lymphedema and applied a gelatin hydrogel system to the site of injury to obtain a controlled release of VEGF-C in combination with injection of human adipose-derived stem cells (hADSCs). Decreased dermal edema depth and increased lymphatic vessel density were observed at all time periods in the mice treated with both hydrogel and hADSCs as compared to mice treated with either hydrogel or hADSCs alone (Hwang et al.)

Similarly, Zhou *et al*. treated rabbits with hind limb lymphedema using bone marrow stromal cells and/or VEGF-C. The rabbits that were treated with both stem cells and the growth factor exhibited a significant decrease in volume of edema in the limb as compared to rabbits treated with only one of the two agents. Vessel numbers increased in the dual treatment group, and VEGF-C expression was also higher in the dual therapy-treated animals. The authors concluded that the treatments enhanced the therapeutic effect of each other (Hu et al.). Therefore, stem cells may play a role in matrix remodeling and lymphangiogenesis, particularly in the setting of upregulated VEGF-C expression; however, further research is needed before concrete conclusions can be made.

3.4 Alternative therapies

As our understanding of lymphatics has improved, additional therapies with effects on lymphangiogenesis have also been identified. One such therapy is extracorporeal shock wave therapy (ECT). ECT is used for the treatment of plantar fasciitis and tennis elbow. Repeated shock waves are localized to an area to produce neo-vascularization. This treatment effectively induces therapeutic angiogenesis and improves myocardial ischemia in pigs and humans as well as hind limb ischemia in rabbits via a mechanism involving upregulation of VEGF. Serizawa *et al*. created a rat tail model of lymphedema and subsequently subjected the animals to serial ECT therapy. Enhanced drainage of lymphatic fluid as well as upregulation of VEGF-C expression were found in the treatment group compared to the controls (Serizawa et al.).

Similarly, skin graft repairs to injuries in a mouse tail model have also been shown to stimulate lymphatic regeneration. In a recent study by Yan *et al*., ingrowth of lymphatic vessels and spontaneous re-connection of existing lymphatics associated with VEGF-C up-regulation was observed following skin grafting (Avraham et al.). In the future, a combination of such therapeutic strategies may be required for optimal management of lymphedema.

3.5 Additional molecular targets

Clearly, VEGF-C plays an important role in the lymphatic system; however, it is also becoming increasingly clear that additional factors are involved in lymphedema. One such factor, hepatocyte growth factor (HGF), has been found to promote lymphatic vessel formation in mice. Furthermore, both HGF and its high affinity HGF receptor (MET) have recently been found to be expressed in lymphatic endothelial cells but not in blood endothelial cells. Finegold *et al*. examined these genes in women with secondary lymphedema following treatment for breast cancer and found that patients with both primary and secondary lymphedema had mutations in HGF/MET, suggesting that mutations in HGF/MET may be a significant risk factor for lymphedema. Thus, HGF may also become a potent therapeutic target for the treatment of lymphedema (Finegold et al., 2008).

Another potential new target for lymphedema therapy is matrix metalloproteinase (MMP)-9. Sustained swelling induced by lymphatic ligation leads to lymphatic hyperplasia and VEGF-C upregulation; however, mice lacking MMP-9 have a larger increase in tail volume with secondary lymphedema as compared to wild-type mice (Rutkowski et al., 2006).

G-protein-coupled receptors are expressed during lymphatic development and function, and thus, these molecules are also potential targets for pharmacological treatment. Genetically engineered mouse models deficient in specific G-protein-coupled receptors have been used to identify several specific G-proteins, such as the adrenomedullin receptor, that are important for lymphatic vascular development and function (Dunworth & Caron, 2009).

Radiation therapy, infections, or extensive surgical resection promote scarring and fibrosis and are, thus, also risk factors for lymphedema. Based on this finding, Avraham *et al*. examined the specific impact of fibrosis, defined as the excessive deposition of extracellular matrix products, on the abnormal regeneration of lymphatic vessels. Inhibition of fibrosis via treatment of the mouse tails with collagen type 1 gel and a moist dressing accelerated lymphatic regeneration and reduced post-surgical acute lymphedema in this animal model. Lymphatic endothelial cell proliferation was also enhanced and lymphatic function was improved. These results were independent of VEGF-C expression (Avraham et al., 2009).

After discovering that fibrosis impairs lymphatic regeneration and function (Avraham et al., 2009), Avraham *et al*. then searched for factors that modulate fibrosis in lymphedemous tissue. Transforming growth factor (TGF)-β is a well-known regulator of extracellular matrix synthesis. Inhibition of TGF-β causes both decreased fibrosis in virtually every organ system and increased lymphatic endothelial cell proliferation, migration, and tubule formation. To study the role of TGF-β, the investigators compared biopsies from lymphedemous limbs of patients to biopsies of the normal contralateral limb. The limbs with lymphedema exhibited a 3-fold increase in the number of TGF-β1-positive cells as compared to the normal limbs. Similarly, a mouse tail model of lymphedema was studied to determine the effect of anti-TGF-β1 treatment on lymphedema. Application of a TGF-β1 antibody (TGFmab) induced a 50-60% decrease in tail volume (a surrogate measurement for lymphedema) in treated mice compared to control mice. The treatment was well tolerated with no evidence of toxicity or wound healing complications. In the future, blockage of TGF-β may lead to further therapeutic options for augmentation of lymphatic regeneration. (Avraham et al., 2010).

Lymphedema has also been found to arise from destructive tissue injury independent of lymph stasis. Based on this observation, inflammatory mediators such as tumor necrosis factor (TNF)-α and cyclooxygenase (COX) have also come under recent scrutiny. TNF-α is prominently expressed in lymphedematous tissue in mouse models and is a known inducer of VEGF-C expression. In a mouse tail model of lymphedema, Nakamura *et al*. examined the anti-inflammatory and possible lymphangiogenic potential of a non-steroidal anti-inflammatory drug (NSAID) and a modified soluble form of a TNF-α receptor R1 (sTNF-R1) on TNF-α expression. Subcutaneous injections of the NSAID ketoprofen, which reduces inflammation by inhibiting COX but increases TNF-α levels, resulted in marked improvement in inflammation, normalization of histological changes, and disappearance of dilated microlymphatics with associated up-regulation TNF-α expression. Treatment with the NSAID also led to upregulation of VEGF-C, VEGFR-3, and Prox1, all factors associated with lymphangiogenesis. Treatment of mice with sTNF-R1, which directly inactivates TNF-α and downregulates its expression, did not result in improvement in lymphedema, and epidermal thickness actually increased in treated mice compared to untreated mice. In these treated mice, both VEGF-C and VEGFR-3 expression decreased as well. Though more evidence is needed, TNF-α activity and its downstream effects on VEGF-C may actually be a protective response to injury-induced lymphedema (Nakamura et al., 2009).

In contrast, another study revealed that increased COX-2 expression was associated with recurrence of lymph flow in wound granulation tissues and with increased formation of lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1)-positive lymphatic-like structures. The authors suggest that these results differed from those of Nakamura *et al*. as a result of the important selectivity of the COX inhibitors (Kashiwagi et al., 2011). Thus, the role of inflammatory mediators, such as TNF-α and COX-2, in the treatment of lymphedema remains both complex and controversial.

4. Larger animal models

Most animal models of lymphedema are mouse models due to the practicalities of establishing new treatment methods; however, utilizing mice carries significant limitations. The microlymphatics in the superficial dermis of mouse tails and the complex macrostructure of human lymphatics, which includes both larger collecting vessels and lymph nodes, have notable differences that preclude direct correlation. Extrapolation of the therapeutic successes achieved in mice to humans, therefore, is dangerous without further experimentation in larger animals.

To that end, Lahteenvuo *et al*. recently investigated the benefits of adenoviral vector-assisted VEGF-C gene therapy in the treatment of lymphedema in pigs. Lymphedema was induced in pigs by excising a 3 cm piece of the inguinal lymphatic vessels that drain distally and proximally from the inguinal lymph node. The pedicular lymph node was then reattached to the remaining tissue 4 cm laterally from its original position, thereby mimicking lymph node transfer in human patients. Adenoviral vectors encoding full-length VEGF-C were then injected into the lymph node. Following injection, expression of VEGF-C was significantly increased. Furthermore, survival and functionality of the transferred lymph nodes was markedly improved in the injected animals as compared to the controls. Lymph node transfer has been used in humans with limited success (22-31%), but in this model, the presence of VEGF-C resulted in better lymphatic vessel function, collecting vessel formation, and lymph node histology compared to controls (Lahteenvuo et al., 2011).

Similar studies have also been performed on other large animals such as sheep. Using such a model, Baker *et al*. tested the effect of lymphangiogenic growth factors delivered via slow-release diffusion on the resolution of lymphedema after lymph node extraction. A single popliteal lymph node was extracted from the sheep to induce distal limb lymphedema. Hydrogel HAMC (a blend of hyaluronan and methylcellulose that facilitates slow protein diffusion) infused with VEGF-C and angiopoietin-2 was then injected into the excision site. The animals that received treatment displayed significantly reduced edema compared with the untreated animals (Baker et al.).

Though no formal testing of targeted therapies for lymphedema has been performed in humans to date, a few isolated case reports have been published. Sorafenib, a synthetic compound produced to block the enzyme RAF-kinase, was found to cause a dramatic reduction in chronic lymphedema in one human case study. As part of a clinical study, the patient took 400 mg of sorafenib twice daily, and the lymphedema was dramatically reduced within a few days of starting treatment. The effect was directly proportional to the dose and was not sustained when the drug was discontinued due to other side

effects. The authors hypothesized that the reduction of lymphedema was due to VEGFR-2 blockade, which reduced vascular permeability but did not affect the VEGFR-3 pathway involved in the proliferation of lymphatic endothelial cells (Moncrieff et al., 2008).

5. Limitations

Though research into targeted therapies for lymphedema is progressing rapidly, significant work is still required. As stated above, there are a number of limitations with the animal models that are currently available. First, the models are based on acute lymphatic damage and not on chronic lymphedema. Lymphedema in humans is slowly progressive and does not recover naturally, whereas the models that are studied progress quickly and often regress naturally. Rats, for example, heal very quickly, and tail lymphedema heals itself. In fact, one of the problems in studying lymphedema treatment strategies is the difficulty in developing a method to sustain lymphedema long enough to study the outcomes of the therapies (Yoon et al., 2003). In addition, the hydrostatic conditions of humans differ from those of small animals, such as mice, rats, and rabbits, that are usually used to study lymphedema. The absolute lymphatic area damaged in humans is greater than in these small animals, and the regenerating lymphatic vessels must span a longer distance. Models using pigs, which are closer in size to humans, will likely be useful in addressing some of these issues. Another major concern is that VEGF-C and VEGFR-3 are known to promote metastasis. Since a large portion of lymphedema patients are breast cancer survivors, an understanding of the metastatic risks associated with VEGF-C treatment of lymphedema is of paramount importance. However, these potentially damaging side effects of VEGF-C treatment are difficult to study in animal models.

6. Conclusion

Lymphangiogenesis is a complex process that involves the interplay of many molecules with redundant mechanisms. VEGF-C and VEGFR-3 are known to be the primary players, but these molecules work in sync with other factors. Currently, no specific molecular treatment options are available for clinical use; however, promising results from animal trials suggest a role for VEGF-C gene therapy in treatment of lymphedema. Other results indicate that other factors such as COX-2, MMP-9, and interstitial flow dynamics may also be important in future management of lymphedema. Combination therapies such as stem cell implantation, skin grafting, and lymph node transfer in conjunction with VEGF-C therapy may further expand the effectiveness of future therapies as well. Design of a drug to treat lymphedema will require an effective animal model that accurately mimics lymphedema in humans. And further evaluation of the metastatic risk of inducing lymphangiogenesis in cancer patients is also needed. However, dramatic progress has been made towards effective targeted molecular therapies for lymphedema. Due to the proliferative efforts of researchers over the last decade, effective treatments for lymphedema in humans may soon be a reality. However, though a solid body of evidence now exists in support of targeted therapies for lymphedema, significant research is ultimately still needed.

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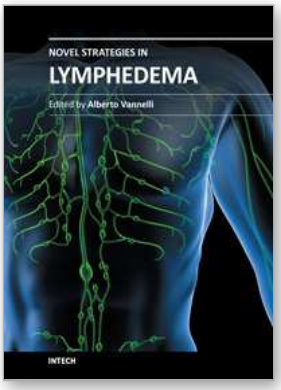

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Lymphedema is a swelling caused by the abnormal accumulation of lymphatic fluid in the skin. Lymphedema can be caused by burns, injury, surgery, radiation therapy or cancer treatment that cancer survivors undergo. Risk of developing lymphedema is high especially in those with breast or prostate cancer. It is hereditary and can appear without warning at any time of life and is related to obesity and circulatory problems. If not treated, lymphedema can be painful and lead to life-threatening infections. This book will help physicians who deal with lymphedema. It will help you understand how the lymphatic system works, how lymphedema is diagnosed, how to cope with the challenges of lymphedema, how to find treatment, and how to deal with insurance issues. Novel Strategies in Lymphedema is for those with, or at risk of, developing lymphedema, and the healthcare professionals who care for them.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821