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Review

Animal Models of Secondary Lymphedema: New Approaches in the Search for Therapeutic Options

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Abstract

Secondary lymphedema is still a worldwide problem. Symptomatic approaches to lymphedema therapy have been mainly used, with complete decongestive therapy as the cornerstone. Due to a lack of regenerative therapy, researchers have established various animal models to obtain insights into pathomechanisms and to reveal the best therapeutic option. Since the first reproducible and reliable animal model of lymphedema was reported in dogs, the technique of circumferential excision of lymphatic tissue has been translated mainly to rodents to induce secondary lymphedema. In these models, various promising pharmacological and surgical approaches have been investigated to improve secondary lymphedema therapy. Imaging modalities are crucial to detect the extent of lymphatic dysfunction and decide the best therapy. The gold standard of lymphoscintigraphy is currently limited by poor spatial resolution and lack of quantification. Animal models could help to bridge a gap in improving morphological correlation and quantifying lymphatic functionality. This review summarizes the animal models used in lymphatic research and focuses on new therapeutic options and requirements for imaging modalities to visualize the lymphatic system.

Keywords: secondary lymphedema, animal models, microsurgery, VEGF-C

Introduction

Breast cancer is the most prevalent cancer and the major cause of cancer-related death worldwide.[1] One in five women surviving breast cancer will develop arm lymphedema after therapeutic lymphadenectomy.[2] In addition to causing a massive volume increase in the edematous limb, the disease modulates the lymphatic-associated immune response, resulting in permanent inflammation. As a consequence, patients suffer from high-risk erysipelas and are affected by major decreases in their quality of life. The previous standard procedure of removing nearly all lymph nodes in the tumor area was subsequently replaced by sentinel lymph node biopsy and selective lymphadenectomy. However, there is still a 4%–10% incidence of lymphedema after breast cancer therapy and sentinel lymph node biopsy.[3] The current generally applied therapy for secondary lymphedema[4] is Complete Decongestive Therapy. The main goal of this symptomatic therapy is to reduce the limb volume using manual lymph drainage, special individually adapted compression garments, exercises, and skin care[4]; however, the disadvantages of this therapy include lifetime restrictions on quality of life. Animal models have played an important role in the search for more effective therapies for secondary lymphedema. The first stable lymphedema animal model was established in dogs in 1968.[5] Innovative circumferential excision with double sectioning of the superficial and deep lymphatics resulted in the first reliable and reproducible acquired lymphedema model.[5] Later, this method of circumferential excision of the lymphatics was translated to the rabbit ear[6,7] and rodent hind limb and tail models.[8–12] In the last decade, the detection of specific lymphatic markers has improved our knowledge of embryonic lymphatic development.[13,14] In particular, the lymphatic-specific growth factor vascular endothelial growth factor (VEGF-C) and the corresponding tyrosine kinase receptor VEGF-R3 (syn. FLT-4) have been used to reveal molecular mechanisms.

Various surgical approaches have been investigated in lymphedema therapy research. For example, tissue transfer and full-thickness skin flaps have been explored, as well as microsurgical reconstruction techniques. In addition, pharmacological therapy options, such as the application of VEGF-C, hyaluronidase treatment, or stem cell therapy, have succeeded in improving lymphedema. Imaging modalities support the evaluation of lymphatic function in this disease.

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Table 1. Second PubMed Search Lymphedema and Surgery

Scheme for the second research
((''lymphoedema''[All Fields] OR ''lymphedema''[MeSH Terms] OR ''lymphedema''[All Fields]) AND (''surgery''[Subheading] OR ''surgery''[All Fields] OR ''surgical procedures, operative''[MeSH Terms] OR (''surgical''[All Fields] AND ''procedures''[All Fields] AND ''operative''[All Fields]) OR ''operative surgical procedures''[All Fields] OR ''surgery''[All Fields] OR ''general surgery''[MeSH Terms] OR (''general''[All Fields] AND ''surgery''[All Fields]) OR ''general surgery''[All Fields]) AND (''rodentia''[MeSH Terms] OR ''rodentia''[All Fields] OR ''rodents''[All Fields])) AND ((''1995/01/01''[PDAT]: ''3000/12/31''[PDAT]) AND English[lang])

Materials and Methods

An investigation was performed using the U.S. National Library of Medicine (NLM) journal citation database (Medline) using PubMed; this free resource was developed and is maintained by the National Center for Biotechnology Information (NCBI) at the NLM (Bethesda, MD). The goal was to identify literature on the topic of lymphedema and animal models using Medical Subject Headings (MeSH).

First search:

1. ''lymphedema''[MeSH] and ''Models, Animal''[MeSH]—183 items
2. January 1, 1995–August 1, 2015—112 items
3. Language: English—106 items
4. Exclude: filariasis, lymphedema mutation (e.g., Fox-2/SOX18), adipogenesis, articles without therapeutic approach, studies in humans, reviews, primary lymphedema, double hits—35 relevant items

A second search concentrated on surgical approaches in animal models.

Second search* (Table 1):

1. ''lymphedema'' AND ''surgery'' AND ''rodents''*—106 items
2. January 1, 1995–August 1, 2015—73 items
3. Language: English—68 items
4. Exclude: same criteria as first search—15 relevant items

In conclusion, 50 relevant items were investigated in this review and additional highly relevant literature that was selected by the author was added.

Results and Discussion

Animal models of acquired lymphedema

The first successful lymphedema model was established in 1968.[5] This breakthrough concerned the circumferential excision of a dog's hind limb to achieve both superficial and deep lymphatics. To create an easy, affordable, and practicable animal model, this strategy was translated to rabbits and rodents. Currently, rodents are the most common animal models in lymphatic research, accounting for $\sim 80\%$ of published models (Fig. 1).

Rodent models of secondary lymphedema

Circumferential full-thickness skin excision is mostly used to induce secondary lymphedema in rodents. The most popular approaches have been rodent limb[8,15] and tail[11,12,16] models (Fig. 2). To achieve successful lymphatic obliteration, lymphatics were stained by lymphatic dyes such as methylene blue by subcutaneous or intradermal injection distal to surgical area.[9,17] In rodent thighs, the medial neurovascular bundle is saved and dissected by the surrounding tissue.[17] The underlining muscle is destroyed to reach the deep lymphatic system either by ligation or electrocauterization.[17]

In rodent tail models, circumferential excision is made in a defined distance distal to the base of the tail.[9] The blue-dyed lymphatics were ligated or cauterized and the wound edges are left for secondary wound healing.[9] Care has to be taken to maintain the integrity of the major blood vessels and tendons to prevent necrosis.[18]

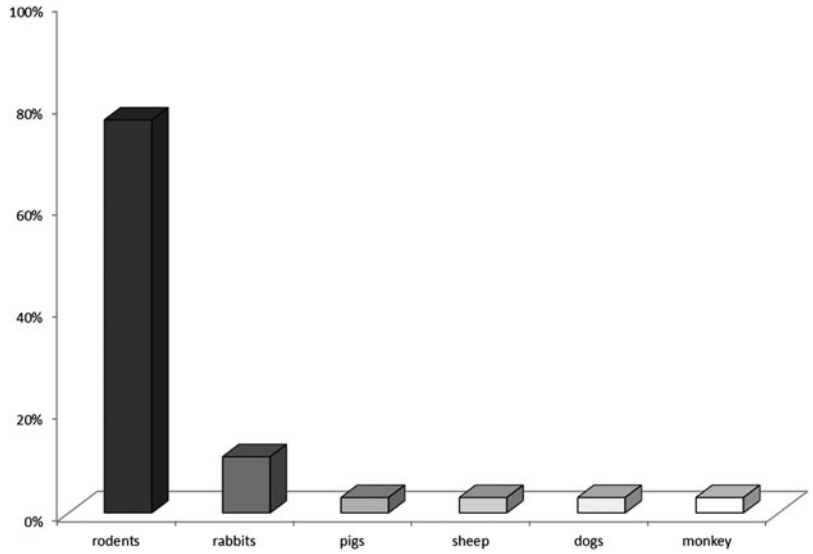

FIG. 1. Distribution of species in lymphedema models ($n = 35$, data from first search).

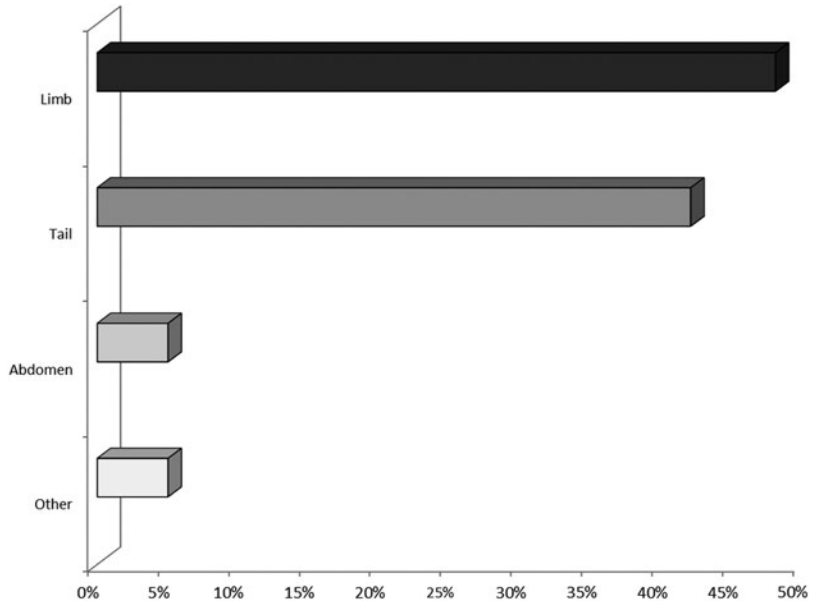

FIG. 2. Proportion of various approaches in rodent models ($n = 42$, data from both searches).

In some cases lymph node dissection was added to lymphatic obliteration,[15] or axillary lymph node dissection (ALND) was used as an animal model for secondary lymphedema.[19] For ALND, the incision is made through the dermis and the axillary lymph nodes were excised with prestaining lymphatic dye as described before.[19] Because rodents in particular experience highly potent endogenous lymphatic regeneration and radiotherapy in cancer treatment increases the lymphedema risk by ~10-fold in humans,[20] it can be used in addition to surgery to establish peripheral lymphedema for a period of 4–8 weeks.[16] The mechanism of action might be destruction of the remaining lymphatics or suppression of spontaneous regeneration of lymphatic vessels.[17]

The main advantage of rodent lymphedema tail models is that these present a standardized, relatively easy method to induce lymphedema. The clinical relevance in rodent models is high in macroscopic and histological changes. Secondary lymphedema in humans provokes tissue swelling, fat deposition exaggerated inflammation, fibrosis, and hyperkeratosis.[21] In most of the animal models, increased tissue swelling and histological changes like fat deposition, increased epidermal thickness, and indication of fibrosis occur. The main difference in rodent models for secondary lymphedema is the stage of disease. In humans, secondary lymphedema does not recover naturally[22] and occurs most frequently months to years after surgery and radiotherapy.[6] In rodents, achieved secondary lymphedema often recovers after a short period of time[22] or gets necrotic.[17]

Rabbit model

Thus, circumferential excision is although used in rabbit's thighs,[15] the most frequently used model in rabbits is the ear model.[6,7,22] When circumferential excision is used in a rabbit ear model, the skin on the rabbit ear base, including superficial and deep lymphatic vessels, is removed until only the neurovascular pedicle is left.[6,7,24] Fu et al. described a restrictive technique of sparing the perichondrium and cartilage while forming a ''skin bridge'' containing the central neurovascular bundle.[24] To prevent the reconnection of lymphatics, the excised wound was left to undergo secondary granulation, whereas the edges of the skin were sutured inversely to the perichondrium. Using this technique, stable lymphedema could be achieved within 2 months. Similar to rodents, rabbit models show compensation mechanisms, but lymphedema was mentioned to achieve for at least 12 months.[15] Typical tissue changes such as fibrofatty tissue deposition, greater skin thickness, hypercellularity, and fibrosis were achieved in rabbit lymphedema model.[6,7] However, in contrast to human secondary lymphedema, tissue modifications occurred already 2 weeks after surgery and recovered naturally.[6]

Large animal approaches

As lymphatic drainage is dependent on muscular activity, body position, breath movements, and capillary permeability, researchers have created large animal models of lymphedema to improve similarity to the human disease pattern. Lymph node dissection is mostly used to induce lymphedema in these models. The advantage of large-animal hind limb models is the single system of lymphatic drainage in the extremities; thus, no circumferential excision is needed. Tobbia et al. were able to maintain limb lymphedema for at least 16 months in sheep using the technique of removal of the popliteal lymph node in combination with ligature of affiliated vessels.[25] In pigs, researchers showed the potential of regeneration after vascularized lymph node transfer and removal of all lymphatic vessels connected to the superficial inguinal lymph node within 2 months.[26] In each group, lymphatic vessel function was significantly improved at 2 months.[26] Thus, similarities to human lymphedema are achieved; chronic stages cannot be displayed. Moreover, Wu et al. created a rhesus monkey lymphedema model by ALND and radiation treatment. The authors established stable lymphedema

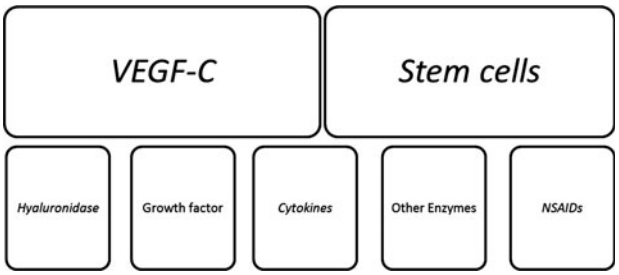

FIG. 3. Pharmacological treatment of secondary lymphedema.

within 24 months in primates with characteristics of human chronic lymphedema.[27]

In summary, circumferential excision in addition to lymph node dissection might currently be the most promising technique for inducing lymphedema. Due to vast endogenous regeneration, it is difficult to establish chronic lymphedema in rodents. Improvements have been achieved with the addition of radiotherapy in surgically treated areas. In large animal models, lymph node dissection without circumferential excision or radiotherapy is a successful approach; however, the experimental setups needed to yield results similar to those in the clinic are very time consuming and labor intensive, and the late effects have to be proofed.

Pharmacological Therapy Options

The most common pharmacological approaches are listed in Figure 3.

VEGF-C in secondary lymphedema therapy

Intensive molecular research performed in the last few decades has revealed important new lymphatic markers, such as the lymphatic-specific growth factor VEGF-C[8] and its tyrosine kinase receptor VEGF-R3, which are specific to lymphatic tissue in adults (Fig. 4).[28] However, the lymphatic-specific hyaluronan receptor LYVE-1 is used to differentiate between vascular and lymphatic vessels. In lymphedema therapy, VEGF-C has shown positive results in reducing edematous volume in the rabbit ear,[7] the rodent tail[8,29] and limb models,[23,30,31] and a pig model.[26] In addition, anti-VEGF-R3 antibody application was found to influence lymphangiogenesis in the mouse tail[11] and a mouse forelimb model.[19]

Combined anti-VEGF-R2 and anti-VEGF-R3 application before surgery and every 5 days postsurgery inhibited resolution of acquired edema.[11] Hence, there are many experimental setups for the concentration and application periods. VEGF-C-based approaches to lymphedema therapy are summarized in Table 2.

Stem cells in secondary lymphedema therapy

As bone marrow stromal cells (BMSCs) differentiate into lymphatic endothelial cells (LECs), they may ameliorate lymphatic edema.[15] Therefore, BMSCs or VEGF-C was administered to rat hind limbs 3 months after circumferential excision and lymph node dissection.[15] The group treated with combined BMSCs and VEGF-C showed the best regeneration, as measured by volumetry, VEGF-C quantification, and LYVE-1-positive vessel counting.[15] Similar results were observed for the application of a VEGF-C hydrogel and human adipose-derived stem cells (hADScs) to mouse hind limbs (Table 2).[32] More LYVE-1-positive vessels were observed after 4 weeks in the group treated with combined VEGF-C and hADScs compared with the other groups.[32] In the case of transplantation of LECs, LEC application exhibited better results than application of human dermal microvascular endothelial cells (HDMECs) or placebo based on tail measurements in a rat tail model.[9]

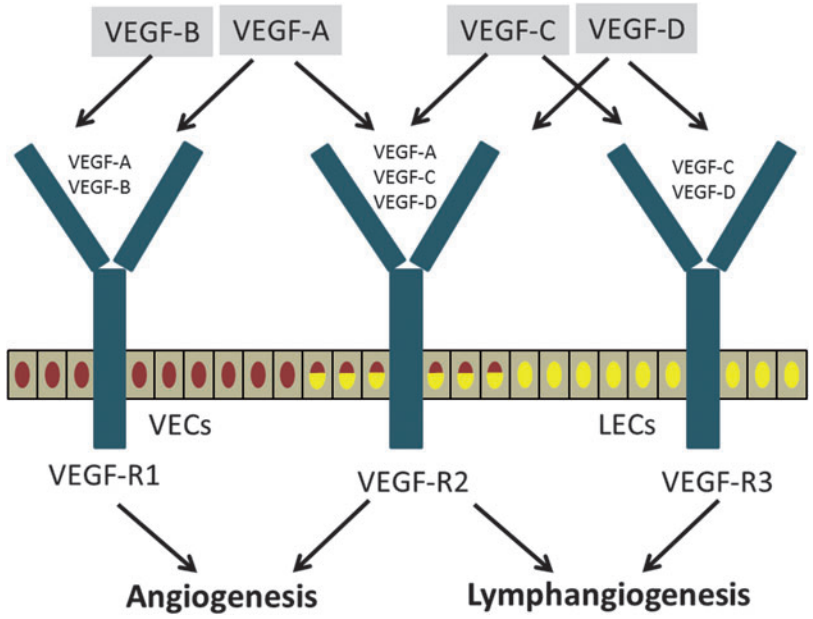

FIG. 4. Molecular pathways in angiogenesis and lymphangiogenesis. *Red* cell nuclei: VECs, *yellow* cell nuclei: LECs, *yellow* and *red* cell nuclei: VECs and LECs. LECs, lymphatic endothelial cells; VECs, vascular endothelial cells.

Table 2. VEGF-C in Secondary Lymphedema Therapy

Author	*Animal model*	*Technique*	*Agent*	*Dosage of agent*	*Application and time*	*Clinical relevance of lymphedema*	*End point, days*
Cheung et al.[8]	Mouse tail	Circumferential excision	Exogenous Human Recombinant VEGF-C	Single application 200 ng	Prox. and dist. tail, once on POD 3	Macroscopic: tissue swelling; Histological: dilated cutaneous lymphatics, acute inflammatory, hypercellularity, increased amount of VEGF-C	71
Jin et al.[29]	Mouse tail	Circumferential excision	Exogenous Human Recombinant VEGF-C, Anti-VEGF-R3	Anti-VEGF-R3 (25 μg/g) VEGF-C: once 200 ng	VEGF-R3 intraperitoneal, daily VEGF-C: subcutaneous, once on POD 3	Macroscopic: tissue swelling; Histological: inflammation, hypercellularity, hyperkeratosis, dilated lymphatics	11
Lähteenvuo et al.[26]	Pig hind limb	Excision of lymphatic vessels	Adenoviral VEGF-C and VEGF-D	1×10^{11} viral particles, or 1×10^{10} VEGF-D	Subcapsulary into lymph node, once in surgery	Histological: accumulation of seroma fluid at the operation site, slight deterioration of lymph node, fibrosis, and fat accumulation	60
Mendez et al.[19]	Mouse forelimb	Axillary lymph node dissection (ALND)	Antibody against mouse VEGF-R3	150 μL in 0.635 mg/ dose against saline	1 day before surgery and every 5 days	Macroscopic: tissue swelling; Fluorescent lymphangiography: increased tracer coverage; Negative: tracer coverage returned normal on POD 10	15
Ongstad et al.[11]	Mouse tail	Circumferential excision	Antibody against mouse VEGF-R3/-2	0.625 mg per dose	Intraperitoneal injection, VEGF-R2/-R3 prior surgery and every 5 days POD	Macroscopic: tissue swelling; Histological: macrophage accumulation; Negative: normalization after 60 days	60

(*continued*)

TABLE 2. (CONTINUED)

Author	*Animal model*	*Technique*	*Agent*	*Dosage of agent*	*Application and time*	*Clinical relevance of lymphedema*	*End point, days*
Sommer et al.[31]	Rat hind limb	Inguinal and popliteal lymph node dissection	VEGF-C	6.67 μg dissolved in 150 μl PBS	Skin next to transplanted fragments, POD 1, 2, 5.	Macroscopic: tissue swelling; Histological: lymph node necrosis after transplantation, vital B and T cells and amount of LYVE-1 positive vessels	28
Tammela et al.[23]	Mouse forelimb	Axillary lymph node dissection	Adenoviral Gene Transfer Vector encoding VEGF-C, VEGF-D	5×10^8 plaque forming units	Under muscle fascia around axillar plexus, once directly postsurgery	Macroscopic: tissue swelling; Histological: inflammatory cell infiltrates in the dermis, low drainage of FITC-dextran, densities of lymphatic vessels	360
Tervala et al.[30]	Mouse forelimb	Axillary lymph node dissection	VEGF-A, VEGF-C, VEGF-C156S, VEGF-D	5×10^8 plaque forming units	Into lymph nodes, once in surgery	Histological: accumulation of inflammatory cells in lymph nodes, low amount of VEGF-C and LYVE-1 pos. vessels; Fluorescent lymphangiography: low uptake	90
Yoon et al.[7]	Rabbit ear and mouse tail	Circumferential excision	Plasmid DNA encoding Human VEGF-C	500 μg/0.5 mL	Intradermal and subcutaneous, POD 1, 6, 11	Macroscopic: tissue swelling; Histological: fibrofatty tissue deposition, greater skin thickness, fibrosis; Lymphoscintigraphy: low lymphatic drainage	Rabbits:84, Mice: 24

PBS, phosphate buffered saline; POD, postoperative day; VEGF, vascular endothelial growth factor.

Hyaluronidase in secondary lymphedema treatment

To reduce lymphedema, various setups, including hyaluronidase treatment, were established.[33] The authors hypothesized a higher-than-physiological hyaluronan level in a lymphedematous extremity, whereas the protein level was irregularly low.[33] Furthermore, the scientists suggested that macromolecules such as hyaluronan inhibit cell traffic and support cytokine release. In a mouse tail model, hyaluronidase treatment decreased lymphatic dilatation, increased the number of neutrophils, increased the number of VEGF-R3- and LYVE-1-positive vessels, and resulted in lymph flow similar to that in the control group.[33] Hyaluronidase gel application resulted in improved outcomes by reducing the tissue fluid content and fostering edema resolution[18] (Table 4).

Growth factors, cytokines, and enzymes in secondary lymphedema therapy

As hepatocyte growth factor (HGF) regulates the motility, growth, and morphogenesis of many cell types, an external HGF supply increases aortic endothelial cell proliferation and migration. In lymphedema models, HGF application caused a decrease in rat tail thickness and the promotion of lymphatic vessel formation independent of the VEGF pathway[34] (Table 3). Pro-inflammatory cytokines, such as interleukin 8 (IL-8), are assumed to stimulate lymphangiogenesis by serving as inflammatory stimuli.[35] A transgenic mouse model that expresses human IL-8 in the skin was used to detect the impact of this cytokine. IL-8-transgenic mice exhibited a faster reduction in their tail volume after lymphedema than wild-type mice did and showed more LYVE-1-positive cells. The authors concluded that IL-8 may promote the proliferation of LECs and tube formation and migration by LECs without VEGF signaling[35] (Table 4). In addition, matrix metalloproteinases such as MMP-9 may play a significant role in matrix changes during acquired lymphedema. Therefore, transgenic MMP-9-null mice were used to investigate the pathophysiological context.[36] In these knockout mice, the collagen density was lower than in the wild type, and greater tail swelling was noted. Interestingly, LEC proliferation began before VEGF-C upregulation.[36]

NSAIDs in secondary lymphedema therapy

Nonsteroidal anti-inflammatory drugs, such as ketoprofen, ameliorates tail lymphedema in mice in comparison to sham surgical controls.[37] Histopathological changes such as inflammatory response, hyperkeratosis, and epidermal thickness were significantly reduced in NSAID treatment group.[37] In addition, ketoprofen leads to an increase of pro-lymphangiogenic factors (esp. VEGF-C incl. receptor and LYVE-1).[37] These results indicate that NSAIDs might ameliorate lymphedema symptoms and histopathological changes by inducing pro-lymphangiogenic factors.

To summarize the most important pharmacological therapy options, the main focus has been on VEGF-C application in combination with stem cell therapy or local application of lymphatic endothelial precursor cells. Interestingly, VEGF-C-independent therapies such as hyaluronidase treatment, growth factor therapies, treatment with matrix remodeling factors, or cytokine application improved lymphedema in animal models, which led to the conclusion that VEGF-C is not the only factor responsible for LEC differentiation in wound-healing lymphangiogenesis.

Surgical Therapy Options

The most common surgical techniques are shown in Figure 5.

Excisional techniques

The previously prevalent method of radical excision of tissue (the Charles procedure) has been replaced by more tissue-restrictive techniques and is only currently used in the worst cases of chronic status.[3] The disadvantages of excisional techniques in lymphedema therapy are massive tissue trauma, subcutaneous morbidities, painful recovery, and failed reconstruction.[3] Pathological subcutaneous fat accumulation additionally occurs in chronically affected edematous limbs in humans. Liposuction can be performed to reduce this accumulation by removing the extra fat enrichment. However, liposuction without surgical intervention cannot restore the flow in the lymphatic system, so lifelong wearing of compression garments is required to avoid recurrent volume increases.[3]

Reconstructive techniques

Lympho-lymphatic anastomosis and lymph vessel transplantation. Lympho-lymphatic anastomosis or lymph vessel transplantations are the most frequently used reconstructive technique of lymphatic vessels in humans. Within these techniques, lymphatic vessels get reconnected to other intact lymphatic vessels to gain new lymphatic drain. Lympho-lymphatic anastomosis could be either transpositions or avascular transplantations.[38]

Lympho-lympho nodular anastomosis. In animal models, several reconstructive techniques have been explored. Lympho-lympho nodular (LLN) anastomosis in a rat peritoneal model produced good results in comparison with the endogenous potential to regenerate lymphatics.[39,40] After 8 weeks, new connections occurred between the left and right sides after an LLN procedure without anastomosis. The researchers determined that lymphatic drainage through the connected efferent lymph node was 100% reconstructed, whereas endogenous regeneration led to 25% restoration.

Vascularized lymph node transfer. To resolve massive tissue damage using flap techniques, isolated vascular lymph node transfers were tested in rats. The vascularized cervical lymph node transfers involved five to six lymph nodes from the rat neck and showed promising results.[41]

In a pig model, vascularized lymph node transfer may improve lymphatic regeneration, especially with the application of VEGF-C.[26] Similar to the case for flap transfers, the length of the vascular pedicle in vascularized lymph node transfer is the determining factor for successful transplantation. However, the microsurgical preparation of major venous and arterial vessels is demanding, time consuming, and risky; so its clinical relevance is controversial.

Avascular lymph node transfer and fragmentation. Since Rothkötter and Pabst reported the viable regeneration of

Table 3. Stem Cells in Secondary Lymphedema Therapy

Author	*Animal model*	*Technique*	*Agent*	*Dosage of agent*	*Application and time*	*Additional therapy*	*Clinical relevance*	*End point, days*
Hwang et al.[32]	Mouse hind limb	Circumferential excision	hADScs VEGF-C Hydrogel	hADScs (1×10^4 cells/mL) VEGF-C hydrogel (100 μg)	Hind limb, once after surgery	—	Macroscopic: tissue swelling; Histological: fibrofatty tissue deposition, Collagen deposition, LYVE-1 staining	30
Park et al.[17]	Rodent hind limb	Circumferential excision	LEC precursor cells	1×10^7 LEC precursor cells	Hind limb; 10 days after surgery	4500 cGy/3 fractions of RT at day 5 after surgery	Macroscopic: tissue swelling; Histological: LYVE-1 staining; Lymphoscintigraphy: surgery and RT have no radioactive uptake; Negative: high incidence of necrosis in hind limbs of mice (surgery+RT)	56
Kawai et al.[9]	Rat tail	Circumferential excision	HDMECs or human LECs	HLECs and HDMECs (both 5×10^6 cells/0.1 mL PBS)	Subcutaneous in operated side; on days 1, 4, 7, 11, 14	—	Macroscopic: tissue swelling; Histological: increase of epidermal thickness, increase of LYVE-1 and Podoplanin pos. vessels; Fluorescent lymphangiography: increased time of transit time	36
Zhou et al.[15]	Rabbit hind limb	Circumferential skin excision and lymph node resection	Bone marrow stromal cells (BMSCs) and exogenous VEGF-C Protein	BMSCs (1×10^7 cells) or VEGF-C (150 ng/kg body weight)	Intramuscular each irritated area; 3 months after surgery	2000 cGy 3 days after operation	Macroscopic: tissue swelling; Histological: VEGF-R3 positive vessels were counted and VEGF-C quantification	28 and 360

hADScs, human adipose-derived stem cells; HDMECs, human dermal microvascular endothelial cells; LEC, lymphatic endothelial cells; RT, radiotherapy.

Table 4. Growth Factors, Cytokines, and Enzymes in Secondary Lymphedema Therapy

Author	*Animal model*	*Technique*	*Agent*	*Dosage of agent*	*Application and time*	*Clinical relevance of lymphedema*	*End point, days*
Choi et al.[35]	Mouse tail	Circumferential excision	Human recombinant IL-8	250 μL Matrigel plug assay with 50 ng/mL IL-8	Subcutaneous in tail; once directly after surgery	Macroscopic: tissue swelling; Histological: decreased lymphatic vessel regeneration without therapy; Negative: no signs of inflammation	14
Jeong et al.[33]	Mouse tail	Circumferential excision	Hyaluronidase	Hyaluronidase (150 IU/0.1 mL)	Dorsal and ventral tail base; on POD 9	Macroscopic: tissue swelling; Histological: increased thickness of dermis and subdermis, increased interstitial fluid volume and number of inflammatory cells, fibrosis; Lymphoscintigraphy: decreased radioactive uptake	23
Nekoroski et al.[18]	Mouse tail	Circumferential excision	Hyaluronidase gel (PH20 SR gel) or Recombinant human hyaluronidase (rHuPH20)	I. Trial group A, B, and C: 20 μL II. Trial: (a)+(b): 30 μL	Intradermal; I. trial: A + B day 12 + 26, C: five times per week start: day 12; II. trial: (a) day 12, (b) day 12 + 27	Macroscopic: tissue swelling	24
Rutkowski et al.[36]	Mouse tail	Circumferential excision	MMP-9-null mice	—	—	Macroscopic: tissue swelling; Histological: collagen degradation, fluid and liquid accumulation, macrophage accumulation, hyperplasia of lymphatic vessels; Lymphoscintigraphy: reduced uptake or radioactive tracer	30
Saito et al.[34]	Rat tail	Circumferential excision	Human HGF gene	200 μg/0.1 mL	Intradermal at distal operated site, Days 1, 7, and 14 of saline/human HGF plasmid	Macroscopic: tissue swelling; Histological: LYVE-1 and Prox1 were increased only in the HGF-injected group.	35

HGF, Hepatocyte growth factor; IL-8, Interleukin-8.

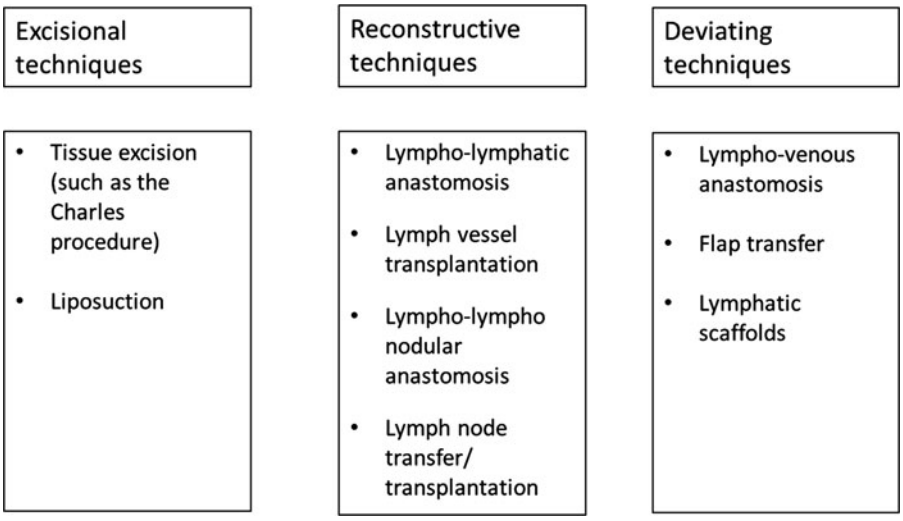

FIG. 5. Surgical treatment of secondary lymphedema.

lymph tissue after avascular lymph node fragment transplantation in pigs,[42] many researchers have tried to replicate this result. The autotransplantation of avascular lymph node fragments showed promising results in rat hind limbs, and these results were exceeded with platelet-rich plasma application.[43] The combination of avascular lymph node transfer and VEGF-C has been explored in rodent hind limb[31] and mouse forelimb[23] models. The major advantage of avascular lymph node transfer is that no vascular pedicle is needed, and transplanted segments show functional recovery.[31] However, further research is needed to investigate which fraction of lymph nodes should be transplanted.

Deviating Techniques

Lympho-venous anastomosis

To investigate lympho-venous anastomosis, several researchers compared various approaches using acquired lymphedema models. Kinjo et al. used a dog retroperitoneal model to compare lymphatic node-to-vein anastomosis with lymphatic vessel-to-vein anastomosis, lymphatic vessel-to-isolated-vein anastomosis, and connection to isolated venous segments.[44] There was no patency of lymphatic node-to-vein anastomosis after day 5, and the overall patency decreased in all groups over time. Vessel-to-isolated-vein anastomosis was the best technique in this surgical setting, with a 180-day patency rate of 71%. Nevertheless, the major problem in lympho-venous anastomosis is high venous blood pressure and the resultant risk of clotting of the incoming bloodstream.[39]

Flap transfer in lymphedema therapy

Since flap transfer was established for lymphedema therapy, various techniques have been used in animal models. A long-pedicled rectus abdominis mucocutaneous tissue flap containing lymph nodes showed the best results in comparison with control and *in situ*-replaced groups in a rodent tail model.[45] Full-thickness skin transferred from transgenic LYVE-1-knockout mice showed spontaneous reconnection with recipient vessels and supported full regeneration in a tail skin graft after 6 weeks.[46]

Nevertheless, high tissue trauma in transplanted areas and the pedicle length of the vascularized fraction limit the use of this technique.

Lymphatic scaffolds

Vascular scaffolds were newly discovered in lymphatic regeneration. Biodegradable synthetic scaffolds serve as temporary matrix to reconstruct new lymphatic vessels. Dai et al. investigated polyglycolic acid (PGA) scaffolds with endothelialization by human LECs subcutaneously transplanted in nude mice.[47] Six weeks after implantation, biodegradable PGA matrices were degraded while layers of LECs were ordered in the inner vessel walls of silicone tubes. In cell-free scaffolds, no LEC-positive layers could be detected.[47] Staining and polymerase chain reaction with lymphatic-specific marker were positive in LEC matrices and absent in cell-free scaffolds.[47] Nonbiodegradable scaffolds have been successful in cardiovascular research and promising use in lymphatic reconstruction.[48] Kanapathy et al. assumed a nanocomposite polymer (polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane [POSS-PCU]) as auspicious lymphatic scaffold as it has been successful in cardiovascular approaches.[48] Wong et al. used the rat epigastric artery model for investigating acellular dermal matrix (AlloDerm) and in a cardiovascular setting found histological evidence for lymphatic vessel formation.[49]

In conclusion, reconstructive techniques are currently the most promising techniques to rebuild the lymphatic drainage, resulting in a significant decrease in the lymphedema volume. However, further research is needed to compare various techniques to standardize surgical secondary lymphedema therapy, and lymphatic scaffolds have to be investigated application-oriented to rebuild absent lymphatic vessels.

Animal Models in Additional Therapy

Shock wave therapy in secondary lymphedema treatment

Because low-energy shock wave (SW) therapy was recently found to ameliorate ischemia-induced myocardial

Table 5. Shock Waves in Secondary Lymphedema Therapy

Author	Animal model	Technique	Total energy flux density	Time of application	Additional therapy	Clinical relevance of lymphedema	End point, weeks
Kim et al.[51]	Mouse hind limb	Circumferential excision	0.05 mJ/mm^2, 500 shots	Start: 3 day POD, tertian	VEGF-C hydrogel (100 μg VEGF-C)	Macroscopic: tissue swelling; Histological: dermal edema, collagen deposition, macrophage infiltration; Negative: acute lymphedema	4
Kubo et al.[6]	Rabbit ear	Circumferential excision	0.09 mJ/mm^2, 200 shots	Start: 2 weeks POD, thrice per week	—	Macroscopic: tissue swelling; Histological: epidermis edema, high cellularity in the dermis, increase in thickness of the tissues; Negative: acute lymphedema, started therapy 2 weeks after surgery	4
Serizawa et al.[22]	Rat tail	Circumferential excision	0.25 mJ/mm^2, 500 shots	Start: 3 day POD, every second day until day 9	—	Macroscopic: tissue swelling; Histological: increase in thickness of the tissues, low average fluorescence intensity; negative: rat tails heal almost completely without treatment	4

dysfunction,[50] researchers have been anxious to apply this method to lymphedema therapy. The mechanism of low-dose SW therapy might be the development of cavitation (the formation of vapor bubbles in flowing liquid),[22] which may induce shear stress. This process would result in increased cell membrane permeability and could lead to upregulation of the expression of genes such as VEGF-A.[50] SWs were applied to acquired lymphedema in various experiments, but the total energy flux density differed among all setups (Table 5). After lymphedema was induced in the rabbit ear, SW therapy achieved significant volume decrease in the treated groups.[6] The amounts of VEGF-C and VEGF-R3 and the density of lymphatics were significantly increased in the therapy group after 4 weeks. Similar results were obtained in rat tail[22] and mouse hind limb[51] models. Interestingly, the combination of SWs and local application of VEGF-C produced the best results compared to the results in the other groups.[51]

In conclusion, SW therapy was very effective in improving angiogenesis and lymphangiogenesis. The upregulation of important VEGF-family factors led to a volume decrease and the rebuilding of lymphatic structures. The disadvantages of SW treatment included no long-term results and an indication that a VEGF-C overdose may induce lymphatic dysfunction, such as hyperplasia.[51]

Modulating diet in secondary lymphedema

Obesity in patients leads to decreased clearance of interstitial macromolecules compared with lean patients[52] and in severe cases with BMI >59 to spontaneously develop lymphedema.[53] Obesity models in animals could help to bare pathological mechanism in secondary lymphedema. In mouse tail models, diet-induced obesity was investigated after lymphatic injury of circumferential excision.[21] Six weeks postoperatively, obese mice showed clinical relevant

changes, including fat deposition, fibrosis, and exaggerated inflammation in comparison to lean mice.[21,54] In addition, lymph nodal uptake of lymphoscintigraphy agent was significantly reduced in obese mice, especially after lymphatic injury.[21]

Lymphangiography in Secondary Lymphedema

The most common procedures for lymphangiography are listed and evaluated in Figure 6.

Macroscopic lymphangiography

Various lymph effluent dyes have been tested. Subcutaneous or intradermal injection of special dyes leads to staining of the draining lymphatics. Patent blue, a triphenylmethane dye, is used for food coloring and for staining sentinel lymph nodes in humans. In animals, blue staining of lymphatics was deployed in rat tail models,[34] rodent hind limbs,[10,31] and pig hind limbs.[26] An alternative dye is methylene blue. This phenothiazine is typically used in antidote therapy after nitrite intoxication, facilitating metabolism from met hemoglobin to hemoglobin. In animal studies, it was used in rodent tail[8,12] and rabbit ear[24] models. Another lymph effluent dye is Evans blue, which was utilized in rodent forelimb[23,30] models. All blue staining dyes are utilized for macroscopic evaluation of lymphatic vessels and lymph nodes in draining areas. Quantification using blood sampling after a defined time of agent application and spectrophotometric analysis has also been performed for evaluation.[26,30]

X-ray lymphangiography

In X-ray lymphangiography, iodine-containing contrast agents such as iopamidol have been used to examine lymphatic flow after subcutaneous application in rabbit ears.[24] Lipiodol, an ethiodized oil-based contrast agent for X-ray imaging, was also used in a pig hind limb model through direct cannulation of lymphatic vessels.[26] The disadvantages of X-ray lymphangiography are poor morphological correlation and low spatial and temporal resolution in addition to ionizing radiation exposure. Furthermore, Isovue, an iodine-containing contrast agent, has occasionally produced allergic reactions or hyperthyreosis, and lipiodol has led to irritation of the lymphatic endothelium and requires direct cannulation. Therefore, X-ray lymphangiography is currently obsolete.

Lymphoscintigraphy

Currently, the gold standard in the imaging of lymphatics is lymphoscintigraphy. A very common agent for lymphoscintigraphy is technetium-99m-radiolabeled sulfur colloid (TC-99m). Detection of lymphatic flow has been conducted with gamma cameras based on the radioactive tracer distribution in the lymph nodes.[16,17,55] This technique has been used to detect dermal backflow and functional transport through the lymphatics. With this method, even quantification of lymphatic flow in edematous extremities has been possible through radioactivity index measurement after injection of TC-99m.[7] In this case, the measured radioactivity at injection sites was subtracted from the total number of counts. Higher ratios indicate more persistent radioactivity and, therefore, less lymphatic drainage.[7] The disadvantages of this method include poor morphological correlation, low tissue penetration, poor spatial resolution, and ionizing radiation exposure.

Fluorescence lymphangiography

High-molecular weight fluorescein isothiocyanate (FITC)-labeled dextran fluorescence has been used in live microlymphangiography[30] with *ex vivo* quantification. Furthermore, near-infrared (NIR) indocyanine green (ICG) fluorescence was used to measure liver function and to perform angiographies.[56] ICG exhibits fluorescence after binding to albumin following intradermal or subcutaneous application.[56]

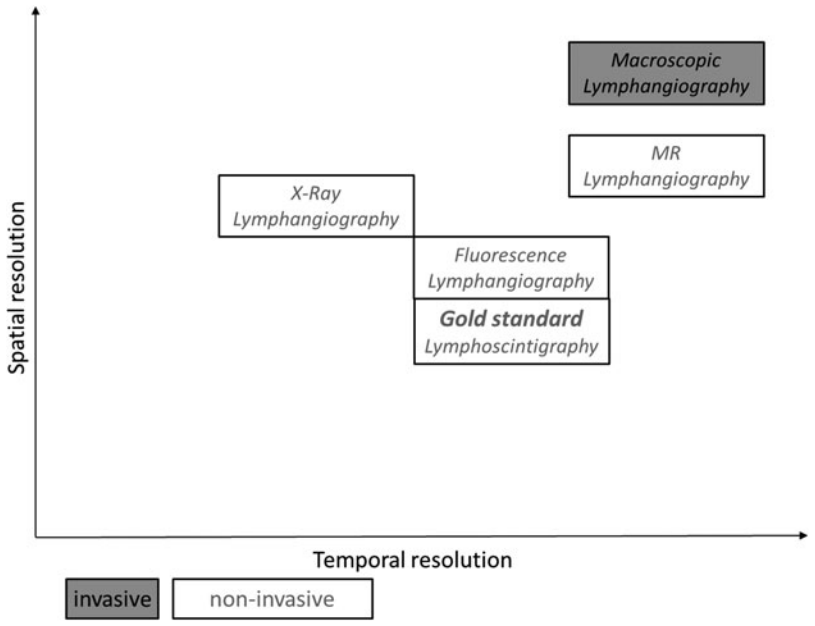

FIG. 6. Assessment of lymphangiography methods in secondary lymphedema.

Drainage through the lymphatics enables its detection at a wavelength of 760 nm.[56] These measurements are translated into a black-and-white signal.[57] Dermal backflow, as a sign of lymphatic dysfunction, can be detected after new pathways are created.[56] Fluorescent lymphangiography can also be implemented using multicolor NIR optical imaging by visualizing the simultaneous drainage of two separate lymphatic vessels using nanoparticles. IgG-conjugated NIR optical agents have also been administered to measure various peak emission wavelengths.[58] ICG fluorescence makes it feasible to image dermal backflow with good temporal resolution. However, its drawbacks include poor penetration depth[57] and morphological correlation, poor spatial resolution and no possibility of quantification. In conclusion, ICG fluorescence can be used to examine the early stages of lymphedema, before extremity swelling occurs.[56]

Magnetic resonance lymphangiography

To improve morphological correlation and spatial and temporal resolution, magnetic resonance (MR) lymphangiography was established.[59] Gadolinium-containing agents were specifically used to visualize lymphatic functionality. Using animal models, Fink et al. showed promising results in illustrating the lymphatic system in rats without acquired lymphedema.[60] After radiation and axillary lymph node dissection, Wu et al. imaged the lymphatic system in rhesus monkey lymphedema using gadolinium-based MR lymphography[27] after intracutaneous injection. The advantages of this technique include good spatial and temporal resolution for the visualization of small lymphatic vessels and clear congruency with the gold standard, lymphoscintigraphy.[61] Gadolinium-containing contrast agents show high skin penetration and direct morphological correlation of lymph nodes and lymphatic vessels.[62]

To sum up, lymphangiography plays an important role in diagnosing secondary lymphedema in humans. In addition, analyzing the functionality of lymphatic vessels is crucial for prospects and to estimate the therapy success. Animal models could help to refine lymphangiography methods to enhance spatial and temporal resolution and investigate reliable methods for lymphatic flow quantification.

Conclusion

Rodent tail and hind limb models are most often used in current lymphatic research. However, especially rodent models suffer from high endogenous lymphatic regeneration, and therefore, only acute lymphedema can be mimicked. Large animal models, such as monkey upper limb lymphedema, are promising, but have to be investigated for late effects. Surgical reconstructive approaches in combination with lymphatic-specific growth factor or stem cell therapy represent the most promising current options among secondary lymphedema treatment models. Several auspicious surgical techniques have been evaluated, but further research is needed to compare techniques to standardize the surgical treatment involved in lymphedema therapy. Furthermore, interference with and the side effects of pharmacological therapies must be monitored. Detailed molecular mechanisms must be elucidated to improve the comparability of animal models to human chronic secondary lymphedema. Further research is also required to enhance current imaging models to yield morphologically correlated and highly temporal and spatially resolved images.

Author Disclosure Statement

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