Disruption of Lymphatic Function following Lymph Node Excision and Irradiation:
Integrating Natural Compensatory Responses with potential Therapeutic Approaches to Facilitate Lymph Flow Restoration

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Introduction: The removal or irradiation of one or more lymph nodes during cancer surgery appears to be a significant causative factor in lymphedema development. In the studies outlined in this thesis our first objective was to define the lymph transport deficit caused by nodal excision and/or the application of radiation to a single node. The second objective was to examine two potential methods to restore function after node removal, autologous node transplantation and the introduction of lymphangiogenic factors into the nodal excision site.

Methods: In both sheep and rabbits, popliteal nodes were removed and/or subjected to radiation. Animals received one of two potential therapies at the excision site; lymph node transplantation or growth factor therapy (VEGF-C and Ang-2). Following surgery/treatment, a tracer was injected into a prenodal vessel and the system’s ability to transport this tracer to plasma was used as a measure of lymphatic functionality. Fluoroscopic and histological examination provided supporting data. Edema was
quantified from the circumferential measurement or bioimpedance of the hind limbs.

Results: Following insult to the popliteal node lymphatic function was reduced significantly in comparison to control limbs. Additionally, in the case of radiation, the pressure-flow relationships differed from controls in a way that indicated enhanced resistance to flow (possibly caused by fibrosis and the thickening of the nodal capsules and trabeculae). Fluoroscopy and studies with Evans blue dye revealed new lymphatic vessel growth and occasionally, these vessels had anastomosed with local veins. Left untreated, limbs undergoing insults regained some lymphatic functionality but did not reach control levels. Both treatments increased lymphatic functionality in comparison to non-treated limbs. Edema was almost completely resolved in animals with successful node transplants, and significantly reduced in those receiving growth factor therapies.

Conclusions: Damage to and/or removal of the lymph node as a result of cancer treatment can significantly impair the lymph drainage in limbs. Regeneration of lymph vessels, the formation of collateral pathways and lymph-venous anastomoses act as natural compensatory mechanisms, although usually only to a limited extent. However, therapies such as autologous lymph node transplantation or growth factor therapy have the ability to restore lymphatic functionality and decrease edema.
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Thank the research gods for coffee and tape! Both of which were invaluable in the completion of this thesis!!
Declaration

The physiological experiments conducted within this thesis were complex in nature and generally required a team effort. Ms. Sara Moore is the senior technician in the Johnston lab and has been involved in all animal related aspects of the studies outlined here. The contributions of key individuals are indicated below.

Study 1 – Functional analysis of lymphatic transport following lymph node removal

I (Amy Baker) was a collaborator on this study and co-author of the publication that resulted from it. I was involved in all surgical aspects of the project, data collection and analysis, as well as manuscript editing.

Study 2 – Functional analysis of lymphatic transport following lymph node irradiation

All aspects conducted by author.

Study 3 – Autologous lymph node transplantation

I (Amy Baker) was a collaborator on this study and co-author of the publication that resulted from it. I was involved in all surgical aspects of this project (excluding transplant microsurgery), data collection and analysis, as well as manuscript composition and editing.

Study 4 – Prolymphangiogenic growth factor therapy

All in vivo experiments were conducted by the author. Dr. Harold Kim assisted with the in vitro experiments.
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## List of Abbreviations

- $^{125}$I-HSA: Iodinated human serum albumin
- $^{125}$I-BSA: Iodinated bovine serum albumin
- Bin: Blood In
- $K_{exp}$: Albumin elimination rate
- $V_p$: plasma volume
- $C_p(0)$: The concentration of tracer at time zero
- $C_p(tf)$: The concentration of tracer at four hours
- CPM: counts per minute
- PBS: phosphate buffered saline
- LEC: lymphatic endothelial cell
- AEC: arterial endothelial cell
- VEC: venous endothelial cell
- SMC: smooth muscle cell
- Gy: gray
- VEGF-C: Vascular endothelial growth factor C
- VEGFR2/3: Vascular endothelial growth factor receptor 2 or 3
- ANG-1/2: Angiopoietin 1 or 2
- Prox-1: Homeobox Prospero-Like Protein 1
- LYVE1: Lymphatic Vessel Endothelial Hyaluronan Receptor 1
- SMA: Smooth muscle actin
- H&E: Hemotoxylin and eosin staining
- Zo-1: Zona Occludens Protein 1
- VE- or N- cadherin: Vascular endothelial or neuronal cadherin
- TGFβ1: Transforming growth factor beta 1
- TGFβRII: Transforming growth factor beta receptor 2
- ALND: Axillary lymph node dissection
- SLND: Sentinel lymph node dissection
- FITC-dextran: Fluorescein isothiocyanate-dextran
Chapter 1
General Background

1.1 Overview

The lymphatics represent a complex circulatory system composed of small initial vessels that coalesce into collecting ducts that ultimately empty into the veins at the base of the neck. Initial lymphatics are composed of overlapping endothelial cells, some of which are attached to the interstitium via anchoring filaments. These vessels merge into collecting ducts, which are larger vessels that transport lymph over long distances in the body. Lymph nodes are interspersed along the lymphatic network. The collectors can be classified into pre- and post-nodal vessels (leading into or away from a lymph node respectively) and are composed of functional units called lymphangions that are essentially 'lymph hearts' arranged in series. Valves interspersed along the system prevent lymph backflow. Collectors eventually converge and lymph is returned into the vascular system mainly via the thoracic vessel, which joins with the venous system at the junction of internal jugular and left subclavian vein. In some species an equivalent right lymph vessel is present although the volume of lymph in this vessel is much less than that of the thoracic duct. Overall, the lymphatic system plays a pivotal role in fluid and protein homeostasis in the body. Other functions include absorption of lipids in the digestive system, trafficking of lymphocytes and antigen-presenting cells to regional lymph nodes and transport of degraded extracellular molecules, cell debris, and lymph fluid.

The hallmark of disordered lymphatic function is edema. Especially important in the North American context is the chronic and debilitating edema that is associated with cancer surgery (lymphedema). The lymphedema associated with breast cancer treatments has been termed ‘the secret epidemic’ [1]. While a lymph transport deficit has been implicated in this disease the exact nature of the deficit remains unclear. It has always been assumed that damage done to the lymphatic vessels is responsible for the impediment to lymph flow in this disease. However, lymphatic vessels are damaged routinely in surgical procedures and no lymphedema develops due to the robust regeneration capacity of the lymphatic system. In contrast it is clear that the removal of lymph nodes, especially when combined with radiation, is a major factor in the development of the disease.
The lymph node has long been considered a 'neutral' element in terms of lymph transport; however there is reason to believe that these structures have an underappreciated physiological role in fluid balance. The objective of the research outlined in this thesis was first to investigate the impact of lymph node excision or irradiation of the lymph node on lymphatic function. These 2 practises represent the key cancer treatment procedures that contribute to lymphedema. The second objective was to examine autologous lymph node transplantation or the delivery of lymphatic growth factors to the excision site as possible therapeutic measures to mitigate the damage to the lymphatic system.

1.2 Lymphatic system function

Lymph is an ultrafiltrate of plasma and is formed when interstitial fluid and solutes are taken up by lymphatics at the absorbing end of the system. The filtration of fluid across capillary membranes is governed by the starling forces \[2,3\]. The sum of the hydrostatic and oncotic forces acting across the capillary membrane results in net fluid movement from the capillary into tissues. In the classic view, the lower pressures in the post-capillary venules cause re-absorption of some of the water at the venular end of the capillaries with the residual fluid in the interstitium, taken up by lymphatics. However, the newer ‘glycocalyx model’ suggests that microvascular absorption is transient in most tissues and that slight filtration prevails in the steady state, even in venules. A greater role for standing plasma protein gradients within the intercellular cleft of continuous capillaries has been proposed for capillary filtration and lymph formation in most tissues \[4\]. The important issue is that the majority, if not all of the filtered solutes and water from capillaries is now believed to enter the initial lymphatic network making lymph drainage even more important in regulating tissue fluid dynamics.

Initial (absorbing) lymphatics are composed of a single layer of overlapping endothelial cells that are anchored to collagen in the interstitium. While the forces necessary for lymph formation are still being debated, it is generally believed that hydrostatic pressure gradients fill the initial lymphatics. In brief, contractions-relaxations of the endothelial cells (or extrinsic compression-relaxation sequences) cause a transient decrease in intraluminal pressure relative to that in the interstitium during the diastolic phase of the cycle. The ensuing pressure gradient favours the movement of
fluid into the initial vessels [5]. Under these conditions, the gaps (~2 µm) between the endothelial cells allow the free movement of water, protein, solutes and even cells into the lumen of the initial lymphatics. Other factors that increase local tissue pressure facilitate lymph formation such as respiration, muscle contraction (e.g., peristalsis, walking), elevated capillary filtration (e.g., venous hypertension, increased capillary permeability), and massage.

1.3 Lymphedema

Lymphedema is broadly defined as the chronic accumulation of protein rich fluid within the interstitium of tissues caused by a deficit in the lymphatic system (whether the concentration of interstitial protein in lymphedema is high or low has been questioned by some) [6]. In any event, once the lymphatic system is compromised, a cascade of events is triggered that leads ultimately to protein/water accumulation and tissue remodelling [7]. There are two different types of lymphedema, primary and secondary.

The primary or heritable form of lymphedema is rare, affecting 1.15 /100 000 persons less than 20 years of age [8]. Multiple mutations have been shown to play a role in the human pathology of this disease including VEGFR3 (Milroys disease), FOXC2 (Lymphedema distichiasis syndrome) and SOX18 (Hypotrichosis lymphedema telangiectasia) [9-13]. In mice, mutations in the genes ANG-2, neurophilin 2, ephrin, Elk, integrin alpha 9, podoplanin, and prox-1 have been associated with lymphedema or abnormal lymphatic morphology [14-20].

The second and by far more prevalent form of lymphedema is the secondary or acquired form. The most common cause of secondary lymphedema is filariasis, a parasitic disease found in tropical and subtropical regions of the world (100 million cases worldwide) [21]. However, in developed nations, lymphedema tends to form more commonly as a consequence of cancer treatment. It is typically associated with breast cancer but also occurs in gynaecological cancers, head and neck cancers, as well as, melanoma and sarcomas [22].

1.3.2 Post-Surgical Lymphedema
Lymphedema following breast cancer is characterized by regional swelling in one or both arms or hands. Swelling can also occur in the chest wall or breast. The reported incidence of lymphedema development following breast cancer treatment is reported to occur in 5-60% of surviving patients [22-26]. The wide variation in the reported results is an indication of differences in study design, diagnostic methods and criteria used, and timing of lymphedema measurement with respect to breast cancer diagnosis and treatment. While all breast cancer survivors are at risk of lymphedema development throughout their lifetime, incidences are linked closely to the extent of treatment [26].

Lymphedema patients suffer from a variety of health conditions including pain, heaviness and loss of mobility in the arm, recurrent infections, psychosocial issues and rarely lymphangiosarcoma [27]. Some patients also suffer economic problems since public and private health insurance plans usually cover only a limited proportion of the costs associated with ongoing management [28]. There is no ‘cure’ for post-surgical lymphedema. Current treatment modalities are conservative and generally employ massage and compression therapy. Compounding this, many sufferers often lack access to qualified health professionals. Increased understanding of this disease is essential for the development of much needed novel therapies for lymphedema.

1.3.3 Major factors influencing lymphedema development: Lymph node excision

A key factor in lymphedema development appears to be lymph node removal [29],[30-32]. In breast cancer, metastatic tumor cells frequently spread to regional lymph nodes (axillary lymph nodes) and this necessitates the need for node resection, which can destroy the lymphatic vessel network and lead to impairment of lymphatic flow [33, 34]. Historically, the removal of these lymph nodes (axillary lymph node dissection; ALND) was done for two reasons: to help stage breast cancer and determine appropriate treatment, as well as to prevent regional recurrence of the disease. The number of lymph nodes in the axilla varies from person to person but usually ranges from 20 to 40.

Until recently it was common practice to remove most or all of these axillary lymph nodes in more advanced cases of breast cancer, however this surgery was associated with significant risk of developing permanent lymphedema (20%–30% of patients) [33, 35]. Due in part to these adverse effects, sentinel lymph node procedures were initiated.
Sentinel lymph nodes are defined as the first lymph nodes receiving lymphatic drainage from the primary tumor and therefore the most likely to harbor metastatic cancer via lymphatic spread. One advantage of sentinel lymph node biopsy (SLNB) is that it reduces the likelihood of developing lymphedema by decreasing the number of nodes removed [36-38]. SLND has reduced the morbidity associated with lymph node excision by approximately four times [39, 40].

1.3.4 The potential role of the lymph node in fluid balance

Lymph nodes are small, kidney shaped organs located throughout the body at various locations along the lymphatic system. They tend to cluster in groups, especially where lymphatic vessels merge to form larger trunks, such as in the groin or axilla. Mice have 22 identifiable nodes, while humans possess approximately 500 [41]. Each lymph node is encased in a capsule of dense irregular connective tissue that radiates inwards at various locations forming trabeculae. The parenchyma of the node consists of a network of reticular fibres (reticular network) that provides structural support, as well as a site for the adhesion of macrophages, dendritic cells and lymphocytes. The parenchyma is divided into outer cortex and inner medulla. The outer portion of the cortex is composed of B lymphocytes arranged in follicles, while the inner portion (paracortex) holds populations of T lymphocytes.

The blood supply of the node is derived from arteries that enter the node at the hilum and branch into the medulla. The number of arteries supplying blood to each lymph node varies between species, the number usually ranges from 1-3, although as many as 12 have been noted [42]. All major structural characteristics of the node are indicated in Figure 1. Lymph flow through the node has been studied extensively in various animal models [43-45]. The pre-nodal or afferent vessels transporting lymph branch extensively upon reaching the surface of the lymph node giving rise to terminal afferents that infiltrate into the capsule at multiple locations. Lymph drains just beneath the capsule into the subcapsular sinus; here the lumen of the afferent vessels are continuous with that of the sinus. In the cortex, the subcapsular sinus drains to trabecular sinuses, and then the lymph flows into the medullary sinuses.
Figure 1. The structure of the lymph node

All major characteristics of the node (purple) are indicted by solid arrows. Lymph vessels are indicated in green and lymph flow direction is indicated by the dotted arrows. Arterial (red) and venous blood supply is also indicated.

The medullary sinuses converge at the hilum and from here lymph leaves the node via the efferent lymphatics. Along this route lymph comes into contact with antigen presenting cells and lymphocytes.

We tend to think of the lymph node solely in immunological terms but there are several ways that these structures can impact fluid balance. While lymph is formed almost entirely at the absorbing end of the system, protein concentrations of lymph can
be altered as it flows through the lymphatic system [46]. As lymph passes through the node, it comes into close contact with the extensive network of blood capillaries within the sinuses [47-50]. Since lymph contains less protein than that in the nodal capillaries, oncotic forces draw water from lymph into the blood with the result that lymph is concentrated on passage through the node [48-50]. As water is removed, the protein concentration of lymph can be increased as much as 400% with one passage through a lymph node [49-52].

This has important implications because the contractile properties of pre- and post-nodal lymphatic vessels appear to be different. Contractions of lymphatic vessels provide a major portion of the force required to move lymph through the system. The lymphangion is the functional unit of lymphatic collecting ducts. Each unit is composed of a segment of vessel between two one-way valves. The walls of the lymphangion are lined with endothelial cells that are seamlessly aligned with each other by tight, zipper-like junctions and ensheathed with the basement membranes and smooth muscle cells. The smooth muscle cells possess a unique ability in that they can undergo spontaneous contraction [53, 54]. A pacemaker-generated action potential, produced by spontaneous transient depolarizations initiates each transient contraction of the lymphatic smooth muscle [55, 56]. The frequency of the spontaneous SMC contractions can be modulated by the lymphatic endothelium [53]. Lastly, there is also some evidence to suggest that the lymph node has the ability to contract and potentially contributes to the transport of lymph [57]. The semilunar valves are directed towards the flow of the lymph and open when the pressure in the upstream lymphangion is greater than the pressure in its downstream counterpart. Pressure in the first lymphangion may increase because of smooth muscle contraction (lymph pump) or because of pressure on the walls from external forces [58-64].

Lymph nodes separate the afferent and the efferent lymph vessels anatomically into higher and lower pressure systems respectively [65]. With this in mind, the removal of a lymph node could have a significant impact on lymph fluid propulsion. Pre- and post-nodal lymphatics have different contractile properties and reach peak pumping performance over different pressure ranges [66]. High pressures of 22 mmHg in rats (mesentery) and 30 mmHg in human popliteal afferents have been measured [67, 68]. In contrast, efferent vessels have considerably lower pressures. Thus the pressure build-up in the afferent vessels is dissipated to some extent in the lymph node.
Therefore, if this nodal function were compromised, the downstream vessels may be forced to function over a non-optimal range of pressures with the result that lymph drainage of the limb could be reduced.

Another issue may relate to lymph flow resistance. The resistance of large lymphatic vessels is generally believed to be low and of little physiological significance [22]. In contrast, lymph nodes are known to provide resistance to lymph transport and this has been estimated to be 50–200 times greater than that provided by the lymph collectors [31]. One might expect, therefore, that the removal of a node would reduce flow resistance. However, there is some evidence that the resistance to flow increases after the removal of a lymph node, at least over a relatively short period [69].

1.3.5 Major factors influencing lymphedema development: Radiation Therapy

In addition to lymph node excision, radiation is a major and independent risk factor for lymphedema development. Axillary radiation alone increases the risk for lymphedema 2 - 4.5 times, while a combination of node removal and radiation in the axilla has an even greater synergistic effect upon disease formation (8 - 10X) [24, 70-72]. Radiation is also associated with an increasing severity of lymphedema [70, 73]. However, linking radiation to lymphatic damage and lymphedema has proven difficult, with studies often yielding contradictory results.

Radiation sensitivities of blood vessel endothelial cells (BECs) have been studied extensively [74-78]. Radiation disrupts cell-cell integrity, inhibits cell growth, and decreases the survival of these cells. Damage to BECs can be detected at very low doses of radiation (<0.25 Gy) [79]. In comparison there are fewer studies on lymphatic endothelial cell (LEC) sensitivities and what is available is inconsistent. Some studies suggest that lymphatics are relatively resistant to ionizing radiation [80] while others support the view that lymphatics are sensitive to radiotherapy [81].

In only a very limited number of studies have investigators incorporated radiation into animal lymphedema models. Initial attempts to study this issue established that radiation alone was not enough to induce any permanent edema [82]. Some were of the opinion that radiation had relatively little to no impact on lymphatic vessels themselves but did cause a significant decrease in node size [82, 83]. In later studies, a combination of surgery and radiation was able to induce lymphedema [84]. A study by Avraham and colleagues has shown that radiation directly causes the death of
lymphatic endothelial cells both in cell culture and in an animal model [85]. With the death of LECs there was also a dramatic loss of lymphatic vessels in the weeks following irradiation. It should be noted however, that inferring function from an increase or decrease in the number of lymphatic vessels in a tissue is difficult. Instead, it is better to measure functionality more directly. For example, Mortimer’s group used the clearance of a tracer injected into the interstitium to estimate lymphatic drainage in a pig model and observed that a radiation dose of 18 Gy reduced lymph drainage [86]. If we assume that radiation impairs lymphatic regeneration (through damage/death of LECs), this would have a negative impact on the restoration of lymph flow at the tumor excision site and on the reconnection of the pre- and post-nodal lymphatics after the removal of the lymph nodes. Very little research has been conducted on lymphatic function following the application of radiation to nodes that have not been removed.

The primary damage to the lymphatic system induced by radiation has not been studied in great detail; however fibrosis has been suggested as a likely contributor to lymphatic dysfunction. This process can lead to tissue hardening and several studies have hypothesized that radiation-induced fibrosis is the major cause of lymphatic dysfunction [85, 87]. It has also been suggested that radiation may play a direct role in the fibrotic process, through an endothelial to mesenchyme transition (EndMT) [87]. This process involves the conversion of LECs into fibroblast-like cells that could contribute and exacerbate the already occurring fibrosis in irradiated areas.

1.4 Overall goal of the studies in this thesis

The aforementioned considerations make it clear that lymphedema is a complex and not very well understood disease. However, the lymph node appears to be a key player in the pathogenesis of this disorder. How do the most common cancer treatment strategies pertaining to the lymph node (excision or irradiation) affect lymph transport in a limb? Can any damage be repaired or lymph function restored? These issues provide the central focus for this thesis.

In the past, a large number of animal models in various species have been developed for the study of lymphedema. The three most popular models were/are the canine hind leg [88-92] (no longer in use), the rabbit ear [93-97] and rodent models [98-100]. Much of current lymphedema knowledge has been gleaned from these models. However it seems prudent to consider whether these approaches duplicate the inciting
stimulus for lymphedema in the clinical sense. Many of the aforementioned animal models rely on significant tissue ablation techniques with the desired outcome being chronic edema. However, it seems clear that the key to lymphedema development in surgical patients relates to the removal of lymph nodes and radiation therapy. In this regard, fewer animal models have been developed which incorporate these components in the experimental design.

Another problem with many animal lymphedema models relates to the difficulty in quantifying appropriate lymphatic parameters. This is due often to the small size of the animals that are most commonly used in these studies. Animals such as the mouse are extremely valuable in understanding the molecular events that give rise to new lymphatic vessels following injury or during development. However, they are less suited for surgical intervention. In the research outlined in this thesis, the general goal was to align an assessment of lymphatic function following lymph node excision or irradiation with appropriate therapies that could be realistically applied to humans. As will be discussed in more detail later, this necessitated the use of larger species such as the sheep and rabbit.

1.5 Specific Objectives

This work comprises 4 specific objectives

1. Assess the impact of lymph node excision upon lymphatic function.
2. Assess the impact of lymph node irradiation upon lymphatic function.
3. Determine the normal compensatory responses of the lymphatic system following injury.
4. Assess potential lymphedema therapies on lymphatic function and edema.
   A) Autologous lymph node transplantation
   B) Growth factor therapy
Chapter 2.  
General methodological approaches

The following material and methods apply to all studies found within this thesis. See each individual chapter for specific methodological issues.

2.1 Animals
A total of 151 randomly bred male and female Dorset sheep (20 - 45 Kg) were used in the studies outlined in this thesis. In addition a total of 170 male and female New Zealand white rabbits (2.5 – 4.5 kg) were used in the radiation study. Animals were given free access to food and water for an observation period of one week preceding surgery. All experiments outlined in this thesis were approved by the ethics committee at Sunnybrook Health Research Institute and conformed to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

2.2 Surgery
All animals were fasted 12 hours prior to surgical procedures. Surgical sites were shaved and prepped with alcohol and betadine. Sheep were anesthetized initially with 20 ml I.V. injection of sodium pentothal. Subsequently, 2.0-3.5 % isofluorane was delivered through an endotracheal tube via a Moduflex, Dispomed machine with Hallowell respirator for surgical maintenance. Rabbits were anesthetized initially with an intramuscular injection of Ketamine (50 mg/ml) and Xylazine (5 mg/ml). Subsequently, 1.5 – 3.0 % isofluorane was delivered through an endotracheal tube. Buprenorphine (0.035 mg/kg) was given for pain management postoperatively and every 12 hours for two days. Duplocillin (0.1 ml/kg; Procaine penicillin 150,000 IU/ml, Benzathine penicillin 150,000 IU/ml) was given intramuscularly on the day of surgery.

2.3 Assessment of edema

2.3.1 Circumference Measurement
In sheep, the hind legs were shaved closely and leg circumference measurements were taken at a point 10 cm distal from the hock (tarsus). This landmark was highlighted pre operatively with a skin marker. Using a blank piece of umbilical tape
circumference measurements were taken daily in the first week post surgery and once a week after that until the animals were sacrificed. Between the hoof and hock/carpal joints the sheep limb is fairly uniform in shape and diameter, eliminating the need for a circumference measurement at more than one point. This assessment was utilized in all sheep studies.

### 2.3.2 Bioimpedance

The tissue water content of affected limbs was monitored using a commercial bioelectrical impedance device (ImpediMed, SFB7, Rochester, USA). This device passes a small current through the tissue and the impedance or the resistance to the flow of this current is directly related to the amount of extracellular fluid in the limb. The greater the water content, the lower the resistance. To take each measurement, 25 gauge needles were placed subcutaneously at the 4 locations outlined in Figure 2. Each animal had a baseline reading before experimental procedures, as well as one additional reading in the same limb before sacrifice. The difference between baseline and the final reading was expressed as a percent change. A positive percent change equated to greater tissue water. A negative percent change indicated a loss of water in the limb in comparison to baseline readings. Bioimpedance was also monitored in the contralateral non-treated limbs. This assessment was utilized in the rabbit radiation study. Each group was compared to a corresponding control group (zero percent change) with paired t-tests. Bonferroni adjustment was used to correct the overall level of significance (because multiple t-tests were run), \( p < 0.007 \).
2.4 Visualizing lymphatics

To visualize the lymphatics in all experiments, Evan’s blue dye was injected subcutaneously into the dorsum of the hind foot. The dye binds to interstitial proteins and is subsequently taken up by initial lymphatics. Additionally, an X-ray contrast agent (1–3 ml of Lipiodol, EZ-EM Canada, Anjou, Canada or 1-3 ml of Visipaque (iodixanol) 320 mg/ml, GE HealthCare, Mississauga, Canada) was injected into a prenodal lymphatic vessel using a 26-gauage cannula and a mobile fluoroscopy system (BV Pulsera, Philips, Markham, Canada) was used to visualize the lymphatic vessels and nodes of the hind limb.
2.5 Quantitative assessment of lymphatic functionality

The main role of the lymphatic vessel is to absorb extravasated vascular derived protein from the interstitial spaces and return it to the venous circulation. Therefore, the ability to transport a known mass of tracer to plasma provides a quantitative measure of the lymph transport effectiveness of a given lymphatic network. Biological tracers are relatively inert molecules that can be introduced into the body and have the ability to be detected easily. Tracers are typically attached to either radioactive isotopes or a fluorescent tag for detection and/or quantification.

A tracer suitable for introduction into the lymphatic system would need to be of sufficient size to be retained within the collecting vessels, but still possess the ability to pass through lymph nodes. Under normal conditions, molecules with molecular weights >6000 Da injected into a limb lymphatic are almost completely recovered in thoracic duct lymph [65]. The two major tracers used within this study were radiolabelled albumin (¹²⁵I-albumin) and fluorescein isothiocyanate-dextran (FITC-dextran).

Albumin is the classical lymphatic physiological tracer and the Johnston lab has extensive experience with its use [101-103]. ¹²⁵I is attached to this protein (70,000 Da) and can be detected by a gamma spectrometer.

Due to the limited availability and stability of radiotracers previously used in the Johnston lab, the use of fluorescent tracers was investigated. Dextrans are polysaccharides and can be chemically linked to fluorophores, such as Fluorescein isothiocyanate (FITC). FITC-dextran is stable in vivo and has been found to be a suitable tracer for use in circulation research [104]. For detection, excitation of this molecule at 490nm and subsequent emission at 520nm can be measured in a spectrophotometer.

Our model assumes that the tracer concentration is not altered by absorption into the initial lymphatic vessels. There is no evidence that such an effect occurs. However, once the tracer is in lymph, it can be concentrated or diluted on passage through lymph nodes depending on the balance of hydrostatic and oncotic forces [52]. Nonetheless, the dilution or concentration of the tracer on passage through the node or the nodal excision site in newly formed vessels would not affect our model since the critical issue is the total mass of tracer (product of flow and concentration) which would be unaffected. The fact that labeled albumins and dextrans have been used as flow
markers is well established in the literature. Dr. Johnston has extensive experience using tracers to quantify various lymphatic parameters [101-103, 105-107].

A schematic illustrating the features of the experimental design is provided in Figure 3. This protocol was adjusted from study to study due to tracer characteristics and its availability. See each chapter for specific details.

2.6 Statistical Analysis

Statistical analysis will be described in each chapter.
Figure 3. Schematic illustrating essential features of experimental design.

 Evan's blue dye was injected subcutaneous into the foot to highlight lymphatics. A 25-gauge cannula was inserted into an afferent lymphatic in the direction of flow. A) In transport experiments, FITC-dextran was infused into the inserted cannula via an infusion pump. B) In resistance experiments artificial lymph was infused at increasing rates via the infusion pump and inflow pressure were monitored with a pressure transducer.
Chapter 3
Assessment of lymphatic function following removal of a single lymph node.

I (Amy Baker) was a collaborator on this study and co-author of the publication that resulted from it. Consequently, the data will be summarized only briefly here.

3.1 Introduction and rationale
While lymph node excision appears to be a major factor in lymphedema development, the nature of the deficit remains unclear. For example it is known that following surgical removal of popliteal lymph node in dogs, reconnection or recannulation of the lymph pathways through the excision region occurs within two weeks [108]. Similarly, reconnection of rabbit lymphatics occurred in animals by week four and the newly formed vessels were maintained for at least a year [109]. However, the functionality of newly these formed lymphatic vessels and the resulting drainage of the limb has not been studied in any detail. In this study we developed a method to quantify lymphatic functionality of a limb following excision of a single lymph node and assessed the functional deficit over time.

3.2 Material and methods

3.2.1 Popliteal node removal in Sheep
A vertical skin incision (approximately 8-10 cm long) was made over the lateral aspect of the popliteal region. The popliteal fossa is triangular in shape and is found by retracting the biceps femoris muscle caudally at the level of the stifle joint. At its distal angle, a single popliteal lymph node lies embedded in a pad of fat. The pre and post nodal lymphatic vessels were tied off with a silk suture and the node was excised. Haemostasis was ensured prior to closure of the surgical site.

3.2.2 Assessment of lymphatic function; Mass transport
The animals were fasted and anesthesia was induced as previously described. A heparinized neckline was inserted into the jugular vein and secured for collecting blood
samples throughout the experiment. Evans blue dye (1% in saline) was injected under the skin to highlight the lymphatics. An incision was made through the skin and subcutaneous tissues over the lower lateral aspect of the hind limb, extending distally from the hoof to expose several pre nodal lymphatic vessels in close proximity to the lateral saphenous vein. A single vessel was dissected free from the surrounding tissues and cannulated with a 26G angiocatheter.

Radiolabeled human serum albumin (\(^{125}\)I-HSA, 2 mg in a 200 µL volume) was injected into a pre nodal lymphatic vessel over a 60 second period with a 250 µL Hamilton syringe and flushed with 100 µL of saline over 30 seconds. The cannula was then capped. Swabs were taken from the around the tip of the cannula as well as the injection site and later analyzed in the gamma counter to indicate whether or not there was a leak or spill of the radioactive tracer.

Blood samples were taken from the neckline to monitor the recovery of radioactivity in the blood over a period of four hours. Samples were taken at time 0, 15 min, 30 min and then every 30 min up to four hours. The animal was sacrificed at the end of the experiment with 20 ml of Euthanyl administered intravenously. The concentrations of the radioactive protein tracer in plasma (cpm/ml) were divided by the amount injected to arrive at percent injected/ml, which was plotted over time. However, once a protein tracer enters blood, it re-filters back into the various tissues of the body and hence, tracer recoveries are inherently underestimated. An albumin elimination rate (\(K_{\text{exp}}\)) has been defined in previous experiments [9] and was used in a mass balance equation (equation 1) to reflect more accurately the ability of the lymphatics system to return protein to blood. The mass balance equation outlined below was used to estimate a single averaged mass transport rate. Quantitative studies were performed at 8, 12 and 16 weeks following popliteal nodectomy as well as in a node-intact group.

\[
B_{in} = \frac{C_P(T_f) \exp(K_{\text{exp}} T_f) - C_P(0)}{\exp(K_{\text{exp}} T_f) - 1} (K_{\text{exp}} V_P)
\]  

\(B_{in}\) (blood in) = the mass transport rate (cpm/hour) was averaged from time 0 to time \(T_f\) (duration of experiment) which in our case was 4 hours. The values for \(B_{in}\) derived from equation (1) were divided by the total radioactivity injected to give % injected/hr. \(C_P(t_f)\) = the concentration of the tracer at 4 hours; \(C_P(0)\) = the concentration of tracer at time 0; \(K_{\text{exp}}\) = is the coefficient of elimination of tracer from plasma. Since
our previous experience indicated that this coefficient did not differ significantly between animals of various ages and weights, an average value was derived from 41 animals used in previous studies [9-12]. Since the volume of distribution of the tracer (plasma volume, \( V_P \)) would differ between animals, we adjusted the plasma recoveries to reflect this. Based on data derived in previous studies from our group [9-12], we plotted the plasma volumes derived from 41 animals against their weights. We used a regression analysis of these data (equation 2) to calculate a plasma volume in each sheep based on the following equation.

\[
y = 21.77x + 649.68
\]  

(2)

3.3 Results

3.3.1 Development of Lymphedema

The removal of a single popliteal lymph node resulted in edema formation in the lower hind limbs of all animals. An example is illustrated in Figure 4.A. Figure 4.B. displays the averaged data. On day one after surgery, the average increase in leg circumference was 19.5%, on day two 29.1% and on day three 33.8% (the peak of the response). The edema declined over time but in most animals did not subside completely and remained elevated until the sheep were sacrificed (up to 16 weeks after surgery). In 3 sheep, the edema subsided completely before the end point of the study. All of the control forelimbs showed no increase in circumference. All animals were ambulatory during the course of the experiments.

3.3.2 Assessment of Lymphatic Function

When \(^{125}\)I-HSA was injected into an upstream prenodal popliteal lymphatic vessel in control limbs, it entered plasma with peak concentrations being achieved approximately 1 hour after injection (Figure 5.A.). Measurement of plasma levels at 8, 12 and 16 weeks following lymph node excision revealed lower blood concentrations. Figure 5.B. illustrates the averaged tracer mass transport rates over 4 hours calculated from equation 1 (B in). A one-way ANOVA revealed that the groups were significantly different. The values obtained at 8 (10.6 ± 1.5), 12 (14.4 ± 1.0) and 16 weeks after surgery (13.9 ± 1.0) were less than that noted for the intact limbs (17.2 ± 0.6) but only
the data at 8 weeks reached statistical significance (Dunnett’s t test). These data suggest that pre- and postnodal lymphatic vessels had regenerated to a considerable extent, since the entry of tracer into plasma showed that some lymph continuity had been re-established. Indeed, at 12 and 16 weeks after surgery, lymphatic function had returned to approximately 80% of the level observed in control limbs. At 8 weeks after surgery, only about 60% of lymph transport had been restored.

Figure. 4. Lymphedema produced after the removal of the popliteal lymph node.

A) Example of edema generation following lymph node excision in comparison with a control limb. B) Quantification of edema after lymph node excision expressed as...
percentage change from pre-surgical levels. The numerals at the top of the graph illustrate the number of limbs that were assessed at various times following lymph node excision.

**Figure 5. Quantification of lymphatic function following node removal.**

A) Radioactive albumin was injected into a prenodal lymphatic vessel and the concentration of the tracer was monitored in plasma for 4 h after injection in 4 groups of animals, i.e. the control group (n=7) and 8 (n=7), 12 (n=7) and 16 weeks (n=6) after surgery. B) Equations 1 and 2 were used to calculate an average mass transport rate for each group of Animals, i.e. controls (n=7) and 8 (n=7), 12 (n=7) and 16 weeks (n=6) after surgery. A one-way ANOVA revealed that the groups were significantly different.
Dunnets’s post hoc revealed that the values obtained at 8, 12 and 16 weeks following surgery were all less than those noted for the intact limbs, only the data at 8 weeks reached statistical significance (*).

3.3.3. **Direct evidence of lymphatic regeneration**

Fluoroscopic and immunohistochemical images supported the view that new lymphatic vessels had formed in the nodal excision site. This issue will be discussed in a following chapter (Chapter 5).

3.4 **Conclusions**

In cancer patients, the removal of one or more lymph nodes often gives rise to acute edema, which resolves successfully. It is, of course, the chronic form of edema that occurs in a subgroup of patients that is most problematic. In this regard, there is no agreed upon time beyond which one considers the edema to be chronic. For practical reasons, we did not follow the animals beyond a 16-week period. It is possible, therefore, that the average edema in our animals would have resolved if the sheep had been permitted to survive for longer periods. Nonetheless, the method we used to assess the tracer transport to plasma provided an effective way to monitor the impact of node removal over a reasonable period of time.

Additionally, the level of impediment to nodal excision in the animal model would be relatively benign compared to that, which occurs in humans with cancer. In the human case, the surgical procedures related to the removal of the cancer and lymph nodes are much more extensive and likely provide a significantly greater insult to the lymphatic system than that occurring in our experiments. Nevertheless, in our model, we can examine the impact of single node excision in isolation without other confounding factors that are frequently present in the clinical setting.

With these qualifications in mind, we can say that the removal of a single lymph node produced a significant lymph drainage deficit in the affected limb. However, regeneration of the lymphatic vessels over a 12- to 16-week period helped to restore a sizeable portion of the lost lymph transport capacity and no doubt facilitated the resolution of a significant portion of the edema. One might speculate that vessel regeneration by itself may not be able to restore the full reserve capacity of the system. Added to this, one would expect that radiotherapy could consume additional functional
reserve, a consequence we will explore in the next chapter (Chapter 4).
Chapter 4
Assessment of lymphatic function following irradiation of a single lymph node.

4.1 Introduction and rationale

Radiation therapy involves the use of ionizing radiation to kill malignant cells, reducing the chance of local reoccurrence as well as preventing the spread of the cancer to distant tissues. Today over 50% of all patients diagnosed with cancer will be subjected to radiation therapy at some point in their treatment regimens.

In breast cancer, radiation is used an adjunctive therapy, meaning it is employed in addition to the primary treatment or the surgical removal of the tumor(s). Typical targets of radiotherapy include breast tissue, chest wall and/or regional lymph nodes. The factors influencing whether one receives radiation generally include the stage of the tumor, the type of surgery to be carried out (lumpectomy vs. mastectomy), the size and location of tumor(s), as well as margin status and node involvement.

While the clinical data suggests that there is a significant relationship between radiation use in breast cancer treatment and lymphedema [110-112], it is difficult to determine the impact of irradiation because it is almost always used in conjunction with other treatments. Therefore there is little functional data available on irradiated lymphatic vessels and nodes.

In this study, our objective was to irradiate a single popliteal lymph node and quantify and compare several lymphatic functional parameters in a rabbit model that permits direct access to the lymphatics of interest. We would have preferred to use sheep to study the impact of nodal irradiation on lymphatic function but we did not have the ability to irradiate an animal of this size and consequently, adapted our methods to a more suitable species, the rabbit. For the most part, the large vessel size in this species allows us to use the methods we developed in sheep to assess lymphatic function. Additionally, this species can be irradiated in devices designed for small animal studies.

We compared 4 groups of animals; non-treated, single lymph node irradiation, node resection alone and node resection with irradiation. Surprisingly, these studies revealed
that any decrease in lymph transport could be attributed largely to the effect of radiation itself.

4.2 Material and methods

4.2.1 Popliteal lymph node removal

The rabbits were anesthetized initially with an intramuscular injection of Ketamine (50 mg/ml) and Xylazine (5 mg/ml). Subsequently, 1.5 – 3.0 % isofluorane was delivered through an endotracheal tube. The surgical site was shaved and prepped with alcohol and betadine. The popliteal node lies in the hind limb posterior to the biceps femoris and can be located with palpitation. A vertical skin incision (approximately 2-3 cm long) was made over the lateral aspect of the popliteal region. The pre- and post-nodal lymphatic vessels were tied off with a 2-0 polysorb ties and the node excised. Hemostasis was ensured prior to closure of the surgical site. Subcutaneous Buprenorphine (0.035 mg/kg) was given for pain management postoperatively and every 12 hours for two days. Duplocillin (0.1 ml/kg; Procaine penicillin 150,000 IU/ml, Benzathine penicillin 150,000 IU/ml) was given intramuscularly on the day of surgery.

4.2.2 Radiation therapy

Rabbits were anesthetized with an intramuscular injection of Ketamine (50 mg/ml) and Xylazine (5 mg/ml) and radiotherapy was applied to the popliteal fossa region in the hind limb using a Faxitron X-ray device (model #43855F, Faxitron Bioptics, LLC, Tuscan, USA). Breast cancer patients typically receive a dose of approximately 50 Gy in 25 fractions of 2 Gy over a period of 6 weeks. Using the linear-quadratic model, we can provide a biological equivalent dose in rabbits by applying fractionated 8 Gy doses (160 kv, 6.3 mA, 33 cm from source, 4 cm depth) on 4 consecutive days. Biological equivalent dose is an established measure for quantifying the expected biological effect of different radiation dose fractionation schedules [113, 114]. In patients, radiotherapy is applied 4-6 weeks following surgery to permit tissue healing. We found that three weeks was sufficient healing time in rabbits. Outcomes were measured at 1 week, 1 and 6 months post radiation. Calibration of the faxitron was done regularly using specialized radiographic film that has the ability to quantify small radiation doses.
4.2.3 Assessment of lymphatic function; Transport of FITC-dextran

As described earlier, the ability to transport a macromolecule to plasma provides a quantitative measure of the lymph transport effectiveness of a given lymphatic network [115-117]. A schematic illustrating the features of the experimental design is provided in Figure 3A.

To measure lymph transport and the integrity of the lymphatic network, Evans blue dye (0.5% in saline) was injected into multiple sites on the dorsal surface of the hock to permit visualization of the popliteal prenodal ducts. One of these vessels was dissected free of connective tissue and cannulated in the direction of flow using a 26-gauge angiocatheter. A fluorescent lymph flow tracer (FITC-dextran 70 kDa, 50 mg/ml, Sigma-Aldrich, Oakville, Canada) was infused via a syringe pump (2 ml/hr, 400 µl, model #260, Kd Scientific Inc, Holliston, USA) and the recovery of the tracer monitored over time in plasma. Blood samples were obtained via a 22-gauge angiocatheter placed in the central auricular artery. Prior to infusion, a blood sample was taken for baseline and then once infusion had begun, a sample was taken every minute to 10 minutes, and then at 15, 20, 25, 30, 60, 90 and 120 minutes. Accumulation of the dextran tracer in plasma was determined using a plate reader at 520 nm wavelength. A concentration (as a percent injected) versus time plot was then generated and the area under the each curve (AUC) was calculated using the trapezoidal rule. As we have demonstrated in our previous work, this dimensionless number is an effective way to quantify lymphatic functionality over time [115, 116].

4.2.4 Assessment of lymphatic function; Resistance through lymphatic network

Hydraulic resistance is an alternate way to assess the functionality through the popliteal lymphatic system. By measuring the inflow pressures during the infusions into the prenodal ducts, quantification of the systems resistance can be deduced. To achieve this, a prenodal lymphatic was cannulated (in the direction of flow) with a 26-gauge angiocatheter connected to a stopcock. One arm of the stopcock was attached to a syringe pump and the second arm was attached to a pressure transducer (Custom CDX3, Cobe, Richmond Hill, Canada; or 042982100AT, Argon Medical Devices, Texas, USA) (Figure 3B). Inflow pressures were recorded at a rate of 10/second on a data acquisition system (Daqview Software, A-tech Instruments, Toronto, Canada). The infusate consisted of ‘artificial lymph’. Lymph on average contains about 40% of the
concentration of plasma proteins [47]. Therefore, heparinized autologous plasma was
diluted with 0.9% saline to achieve a similar protein concentration. Based on past
experience [101, 118], infusion rates were varied incrementally from 0.05 to 1.0 ml/h
and the inflow pressures recorded continuously. Pressures were monitored for a
minimum of 3 minutes at each inflow rate. The equilibrium pressures were plotted
against the flow rate with the slopes of the relationships (an estimate of resistance to
flow) determined from regression analysis. To simplify comparison between treatment
groups, pressures were estimated from the regression graphs for the flow rates 0.2, 0.4
and 0.6 ml/hr for all studies. Additionally, the extrapolated y-intercepts were measured.
All data was expressed as the mean ± SEM.

4.2.5 Assessment of edema; bioimpedance

Tissue water content of effected limbs was monitored using a commercial
bioelectrical impedance device. See earlier section for details.

4.2.6 Histology

Following sacrifice, nodes and surrounding fatty tissues were placed in 4%
paraformaldehyde. Tissue was then sectioned and stained using hematoxylin
and eosin. To visualize fibrosis, Masson’s Trichrome stain was used.

4.2.7 Experimental groups and data analysis

Four groups were compared in this study.
1. Non-treated animals provided baseline measurements for all experiments.
2. Irradiation of a single popliteal lymph node. Outcome measures were performed at 1
   week, 1 and 6 months post-surgery.
3. Node resection alone. Outcome measures were performed 1 and 6 months post-
   surgery.
4. Node resection with irradiation. Outcome measures were performed 1 and 6 months
   post-surgery.

Transport and resistance data were analyzed with one-way or repeated measures
ANOVA with Dunnett’s post-hoc test as appropriate. Bioimpedance data were analyzed
with paired t-test. Data are presented at mean ± SEM. We interpreted p<0.05 as
significant.
4.3 Results

4.3.1 Radiation effects - general observations

Radiation was well tolerated by all animals. Slight to moderate erythema developed immediately following the completion of treatment but generally subsided by 3 months post-irradiation (Figure 6.A). Desquamation also occurred in some cases (Figure 6.B). The extent of radiation-induced scarring varied greatly from animal to animal. In some cases no visible scarring was noted, while in others extensive scarring was noted at both the 1 and 6-month time points (Figure 6.C). In 2 animals, tissue hardening developed directly upstream of the irradiated area (not shown).

Figure 6. Radiation induced skin effects


4.3.2 Transport of FITC-dextran

Lymphatic function data is illustrated in Figure 7. In intact limbs, the plasma accumulation of the tracer increased over time, peaking at approximately 20 minutes after the start of tracer infusion. This group generated a mean AUC of 667.32±104.18
There was a significant decrease in functionality observed in limbs subjected to radiation treatment alone (Figure 7. A). AUCs for 1 week and 1 month were 207.9±79.87 and 191.9±62.95 respectively (p=0.018 for both). Lymphatic functionality was only slightly increased at the 6-month time point (250.44±46.45, p=0.034) indicating a relatively long-term deficit in lymph transport in this group. It should be noted there were a significant number of animals in each of the early radiation groups that lacked suitable lymphatics to cannulate (15 animals). In these cases the vessels present were simply too small to carry out the procedure.

On average, node removal caused only a modest decline in function at 1 month (590.28±139.88, p=0.741). However, this appeared to be an anomaly as 2 animals had very high AUCs (938 and 839) both of which were greater than the average for intact (control limbs). If we omit these 2 animals, the average AUC would be 341.97±142.38. Lymph transport at 6 months was less than controls but did not reach significance (AUC 274.03±142.58, p=0.183) (Figure 7. B).

As expected, limbs that underwent both node removal and radiation also saw a significant decrease in accumulation at one month (AUC of 227.18±105.92, p=0.019) (Figure 7. C). This deficit was somewhat resolved over the next 6 months (AUC 311.79±146.96, p=0.092) to the extent that these data were not significantly different from controls. In this regard, there were four animals subjected to node removal and radiation (two at 1 month and two at 6 month time points) that had functionality levels that were similar to the intact limbs. As will be discussed below, we observed some unusual patterns of new lymphatic vessel growth upstream of the site of injury in these animals and lymph-to-venous anastomosis may have occurred.
Figure 7. Transport of FITC-Dextran following radiation therapy.

The transport results are displayed as area under the curve of the transport vs. time graph. Data were analyzed with a one-way ANOVA with Dunnets post hoc. Significance from control (p<0.05) is denoted by star. A) Radiation only treated limbs had significantly reduced lymphatic transport in comparison to untreated limbs; 1 week (AUC of 207.9±79.87, p=0.018, n=7), 1 month (AUC of 191.6±62.95, p=0.018, n=6) and 6 months post-irradiation (AUC of 250.44±46.45, p=0.034, n=7) in comparison to controls (667.32±104.18). B) Limbs undergoing node removal did not have transport levels that differed significantly from controls; 1 month (AUC of 590.28±139.88, p=0.741, n=5), and 6 months post-surgery (AUC of 274.03±142.58, p=0.183, n=7). C) Limbs undergoing combined treatment had significantly impaired transport at 1 month (AUC of 227.18±105.92, p=0.019, n=8) and reduced function at 6 months but this was not significant (AUC of 311.79±146.96, p=0.092, n=6).

4.3.3 Resistance through the lymphatic network

These studies presented a considerable experimental challenge. In the radiation treatment groups, the pre-nodal lymphatics were often much smaller than controls and
we had difficulty cannulating a suitable duct. This was especially an issue in the node excision and combination treatment groups, in which 3/12 and 6/15 respectively were unsuccessful. Additionally, we had multiple experiments in which the treated lymphatic networks began to leak midway through the experiment and we were unable to obtain any useful pressure recordings. This was a problem in 2/15 in nodectomy limbs and 3/12 in the combination groups. Ultimately, we were able to obtain data for 24 limbs. A representative example from each experimental group can be viewed in Figure 8. A-D. As the 6-month data for the 3 treatment groups did not differ significantly from 1-month data, the results for the 2 times were combined to facilitate statistical analysis.

The pressure-flow relationships in all treatment groups were significantly different from controls (radiation p=0.049; nodectomy p=0.045; and combination treatment p=0.005) (Figure 8.E). The slopes for all treated groups were greater than that of the control (1.70±0.24), although only the combination treatment group reached significance (combination treatment 4.70±2.83 p=0.027; radiated 3.84±0.53 p=0.0.84; nodectomy 3.27±1.54 p=0.266).

The most striking observations in the resistance studies were that 1) the pressure-flow relationships were shifted upwards in all of the treated groups and 2) that the 3 treatment groups were quite similar to one another. In the first instance, the y-intercepts for radiation alone (16.55±2.03 cm H₂O, p=0.002), nodectomy alone (17.18±5.02 cm H₂O, p=0.009) and radiation plus nodectomy (17.18±2.02 cm H₂O, p=0.002) were all significantly higher than controls (5.04±0.85 cm H₂O). The y-intercepts can be used to denote a theoretical ‘opening pressure’ for the lymphatic system. Therefore, it would appear that a greater lymphatic pressure is required to establish flow in limbs subjected to any treatment. Second, as is indicated above, the y-intercepts for the treatment groups are all very similar. This suggests that the insult to the lymphatic system was relatively greater with radiation injury than that due to the removal of the lymph node.
Figure 8. Resistance to flow following radiation therapy.

A) Control. B) Radiation 1 month. C) Nodectomy 1 month. D) Combination treatment 1 month. E) The pressure-flow relationships in all treatment groups were significantly different from controls (radiation p=0.049; nodectomy p=0.045; and combination treatment p=0.005). Additionally, the average slope of the pressure-flow
relationship was greater for the irradiated limbs (3.84±0.53 vs. 1.70±0.24 in non-treated animals). The y-intercepts (16.55±2.03 for radiation treated, 16.34±5.02 for nodectomy group and 17.18±2.02 for combination treatment group) were significantly higher than those of controls (5.04±0.85). Control limbs (black, n=6). Radiation (red, 1-month n=5 and 6-months n=3 were combined). Nodectomy (green, 1-month n=4). Combination Treatment (blue, 1-month n=5 and 6-months n=1 were combined). Pressure-flow relationships were analyzed with repeated measures ANOVA and Dunnets post hoc. Significance from control is denoted by a star. Slope and Y-intercept data was analyzed with one-way ANOVA with Dunnet’s host hoc. Significance from the control (p<0.05) is denoted by multiple stars (y-intercept).

4.3.4 Tissue water

In limbs subjected to irradiation, nodal excision or a combination treatment, visible edema was only rarely observed. This is generally supported by the bioimpedance data as typically, there was not more than a 250 ohm difference between pre and post treatment measurements regardless of treatment. To put this into perspective, baseline bioimpedance measurements in untreated limbs ranged from 1300 - 1900 ohms. Untreated limbs underwent slight changes (less than ± 2 percent) over the course of the study. This is most likely due to slight variations in hydration level of the animals at the time of measurement.

Following node removal there was an increase in tissue water found at one month (p=0.028, Figure 9), however this edema resolved over the next six months. While no other treatment group demonstrated a large increase change in water content there was an unexpected trend. Following radiation treatment, tissue water declined especially at 6-month time point (p=0.094). This suggested that these limbs had less water than the pre-treatment level. Also, when the nodal excision site was irradiated, the trend was to a decrease in tissue water in comparison with nodectomy alone.
Figure 9. Assessment of tissue water with bioelectrical impedance.

Following nodal excision (1 month) an increase in tissue water was observed (p=0.028). This increase appeared to resolve over 6 months. In the radiation and combination groups (at 6 months) there was a decrease in water in the treated limbs, although it did not reach significance. Radiation 1 week (n=10), 1 month (n=4), 6 months (n=8). Nodectomy 1 month (n=6) and 6 months (n=8). Combined treatment 1 month (n=9) and 6 months (n=8). Paired t-test with a bonferroni correction p<0.007.

4.3.5 Fluoroscopy

Intact, non-treated preparations revealed the cannulated afferent vessel emptying into the popliteal node and a single large diameter efferent vessel draining it (Figure 10A). Limbs subjected to radiation were prone to leakage from both the afferent vessel as well as from the node. At the 1-week time point leakages were noted and by 1 month, channels were visible within the node (Figure 10B). Six months following irradiation additional lymphatic vessels branching around the periphery of the popliteal node could be seen (Figure 10C). At 1 month following node removal continuity was been re-established and leakage into the node excision site was often seen. In rare cases collateral pathways had developed at 6 months following nodectomy. Combined
treatment limbs also displayed fluid continuity by 1 month post-treatment however abundant branching and the formation of multiple collateral pathways were often present (Figure 10D).

![Fluoroscopy of popliteal region following radiation therapy.](image)

**Figure 10. Fluoroscopy of popliteal region following radiation therapy.**

A) Control. Afferent lymphatic (red arrow) leading into popliteal node (circled) and efferent vessel leading downstream (white arrow). B) At 1 month post-irradiation, channels within the node can be visualized. C) At 6 months post-irradiation, increased lymphatic branching can be seen surrounding the node. D) 1 month combination treatment showing increased lymphatic vessels/collateral pathways (yellow arrows) around the nodal excision area. Some effusion of the contrast agent can be seen in the area originally occupied by the node (dotted circle). All animals were subjected to fluoroscopy.

### 4.3.6 Histology

The most obvious change in the lymph nodes following irradiation was an increase in collagen distribution at the 1 and 6-month time points (Figure 11). Most evident was a thickening of the node capsule and trabeculae.
4.4 Conclusion

4.4.1 Radiation of the lymph node induced a deficit in lymphatic function

Irradiation of a single popliteal lymph node reduced the lymphatic to blood transport of the lymph flow tracer to about a third of that observed in non-irradiated controls. In addition, the inflow pressure-flow relationship in irradiated limbs was shifted significantly upwards. This indicated that much higher pressures were needed to move...
lymph through these limbs in comparison to controls. For example, at 0.4 ml hour, a pressure of $8.3\pm0.92$ cm H$_2$O was required to move lymph in non-treated controls whereas $26.64\pm3.9$ cm H$_2$O pressure was necessary in the irradiated preparations. The latter result suggested a greater resistance to flow following radiation treatment. Perhaps surprisingly, the inflow pressure-flow relationships in the nodectomy alone or combined treatment groups were very similar to those in the radiation treated limbs. We expected that the removal of a lymph node or the combination of nodal excision plus irradiation would lead to a greater functional deficit but this did not appear to be the case. *These data suggested that that radiation itself was a main factor in reducing lymph function in this model.*

Radiation undoubtedly contributes to the development of lymphedema and evidence suggests that it may occasionally be sufficiently damaging to cause the disease by itself. For example, in a clinical study on a group of breast cancer patients unable to undergo surgical treatments (inoperable tumors, unable to undergo general anesthetics etc.) 11% of women developed arm edema in the following 3 and half years [119], suggesting that ionizing radiation alone has the ability to impact the lymphatic system negatively.

Previous studies utilizing radiation in the study of lymphedema have been limited and the results contradictory. Several authors have concluded that radiation has relatively little impact on the lymphatic system apart from a reduction in node size [8, 9]. A recently conducted study by Avraham and colleagues has shown that radiation directly causes the death of lymphatic endothelial cells both in cell culture and in an animal model [10]. With the death of LECs there was also a dramatic loss of lymphatic vessels in the weeks following irradiation. In contrast, others have observed that the application of radiation in humans seems to increase the number of small lymphatics [11]. It should be noted however, that inferring function from an increase or decrease in the number of lymphatic vessels in a tissue is difficult. Instead, it is better to measure functionality more directly as we have done in the above study. Here we show that a clinically relevant radiation schedule is sufficient to cause significant lymphatic dysfunction. Similarly, Mortimer’s group has used the clearance of a tracer injected into the interstitium to estimate lymphatic drainage in a pig model and observed that a radiation dose of 18 Gy reduced lymphatic function [20].

Radiation is likely to have a complex effect on the interstitial-lymphatic system. In
the study reported here, we observed that much higher pressures were required to establish a given flow rate through lymph nodes after the application of radiation. This would imply that the initial insult to the node (possibly radiation induced fibrosis) impaired the velocity of fluid flow from the interstitium into the initial lymphatic vessels, a phenomenon that is a central tenet of a novel interstitial lymphedema hypothesis [120]. Consequently, elevated hydrostatic pressure differences between the interstitium and lymphatics would be necessary to maintain lymph flow at a given level through the system. Additionally, the functional deficit caused by radiation may introduce hemodynamic factors, which could exacerbate the accumulation of fluid in the interstitium. Increased capillary filtration due to vasodilation or the formation of new blood vessels has been suggested to be a significant contributor to edema in cancer related lymphedema [6, 120].

4.4.2 How does radiation affect lymph transport through a lymph node?

While irradiation of the node may affect several parameters related to the lymph nodes normal physiological parameters (for example the permeability of the nodal vasculature), excess collagen deposition in the node is a factor to consider. Fibrosis is a well-documented consequence of ionizing radiation and in the study reported here, one of the most obvious changes we observed following radiation of the node was the deposition of collagen with a thickened node capsule and interior trabeculae. Presumably, transforming growth factor beta 1 (TGFβ1) [121] plays a role in this process by promoting myofibroblast proliferation and extracellular matrix deposition. TGFβ1 has also been shown to cause endothelial cells to undergo a phenotypic conversion into fibroblast-like cells (endothelial-mesenchymal transitional (EndMT) event [122], which would exacerbate the ongoing fibrotic process in irradiated tissues. There is some evidence that the EndMT phenomenon occurs in lymphatic endothelium following radiation exposure [85, 87] but whether such an event occurs within the node parenchyma is unknown.

In addition, based on studies using lymphatic congestion lymphoscintigraphy, it is evident that lymph pump failure occurs in breast cancer-related lymphedema [123]. Surprisingly, the lymph node itself is capable of contracting and radiation may negatively impact this function as well [57].
4.4.3 Paradoxical findings related to bioimpedance measures of tissue water

Once the lymphatic system’s capacity to remove protein and water from the interstitium is compromised, edema develops [124, 125]. In the radiation study, radiation impaired lymphatic function as evidenced by reduced transport of the intra-lymphatic tracer to plasma and as altered inflow pressure-flow relationships. Nonetheless, the association between lymphatic dysfunction and tissue edema was less clear. While not statistically significant, our data demonstrated a counter intuitive trend to reduced tissue water after radiation was applied to the tissues in concert with impaired lymphatic function as noted above. A number of factors may have contributed to this effect.

A major issue was the variability of the bioimpedance data. While measurements of tissue water with bioimpedance is well accepted [126, 127], the method in our hands was quite sensitive to needle location and it was difficult to ensure the identical placement of the needles between the pre- and post-treatment measurements. However, it should be noted that we rarely observed visible edema in the rabbit limbs of the treated animals (all groups). This is in contrast to our experience with sheep where the removal of the popliteal lymph node produced a discernible edema consistently. We must also reflect on the biological variability and the individual compensatory mechanisms recruited in response to the radiation or surgical insult.

Second, in some dog and rat hind limb studies, the authors concluded that radiation alone was not sufficient to induce any permanent edema [82, 84, 128, 129]. Therefore, in our study it is possible that any elevation in tissue water in the irradiated group was largely subclinical. Additionally, the reduced tissue water observed at 6 months may be due to the presence of large scar in the treatment area as collagen binds less water [130]. Tissue fibrosis in one part of the limb might mask or cause an underestimation of elevated water (possibly subclinical) in other areas.

A final point to consider is the possible disconnect between our measurements of lymphatic function and global limb drainage. Our lymph function measurements were based on the intra-lymphatic infusion of tracer or artificial lymph. We observed a significant reduction in transport capacity and changes in the pressure-flow relationships that signalled increased resistance to flow. Nonetheless, at the same time, we found evidence of vigorous new vessel formation in the limb and new lymph-venous connections both of which would work to bypass the obstruction in the popliteal
fossa. Therefore, it is possible that the capacity of the lymphatic system to regenerate new drainage pathways and find new upstream venous connections helped to maintain tissue fluid removal from the limb in the face of compromised flow through the vessels from which we were taking measurements. This may be especially true in the combination treatment groups (radiation plus node removal) in which one would expect to see the greatest degree of lymphatic dysfunction. This group exhibited the greatest evidence for new lymphatic vessel growth.

One of the dominant observations we made with lymph node excision with or without radiation was the ability of the lymphatic system to adapt to a downstream impediment to flow. The formation of new lymphatic vessels and the connection of upstream lymphatics to proximal veins will be examined in the next chapter (Chapter 5).
Chapter 5
The lymphatic system: Natural compensatory mechanisms.

5.1 Introduction and rationale

Over the course of the studies outlined in this thesis, we have been impressed by the natural lymphatic compensatory mechanisms in response to lymph node excision and irradiation. These include robust lymphangiogenesis in sheep with lymphatic function restored to 80% of normal after 12-16 weeks. In rabbits, functional restoration was less at about 40% at 24 weeks although this difference was not significant due to the wide variation between animals. However, in this species, in addition to lymphangiogenesis we observed evidence of lymph-venous anastomoses. As the following chapters will deal with potential therapeutic approaches to restore lymphatic function after excision/irradiation, we thought that it was worthwhile to emphasize the normal endogenous responses of the host and consequently, these are presented as a separate chapter.

5.2 Material and methods

5.2.1 Visualization of lymphatic system

Evan’s blue studies and fluoroscopy images in this chapter were obtained from all studies found within this thesis.

5.2.2 Immunohistochemistry

In 10 animals, the growth of new lymphatic vessels (lymphangiogenesis) was assessed at the nodal excision site. Briefly, 2 methods were used to identify the lymphatic vessels. First, 5- (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFDA SE; Molecular Probes No. C-1157) was injected into an upstream prenodal lymphatic. This molecule diffuses passively into cells and remains non-fluorescent until its acetate groups are cleaved by intracellular esterases (it fluoresces green). This provided unequivocal identification of the lymphatics since the dye was introduced directly into the lumens of the vessels. In addition, the sections were co-stained with antibodies to the lymphatic endothelial receptor for hyaluronan (LYVE-1; tagged wit
Cy3 red). Tissue blocks from the popliteal region were removed surgically. The samples were embedded in Tissue-Tek OCT compound, placed in a base mold and frozen immediately at −80 °C. Sections (7 µm thick) were cut using a Leica cryostat (model Leica CM 3050S-3-1-1) and were placed on glass microscope slides. The slides were washed 5 times in phosphate-buffered saline (PBS; 0.1M) and blocked for 1 h at room temperature with 10% goat serum in PBS. After washing with PBS, the sections were incubated overnight at 4 °C with 1: 50 or 1: 100 dilutions of rabbit, antihuman LYVE-1 primary antibody (Research Diagnostics Inc.). The next day, the sections were washed with PBS and incubated with 1: 100 dilutions of goat, antirabbit IgG antibody tagged with Cy3 (Jackson Immuno Research). In controls, the primary antibody to LYVE-1 was omitted. Finally, the slides were mounted in aquapolymount and coverslipped. Immunofluorescence microscopy was performed with a Zeiss Axiovert 100M laser scanning confocal microscope. The argon and helium/neon lasers were set to wavelengths of 488 and 543 nm for excitation of CFDA-SE and Cy3, respectively. When both techniques were combined in the same tissue sample, the lymphatic vessels appeared yellow-orange.

5.2.3 Physiological analysis of lymph-venous anastomoses

The visualization of limbs that received Evans blue dye revealed an unexpected finding. In some preparations, lymph-venous connections were observed upstream of the irradiated/excised lymph nodes. For further confirmation of these anastomoses, in a limited number of animals, FITC-dextran tracer was infused into a popliteal pre-nodal lymphatic as described earlier and blood samples were taken from both femoral veins simultaneously at 0, 2.5, 5, 10, 15, 20, 25, 30, 60, 90 and 120 minutes. Limbs containing a lymph-venous connection should display enriched tracer concentrations in the ipsilateral side.

5.3 Results

5.3.1 Visualization of the lymphatic system – Generalizations

Observations made during the all studies using Evans’s blue dye have been summarized in Figure 12. In intact, non-treated controls it is typical to see two large
afferent lymphatic vessels that run parallel to the saphenous vein on both the medial and lateral aspects of the lower limb leading to the popliteal node (Figure 12. A). There are no connections between the medial and lateral vessels in either sheep or rabbits until they coalesce into the popliteal node.

In limbs subjected to radiation (Figure 12. C), lymphatic vessels tended to be very delicate and prone to leakage (at all times analyzed). Afferent vessels leading into the node had a lattice appearance (<0.5 mm, 1 month and 6 month time points). The lymphatics were typically shrunken in appearance in comparison to control limb vessels.

Following removal of the popliteal node, it was typical to see dilation of the afferent lymphatics in comparison to controls, no doubt due to the impediment to flow caused by the removal of the node (Figure 12. B). Some fluid continuity had been re-established by the 1-month time point and to support this, the regenerating lymphatic network showed a lattice appearance.

Lastly, a combination of both node removal and radiation appeared to induce the most radical compensatory response (Figure 12. D). A large increase in number and/or branching of lymphatic vessels was observed. In some cases underlying vessels were found to branch into the dermis. Instead of branching into the popliteal region, lymphatics tended to do one of two things. In some animals, collateral pathways appeared to form around the popliteal region. In others, vessels upstream of the popliteal region (both on the lateral and media aspects of the limb) would frequently branch at right angles towards the saphenous vein on the lateral side of the limb. In some cases medial and lateral vessels would coalesce and in others it appeared that there were lymph-to-venous connections formed between an afferent vessel and a vein.
Figure 12. Schematic illustrating patterns of lymphatic regeneration following irradiation and lymph node excision with irradiation

A) Control. The rabbit hind leg is displayed with afferent lymphatics (yellow) leading to the popliteal node (orange) and a large efferent lymphatic vessel draining lymph from the node. Major veins are shown in blue. The image to the right of the schematic illustrates the lymphatics filled with Evans blue dye on the lateral aspect of the limb. B) Radiation appears to induce new vessel growth with increased branching of lymphatic vessels upstream of the popliteal node (at 1 month and 6 months post-irradiation). C) Following nodectomy, lymphatic vessels appear to reconnect in a few weeks (1 month rabbits, 4 weeks sheep), although upstream vessels are dilated, suggesting an upstream lymph flow obstruction. D) Combined treatment group often displayed excessive branching of upstream lymphatic vessels, as well as an increased occurrence of potential anastomoses (red circles). Arrow in image on the left indicates a collateral pathway branching off of an afferent lymphatic. Arrow in the image on the right indicates a new connection between lateral and medial afferent lymphatics.

5.3.2 Visualization of the Lymphatic System – Fluoroscopy

Intact, non-treated preparations revealed the cannulated afferent vessel emptying into the popliteal node and a single (or two) large diameter efferent vessels draining it (see Chapter 3, and 4 for examples).
Fluoroscopic analysis at 6 weeks in sheep and 4 weeks in rabbits after nodal excision confirmed that some degree of fluid continuity had been established between the prenodal and postnodal lymphatic vessels in the absence of the lymph node. The contrast agent injected into one of the prenodal lymphatics in the lower limb could be observed in the relatively large duct that would normally collect lymph from the popliteal node (postnodal vessel; example illustrated in Figure 13. A). Small lymphatic vessels were observed in the nodal excision area. In limbs assessed six months post-node removal collateral pathways had developed around the excision region. Limbs that were subjected to harsher treatment (combination of node removal and radiation) and therefore potentially possessed a greater lymphatic functional deficit, still displayed some fluid continuity by 1 month post-treatment. However abundant branching and the formation of multiple collateral pathways were often present (Figure 13. C).

5.3.3 Visualization of the lymphatic system - Immunohistochemistry

Confirmation that the vessels in the nodal excision area were newly formed lymphatics attempting to bridge the gap left by the absent node (noted in the above section) was supported by immunohistochemistry. We observed co-localization of LYVE-1 (red) and CFDA-SE (green), indicating that lymphatic vessels were present at the area vacated by the popliteal lymph node. An example is provided in Figure 13 B. The newly formed lymphatics observed at 6-12 weeks after surgery were variable in size, but at the upper end were about 40 µm in diameter in sheep. These vessels were the conduits that connected the originally placed pre- and postnodal lymphatics and contributed to the restoration of lymph transport that was observed in the tracer and fluoroscopic studies.
Figure 13. Morphological evidence of lymphatic vessels regeneration following popliteal lymph node excision.

A) Fluoroscopy performed at 12 weeks after lymph node removal illustrating some degree of continuity between the lymphatic vessels that were originally pre- and postnodal to the popliteal lymph node. B) Confocal microscopy image of regenerated postnodal lymphatics in the nodal excision site. For unequivocal identification of these vessels, a nonspecific cell dye CFDA-SE (green) was infused into an upstream prenodal lymphatic and, at the same time, the tissues were stained with antibodies to the lymphatic endothelial marker LYVE-1 (red). The stained sections appeared yellow-orange, indicating coexistence of the two stains. At 12 weeks, an irregular network of small interconnecting lymphatics could be observed in the area originally occupied by the popliteal lymph node. We presume that all vessels in this image are newly formed in response to the removal of the node. C) 6 month Nodectomy, collateral pathways
(indicated by yellow arrows) have developed to bypass the nodal excision region. Some effusion of the contrast agent can be seen in the area originally occupied by the node (circle).

5.3.4 Lymph-venous anastomoses

The development of potential lymph-venous anastomoses was not seen in any study we conducted with sheep and therefore was unanticipated in the rabbit experiments. While it did appear to be a rare phenomenon, its occurrence was noted in the rabbit hind leg.

Our first indication that a lymph-venous connection could be occurring was the observation of Evan’s blue dye in veins running parallel to afferent lymphatics of treated limbs (Figure 14 A). When Evan’s blue dye is injected subcutaneously it binds to interstitial protein and is then absorbed into the lymphatic system. Normally, no dye enters the local vasculature but we identified 10 limbs in which the dye was observed in a lower limb vein connecting with a nearby lymphatic vessel. Eight potential anastomoses were identified in the combination treatment group and 2 in the node excision animals.

We attempted to obtain physiological evidence to support the presence of anastomoses but conditions were not favourable for routine analysis. An upstream afferent lymphatic that was large enough for cannulation in combination with the ability to sample blood from both ipsilateral and contralateral femoral veins was only achievable in 2 treated animals. While it is difficult to draw any conclusion, the results are intriguing nonetheless. In one of the two of these animals (combination treatment), the tracer appeared in the ipsilateral blood (with suspected anastomosis) before appearance in the contralateral side (Figure 14. C). In addition, the tracer began to accumulate in the blood earlier (5 vs. 15 minutes) and also peaked earlier (15 vs. 30 minutes) than in control limbs. In contrast, in the second animal with a potential anastomosis, as well as in untreated limbs with no suspected anastomosis, we observed simultaneous accumulation of tracer into blood from both the infusion limb and the contralateral limb (Figure 14. B).
Figure 14. Evidence of lymphatic-venous anastomosis.

A) In this example, Evan's blue was taken up by an afferent lymphatic vessel (black arrow) flowing towards the popliteal node region (direction of flow is towards the top of image) and due to the presence of an anastomosis (circle) the dye is also seen in the saphenous vein (white arrow). Some backflow of Evan's blue dye in the vein can be observed. B and C) To identify anastomoses FITC dextran was infused into an afferent vessel upstream of the potential anastomosis site and blood was sampled from ipsilateral limbs and the contralateral limb. In a normal limb, the tracer enters the venous system through the thoracic duct and appears in the blood of femoral veins simultaneously (B). In the limb data illustrated in (C), the tracer enters the venous system in the ipsilateral side earlier than its appearance in the contralateral limb. This suggests the presence of a lymphatic-venous anastomosis. Ipsilateral limbs are shown in black and the contralateral limb in grey.
the femoral veins of both hind limbs. In a normal limb, the tracer enters the venous system through the thoracic duct and appears in the blood of femoral veins simultaneously (B). In the limb data illustrated in (C), the tracer enters the venous system in the ipsilateral side earlier than its appearance in the contralateral limb. This suggests the presence of a lymphatic-venous anastomosis. Ipsilateral limbs are shown in black and the contralateral limb in grey.

5.4 Conclusions

The pre- and post-nodal popliteal lymphatic vessels are normally well delineated in the sheep and rabbit hind limbs and follow a relatively linear course. With lymph node excision, there is vigorous new vessel growth in an attempt to restore fluid continuity. In chapter 3, vessel regeneration was capable of restoring lymphatic drainage to about 80% of control values over a 4-month period [117] and in chapter 4, new vessel formation returned function to about 40% of control levels in rabbits at 6 months. From the literature, we learn that the number of lymphatic vessels is altered by radiation but results have been inconsistent. For example, in one study, the number of lymphatic vessels decreased one week following a large dose of radiation (15 or 30 Gy) in a mouse-tail model [85]. This decrease continued over the course of the study, reaching a 4-fold loss of vessels in comparison to controls by 6 months. Alternatively, in humans, patients receiving conventional delivery (small dose five days a week) of 30-40 Gy had an increase in the total number of lymphatics (mainly those of diameter <10 \( \mu \)m) at 1-year time point [131].

In the studies reported here, both Evans blue and fluoroscopy demonstrated that the number/density of lymphatic vessels increased following node removal and/or irradiation even though fibrosis has been shown to inhibit lymphangio genesis [87]. The pattern of new vessel growth was however, extremely variable. Both densely and sparsely packed vessels were observed upstream of the irradiated popliteal node or its excision site. Heavily branched vessels were also commonly noted. The upstream existing lymphatics (up to 15 cm away) were often observed to branch laterally, apparently using the vasculature for guidance. While some function could be attributed to these vessels as indicated by the tracer transport to plasma in all treatment groups at all times, the irradiated lymphatics were delicate and much more prone to leakage in comparison to controls.
Lymphatic vessels are routinely damaged during surgery and no long-term edema develops. This is because the lymphatic system possesses a great capacity for regeneration. It is know that damage creating small gaps (< 1 mm) in lymphatic vessels will reconnect and display fluid continuity as early as two weeks following the insult [109]. When a lymph node is removed the pre and post nodal vessels sprout and regrow along the original fluid route. In some cases, growth along the original region is reduced or inhibited. The inhibition could be due to the development of fibrosis or scarring in that region. Increased collagen deposition could compress or collapse existing lymphatics in the area. The increased density of collagen in scars may not permit new vessels to invade. Secondly, it has also been suggested that lymph stasis inhibits lymphangiogenesis through the affected region [109]. This effect of lymph fluid may be, in part, explained by (1) directing a route for recanalization [132], (2) growth factors in the lymph fluid [133] and (3) growth factor(s)-secreting cells, such as mononuclear cells and dendritic cells, and angioblasts, if they exist, in the lymph fluid [134].

Whatever the cause of the inhibition of lymphangiogenesis along its original route, collateral lymphatic pathways result. These collaterals originate by branching from existing lymphatic vessels upstream of the impediment to flow (up to 15 cm upstream in rabbit hind legs). These vessels are often found running in parallel with veins, which is expected because vascular derived VEGF-C is known to direct lymphatic growth [135]. Fluoroscopy images from studies conducted in this thesis reveal that these collateral pathways eventually connect with original efferent vessels downstream.

In rare events, perhaps when lymph flow from the limb is extremely impaired, lymphatic vessels branch laterally (upstream of impediment) and connect to a local vein. This is seen in the rabbit hind leg following node removal and radiation. Under normal conditions, almost all lymph is returned to the bloodstream at the major lymph-venous junctions in the neck. However, lymph-venous anastomoses in unconventional locations have been reported in various species (rats, cat, dogs, monkeys) as well as in humans in response to lymph obstruction or edema [136-140]. In a study conducted on 552 patients with primary and secondary lymphedema, as well as lymph node malignancies, 16 anastomoses were identified by lymphography [141]. Iodinated (125I) human serum albumin was injected intra-lymphatically to detect lymph-venous
connections physiologically in 40 post-mastectomy patients [142]. Blood was taken from both basilic veins simultaneously and an increased quantity of the labeled protein transferred locally to the ipsilateral venous system in the non-edematous group. The presence of a lymph-venous connection was confirmed radiographically in 2 of the non-edematous patients. Little local transfer of the tracer was noted in the edematous patients or volunteers. Similar connections have been suggested in more recent studies involving subcutaneous injection of radiolabelled human IgG in patients with established breast cancer related lymphedema [143-145].

Natural compensatory mechanisms of the lymphatic system, such as lymphangiogenesis and lymphovenous anastomosis, work to ensure that lymphatic function is sufficient to maintain viability of upstream tissues. Understanding these mechanisms is important, as it may be possible to enhance natural compensatory mechanisms in the treatment of lymphedema.
Chapter 6
Potential therapies of lymphedema

6.1 Introduction and rationale

The studies thus far outlined in this thesis have revealed the functional deficit in the lymphatic system that results from the removal or irradiation of lymph nodes. These deficits most likely contribute significantly to the development of lymphedema in cancer patients. As such, several important issues are unresolved. If one replaces a lymph node, is the magnitude of lymphedema reduced? Of course, there are a few reports of lymph node transplantation in humans but the results are mixed. Presumably, the timing of the transplant is critical. If the transplant occurs early (even during the original cancer surgery), perhaps the sequelae of events leading to edema can be prevented. Second, given the interest in the molecular factors that control lymphangiogenesis, would the placement of selected growth factors in the nodal excision site improve lymphatic function and prevent lymphedema? Finally, would a combination therapy of nodal transplantation plus growth factors be effective?

In the studies outlined below we will assess the effectiveness of A) lymph node transplantation and B) growth factor therapy on their ability regain lymphatic functionality following the removal of a single lymph node. As will be seen in the results, combination therapy is probably not necessary as will be explained in due course.
6.2 Part A) Autologous lymph node transplantation.

I (Amy Baker) was a collaborator on this study and co-author of the publication that resulted from it. Consequently, the data will be summarized only briefly here.

Removal of a lymph node creates a functional deficit in the lymphatic system of the affected limb (Chapter 3). In the weeks following node removal, lymphatic vessels regenerated into the excision region (Chapter 3) and fluid continuity was re-established. Even so, some functional deficit remained. This reduce lymph drainage was correlated with a significant increase in limb edema (Chapter 3). Theoretically if the deficit is the direct result of the missing lymph node then transplantation of an autologous lymph node from an unaffected region of the body into the excision region may eliminate the loss of lymphatic functionality and prevent edema formation.

6.2 Material and methods

6.2.1 Nodectomy followed by Lymph Node Transplant by Micro-vascular Anastomosis

The lymph nodes were harvested together with the intact fat pads. The vascular pedicle formed by the lateral saphenous vein and the medial circumference femoral artery was identified and the pedicle was dissected free from the surrounding tissues before ligation and clipping of the vessels. The animal was then turned over and the contra-lateral popliteal node was excised. Recipient vessels of a suitable size and location were exposed and the transplant vessels approximated to these vessels with bridging micro clamps (Figure 15.A). The vessels were sutured together micro-surgically with the use of a Carl Zeiss Microscope (OPMI 1-FC) before removal of the clamps. The animals were returned to their holding pens after recovery from the anaesthetics.

As an additional control avascular/node graft insertions were also completed. A small opening was made in the popliteal fat and pre and post nodal lymphatic vessels were tied off with a silk suture and the node excised. Once the nodes from both hind
limbs were removed, each was placed into the contra lateral popliteal fat pad as a free graft without vascular reconnection.

In sham control animals, the popliteal fat pad was exposed but undisturbed. A saline soaked piece of gauze was applied to the wound and it was left in this state for 3 hours to duplicate the time needed for the vascularized node transplants.

Popliteal node removal was carried out as described in chapter 1.

6.2.2 Lymphatic function
Carried out as described in Chapter 3.

6.2.3 Edema Assessment
This study was performed before we acquired the bioimpedance device. Consequently, changes in leg circumferences were used to assess edema development. The hind legs were shaved and leg circumference measurements were taken at a point 10 cm distal to the hock (tarsus). This landmark was highlighted with a skin marker for circumference measurements postoperatively. The limb circumferences were divided by the original (presurgical value) and expressed as percentage change over time. To compare the edema outcomes in the various groups, the percentage change in limb circumference was plotted against time, and graphical integration of the area under the curves was calculated using the trapezoidal rule. To allow comparisons of experiments conducted over different times (8 and 12 weeks), an edema coefficient was calculated by dividing the areas under the curves with the duration of the experiment in days.

6.3 Results

6.3.1 Lymphatic Function; Transport of tracer to plasma
Measurements of lymphatic function for all groups are illustrated in Figure 15. B. The transport rates of the protein tracer for the sham group averaged 16.6 ± 0.7 percent per hour at 8 weeks and 16.1 ± 0.7 percent per hour at 12 weeks. These values were similar to those observed in limbs that had not been subjected to any surgical procedures (17.2 ± 0.6 percent per hour, taken from our node removal study—chapter 1) and indicated that all of the surgical procedures excluding the actual removal
of the nodes had little impact on lymphatic function. The removal of a lymph node and replacement with an avascular node from the contralateral side gave lymphatic function values at 8 (12.3 ± 0.5 percent per hour) and 12 weeks (12.6 ± 0.8 percent per hour) after surgery that were significantly lower than those of the sham group (p < 0.001, unpaired t test). In contrast, the replacement of the excised node with a vascularized node transplant resulted in lymph transport that was significantly greater than that of the avascular group (p < 0.001, unpaired t-test). Indeed, lymphatic function approached sham levels (15.8 ± 0.9 percent per hour at 8 weeks and 15.7 ± 1.0 percent per hour at 12 weeks).
Figure 15. Impact of node transplantation on lymphatic function.

A) Popliteal lymph node with associated fat pad and vasculature, microsurgically transplanted into the contralateral hind limb. The arrow indicates the anastomosis between the popliteal and a local artery, while double-headed arrow indicates the venular anastomosis location. B) The transplantation of autologous avascular popliteal lymph nodes into the nodal excision site (white bars) resulted in lymphatic function at 8 (n=6) and 12 weeks (n=6) that was significantly less than that observed in the sham group at similar times (grey bars, n=12). In contrast, the transplantation of vascularized nodes (black bars) produced lymph transport values that were similar to sham controls but significantly greater than those in the avascular transplant series (8 weeks, n=8; 12 weeks, n=10). The hatched bar illustrates data from limbs that had no surgery (i.e., with lymph nodes intact) (n=7) taken from Chapter 1. For statistical analysis, the data from 8 and 12 weeks were averaged together for each experimental group because there were no time-dependent differences in any of the experimental series. Numbers in parentheses represent the number of animals in each group. HSA, human serum albumin.

6.3.2 Edema

In these experiments, lymph nodes from both hind limbs were excised; one of the limbs received a vascularized node transplant (n = 22) and the other limb (n = 22) did not. In both groups, the limb circumference changed significantly over time (p < 0.001, repeated-measures linear regression). In the limbs that received a vascularized transplant, the average percentage increase in leg circumference at postoperative day 1 was 12.1 percent, with peak edema also occurring during the first week. Edema in the contralateral nodectomy limbs peaked at the same time. Thereafter, limb circumference declined steadily in both limb groups, with values from the vascularized transplant limbs significantly less than those in the “untreated” limbs (p = 0.023, repeated-measures linear regression). Figure 16 illustrates averaged edema coefficients for these groups in addition to the limbs from which the nodes were harvested for transplant (labeled node excision). These comparisons indicate that the vascularized node transplants (n=18) were associated with the lowest levels of edema. The edema coefficients in this group were significantly lower than those in the nontreated limbs (node excision) (p=0.039, unpaired t test). On average, the
vascularized group had considerably lower edema coefficients than the avascular series (n = 24), but these effects just failed to reach statistical significance (p = 0.055, unpaired t test).

![Edema Coefficient Chart]

**Figure 16. Edema assessment following lymph node transplantation**

The average edema coefficient for the vascularized transplant group (n=18) was lower than that in the untreated node excision series (n=18) (p=0.039, unpaired t test). On average, the vascularized group had lower edema coefficients than the avascular series (n=24), but these effects were not significantly different (p =0.055, unpaired t test).

### 6.4 Conclusion

It is well documented that removal of the lymph nodes greatly increases the chance of developing lymphedema especially if this is combined with radiotherapy [33]. In the study mentioned above we demonstrated that a successful vascularized popliteal node transplant could effectively restore lymphatic function to normal levels and limit or prevent edema development [2]. This result has important theoretical and practical implications in the lymphedema field. From a conceptual perspective, these data confirm the importance of the lymph node in lymphedema development in the absence
of other factors as has been previously suggested [146]. Removal of the node induced a lymphatic deficit and its replacement restored lymphatic function to control levels and essentially prevented the development of chronic edema.

However, the practical inferences from this study are more complicated. It is important to emphasize that our results were based on transplantation of the node immediately after nodal excision. In the clinical applications of the method, surgeons have generally transplanted nodes some time after the original cancer surgery when lymphedema is already established. For example, Becker and colleagues [34] transplanted femoral nodes into the axillary region of 24 postmastectomy patients who had lymphedema for over 5 years using microsurgical procedures followed by manual drainage (physiotherapy) for 3 months. In 10 of 24 cases, the edema appeared to be resolved. However, it is difficult to separate the effectiveness of transplantation alone given the other treatment provided. Additionally, no functional studies were attempted. Isotopic lymphangiography in 16 patients suggested that node transplants were successful in only 31% of individuals. The best results were attained when the lymphedema was of the shortest duration.

According to existing clinical studies (of which there are few), LNT is able to alleviate postmastectomy upper extremity lymphedema in the majority of patients [147-153]. However, the improvement is variable and no conclusions have been drawn regarding which technique (flap origin and vascular connections), group of donor lymph nodes, or recipient site (wrist, elbow, or axilla) can ensure the maximum reduction of the affected limb.

One major hindrance to the mainstream use of this treatment in lymphedema is donor site morbidity. In a study where 26 individuals received ALNT, 38% of those patients develop chronic lymphedema in the donor limb [154]. Therefore, while it is undeniable that lymph node transplantation for lymphedema treatment has potential, surgical complexities, low node survival rates and potential donor site morbidity issues complicate the application of lymph node transplantation in clinical practice. Perhaps the most important concept is that the lymph node is one of the critical focal points for lymphedema development and it may be prudent to direct new therapies with this in mind.
Part B) Lymphangiogenic growth factor therapy.

6.5 Overview

While lymphatics normally have an impressive capacity to regenerate following injury, it is possible that this process fails to compensate fully and that non-optimal lymph transport conditions predispose the patient to edema formation. With this in mind, various groups have found that the induction of lymphangiogenesis can reduce secondary lymphedema in animal models [96-98, 155, 156]. However, the majority of these studies applied single growth factor therapy only (usually VEGF-C) and then used a rather crude method to accurately quantify lymphatic functionality following treatment. These methods usually employ the injection of a tracer (usually a radiocolloid or fluorescently tagged molecule) and the visualization of this tracer through the site of growth factor application as a measure of functionality. Sometimes quantification of the tracer remaining in the injection site was also conducted [97]. One study utilized the transport of Evan’s blue dye to blood as a measure of lymphatic function [157]. Additionally, apart from one study in the mouse forelimb [157], the major perturbation to the system afforded by nodal excision (a critical contributor to lymphedema development in humans) was not employed in the experimental design (rabbit ear and mouse tail models do not possess lymph nodes).

Thus the objective of this study was to test the principle that the delivery of lymphangiogenic growth factors specifically into the nodal excision site would enhance vessel regeneration, re-establish lymph transport capabilities and reduce edema formation.

In terms of identifying which factors to inject, we identified two major players in the process of lymphangiogenesis. Vascular Endothelial Growth Factor-C (VEGF-C) seemed like an obvious choice given its role in regulating new lymphatic vessel growth [158, 159]. Another factor that we decided to introduce into the lesion site was Angiopoietin-2 (ANG-2), which appears to play a role in smooth muscle cell recruitment and the maturation of newly formed lymphatic networks [20].

Having determined the animal model (the sheep) and the therapeutic molecules to deliver, we then focused on the optimal delivery strategy. Osmotic mini pumps or adenoviral vectors are commonly employed to introduce molecules into the tissues.
However, an evolving trend in drug delivery is the use of minimally invasive, injectable drug delivery strategies. One such drug delivery system is the hydrogel HAMC- a physical blend of hyaluronan (HA) and methylcellulose (MC). Proteins diffuse readily through HAMC, which also degrades over time [160]. Until now the use of HAMC has mostly been restricted to delivery of therapeutic molecules into the spinal cord (but also to retina [161] and brain [162, 163]) of injured rats [160, 164-166], but its capacity for sustained, localized release makes it an attractive candidate to deliver lymphangiogenic factors as well.

6.6 Materials and Methods

All media and reagents were purchased from Sigma Aldrich (ON, CA) unless otherwise stated.

VEGF-C is a ligand and activator of the receptor VEGFR3, found on LECs [159, 167, 168]. It also has the capability to bind VEGFR2, found predominantly on vascular endothelial cells, inducing vascular permeability [159, 169]. In addition, at higher concentrations VEGF-C also stimulates proliferation and migration of vascular endothelial cells in culture [159, 169]. To minimize the vascular effects of VEGF-C, we utilized the mutant VEGFC156S, that has lost its ability to bind VEGFR2 [170]. VEGFC156S was utilized exclusively throughout this study and will be referred to as VEGF-C throughout the remainder of the document.

6.6.1 Formation and sterilization of HAMC

The formulation and sterilization of HAMC was modified from Gupta's description [160]. To prepare the HAMC components, a 0.5 w/v solution of methylcellulose (MC; M7140) in distilled water was created using a dispersion technique. Briefly, MC was added to 1/3 of distilled water at boiling, and once completely wetted, the remaining 2/3 of distilled water (cold) was added. The solution was placed in 4°C until completely dissolved. Sodium hyaluronanate (HA; MW 1500kDA, NovaMatrix, Akershus, NO) was dissolved in distilled water to obtain a 0.1 w/v solution. Both solutions were sterile filtered through a 0.22 µm polyethersulfone membrane filtration system (Nalgene, NY, US) and lyophilized under sterile conditions. To produce growth factor supplemented HAMC, 3 µg/ml of VEGF-C (R&D Systems, ON, CA) and ANG-2 (R&D Systems) were
combined with sterile filtered phosphate buffered saline (PBS) in a laminar flow hood and mixed to ensure homogenous dispersion of proteins within the polymer matrix. Methylcellulose was then added and the mixture was vortexed to dissolve the MC. Lastly, hyaluronan was added and the solution was vortexed and incubated overnight at 4°C to allow HA to dissolve into the MC solution. Final concentrations of either 1 or 3 µg/ml of growth factors were used throughout the study as indicated.

6.6.2 Release rates

To determine the release profile of the growth factors from HAMC, 1 ml of growth factor infused HAMC (1 or 3 µg/ml) was loaded manually into a 3 ml tuberculin syringe. The gel solution was then injected into 9 ml of Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 0.5% Fetal Bovine Serum (FBS) in a 15 ml falcon tube. Growth factor release was assessed at 37°C over a 256 hour time period. At each time point all media was removed and stored at -20°C for further analysis by Enzyme-Linked Immunosorbent Assay (ELISA; R&D Systems). Fresh media was then added to each tube and the experiment was continued. As an internal control 1 µg/ml and 3 µg/ml samples of each growth factor (in 0.5% FBS supplemented media) were kept at 37°C for 256 hours.

6.6.3 Release rate in presence of Hyaluronidase

The above release rate experiment was repeated in the presence of the hyaluronan degrading enzyme Hyaluronidase (0.5 U/ml to 50 U/ml) in PBS at pH 5.5, the optimum pH for this enzyme (HAase; Bovine Testicular Hyaluronidase).

6.6.4 Cell culture of bovine lymphatic endothelial cells

The primary bovine lymphatic endothelial cells utilized in this study were generously provided by Dr. Dumont (Sunnybrook Health Science Center, ON, CA). Cultures were maintained in 10% FBS in DMEM on uncoated plates at 37°C and 5% CO₂. Cultures were supplemented with 10 n/g of VEGF-C to stimulate cell growth. Once cultures reached 70% confluency they were used in bioactivity experiments.
6.6.5 Bioactivity of VEGF-C156S and Ang-2 released from HAMC

To ensure that the concentrations of VEGF-C and Ang-2 introduced into the drug delivery system were capable of activating target receptors in lymphatic endothelial cells, we performed the following experiments. Bovine LEC cultures were serum-starved overnight in DMEM supplement with 0.5% FBS to minimize signalling through the lymphangiogenic pathways of interest. Following the starvation period, cultures were stimulated in one of two ways. In the case of western blots of VEGFR-3, HAMC (containing 3 µg/mL of both VEGF-C and ANG-2) was added directly on to the plates, and cultures were stimulated for 24 hours. Background signalling through the Tie-2 in this system was high and therefore to definitively see an increase in signalling from our drug delivery released Ang-2, we needed to reduce the background signalling. To do this, cultures were incubated with release media for 10 minutes. To form this release media, VEGF-C and ANG-2 (loaded at 3 µg/mL) were released from HAMC for 8 hours and the resultant supernatant was centrifuged to remove HAMC. As controls, cultures were also treated with VEGF-C (200 ng/ml) and ANG-2 (800 ng/ml) in 5 mls of 0.5% FBS in DMEM to determine the baseline signaling levels through each receptor. Furthermore, 1 ml of HAMC not loaded with growth factors was included as a vehicle control. Following treatment, cells were lysed for 30 minutes in Phospholipase C-γ lysis buffer (50 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH7.5, 150mM Sodium Chloride, 10% Glycerol, 1% Triton X-100, 1 mM ethylene glycol tetraacetic acid, 1.5 mM Magnesium Chloride, 10 mM Sodium Fluoride, 10 mM Sodium Pyrophosphate, 1mM Sodium Orthovanadate and protease inhibitor cocktail, Roche Diagnostics, DE) for 30 minutes on ice, centrifuged and the supernatants of each sample were collected and immunoprecipitated for Vascular endothelial growth factor 3 (VEGFR-3) (C-20, SC-321, rabbit polyclonal, Santa Cruz, CA, USA) and Tie-2 (C-20, SC-324, rabbit polyclonal, Santa Cruz) overnight at 4°C. Samples were resolved on 8% SDS-PAGE gels and transferred to a Polyvinylidene Fluoride membrane (GE Healthcare, ON, CA). Blots were blocked in either 5% BSA or 3% milk overnight. The following day, samples were incubated in primary antibody (1/1000; anti-phosphotyrosine 4G10, Millipore, ON, CA), Tie-2, and VEGFR3 at 4°C overnight. Blots were then incubated in a horseradish peroxidase linked secondary (1:10000, Biorad,
ON, CA) for 45 min at room temperature and developed using a chemiluminescence Kit (Supersignal West Pico, Thermo Scientific, ON, CA).

6.6.6 In vivo biocompatibility of HAMC

Sheep were fasted 24 hours prior to anesthesia induction. The animals were anesthetized initially by 15 ml intravenous injection of sodium pentothal. Subsequently, 2.0-3.5% isofluorane was delivered through an endotracheal tube via a Moduflex, Dispomed machine with Hallowell respirator for surgical maintenance. The dorsal surface of each animal was shaved, washed with soap and water and prepped with alcohol to remove all existing contaminants from skin. Intradermal injections of 100 µl of HAMC and HAMC dually infused with VEGF-C and ANG-2 were administered through 25 gauge needles. Equal volumes of saline and bradykinin (1 mg/ml) served respectively as the negative and positive controls. Injections were administered, in duplicates, once an hour for the first 3 hours (255, 195, 135, 75 minutes) and then at 45, 30 and 15 minutes. Following injections at 15 minutes 100µL of ¹²⁵I-Bovine Serum Albumin (¹²⁵I-BSA) (0.364 mg/ml; Perkin Elmer, ON, CA) and 25 ml of Evan's blue dye (EB) were introduced through a catheter in the cephalic vein following which, the catheter was flushed with 5 ml of heparinized saline. A 15-minute time period was then allowed to elapse to allow radioactivity and dye to accumulate at points of inflammation at which point, the animal was sacrificed.

Skin encompassing the injection points was removed and photographed with a Nikon digital camera (model CoolPix 4500). Each injection area was then punched out individually using a 2.54 cm cork borer, placed in 10% formalin and measured for radioactivity in a gamma counter (1282 Compugamma CS Universal gamma counter, LLK Wallas, Turku, FI) to quantify ¹²⁵I-BSA accumulation. Hematoxylin and Eosin staining was performed on 7 µm paraffin-embedded cross-sections of the injection areas to determine neutrophil infiltration. Based on preliminary results it became necessary to assess inflammation induction over a longer period of time and as a result intradermal injections of HAMC (with and without growth factors) were introduced into non-anesthetized sheep at time points corresponding to 24 and 128 hours prior to sacrifice.
6.6.7 Induction of lymphedema and growth factor therapy

Fasting and anesthetic induction was performed as described in the previous section. Surgical areas were shaved, washed with soap and water and disinfected with 70% alcohol. A vertical incision (6-8 cm) was made over the lateral aspect of the popliteal region in the hind limb and the popliteal fossa exposed. Following identification of the popliteal node in its surrounding fat pad, the pre- and post-nodal lymphatics were ligated and the node was excised. At this time growth factor therapy was introduced. Pre-prepared HAMC (1 ml pre-loaded into a 3 ml syringe with a blunted 18 gauge needle) was introduced into the area of nodal excision. To increase the retention of HAMC at this site, the fat pad pocket was then carefully sealed with absorbable suture. In the case of sham surgeries, the popliteal fat pad was exposed but undisturbed. A piece of saline soaked gauze was applied to the area for ten minutes to duplicate the length of time needed to perform the growth factor treatment surgery. The animals were returned to their holding pens after recovery from the anesthetics.

6.6.8 Edema Assessment

This study was performed before we acquired the bioimpedance device. Consequently, changes in leg circumferences were used to assess edema development as described in Chapter 2.

6.6.9 Assessment of lymphatic function; Transport of tracer to plasma

All animals were fasted and anesthesia was induced as described above. Evan’s Blue dye (1% in saline) was injected subcutaneously above the hind leg hoof to enhance visualization of the prenodal lymphatic vessels. An incision was made through the skin and subcutaneous tissues over the lower lateral aspect of the hind limb and a single vessel was cannulated with a 26-gauge angiocatheter. Saline (100 µl) was injected into the cannula over 30 seconds to check for leaks. Radiolabeled BSA (\(^{125}\)I-BSA, 0.364 mg/ml; in a 100-µl volume) was then injected over a 60-second period into the prenodal lymphatic vessel and flushed with 100 µl of saline. Blood samples were taken from a neckline inserted into the jugular vein at time 0, and every five minutes up to one hour and then every 30 minutes up to 2 hours. Fluoroscopic imaging was then
conducted and then animals were sacrificed (20 ml Euthanyl, 240 mg/ml, administered intravenously). It was determined that our radiolabeled tracer was undergoing some breakdown upon introduction into the animal. To determine the extent of the breakdown, trichloroacetic acid precipitations were performed on 2-hour plasma samples from each animal. Supernatant and pellet samples were compared to equivalent volumes of unprecipitated plasma to determine the amount of bound iodine. Plasma samples (1 ml) were then placed in the external gamma counter to determine $^{125}$I-BSA accumulations in blood over time. These values were expressed as a percentage of the total value of radioactivity (counts per minute) injected and corrected to reflect only BSA still bound to its radiolabel. These values were then plotted versus time. To allow comparison between the experimental groups, the graphical integration of the AUC was calculated using the trapezoidal rule.

6.7 Results

6.7.1 Release rates

6.7.1.1 Release rates in static cultures

Figure 17 displays cumulative release rate profiles in which, each VEGF-C (Figure. 17.A) and ANG-2 (Figure. 17.B) (1 or 3 µg/ml) were continuously released from HAMC over a period of 128 hours. Within this time, growth factor recovery was approximately 50% with the majority of release occurring by hour 32, indicating that about half of the initially loaded protein can be released from HAMC through diffusion. These results correspond to those previously reported for release profiles of growth factors from HAMC [160, 171]. When HAMC had completely degraded/dissolved into the media over the 2-week period, growth factor recovery was approximately 100%, suggesting that matrix degradation may be needed for complete growth factor release.

6.7.1.2 Release rate in the presences of Hyaluronidase

Hyaluronidase (HAase) is one of the primary enzymes involved in hyaluronan degradation, cleaving the (β1-4) glycosidic bonds of this co-polymer. This enzyme can be found both on the cell surface (HYAL2) and within the lysosome (HYAL1) and high
levels of this enzyme are often associated with high levels of hyaluronan turnover [172, 173]. Since it was unknown whether the popliteal fat pad would contain relatively low or high levels of HAase, a dilution series of HAase activity levels was used to assess the potential effect this enzyme could have on release rates from HAMC. The activity level, 50 U, is representative of the highest natural tissue level of this enzyme found within the human body (liver). In the presence of hyaluronidase, HAMC displayed dose-dependent release of growth factors that correlated with increasing hyaluronidase concentration (Figure 18). Therefore the presence of hyaluronidase at the site of HAMC injection will likely enhance growth factor release.

Figure 17. Percentage cumulative release of angiopoietin-2 (ANG-2) and vascular endothelial growth factor (VEGF-C) from HAMC.
A) VEGF-C and B) ANG-2 display similar release profiles in which approximately 50% of initially loaded protein (1 or 3 µg) is released into the surrounding media in the first 32 hr. Following this initial diffusion, controlled phase release rates then plateau. Complete dissolution of HAMC (128 hr) increases recovery of both factors to 100%, suggesting that matrix degradation also plays a role in release. Data are shown as means ± SEM (n = 3). Control (square), 1 µg (triangle), 3 µg (circle).

Figure 18. Hyaluronidase increases the release rate of VEGF-C and ANG-2.
Increasing levels of hyaluronidase activity (U/ml) is accompanied by increasing release of (a) VEGF-C or (b) ANG-2 from HAMC. Data are shown as means ± SEM (n = 3). Fifty units (red), 5 U (blue), 0.5 U (green), and no enzyme (black) are shown. Significant differences in ANG-2 release were reached by hour 24 in both the 50 U and 5 U enzyme concentration solutions. HAase appeared to enhance VEGF-C release in a dose-dependent way but due to the variability, these data did not reach significance. Significance in comparison to control levels (no HAase) was assessed by ANOVA and Dunnett’s one-sided t-test and is indicated by asterisks. *P < 0.05.

6.7.2 Bioactivity

6.7.2.1 Sequence homologies

It was necessary to determine if the growth factors released from HAMC remained bioactive - that is could bind to their target receptors and activate their respective signaling cascades in sheep. The growth factors employed in this study are human derived and thus share only partial homology with those of sheep. Specifically, human ANG-2 protein shares 89.6% homology with its sheep counterpart (HomoloGene, NCBI). While no data was available for ovine VEGF-C, it was determined that bovine (also a ruminant animal) and humans share 88.3% homology (HomoloGene, NCBI). The receptors for each of these ligands share similar homologies at 86.8% for VEGFR-3 (between bovine and human) and 95.4% for Tie-2 (between bovine and human) (HomoloGene, NCBI). The homologies appear to be sufficiently similar that one could expect reactivity between the sheep receptors and their human ligands.

6.7.2.2 Stimulation of LEC cultures

On lymphatic endothelial cells ANG-2 acts through the receptor Tie-2, while VEGF-C165S is known to associate exclusively with the receptor VEGFR3 [170]. Lymphatic endothelial cell cultures [174] were stimulated with conditioned media from either HAMC alone or HAMC infused with ANG-2 and VEGF-C. Significantly, HAMC infused with ANG-2 and VEGF-C induced an increase in cognate receptor phosphorylation over HAMC conditioned media alone (Figure 19). Interestingly, HAMC conditioned media alone can induce receptor activation of Tie-2, although levels did not reach those
induced by ANG-2. These results demonstrate that both ANG-2 and VEGF-C when released from the HAMC vehicle can activate their cognate receptors in vitro.

Figure 19. Growth factors released from HAMC possess the ability to stimulate lymphatic endothelial cells.

Purified bovine lymphatic endothelial cells (LECs) were stimulated with conditioned media from HAMC alone or HAMC infused with ANG-2 and VEGF-C. As controls, cultures were also treated with VEGF-C156S (200 ng/ml) and ANG-2 (800 ng/ml). Immunoprecipitated (a) Tie-2 or (b) VEGFR-3 activation was assessed by phosphotyrosine (pTyr). Cultures treated with growth factor release media displayed increases in receptor phosphorylation levels in comparison to negative controls (unstimulated and HAMC alone). Blots were stripped and reprobed to confirm similar receptor levels across treatment groups. n = 2. Molecular weights of each receptor are indicated.
increases in receptor phosphorylation levels in comparison to negative controls (unstimulated and HAMC alone). Blots were stripped and re-probed to confirm similar receptor levels across treatment groups. n = 3. Molecular weights of each receptor are indicated.

6.7.3 Biocompatibility

Previous studies have shown that HAMC was biocompatible when used in rats [160]. We felt it necessary to determine if similar results could be expected upon introduction of human growth factor infused HAMC into our sheep model.

6.7.3.1 Qualitative and quantitative assessment of vascular permeability: Evan’s Blue and $^{125}$I-BSA study

An effective method to measure the induction of inflammation is to quantify changes in vascular permeability. This is achieved by monitoring the accumulation of intravenously administered dye or radiolabeled protein at the site of interest. In this study we employed the use of EB and $^{125}$I-BSA accumulation following intradermal injections of HAMC. Following sacrifice, injection areas were first examined for EB accumulation. The outer and under-surface of the skin displayed slight blue staining corresponding to HAMC injection areas at 1 through 4 hours (Figure 20.A, inlay). This colour development was greater than that exhibited by the saline controls (no colour) but not reaching the blue staining level visualized in the bradykinin injections points (positive control) at 0 min and 15 min. Injection areas corresponding to 1 day and 1 week did not show any dye accumulation, suggesting that vascular permeability had subsided by these time points (Figure 20.A, inlay).

Next, analysis at injection was conducted for $^{125}$I-BSA. These results suggested that HAMC (regardless of growth factor presence) induced a minimal level of inflammation. Levels of radioactive BSA were approximately double that observed in saline-injected sites but never reached the levels attained by bradykinin injection (Figure 20.A). Over a one-week period the skin BSA levels induced by HAMC remained relatively constant and slightly elevated related to saline controls. We concluded that HAMC did not induce any significant inflammation over the monitoring period.

6.7.3.2 Histology
An increase in the presence of neutrophils was observed compared to control injections at hour 4 (Figure 20.B). However, by the end of week 1 HAMC injection areas resembled that of controls. This result suggested that the mild inflammation induced by HAMC was temporary.

**Figure 20. HAMC induces minimal inflammation in sheep.**

Introduction of HAMC (HAMC alone (green)) and HAMC infused with 3 µg/ml of ANG-2 and VEGF-C (blue) resulted in an increased accumulation of A) $^{125}$-iodine radiolabeled bovine serum albumin ($^{125}$I-BSA) in comparison to saline (black) injections at corresponding time points. These vascular permeability increases reached significance at 3 and 4 hr post-injection; however, they appeared to resolve over a 1-wk
period. Bradykinin, a transient vascular permeability inducer, was used as a positive control (red). Data are shown as means ± SEM (0-4 hr, n = 5; 24 and 168 hr, n = 2). Double lines indicate a change in scale. Significance in comparison to saline was assessed by ANOVA with accompanying one-way Dunnett’s t-test. *P < 0.05. Similar results were obtained when Evans blue dye (EB) accumulation was examined ((a) inset), saline 4 hr (1), HAMC 4 hr (2) HAMC + GFs 4 hr (3), HAMC 1 wk (4), HAMC + GF 1 wk (5). B) Hematoxylin and eosin-stained skin sections of injection areas were examined using a bright-field microscope at 10X magnification. There were areas of heavy neutrophil infiltration in HAMC injection areas at 4 hr (2) in comparison to saline controls (1). Neutrophil infiltrations appeared to resolve over the next week in HAMC injection areas (3). Circle indicates an area of heavy neutrophil infiltration. Inset: Enlargement of cell infiltration area depicting classical morphology of neutrophilic cells, 100X magnification.

6.7.4 Lymphatic transport function

The average tracer accumulation in blood is illustrated for all groups in Figure 21A. The black line (intact limbs) provides the reference point for the various interventions. At the other extreme (orange line), very little BSA enters the blood immediately after lymph node excision because the lymphatics have been disrupted. It is clear that sham surgical procedures impact lymphatic function negatively, presumably since some of the fragile vessels have been damaged (red line). By 6 weeks after excision, the normal lymphatic regeneration has improved lymph transport somewhat (grey line) and this is similar to that observed with HAMC alone (green line). Improvement in lymphatic function was noted in the group receiving HAMC plus growth factors (blue line).

When the AUCs were compared, lymph node removal was found to significantly reduce functionality in the non-treated groups (Nodectomy 1.9 ± 0.9 or HAMC alone 1.7 ± 0.8) in comparison to intact controls (3.2 ± 0.7)(Figure 21.B). Alternatively, growth factor treated groups (2.3 ± 0.7) were not significantly different from the values observed in both sham and control groups indicating functional improvement after the application of the VEGF-C/ANG-2. Unfortunately, growth factor treated limbs did not return completely to the functionality levels measured in intact limbs over the 6-week period.
Figure 21. Lymphatic functionality following growth factor treatment.

A) Removal of the popliteal lymph node results in lymphatic vessel damage and immediate assessment (Nod T0) reveals that minimal $^{125}\text{I}$-BSA transport to plasma occurs (orange, n = 7). Growth factor treatment (blue, n = 8) at the time of node removal resulted in an increase in $^{125}\text{I}$-BSA accumulation levels in plasma over 2 hr in comparison to non-treated groups, nodectomy (gray, n = 8) and HAMC (green, n = 6). Growth factor treatment accumulation levels did not return to those seen in sham (red, n = 7) and intact control groups (black, n = 7). Points represent means. SEM was omitted for clarity. (B) The AUC was then tabulated for each individual
animal using the trapezoidal rule. Following node removal, non-treated groups (nodectomy, HAMC, Nod T0) had significantly less functionality at 6 wk in comparison to the intact group. In contrast, functionality in the growth factor treatment group was similar to the intact group. Each bar represents mean ± SEM. Significance in comparison to control levels (intact limbs) was assessed by ANOVA and Dunnett's one sided t-test. *P < 0.05. BSA, bovine serum albumin.

6.7.4.1 Fluoroscopy

In intact and sham surgical groups there were clearly visible popliteal lymph nodes, with identifiable prenodal and postnodal vessels (Figure 22.A). Six weeks after lymph node excision, some fluid continuity had been re-established since both the afferent and efferent vessels were visible and connected by an entanglement of newly regenerated vessels (example in Figure 22.B). These newly formed vessels appeared to be fragile as indicated by the frequent leaks in this area upon contrast agent infusion. With HAMC alone, the pattern was similar to that observed in the nodectomy group (example in Figure 22.C). In the limbs receiving HAMC plus growth factors, there appeared to be an increase in the number of regenerated vessels (Figure 22.D).
Fluoroscopy of popliteal fossa region following growth factor treatment.

Fluoroscopic images of the lymphatic networks 6 wk following surgery were taken. Representative images from each group are shown above. In sham preparation (a) at 6 wk, the popliteal lymph node (arrow) is visible as well as several pre- and postnodal lymphatics. In the cases where nodes were removed (nodectomy (b) and HAMC (c)), at 6 wk a tangled network of lymphatics is visible at the surgical site, suggesting some lymph continuity has been reestablished through natural lymphangiogenesis. Growth factor-treated animals (d) displayed an increase in the number of vessels in the excision site. This increase in vessels may be responsible for the increase in lymphatic functionality seen in this group. Circles indicate popliteal fossa region.

6.7.4.2 Edema

As expected, limb circumferences did not change over time in the sham treated animals. In contrast, the removal of a popliteal lymph node resulted in edema formation.
in 100% of animals. The presence of HAMC plus growth factors appeared to produce the best results in terms of reducing edema levels. Examples are illustrated in Figure 23A.

Quantitative data is illustrated in Figure 23B. Growth factor treated groups displayed edema magnitudes similar to sham levels (107.4 ± 51.3 and 59.2 ± 62.5 respectively). Non-treated groups displayed significantly greater edema levels in comparison to the sham group (Nodectomy 219.8 ± 118.7, and HAMC 162.6 ± 141). This result suggests that growth factor treatment has the ability to reduce edema magnitude following lymph node removal.

When we plotted all of the individual lymphatic function data versus their corresponding edema measurements at 6 weeks for all groups, we observed a significant negative correlation (p=0.001) (Figure 24). That is, the highest levels of lymphatic function correlated with the lowest levels of edema supporting the notion that improvement in lymph transport capacity with growth factors has potential for lymphedema treatment.
Figure 23. Development of edema following popliteal lymph node excision in the presence and absence of growth factor therapy.

(a) Typical examples of edema formation in each treatment group (n = 1) are displayed. (b) The area under the curve (AUC) was calculated for animals using the trapezoidal rule. Node removal (nodectomy (2)) and node removal with HAMC (3) usually resulted in a sharp increase in edema formation over the first week and decreased marginally over the next 5 wk. In contrast, growth factor-treated groups displayed reduced edema levels by 6 week (4), statistically similar to sham levels (1). Each bar represents mean ± SEM. Sham n = 18, nodectomy n = 36, HAMC n = 7, HAMC + GF n = 7. Significance in comparison to sham levels was assessed by ANOVA and Dunnett’s one sided t-test. *P < 0.05.
ANOVA and Dunnett’s one sided t-test. *P < 0.05.

Figure 24. Relationship between lymphatic functionality and edema magnitude.

Functionality data (AUC at 2 hr) were plotted versus their corresponding edema values at 6 week, n = 36. The trend shows a negative correlation, suggesting that as lymphatic functionality increases, edema magnitude at 6 week decreases. Linear regression analysis revealed this trend to be significant, P = 0.001.

6.8 Conclusions

We report that the addition of two lymphangiogenic growth factors to a nodal excision site enhances lymphatic transport function and reduces the edema burden in a sheep model of lymphedema. These data suggest that molecular therapeutic approaches may ultimately be effective in improving outcomes for breast cancer patients with lymphedema. For this concept to be valid, choice of molecules and drug delivery systems and comparisons with other potential therapeutic approaches is warranted.
6.8.1 Drug delivery systems

There are many potential drug delivery approaches that could be used to introduce lymphangiogenic growth factors. The HAMC system offers several advantages in this regard. A hydrogel represents a network of water-swollen polymer chains, in which water is the dispersion medium. When HAMC is placed within the body, it absorbs water from surrounding environment and the pore sizes within the matrix expand and proteins are released [175]. This characteristic property of hydrogels, accounts for the rapid burst release of growth factors at the beginning of each release study. In the case of our system, following this initial rapid release, rates appeared to slow until HAMC had undergone dissolution. This suggests that growth factors incorporated into the outer regions of HAMC could readily diffuse into media but the diffusion distance may have been too great for those incorporated into the core of HAMC, causing release to plateau. Degradation of HAMC then allows for the release of remaining factors. Previous HAMC studies saw almost complete diffusion-mediated release of a single growth factor from this system over a 16-hour period [171] but in these experiments the volume of HAMC used was 1000-fold less than those utilized in our report.

Hyaluronan is metabolized by Hyaluronidase in vivo, and it has already been shown that this enzyme can affect the degradation of HAMC [160]. As anticipated, increasing activity levels of HAase in our study were associated with increasing release rates of both growth factors. This result suggests that endogenous HAase activity in the popliteal fossa may result in more rapid release of lymphangiogenic factors in vivo than predicted from the in vitro experiments. While we do not know the release profiles of VEGF-C and ANG-2 in vivo, we expect that the release rates will depend upon consumption of the factors (maintaining a concentration gradient favoring release) in the popliteal fossa and the presence of degrading enzymes, such as hyaluronidase. We anticipate that the bioactivity of released factors will be maintained over the course of the release, due to their incorporation into HAMC. The half-life of VEGF-C in the circulation is less than 15 minutes, thus HAMC will likely provide some protection to the growth factors by controlling its release over time [176].

The HAMC drug delivery system contains numerous components that could potentially invoke a reaction from the host; the methylcellulose is synthetically manufactured, hyaluronan is produced in bacteria, and the growth factors are all human derived. Nonetheless, the evidence presented in this report including
assessments of vascular permeability and leukocyte infiltration indicates that any host response is minimal. This is supported by the literature where HA has been shown to attenuate the inflammatory response in several tissues [177].

6.8.2 Choice of growth factors and in vivo bioactivity

One of the most important questions in devising a molecular strategy for lymphedema treatment is the selection of the lymphangiogenic growth factor. The following factors have been reported to stimulate lymphangiogenesis of both cultured LECs and animals, including VEGF-A, VEGF-C, VEGF-D, FGF-2, PDGF, IGF-1, IGF-2, Angiopoietin-1 and -2, and HGF [174, 178-182] and the list is growing rapidly. Even with so many to choose from, VEGF-C seemed an obvious choice. VEGF-C is required for the development of the lymphatic system as well as for proliferation, migration and survival of LECs [158]. It is also known that blocking signaling through its receptor, VEGFR-3, results in regression of pre-existing lymphatic vessels. Recent studies in mice have revealed that VEGF-C introduction increases the rate of damaged vessel reconnection in comparison to controls [183]. Furthermore, evidence has been found that directly links VEGF-C signalling and lymphedema. Point mutations identified in VEGFR-3 lead to a hereditary form of lymphedema, known as Milroy’s Disease that presents with edema of the lower extremities [184, 185].

Another factor we decided to introduce into the lesion site is Angiopoietin-2. ANG-2 has been implicated in the organization of the lymphatic system through the activation of the Tie-2 receptor. ANG-2 knockout mice display abnormal lymphatic patterning, disrupted lymphatic endothelial-smooth muscle cell interactions as well as subcutaneous edema and chylous ascites indicting an obligatory role for ANG-2 in lymphatic system development [20, 174].

Overall, this study revealed that both growth factors appear to be protected from inactivation while incorporated in HAMC until their release, when they can in turn, bind and activate target receptors involved in lymphangiogenic signalling. Due to close homology between both bovine and ovine ligands and receptors, these results suggest that similar results would likely be obtained with ovine cells, which was supported by new vessel growth in the fluoroscopy images.

Additionally, we found that HAMC appears to potentiate Tie-2 phosphorylation by ANG-2. Specifically, there appears to be an increase in Tie-2-ANG-2 association
demonstrated by the increased presence of a second band in these cultures. As HAMC undergoes dissolution, the viscosity of media surrounding the cells increases, which may alter the interaction of Tie-2 with ANG-2 dimers (which form spontaneously in solution), perhaps increasing their association time, explaining the above result.

This study also indicated that HAMC might possess the surprising ability to activate the receptor tyrosine kinase (RTK), Tie-2. It is well known that low molecular weight hyaluronan fragments play a role in endothelial cell proliferation and migration, through activation of particular RTKs [186]. Typically these fragments range from 3 to 16 oligosaccharides in length [187]. However, the hyaluronan utilized was initially 1500 kDa and while small amounts of random cleavage may occur, this seems like an unlikely cause of receptor activation. A second, more plausible theory is that through an association with CD44, hyaluronan is indirectly influencing Tie-2 signalling. Hyaluronan can directly bind CD44 causing the cell surface receptor to cross-link [188]. This cross-linking has been shown to influence integrin signalling [188] which in turn can influence Tie-2 activation [189]. All three are common receptors found on the surface of endothelial cells, and therefore association with CD44 could indirectly affect Tie-2 signalling. The ability of HAMC to independently activate a RTK such as Tie-2 may provide an additional advantage to the lymphangiogenic process, working synergistically with the ligand ANG-2 to improve functionality.

6.8.3 Other factors to consider

Our results suggest that pro-lymphangiogenic growth factors can have a positive impact on lymphatic function and improve edema outcomes. However, there are several conceptual issues that may need refinement in future investigations. First, there is evidence that lymphangiogenesis is a two-phase process. The initial phase involves activation of endothelial cells and sprouting, while the second phase involves maturation of these vessels. Maturation includes smooth muscle cell recruitment, which appears to play an important role in regulating vessel phenotype and the orientation of the newly formed vessels [20, 157]. With this in mind, it may be possible to build a system to control the temporal release of the growth factors to mimic more closely, the endogenous realities. It should be noted however, that the dual factor approach and the doses we utilized in this study had a significant impact on lymphatic function and
edema resolution. This information provides a useful framework on which to optimize lymphangiogenic therapeutic approaches in future studies.

A second issue involves the signalling ability of VEGFC156S (utilized here) versus its wild-type counterpart. The wide-type ligand has the ability to bind both VEGFR2 and VEGFR3 receptors, while VEGFC156S is an agonist only to VEGFR3. Signalling through VEGFR2, which is found predominantly on vascular endothelium, causes increased permeability, an event that we wanted to minimize in our study. However, upon initial characterization, it was noted that VEGFC156S signalling in LECs was different from VEGF-C [170]. It was postulated that activation of VEGFR2 on LECs might be needed to induce maximal lymphangiogenic signaling. In any event, it is clear that manipulation of lymphangiogenesis in vivo is a complex issue and that optimal conditions (choice of factor, dosage, and timing of application) require further investigation.

The HAMC drug delivery system has been successfully adapted for the introduction of pro-lymphangiogenic molecular factors to a nodal excision site. The growth factors employed in this study, VEGF-C and ANG-2, enhanced the naturally occurring lymphangiogenesis, significantly increased lymph transport and reduced the magnitude of edema formation. Results from this study highlight the potential of molecular therapies for lymphedema patients.

6.8.4 Combination therapy

The combination of nodal transplantation with growth factor therapy in lymphedema treatment would appear to be an appealing concept. We considered testing this concept but our results suggested that these experiments may not be informative. This is due to the fact that successful autologous, vascularized lymph node transplantation by itself was able to restore lymphatic function and tissue water levels to essentially normal levels. The addition of growth factors could not improve on this record. One might argue that lymphangiogenic factors would have improved the survivability of the transplanted nodes by enhancing the connection of pre- and postnodal vessels to the node capsule and hilum. However, the evidence suggests that the unsuccessful transplants were due to failure of the vascular supply and not to the regeneration of new lymphatic vessels. One might make the case for the addition of molecular angiogenic elements to facilitate blood vascular connections to the node but this was
beyond the scope of the thesis. In any event, it seems likely that microsurgical issues associated with the connections of the very small blood vessels during the transplant procedure were the limiting factor in the success of the transplants.
Chapter 7
General Discussion

Damage to the lymphatic system occurs frequently during the innumerable surgical procedures that occur in hospitals around the world. However, while acute edema is a natural response to tissue injury, chronic tissue swelling (lymphedema) is not a characteristic feature of most of these surgeries. However, the removal of one or more lymph nodes appears to be a significant causative factor in lymphedema development. The data in this thesis highlight a number of key lymphatic functional deficits that are associated with removal or damage to a lymph node. We believe that one of the strengths of this work relates to the use of larger animals for studies on lymphedema, which allows us to assess more effectively, important lymphatic functional parameters. Additionally, with the larger experimental subjects, we were able to examine several ‘human-sized’ approaches to reverse the deficits in function including autologous, vascularized lymph node transplantation and the introduction of lymphangiogenic growth factors using a novel gel-delivery system. Both of these approaches could have relevant clinical applications. Regarding the latter, the gel-delivery of lymphangiogenic factors seems entirely amenable to the clinical setting since the gel itself is biocompatible and biodegradable and it utilizes growth factors that exist naturally in humans.

7.1 Animal models

At first glance, the use of sheep would seem an unlikely choice for lymphedema research. However, there are some significant advantages to using this species. First, sheep possess a well-developed lymphatic system with vessels that are large enough to be manipulated individually, which is very difficult to achieve in smaller animals. The popliteal system in sheep is also unique in that all the afferents of the lower limb drain through the single popliteal node. Resection of this node results in consistent edema development in the lower limb [117] making this an ideal model in the study of postsurgical edema. Also, the vessels and lymph nodes in this species are 'human-size' in their perspective, which makes this model useful for the investigation of realistic clinical intervention methods.

Radiation with or without surgical intervention has been applied to dogs [84], rats [82] and mice [85] with varying results. In these models, the major focus was on inducing lymphedema rather than assessing lymphatic function. For reasons noted
earlier, we used rabbits to assess the impact of nodal irradiation on lymphatic function as we were not able to utilize sheep for this purpose. Rabbits provided some of the advantages of the larger species and allowed us to apply radiation to the hind limbs. Overall, the two species responded similarly to nodal excision although there were a few differences, which will be noted below.

One major difference was the functional deficit measured following node removal. In rabbits, while functionality was reduced it did not reach significance in comparison to control animals (as was found in sheep). In rabbits there was also a lack of measureable edema in hind limbs following node removal. These variations can likely be attributed to the anatomical differences between the species. The rabbit popliteal node is located very superficially and only requires a small incision in the skin to expose the popliteal fossa. In sheep the popliteal node is located significantly deeper, requiring a larger skin incision and retraction of the biceps femoris muscles. We know from our own studies [115, 116] that lymphatics are damaged and functionally affected by this skin incision and muscle retraction alone. Therefore the greater functional deficit seen in sheep might be attributed to greater damage inflicted during the node removal surgery. Consequently a greater functional deficit may have resulted in the greater edema measurements seen in the sheep model.

One final point about our animal models; it should be noted that we were not attempting to replicate the complex conditions experienced by cancer patients in our animal models. In all likelihood, lymphedema is the result of a complex interplay between many factors that lead to the development of this condition. Instead, our models allow the measurement of lymphatic-related physiological changes over time and can provide a realistic framework on which to develop therapeutic measures and methods of administration that may be directly applicable to humans.

7.2 Assessment of lymphatic functionality and edema

The assessment of lymphatic drainage provides several challenges not the least of which relates to the relatively small size of the vessels. While there is no perfect method, one of the best indicators of a functional lymphatic system is the ability to transport a lymph flow tracer across an area of injury back to the blood stream. We used this technique to assess the reconnection of damaged lymphatics over time after lymph node removal or irradiation. This approach is of course, most amenable to
studies in larger animals. Most reports in the literature focus on identifying the presence of lymphatic vessels using some sort of contrast agent. For example, lymphoscintigraphy has been used commonly to diagnose and characterize the severity of lymphedema. This minimally invasive procedure employs the intradermal or subcutaneous injection of a chosen radiolabeled colloid and with the application of a gamma-camera, can provide a quantitative measure of lymph drainage. A commonly used tracer for such studies is 99m Tc-Filtered sulfur colloid. While a useful technique, the disappearance of this tracer from the injection depot can be unpredictable as the inevitable differences in the injection location can make reliable calculation of tracer disappearance rates difficult. In smaller animals, immunohistochemical methods are quite commonly used to identify lymphatic endothelial cells but it is sometimes difficult to relate the presence of endothelial markers with function.

While on the subject of methodology, the measurement of edema is problematic for several reasons. First, the criteria for the diagnosis of lymphedema are not clearly defined. It may be based upon changes in limb volume, although in the case of breast cancer, swelling may occur at one point or multiple (hand, arm, breast, chest, underarm and back). A diagnosis may also be based on functional studies or on a patient’s self-reported symptoms (heaviness, pain). Secondly, there is a lack of sensitive modalities to detect lymphedema. The wide range in incidence of lymphedema reported in the literature is a reflection of these various methodologies. The circumference measurement is the simplest approach, in which the limb girth is measured using a non-elastic tape or string. A change in limb girth of 2 cm or more represents a lymphedema situation. However, this method is not sensitive in relation to other methods and is plagued by high error rates. Since Guyton has demonstrated that visible edema is not evident until limb weight was increased about 10% this method is unable to measure any differences in water content until significant accumulation has occurred [190].

Bioelectrical impedance in contrast has been shown to be reliable, sensitive and specific at detecting localized edema changes [191-195]. This method uses a small, low frequency electric current that is passed through the body and resistance to current movement is measured. Impedance measures give an estimate of extracellular water volume that is independent of arm volume. Additionally, with water measurements in cancer patients (perhaps with impedance technology), we may be able to identify
patients that are at greater risk of developing lymphedema (at a subclinical level) and intervene early in the treatment process.

While emerging evidence suggested that bioimpedance had a clear advantage over its predecessors in the quantification of edema, however, our experience with this technique was disappointing. This technique required animals to be under anaesthesia, and needles had to be inserted intradermally at probe placement locations. Measurements were very sensitive to needle placement (location and depth). Electrode pads that stick to the skin, as used in human use, may help to eliminate this problem. Issues with this technique have also been noted in clinical use. Infrequently, a limb cannot be assessed by bioimpedance, although reasons remain unclear. It has been noted that this technique cannot be used in limbs affected by advanced fibrotic forms of lymphedema [196]. Newer techniques utilizing MRI [197, 198], CT [199] and ultrasound technologies [200, 201] have demonstrated potential in visualizing lymphatic parameters in lymphedema, although their use is not yet common practise.

7.3 Clinical treatment of lymphedema

The gold standard of treatment for this disease is complete decongestive therapy. This treatment regimen involves manual lymph drainage, which is a type of massage to “milk” the lymph from the tissues into the lymphatics. In addition compression garments, which raise interstitial fluid pressure and reduce the extravasation of fluid and proteins into the tissues, are used. These treatments are associated with partial symptom relief and must be performed continuously to prevent relapse.

7.4 Surgical methods of treatment

The two main categories of surgical treatment proposed for the treatment of lymphedema lymphatic microsurgeries and tissue transfer procedures. Lymphatic microsurgeries involve the microsurgical reconnection of the lymphatic system through the damaged region. Studies have attempted to reconnect distal and proximal lymphatics to bypass the affected region [202, 203]. There have also been many reports involving surgical connections between lymphatics to local vasculature [204, 205]. Lymph-venous anastomoses involve the microsurgical connection of multiple (10-15) lymphatic collectors directly to veins upstream of the lymphatic obstruction. This technique has been met with some criticism in the past due to poor results and frequent
complications. Further study of the characteristics of the lymphatic-vein interface and the physiological parameters that allow such connections to form and be maintained should be conducted to allow refinement of this technique.

Tissue transfer surgeries involve the introduction of ‘normal’ tissues from alternative places in the body to assist/replace the damaged portions of the lymphatic system within the edematous region. The types of tissues transferred include: omental grafts that contain lymphatics [206, 207], implantation of lymph node fragments [208-213], avascular and vascularized lymph node transfers [146, 148] as well as microfat grafting. Lymph node fragments and avascular node transplants have demonstrated the ability to induce vascular and lymphatic proliferation and appeared to regain some ability to filter peripheral lymph. Significant regeneration of these fragments has not been observed and in comparison to other treatment mythologies this therapy has yet to show it has any beneficial effect on lymphedema.

In terms of novel therapy development for the treatment of lymphedema, vascularized lymph node transfer has been shown within this thesis to have the ability to prevent and/or reduce the lymphatic dysfunction associated with node removal. As noted earlier, transplantation has also been shown to provide at least some benefit in human lymphedema patients. Despite problems, autologous lymph node transplantation for the treatment of lymphedema has been gaining popularity, especially in the USA and Europe. We like to feel that our publication on this procedure in 2009 helped to facilitate enthusiasm for this procedure in Canada [116]. In our hands, the immediate replacement of a node with another has the potential to complete reverse edema development. Indeed, the data from the transplant studies has encouraged Dr. John Semple (Chief, Department of Surgery at Women’s College Hospital) to initiate preliminary lymph node transplantation trials in breast cancer patients. This method involves the harvesting of a small cluster of lymph nodes, usually from the groin region, and transplantation into the axilla of breast cancer patients. This treatment has shown to be beneficial however the procedure itself comes with the risk of lymphedema formation as a result of node removal at the donor site. Additionally, the timing of the transplant is an important consideration. It is likely that early replacement of the node will be more effective that transplantation after lymphedema has become established.

In a review of studies pertaining to the surgical treatment of lymphedema,
marked reduction of limb volume or circumference was noted in some studies [214]. However most of these were conducted on a small number of select patients. In addition, almost all studies lack comparison to control limbs and there was a general lack of standardized edema measurements and long-term follow up. Therefore, attempting to generalize the results from each study across all types of lymphedema is difficult. Large, randomized studies, with long-term follow-ups are needed before realistic outcomes of specific surgical procedures are known.

To get around some of these problems in the future, one might envisage the development of an artificial lymph node. It might be possible for example, to engineer some type of scaffold impregnated with the appropriate host cells and/or molecular factors to provide the anatomical and molecular cues necessary for the development of a nodal structures in vivo. Some success has been made in the development of a bioreactor for the engineering of a human lymph node in vitro [215]. However, the products do not appear to be useful for implantation in patients. On balance then, the development of an ‘artificial lymph node’ is conceptually promising but in practical terms, represents a challenge to implement in the foreseeable future.

7.5 The application of lymphangiogenic growth factors

It is clear from our study that the surgical removal of a lymph node is followed by lymphatic vessel regeneration and reconnection with downstream and upstream ducts to re-establish flow. Presumably, the growth of new vessels from pre-existing lymphatics follows many of the same principles as those involved in the original development of the system in the fetus. In the embryo the trans-differentiation of venous endothelial cells to those of a lymphatic phenotype, the separation of the two systems and subsequent spouting and maturation of lymphatic vasculature have been well characterized [216, 217]. Adult lymphangiogenesis, or the formation of new lymphatic vessels from pre-existing ones occurs routinely in adult tissues during wound healing and inflammation. In severed lymphatics, lymph flow continues between the divided ends for 24-48 hours before a clot is formed [47, 90, 218]. Lymph contains all the coagulation factors of blood however in much lower concentrations [219, 220]. After the clot formation, lymphatics regenerate and lymph flow is quickly re-established [221].

Nonetheless, while the ability of lymphatic vessels to regenerate is well established, we were somewhat surprised by the magnitude of the natural compensatory responses
to nodal removal or injury. Clearly, lymphatic vessels have a remarkable capacity to regenerate. Key regulators of this process are still being debated, however VEGF-C/D and their cognate receptor VEGFR-3, are required for proliferation, migration and survival of lymphatic endothelial cells (LECs) [158]. Additionally, evidence has been found that directly links VEGF-C signalling and lymphedema. Point mutations identified in VEGFR-3 lead to a hereditary form of lymphedema, known as Milroy’s Disease that presents with edema of the lower extremities [11, 12, 185]. Another, far less studied growth factor in terms of its role in lymphangiogenesis, is angiopoitin-2. Ang-2 has been implicated in the organization of the lymphatic system through the activation of the Tie-2 receptor. Ang-2 knockout mice display abnormal lymphatic patterning, no valves, disrupted lymphatic endothelial-smooth muscle cell interactions as well as subcutaneous edema and chylous ascites indicating an obligatory role for Ang-2 in lymphatic system development [20, 222].

In any event, new lymphatic vessel growth is a complex process and the decision as to which factors to apply to the nodal excision site was a difficult one. Several of these have been reported to stimulate lymphangiogenesis of both cultured LECs and animals, including VEGF-A, VEGFC, VEGF-D, FGF-2, PDGF, IGF-1, IGF-2, Angiopoietin-1, and HGF [178, 179, 182, 223]. While these studies have shown some promising results they have not yet progressed into clinical setting. In our studies, we chose a combination of VEGF-C and Ang-2 in the gel delivery system; the first to stimulate new vessel growth and the second to help in the maturation of the newly formed vessels. This approach yielded impressive results but we are the first to admit that much more work is necessary. While growth factor therapy application increased lymphatic function and decreased edema, these characteristics never reached control/pre-treatment levels.

This result was in direct contrast to our study in which limbs that received successful lymph node transplants, regained full functional capacity and edema was eliminated. This supports the theory that the lymph node is a vital component of lymphatic function and depressingly, of lymphedema development. As a treatment, lymphatic vessel regeneration alone, without accompanying lymph node transplantation, may be not be capable of fully restoring the system to an optimal functioning level. Future experimentation to optimize growth factor treatments is needed to confirm this theory. For example, it might be advantageous to have a system
that delivers the ‘drugs’ over a longer period of time although the most appropriate window of action for this therapy is not clear at present. Additionally, the doses of the individual agents have not been optimized.

One caveat regarding growth factor therapy must be made. Prolonged VEGF-C over-expression is a hallmark in many tumor types including breast cancer and is a known promoter of metastasis [224-226]. Therefore long-term delivery of this factor (for example with viral growth factor treatment) would likely be limited in clinical uses to prevent increased cancer reoccurrence. A drug delivery system that is both localized and very short-term, such as HAMC utilized in this thesis, may allow treatments to be conducted in the clinical setting.

Stem cells are consistently in the news and there is some excitement that these can be utilized in lymphedema therapy [227]. Until recently it was thought that all lymphatic vessel formation in adults occurred by lymphangiogenesis, however new evidence suggest that lymphvasculogenesis may also contribute [228, 229]. Lymphvasculogenesis is the process of new vessel generation through stem or progenitor cells. It was first noted that a subpopulation of human fetal liver cells co-expresses VEGFR3, and two known markers of hematopoietic stem cells, and endothelial progenitor cells CD34 and CD133 [230, 231]. In culture, this specific cell population displays a robust proliferative potential, becomes adherent, and expresses other lymphatic markers, such as LYVE-1 and podoplanin [232]. In addition, Kerjaschki et al. (2006) reported the presence of male donor-derived LECs in the lymphatic vessels of transplanted kidneys [229]. Taken together these studies support the potential of lymphvasculogenesis in the development of new vessels postnatally, with precursor cells being derived from either bone marrow or other organs.

A combination of node transplant and growth factor therapy has shown promising results in mouse and pig models. Several groups have combined autologous lymph node transplantation with adnoviral VEGF-C treatments [157, 179]. The experimenters observed that growth factor treatment appeared to facilitate lymph vessel connection to the transplanted tissue and increase transplant survival. However, there are several concerns related to the interpretations of these studies. For example, the mouse lymph node is small enough to be maintained on diffusion of oxygen and nutrients alone, unlike human lymph nodes, which need an intact vascular supply for survival. Thus any conclusions regarding survivability of mouse lymph nodes irrespective of VEGF-C
application is not relevant to the human situation. In a pig study that appeared to support a combination of transplantation and growth factors, the authors did not actually transplant nodes but ‘simulated’ the procedure by damaging the lymphatic vessels leading to and from a single inguinal node [179]. They noted that there was increased number of lymphatic vessels and increased flow through the growth factor treated limb. However, increased function could be attributed to the increased lymphangiogenesis in these animals (reconnection of vessel to the node and collateral formation around the node), but is no way an indication of graft survivability. As such, little information can be drawn from these alleged combination experiments.

7.6 Efforts to reduce lymphatic damage

Considerable effort has been made to reduce the damage sustained by normal tissues during breast cancer treatment. Sentinel lymph node dissection has eliminated the need for axillary dissection in patients whose sentinel node is tumor-free, thus greatly reducing the probability of lymphedema development. Going one step further, recent studies have shown that removing axillary lymph nodes, even in patients with tumor-positive sentinel lymph nodes, offered no survival benefit in early stage breast cancer [233-235]. If this becomes a widespread practice, this will likely reduce the number of patients developing lymphedema even further. However, it seems possible that breast and axillary radiation will replace the need for further node dissection in these patients. The debate may evolve around the location and the dose of radiation that should be given to these patients. Results from this thesis suggest that radiation alone to a single normal lymph node can impact lymphatic function negatively in the entire limb. Therefore radiation therapy application should be reduced when possible and given with caution when needed. In addition patients need to be made aware of the risk of lymphedema development even without lymph node removal.

As these factors play out, efforts are being made to minimize the collateral damage associated with radiotherapy. For example, there is a trend towards more conservative treatment, trying to reduce overall doses and volumes of tissue that need to be irradiated without affecting the desired outcomes. From a lymphatic perspective, these could include an understanding of the doses that kill cancer cells but spare the lymphatic vessels. Alternatively, research is also being conducted into treatment of breast cancer tumour cells to make them more sensitive to radiation. This would allow
delivery of smaller doses of radiation to kill tumour cells with the possible side effect of decreasing the damage sustained by surrounding tissues and organs [23]. In the lab, researchers are looking for methods to combat radiation-induced fibrosis. For instance, blockade of the major fibrosis pathway (TGF-β1) has shown to result in decreased fibrosis and improved lymphatic functionality following radiation treatments [10]. Additionally, radio-protectant drugs have the ability to save non-cancer cells. In a rodent radiation therapy dependent lymphedema model, the radio-protectant amifostine reduced the magnitude of edema suggesting that the further development of such drugs may have some merit in helping lymphatics survive radiotherapy-induced damage [24].

7.7 Integrated view of lymphedema development

While the studies within this thesis have focused on the removal of, or injury to the lymph node, numerous other factors have been implicated in the development of lymphedema. Other contributors include but are not limited to trauma to the ‘at risk’ limb, obesity and hypertension, air travel, infection and inflammation, as well as malignant and venous diseases [236]. A genetic disposition to the development of chronic edema may also apply, although no susceptibility genes have been identified [33, 34, 237]. However, it has been suggested that women with greater than average peripheral lymph flows were more likely to develop lymphedema following breast cancer surgery [21]. The authors postulated that this predisposing factor might explain why lymphedema develops in patients in which only a few nodes have been removed.

Based on the aforementioned considerations, we might consider a ‘multi-hit’ hypothesis for lymphedema development. In this regard, it is important to link the various perturbations in lymphatic function to tissue compliance. The issue of limb compliance has been largely neglected in lymphedema studies and yet, pressure-volume relationships are important in understanding the basic pathology of this disorder. Guyton described the concept of a tissue ‘safety factor’ that works to prevent increases in interstitial fluid volume that can lead to edema formation [190]. Pressure-volume analysis in limbs indicates that normal interstitial fluid pressures are negative with respect to atmospheric pressure. As long as pressures remain at the sub-atmospheric level (in part due to lymphatic drainage), tissue compliance is low providing a buffering effect to protect against edema formation. However, as fluid
accumulates, pressures increase and at a critical ‘threshold’ pressure (around atmospheric pressure), compliance becomes very large and allows the accumulation of considerable volumes of water.

Presumably, each insult to the lymphatic-lymph node axis would consume some of this reserve and move the limb closer to the threshold where clinical edema may occur. In the context of cancer surgery, each step in the treatment process (tumor and node removal, as well as, chemo- and radiation therapy) could shift a patient closer to edema threshold. This concept along with possible genetic susceptibility may also help to explain why some patients develop edema while others do not, and help to explain the erratic onset time of edema which is anywhere from immediate, to years following treatment. Each patient undergoing treatment may start with a different level of available interstitial fluid buffering capacity (further away from or closer to the threshold pressure beyond which chronic edema develops). For those patients in whom the ‘buffering capacity’ is largely used up, it may only take a single insult to the system to trigger edema formation. On the other side of the ledger, the ability to form new lymphatics and generate lymphatic-venous anastomoses appears to compensate to some extent for the pathological events that facilitate edema formation.

7.8 Possible future studies

7.8.1 Radiation and lymphatic endothelial cells

A recent study revealed that the loss of LECs following irradiation (due to apoptosis) occurs in a dose dependant manner [85]. However the proliferative ability of the surviving endothelial cells has not been studied in any detail. In addition the phenotypic changes of LECs following irradiation also needs further exploration. Do they still possess key endothelial (CD31, VE-cadherin, ZO-1) and lymphatic markers (Prox-1, VEGFR3, podoplanin)? Preliminary data suggests that LECs downregulate VE-cadherin, and LYVE-1 while upregulating mesenchymal proteins n-cadherin and collagen type 1 [85]. These results suggest that LECs may undergo an endothelial to mesenchymal transition when exposed to radiation. This phenomenon would not only lead to a decrease in existing lymphatic vessels but also exacerbate the ongoing fibrosis in tissues subjected to radiation.
7.8.2 Facilitating lymphatic-venous anastomoses

Recently, studies have revealed the conditions that allow for the separation of the blood and lymphatic systems. Following development, all direct connections are lost between vascular and lymphatic system except for thoracic duct-subclavian vein connection (and corresponding right lymph duct where present). The exact physiological/molecular setting to form these connections is yet unknown although the involvement of the proteins Syk and Slp76 have been suggested. It is known that mice with homozygous mutations in either of these proteins develop abnormal lymphatico-venous connections [238]. Syk, a tyrosine kinase and SLP-76 (Lcp2), an adaptor protein are expressed almost exclusively in hematopoietic cells, suggesting that these cells contribute to the separation of the two vascular systems [238, 239]. It is also known that separation of the two systems is dependent upon platelet activation by podoplanin. During development, podoplanin activates C-type lectin receptor 2 (CLEC-2) in platelets [240]. This activation leads to the activation of Syk via SLP76 in the platelets.

Can one take advantage of our knowledge of the molecular basis of lymph-venous separation to reverse this process locally? Utilizing the rabbit hind leg model would offer an invaluable opportunity to study the physiological and molecular factors that facilitate the formation of these new junctions, which presumably work to enhance fluid clearance in response to the stress of downstream lymph flow obstruction.

7.8.3 Microfat grafting and its impact on lymphatics

Microfat grafting is routinely conducted in breast reconstruction and the reconstructive properties of adipose tissue have become apparent. The benefit of this treatment is potentially two-fold. First there is some evidence that the stem cells found in adipose tissue can reduce radiation damage [241, 242]. Secondly, injections of adipose tissue into scarred regions may allow local regenerating lymphatic vessels to penetrate into the densely compacted fibrotic regions left by surgery and radiation [241]. In addition it appears that stimulation of adipose stem cells with VEGF-C can induce factors (PROX-1 and podoplanin) that are associated with an increase in lymphangiogenesis [243]. This treatment type is still in the very early stages and further refinement of the techniques is needed.
Summary of Conclusions

- In most experimental lymphedema studies, investigators have induced chronic edema with tissue ablation approaches, an approach that does not mimic clinical realities. In this study, we approached the lymph node as a critical factor in lymphedema development and focused our thoughts about potential new therapeutic approaches at the node or its excision site.
- Lymph node excision or irradiation alone caused a significant lymphatic functional deficit.
- Lymphatic vessels demonstrated a remarkable ability to regenerate after injury through the process of lymphangiogenesis.
- When a lymph node was removed, the new vessel formation in some cases was able to restore lymphatic function to near pre-injury levels. However, the application of radiation to the node appeared to compromise function to a greater extent with lymph transport reduced to a fraction of that of controls. Contributing to the lymphatic functional deficit were tissue fibrosis, abnormal collagen formation in the lymph node and elevated resistance to flow. We also observed new lymphatic vessel growth in irradiated areas and the development of lymph-venous anastomoses upstream of injury to the node in a few animals.
- Radiation injury to a lymph node may be more relevant to lymphatic dysfunction than has been appreciated in the past. The data in this study demonstrate that radiation of a lymph node inhibits lymph transport significantly. The fact that the magnitude of functional suppression following lymph node excision plus irradiation is similar to irradiation alone, suggests that a major portion of the injury to the system was caused by the radiation itself. Surprisingly, there was a tendency for tissue water to decrease in the irradiated limbs. This was likely due to fibrosis in the affected tissues.
- Autologous lymph node transplantation has the potential to restore normal lymph fluid dynamics if this surgery is applied very early after nodal excision. Due in part to these results in our experimental model, this approach is being applied to breast cancer patients at Women’s College Hospital in Toronto.
• The use of pro-growth lymphangiogenic agents in a novel gel delivery system was effective at facilitating lymph transport after the removal of a node. With further optimization of this system, this approach could have direct clinical applicability.

• We are unaware of any other animal models beside the rabbit in which lymph-venous connections occur consistently. This model may be helpful in studying the cellular/molecular mechanisms that facilitate these connections.
References

18. Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N, Williams M, Dvorak AM, Dvorak HF, Oliver G *et al.: T1alpha/podoplanin deficiency disrupts...*


172. Stern R: Devising a pathway for hyaluronan catabolism: are we there yet? Glycobiology 2003, 13(12):105R-115R.


