Lymphatic Pathophysiology of Tumors

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SUBMITTED TO THE HARVARD-MIT DIVISION OF HEALTH SCIENCES AND TECHNOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN MEDICAL ENGINEERING
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
JUNE 2003

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Lymphatic Pathophysiology of Tumors

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Submitted to the Harvard/MIT Division of Health Sciences and Technology on May 23, 2003 in Partial Fulfillment of the Requirements for the degree of Doctor of Philosophy in Medical Engineering

ABSTRACT

Lymph node metastases have a negative impact on cancer survival, but the mechanisms for lymphatic metastasis are not well understood. The universal finding in solid tumors of an absence of functional lymphatic vessels seems paradoxical, as cancer cells do travel through lymphatics in order to disseminate. In order to address some of these issues, this thesis proposes two etiologies for the absence of functional lymphatic vessels in solid tumors. The first hypothesis addresses whether Vascular Endothelial Growth Factor-C (VEGF-C), a lymphangiogenic factor, was sufficient to induce lymphatic function in tumors. The overexpression of VEGF-C in tumors leads to an increase in lymph node metastasis as well as structures that positively stain for lymphatic markers, but does not induce functional lymphatics within the tumor. Thus VEGF-C is not sufficient to grow functional lymphatic vessels in tumors. The second hypothesis addresses whether mechanical forces generated by the proliferation of cancer cells in a confined space compress lymphatic vessels in tumors. The mechanical forces inside of the tumor were reduced by the selective killing of human cancer cells grown in mice by Diphtheria Toxin. Tumor cell death leads to an increase in the fraction of lymphatics with open lumen. In addition, lymphatic vessels with open lumen are surrounded by a lower cellular density than collapsed vessels. Thus, relieving solid stress allows lymphatic vessels to open. However, function was not restored in these vessels. This is presumably due to the inability of the lymphatic vessels to completely open along its entire length, leaving focal areas of lymphatic collapse. Compressive forces are common to all growing tumors, giving credence to the mechanical etiology of the absence of functional lymphatic vessels in tumors, regardless of tumor type or organ site. These findings lead to an interesting question: Does cancer treatment in humans relieve the mechanical compression allowing lymphatic and blood vessels to open? Furthermore, would the resumption of function of compressed blood and lymphatic vessels lead to a paradoxical increase in metastasis? These questions require further investigation.

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Intratumor Lymphatics.” *Science*, **296**: 1883-1886 (2002); Published online April 25, 2002; 10.1126/science.1071420.


**INVITED TALKS**


**ABSTRACTS**


Acknowledgements

I would like to start by thanking my thesis advisor, Professor Rakesh Jain. His guidance, advice and energy are inspirational. He encourages me and pushes me to reach my full potential. He has provided me with countless opportunities and given me the foundation to succeed in my career. His support for me has been boundless and I am deeply grateful.

I would like to thank the members of my thesis committee, Professors Robert Langer (MIT), Alan Grodzinsky (MIT), Annick Van den Abbeele (HMS), and Brian Seed (HMS). Their input, advice, and support have been invaluable. I would also like to thank the Harvard-MIT Division of Health Sciences and Technology and the MIT Chemical Engineering Department for opening many educational avenues and building solid and rigorous foundation for my career.

My colleagues and friends in the Steele Lab have always been generous with their time and talents, and have been patient with me as I learned many new things. In particular I would like to thank Sylvie Roberge, Emmanuelle di Tomaso, Edward Brown, and Ananth Kadambi from whose time and effort I benefited the most. I would also like to thank Julia Khan, Chelsea Swandal, Diane Capen, Russell Delgiacco, and Jessica Tooredman for their outstanding technical support and dedication to the projects. And Phyllis McNally for never being “bothered by me” and for all of her help throughout the years. I would like to thank Dai Fukumura, Lance Munn and Yves Boucher for their expertise, support, and input into my work. I am grateful to Peigen Huang and Alan Hartford for help with the animal work. I would also like to thank Melody Swartz, David Berk, Gerald Koenig, Saroja Ramanujan, Carla Mouta Carreira, Chae-Ok Yun and Nils Hansen-Algenstaedt for helping me as I started in lab.

I would especially like to thank Brian Stoll. We started at MIT together and are finishing up together, so we have shared our experience. Brian is truly a man of uncompromising ethic, loyalty, intelligence and generosity. I thank him as a collaborator, but mostly I thank him as a friend.

I would like to thank my outside collaborators, Peter So (MIT), Noah Choi (MGH), Eugene Mark (MGH), Douglas Matheson (MGH), and John Wain (MGH) for their time and effort. I would like to thank Kari Alitalo (Ludwig Institute) for VEGF-C constructs, David Jackson (Oxford University) for LYVE-1 antibodies, Stanislav Tomarev (NIH) for Prox-1 antibodies, and Isaiah Fidler (M.D. Anderson) for B16-F10 melanoma cells. I am grateful to the NIH, NSF, Whitaker Foundation, and the National Foundation for Cancer Research for funding my research.

Mostly I would like to thank my family and friends. Torunn, thanks for supporting me and helping me through this. You are the best and I love you! My parents, Bob and Eileen, give me every opportunity to succeed and support and love me fully and unconditionally. Their sacrifice allowed me to follow my dreams and I am truly grateful. You are my inspiration and I love you. My brother Bobby is always there for me and is great role model and friend. My sister Beth always keeps me in my place and is truly one of my best friends. And of course the people who shared the highs and lows with me, Ricker, Andrew, Kary, Ellen, Steph, Scuvry, Matt, Justin, Mark, Stephanie, Jason, Erin, Chase, Liz, Dave, Ann, Ed, and Doug.
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Chapter 1: Original Contribution
The primary goal of this thesis is to answer the question "Why is lymphatic function in a tumor impaired?" The answer to this question has long been sought, eluding scientists for about a century. The answer will drive our understanding of general tumor pathophysiology and may implicate a lymphatic etiology for deficiencies in tumor drug delivery and the poor immune response to cancer. Furthermore the spread of cancer to lymph nodes relies on the lymphatic system, bringing further urgency to lymphatic research. But how can a tumor that does not have functioning lymphatic vessels spread to lymph nodes via lymphatic vessels? This paradox is also addressed in this thesis.

One of the limiting factors in answering these questions is the lack of techniques to identify lymphatics, both functionally and molecularly. To be able to look into the depths of tumors and determine if functional lymphatics are present, an imaging technique with three-dimensional resolution at depth in tissue is needed. In this thesis such a technique, Multiphoton Laser Scanning Microscopy (MPLSM), is applied for the first time to image lymphatic vessels in living tissue (Chapter 4). A limit of MPLSM is its slow imaging speed, which can prevent the study of rapidly dynamic systems. *In vivo* video-rate MPLSM is also demonstrated for the first time in this thesis.

With the development of these novel imaging techniques, and the subsequent discovery of putative lymphatic markers and growth factors by other researchers, the following question is posed. Can the overproduction of a lymphatic growth factor induce lymphatic function within tumors (Chapter 5)? By the genetic alteration of tumor cell lines to overproduce a lymphatic growth factor, a higher incidence of lymphatic metastasis is demonstrated. This suggests that these tumors might have functional lymphatic vessels inside. Surprisingly, lymphatic function is not demonstrated inside these tumors by four different functional assays. Based on the increases in size of lymphatic vessels in the tumor margin of tumors overproducing a lymphatic growth factor, it is concluded that lymphatic metastasis occurs through functional lymphatics in the tumor margin, even in the absence of functional lymphatic vessels inside the tumor (defined as greater than 100 \( \mu m \) from the tumor edge). These results are obtained by the first combination of high-resolution functional lymphatic assays with high-resolution molecular lymphatic identification.
But are these experiments in mice relevant to cancer patients? A group of lung cancer patients, who had surgical resections of their tumors, is studied to answer this question (Chapter 5). A functional lymphatic parameter is measured in tumors in situ and molecular analysis performed on their excised tumors. Confirming the results from the animal studies, the human tumors show no evidence of functional lymphatics. Surprisingly, the lung tumors do not even have a molecular trace of lymphatics inside, even though nearly half of the patients have lymph node metastasis. This further confirms that cancer cells invade functional lymphatics in the tumor margin in order to metastasize to lymph nodes. Another surprising discovery is that the small blood vessels of the lung also expressed a marker of lymphatic vessels.

Since there is no molecular explanation for the absence of functional lymphatics in tumors, a mechanical hypothesis is proposed (Chapter 6). Do the mechanical forces that build-up in tumors due to the growth of a tumor in a confined space cause the collapse of lymphatic vessels and render them non-functional? By specifically killing tumor cells, the fraction of lymphatic vessels that are open, as opposed to compressed, increased. However, lymphatic function is not resumed in tumors, probably due to compression of lymphatic vessels in some parts of the tumor that have not undergone significant cell death. The evidence gathered however does support the hypothesis that mechanical forces collapse lymphatic vessels in tumors rendering them non-functional. Based on this finding, one may predict a paradoxical increase in both drug delivery and metastasis when cancer therapy kills tumor cells and reduces the compressive forces.

This thesis enhances the ability to perform lymphatic research by combining functional lymphatic studies with both state-of-the-art imaging techniques and high-resolution molecular identification. These techniques allow many more discoveries to be made involving the lymphatic system and cancer. This thesis also resolves two unanswered questions in the field. First, lymph node metastases occur from the invasion of functional lymphatic vessels in the tumor margin, even in the absence of functional lymphatic vessels inside of the tumor. This poses an opportunity to use the intact lymphatic vessels draining the tumor to deliver drugs locally to lymph nodes with metastatic disease.
Second, mechanical forces generated by the growing tumor in a confined space collapse lymphatic vessels, explaining the lack of functional lymphatics in tumors. When cancer therapy kills tumor cells and reduces the compressive forces, a paradoxical increase in both drug delivery and metastasis may occur. There remain many unanswered questions associated with lymphatics and cancer that will continue to be investigated. The continued progress in this field will impact the clinical treatment of cancer in the coming years.
Chapter 2: Introduction and Thesis Aims

Lymphatic Pathophysiology of Tumors

Timothy P. Padera

Portions of this chapter have been taken from:

Since 1938, the United States government has invested over $45 billion (1) dollars in cancer research in the hope of finding an effective treatment for the second leading cause of death in the U.S. The American Cancer Society predicts about 556,500 Americans (about 215 per 100,000) will die of cancer this year and 1,334,100 (about 500 per 100,000) new cases of invasive cancer will be diagnosed, not including skin cancer, which would account for about 1 million more cancer cases. The nation’s investment in cancer research has led to significant improvements in cancer treatment (reducing morbidity) and to discoveries that have had clinical impact in many other areas of medicine. However, the cancer associated mortality rate has remained virtually the same over the years (ignoring cases associated with tobacco abuse)(2)(Figure 2.1). The impetus for our nation’s investment in cancer research is the fact that approximately one-fourth of all deaths in the U.S. are due to cancer, and half of the patients diagnosed with solid tumors die from their disease. Metastases, the dissemination and growth of the primary tumor in distant sites throughout the body, are usually the cause of death. Treatment thus requires systemic approaches, which currently have high co-morbidity.
Age-Adjusted U.S. Cancer Death Rates by Site, 1930-1996

Males

Females

Figure 2.1: U.S. cancer mortality rate from 1930-1996. Figure courtesy of the American Cancer Society. *Per 100,000, age-adjusted to the 1970 U.S. standard population.

Recently, it is has become clear that the local tumor environment, pathophysiology and biology are critical components in determining the success of cancer treatments that years of research have promoted (3-6). Pharmacological treatments all require the therapeutic agent (i.e. chemotherapy, gene therapy, immunotherapy, or other novel molecular medicine) to: i) localize in the tumor in quantities large enough to promote anti-tumor activity without causing systemic tissue toxicity; ii) remain active in the tumor microenvironment for optimal duration of time; and iii) interact with tumor cells in the intended way. Focusing on item i), the local accumulation of therapeutic agents in
tumors, with the exception of a few novel local treatments (7-9), depends on vascular perfusion, which brings systemically delivered drugs to the tumor mass. Molecular transport then brings the agent from the vasculature or controlled release device to the tumor cells where it acts. Both of these steps are required for a systemically administered drug to distribute throughout the tumor and both are compromised in tumors. Tumor vasculature, the proposed target for promising anti-angiogenic cancer therapies (10), is heterogeneous and furthermore, so are its blood flow characteristics and permeability (11, 12). These properties of tumor blood vessels lead to regions of nutrient deprivation and necrosis, and also inhibit homogenous drug delivery, with regions of tumor not sufficiently accumulating systemically administered anti-tumor agents. In addition, tumor endothelial cells are activated and highly express survival and angiogenic factors, making them resistant to apoptosis (13), making anti-vascular therapy more difficult.

Molecular transport within the tumor interstitium, the second step in tumor drug delivery, is also inhibited due to the severe reduction in fluid convection, a key in vivo transport mechanism for macromolecules. Molecular transport in tumors thus relies upon diffusion, which, for large molecules, is relatively slow compared to convection. The lack of fluid convection through tumor tissue is a result of elevated and spatially uniform interstitial fluid pressure (IFP) (14-17). Elevated IFP can be traced to two main pathologies in tumors. The first is the overall blood vessel hyperpermeability that results in excess fluid exudate leaving the vascular space and entering the tumor interstitial space. The second is the lack of a functional lymphatic network, preventing effective removal of excess fluid from tumor tissue (18, 19). This thesis probes the etiology of the lack of lymphatic function in tumors (Chapters 5 and 6).
The lack of functional lymphatics in tumors leads to an interesting paradox. Cancer cells are known to leave tumors through lymphatic vessels and form metastases in regional lymph nodes. So how can tumors spread to lymph nodes through lymphatic vessels if tumors lack functional intratumor lymphatic vessels? This thesis aims to resolve this paradox (Chapter 5).

**Specific Aims**

Elevated interstitial fluid pressure is a major barrier to the delivery of macromolecular therapy to solid tumors. One key cause of elevated IFP is the dysfunction of the lymphatic system in tumors. The pathophysiology of this dysfunction and the implications for lymph node metastasis have not been extensively investigated.

**Hypothesis:** Tumors lack a functional lymphatic network due to insufficient production of lymphatic growth factors and pathologic mechanical properties of tumors.

**Specific Aim 1:** Vascular Endothelial Growth Factor–C (VEGF-C) is produced in many different tumors, is correlated to lymph node metastasis in patients, and is known to be trophic for lymphatics. **Hypothesis:** VEGF-C can induce functional intratumor lymphatics, alter tumor margin lymphatic vessels, and increase the rate of lymph node metastasis from solid tumors (Chapter 5).
**SPECIFIC AIM 1A:** *Quantify differences in the number of functional intratumor lymphatic vessels in VEGF-C overexpressing and mock-transfected tumor lines.*

VEGF-C overexpressing and mock-transfected (MT) tumor lines were grown in the ear and tail of nude mice and three different functional lymphatic assays were performed. Interstitial fluid pressure (IFP) was measured using the wick-in-needle technique. It was predicted that the growth of intratumor functional lymphatic vessels will decrease intratumor IFP. Fluorescent intratumor lymphangiography was preformed and imaged intravitaly and non-invasively using multi-photon laser scanning microscopy (MPLSM). Ferritin intratumor lymphangiography with immunohistochemistry (IHC) and Prussian Blue staining was preformed to screen the entire depth of the tumor for functional lymphatics.

**SPECIFIC AIM 1B:** *Quantify differences in tumor margin lymphatic architecture with and without VEGF-C overexpression in tumors.*

VEGF-C overexpressing and MT tumor lines were grown in the ear and tail of nude mice. Tumor margin lymphatic architecture was monitored for VEGF-C induced differences using peri-tumor microlymphangiography and intravital microscopy.

**SPECIFIC AIM 1C:** *Quantify the effect of VEGF-C on the rates of lymphatic metastasis.*

VEGF-C overexpressing and MT tumor lines were grown in the hindlimb of the mouse. When the primary tumor reached a specific size, it was resected. Animals were sacrificed at a specified time after primary tumor resection and autopsies performed. The amount and pattern of metastasis were quantified.
**SPECIFIC AIM 2:** Solid stress in tumors is produced by proliferating tumor cells in a confined space. **Hypothesis:** *Solid stress induced by proliferating neoplastic cells causes the collapse of lymphatic vessels in tumors, rendering them non-functional* (Chapter 6).

**SPECIFIC AIM 2A: Quantify differences in the number of functional lymphatics in tumors before and after a reduction in solid stress caused by tumor cell specific apoptosis.**

Tumor cells of human origin were treated with Diphtheria Toxin (DT), which selectively killed tumor cells. Functional intratumor lymphatic vessels were quantified using fluorescent intratumor lymphangiography with MPLSM and ferritin intratumor lymphangiography with IHC and Prussian Blue staining.

**SPECIFIC AIM 2B: Quantify differences in the number and architecture of functional lymphatics in the tumor margin before and after a reduction in solid stress caused by tumor cell specific apoptosis**

Tumor cells of human origin were treated with Diphtheria Toxin (DT), which selectively killed tumor cells. Functional tumor margin lymphatic vessels were analyzed using fluorescent peri-tumor lymphangiography with MPLSM.

**SPECIFIC AIM 2C: Quantify differences in the morphology of structures stained with a lymphatic marker as a function of local cell density.**

Tumor cells of human origin were treated with Diphtheria Toxin (DT), which selectively killed tumor cells. Tumors were removed at different times after treatment and stained by IHC with LYVE-1. Using image analysis, the number of LYVE-1 positive structures
with open lumen and their morphology were measured. Cell density was counted around LYVE-1 positive structures.

**Background**

**The role of lymphatics**

In normal physiology, the lymphatic system maintains interstitial homeostasis by removing fluid and proteins from the interstitium and returning them to the blood. This allows the interstitium to remain at constant IFP and oncotic pressure. The lymphatic system also carries white blood cells and other antigen presenting cells (APC’s) from the tissue to lymph nodes where surveillance for foreign antigen can take place. Lymph node architecture is critical for communication between antigen specific lymphocytes and APC’s, allowing identification of foreign antigen and initiation of an appropriate response. Thus, lymphatics are important in maintaining both the tissue fluid homeostasis and proper function of the immune system (20-23).

Fluid is removed from the interstitium either via resorption by venous capillaries or by collection in lymphatics. The balance of venous resorption and lymph formation depends on Starling forces and lymphatic pump activity. Starling’s law describes fluid flux through a semi-permeable barrier based on hydrostatic and oncotic driving forces and is written as follows:

\[
J_v = K_p \left[ (P_i - P_o) - \sigma (\pi_i - \pi_o) \right]
\]

(2.1)

where: \( J_v \) = fluid flux, \( K_p \) = hydraulic conductivity, \( P_i \) = hydrostatic pressure inside the lumen, \( P_o \) = hydrostatic pressure outside the lumen, \( \sigma \) = oncotic reflection coefficient, \( \pi_i \)
= oncotic pressure inside the lumen, and \( \pi_o \) = oncotic pressure outside the lumen. Starling’s law applies to venous resorption at all times, but lymphatic formation is regulated by the presence of lymphatic endothelial microvalves (LEM) (24) and Starling’s law can only be applied when the LEM’s are open. As lymphatic pump activity increases, so does the amount of time LEM’s are open and thus more fluid is taken into lymphatics, reducing the relative amount of venous resorption (20, 21).

Lymph formation requires lymphatic dilation because lymphatic fluid pressure (LFP) is normally higher on average than IFP. When LFP exceeds IFP, LEMs are closed, preventing the flow of lymph back into the interstitium (Figure 2.2). When lymphatics dilate, two phenomena occur which allow for the formation of lymph. Structurally, dilation pulls open LEM’s. LEM’s are formed by overlapping lymphatic endothelial cells (LEC) that are tethered to the extracellular matrix by anchoring filaments and have an unattached flap that constitutes the intracellular junction (Figure 2.2). As the ECM expands due to excess fluid, adjacent pulsatile blood vessels, adjacent muscle contraction, or some other reason, the anchoring filaments pull LEC’s apart, increasing intracellular channels. These channels are non-selective, resulting in an oncotic reflection coefficient of zero in Starling’s Law (20, 21, 24).

The second phenomenon that occurs during lymphatic dilation is the increase in lumen size of the lymphatic vessel. This reduces LFP below the level of IFP, allowing Starling forces to push the unattached flap of the LEM away from the attached adjacent cell (Figure 2.2). The LEM is now open and fluid can move through the LEM into the lymphatic lumen, forming lymph. As fluid moves into lymphatics, LFP equalizes with
IFP. When the lymphatic vessel recoils to its original state, LFP increases above IFP and
the LEMs close, preventing backflow of lymph into the interstitium. The presence of
LEM allows the LFP to be greater than IFP on average, but stills permits the formation
of lymph (20, 21, 24).

Besides maintaining tissue homeostasis, the lymphatic system plays an important role in
immune surveillance. Lymph nodes are the primary sites of naïve lymphocyte activation
and subsequent differentiation into effector cells. This activation depends upon the
interaction of B-cells, T-cells and APC’s. The lymphatic system allows APC’s to travel
from tissue to lymph nodes and elicit an immune response if they present foreign antigen.
The lymphatic system also permits circulating lymphocytes to travel from the blood to
tissue, through lymph nodes and then back into the blood. This ability of lymphocytes to
recirculate from tissue to lymph nodes is crucial in finding and combating infectious
pathogens. Thus, a functional lymphatic system is required to maintain resistance to
infectious disease and remove cancerous cells (22, 23).
Figure 2.2: Illustration of Lymphatic Endothelial Microvalve (LEM). A) When IFP<\text{LFP}, the valve is pushed closed. B) When the IFP>\text{LFP}, the valve in the endothelial wall is pushed open and lymph is formed.
Role of IFP in drug transport

It is well documented that the delivery of therapeutic agents is severely compromised in solid tumors (5, 25). One reason for the reduced delivery is the elevated IFP found in tumors. The primary mode of molecular transport in normal tissue is convection, driven by pressure gradients in the tissue. Elevated IFP in tumors reduces the gradient between the vascular space and interstitium, thus reducing convective flow from blood into the tumor. Furthermore, the IFP gradient throughout the tumor mass is near zero (14, 16), severely limiting intratumor convection. This lack of convection leaves diffusion as the primary transport mechanism. The extremely slow process of diffusion for large molecules significantly limits the effectiveness of many cancer therapies.

Fluorescence recovery after photobleaching (FRAP) measures effective diffusion coefficients by bleaching a small spot in an otherwise uniformly fluorescent media. FRAP can be used to measure effective diffusion coefficients in both in vitro gels and in living tissue in vivo (26). The effective diffusion coefficient in tumor tissue is inversely related to the molecular hydrodynamic radius and the amount of collagen (27-29), with collagen content being tumor site dependent (28). The diffusion of liposomes (D=90-150 nm) in high collagen tumors is undetectable using photobleaching techniques. Thus, the slow diffusion-mediated transport prevents effective delivery of liposome-encapsulated agents to many tumors, and severely reduces the ability of conventional chemotherapeutics bound to plasma proteins to distribute uniformly in the tumor. FRAP can also be used to calculate the binding affinity and receptor distribution in vivo (30). FRAP is thus able to provide powerful information about drug delivery to tissue in
general and to tumors in particular, due to the high resolution of the technique and the small-scale heterogeneity of tumors. Using FRAP technology, many of the barriers to drug delivery have been characterized and continue to be studied.

In normal tissue, fluid leaks out of blood vessels and into the interstitial space where it bathes the cells. Lymphatics collect the interstitial fluid and return it to the circulation through lymph nodes in the neck. The lymphatics' ability to drain tissues prevents fluid accumulation (edema), thus maintaining a low IFP in normal tissues. In tumors the elevated IFP results from the increased permeability of the blood vessels and also from the absence of functional lymphatics, leading to accumulation of interstitial fluid.

**Lymphatic development**

Our body has two distinct vascular networks made of blood vessels and lymphatic vessels. These two networks separate in embryonic development from their common origin to provide complementary, yet distinct roles in maintaining normal tissue function. Sabin (31) showed that lymphatic originate from “empty sacs” that arise from veins and are preceded by a plexus of veins. These sacs are found near the jugular vein, the sciatic vein, the cisterna chyli, and the mesenteric vein. Lymphatic vessels sprout from these sacs to form the lymphatic system. Mesodermally derived lymphangioblasts may also contribute to the development of the lymphatic system (32), but embryonic lymphatic vessels primarily originate from blood vessels (Figure 2.3) (31, 33-38). In the early embryo, endothelial cells of the cardinal vein express LYVE-1 and VEGFR-3, molecules observed primarily on lymphatic vessels in normal adult tissues (*step 1*). A yet unknown signal triggers the expression of the homeobox gene *Prox* 1 in a polarized fashion in the
endothelial cells of the cardinal vein. This marks the first stage of commitment to the lymphatic lineage (step 2). These LYVE-1/VEGFR-3/Prox 1 positive cells then start to bud, again in a polarized fashion (step 3). At this stage these early lymphatic endothelial cells start expressing SLC and increased levels of VEGFR-3 (step 4), markers of mature lymphatic endothelial cells, and start forming the lymphatic system (step 5).
Figure 2.3. Lymphatic development (38). Molecular events in the development of the lymphatic system are becoming defined. The molecular players known to orchestrate lymphatic processes include: members of the VEGF (Vascular Endothelial Growth Factor) family and their cognate receptors (VEGF-A, -C, -D, VEGFR-2, VEGFR-3), members of the angiopoietin family and their receptor (Ang-1, Ang-2, Tie-2), neuropilin 2 (a co-receptor for VEGFR-3), Prox 1 (a homeobox protein), LYVE-1 (Lymphatic Vessel Endothelial HA receptor), SLC (secondary lymphoid chemokine, CCL21), FOXC2 (forkhead/winged helix C2), α9 integrin, SLP-76 (SH2 domain containing leukocyte protein with molecular mass of 76 kDa) and Syk (spleen tyrosine kinase)(36, 37, 39, 40). SLP-76 is an adapter protein that acts as a substrate for the tyrosine kinases Syk and ZAP-70 (41).

Members of the angiopoietin family (Ang 2) and its receptor (Tie 2) are presumably involved in the maturation and patterning of these nascent vessels as Ang 2 null mice have a poorly functional and abnormally patterned lymphatic system (39). Neuropilin-2 (NRP-2), a potential VEGFR-3 co-receptor, also plays a role in early lymphatic development (42). NRP-2 deficient animals have fewer lymphatic capillaries at birth, but in older animals the capillaries eventually form with some patterning abnormalities. In slp-76 and syk deficient mice (40), lymphatic development appears to progress normally through at least step 2, after which the development of the lymphatic vascular system becomes aberrant and connects with blood vessels. SLP-76 and Syk are not found on LECs and the vascular malformation phenotype can be transferred to wild-type animals through SLP-76 or Syk deficient bone-marrow derived cells (40). This could be mediated through deficiencies in secreted molecules necessary for lymphangiogenesis.
from bone marrow derived cells (38) (Figure 2.3, step 5). Another possibility is that the bone-marrow derived cells include lymphatic endothelial progenitor cells (LEPC) (Figure 2.3, step 5) (43). LEPC are circulating cells that can differentiate into endothelial cells expressing lymphatic markers in vitro. These cells may play a role in lymphatic development as well as in post-natal lymphangiogenesis in physiological and pathological conditions. Signaling through SLP-76/Syk pathway could trigger the early differentiation of a multipotent stem cell into a more specified LEPC. Since slp-76 or syk expression could not be detected in wild-type endothelial cells, the expression of these molecules could be down regulated before LEPC incorporation in the developing lymphatic vessel (38). Understanding the origin and biology of LEPCs may provide further insight into the mechanisms of lymphangiogenesis and lymphatic disease.

It is becoming clear that multiple molecules are necessary to ensure proper differentiation and patterning of lymphatic endothelial cells into a functional lymphatic system (Figure 2.3). By expanding the roster of molecules, many more targets for therapeutic intervention will emerge beyond VEGFR-3 and its ligands, bringing more hope for treatments of lymphedema. Furthermore, by understanding molecular lymphangiogenesis, insight into the dysregulation of this process in solid tumors may emerge.

**Role of VEGF-C and VEGFR-3**

VEGF-C (Vascular Endothelial Growth Factor-C) is a member of the VEGF family of growth factors that consists of six members: VEGF (also known as Vascular Permeability
Factor-VPF) (44-46), VEGF-B (47), VEGF-C (48, 49), VEGF-D (50), VEGF-E (Orf virus VEGF) (51, 52), and PlGF (53). The family also contains three tyrosine kinase receptors: VEGFR-1 (54), VEGFR-2, and VEGFR-3 (55). Additionally, members of the VEGF family bind to the neuropilin receptors, NRP-1 and NRP-2, which do not have kinase activity (56). Each member of the VEGF family has a characteristic VEGF homology domain consisting of eight cysteine residues that are conserved across family members (45). VEGF binds to receptors VEGFR-1 and VEGFR-2 and is a potent angiogenic and permeability-inducing factor. VEGF has a differential tissue specific effect on non-endothelial cells (46). VEGF is also the target in many anti-angiogenic therapeutic strategies (57, 58). VEGF-B and PlGF bind only to VEGFR-1 and can also form heterodimers with VEGF. VEGF-C and VEGF-D bind to both VEGFR-2 and VEGFR-3, with a higher binding affinity to VEGFR-3. VEGF-E appears to bind to VEGFR-2 only. All three VEGF receptors are mainly expressed on endothelial cells. They all have an extracellular domain with seven immunoglobulin homology domains and an intracellular domain with a tyrosine kinase domain that is responsible for signaling (55). VEGFR-3, however, is slightly different, as its extracellular domain is cleaved and the two peptides are held together by a disulfide bond (59). NRP-1 binds VEGF-165, VEGF-B, VEGF-E and PlGF, and acts as a co-receptor to increase the activity of VEGFR-2 signaling (56). NRP-2 binds VEGF-145, VEGF-165, VEGF-C and PlGF, and is thought to act as a co-receptor to increase the activity of VEGFR-3 signaling (42).

VEGF-C undergoes significant post-translational processing. Two full pro-VEGF-C peptides will orient in an anti-parallel fashion after the signal peptide is cleaved, leaving
the pro-peptide in the secretory pathway. Disulfide and non-covalent bonds stabilize the dimer. Each peptide in the dimer is then cleaved into a 29 and 31 kDa peptide between Arg227 and Ser228, making a tetramer complex. The N-terminal 31 kDa peptide contains the VEGF homology domain and is non-covalently bonded to the 31-kDa piece from the anti-parallel pro-peptide. The cysteine-rich C-terminal 29 kDa piece contains BR3 motifs and is disulfide bonded to the 31-kDa piece of the anti-parallel pro-peptide. The molecule is then excreted as a tetramer. Extracellularly the N-terminal part of the 31-kDa piece is cleaved, removing the S-S binding site so only a dimer of 21 kDa molecules containing the VEGF homology domain remains. This dimer is the mature VEGF-C molecule, which is held together by non-covalent bonds. VEGF-C can cause the phosphorylation of both the VEGFR-2 and VEGFR-3 tyrosine kinase domain, but there is a larger effect on VEGFR-3 than VEGFR-2 with equal levels of fully processed VEGF-C (59). With forms of VEGF-C that are less mature, the affinity for VEGFR-2 is increased (59). By switching the cysteine at position 156 in the 21 kDa form of VEGF-C with a serine makes a VEGFR-3 specific agonist, without any VEGFR-2 activity (60). It is not known whether VEGF-C processing alters NRP-2 binding affinity (42).

VEGF-C and VEGFR-3 mRNA is expressed in mouse day 8.5 and 12.5 embryos, specifically near the dorsal aorta and cardinal vein, and in the jugular region (33). It is in these same regions that Sabin described the embryonic development of lymphatics from sacs of venous endothelial cells (31). In addition, mRNA for both VEGF-C and VEGFR-3 are found in the embryonic mesentery, which is rich in lymphatic vessels in an adult animal (33). In the region of the thoracic duct and the cervical, submandibular, and axillary lymph nodes, VEGFR-3 mRNA is also found in day 14.5 and 16.5 embryos,
while in day 8.5 to 11.5 embryos these structures also have Tie-1 receptor mRNA, a marker of vascular endothelium (61). However in adult lung, mesentery, and appendix (61) and in fetal skin (62), only VEGFR-3 mRNA is found on vessels thought to be lymphatics. From these data it has been suggested that VEGFR-3 and VEGF-C play a role in lymphatic development, and that VEGFR-3 becomes restricted to the lymphatic endothelium, which loses markers of vascular endothelium (33, 61).

In normal physiology, VEGF-C causes dermal lymphatic hyperplasia without affecting blood vessels in transgenic mice overexpressing VEGF-C in their skin (63), but the lack of angiogenic effect is likely due to production of the fully processed form of VEGF-C (59). VEGF-C induces angiogenesis in the mouse cornea, the chick chorioallantoic membrane (64), and in ischemic rabbit hindlimbs (65). In addition, VEGF-C increases blood flow in mice (65) and vascular permeability (by Miles Assay) in mice (59), in guinea pigs (65), and in transplanted tumors in mice (66). The affects of VEGF-C on blood vessels are mediated through VEGFR-2 signaling (67).

**VEGF-C and VEGF-D in cancer**

VEGF-C is expressed in a variety of tumors, including human gastric tumors (68), breast tumors (69), colon tumors (70), Kaposi’s Sarcoma (71, 72), acute myeloid leukemia (73), and other human tumors (74). In addition, VEGFR-3 is found in a variety of tumors (62, 69, 70, 72, 75), suggesting that VEGF-C and VEGFR-3 may play an important role in the clinical course of cancer patients. The expression of VEGF-C and VEGF-D are correlated to lymph node metastasis and poor outcomes in a variety of human cancers (Table 2.1)(76).
<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Method</th>
<th>Significant Association</th>
<th>VEGF subtype</th>
<th>Endpoint</th>
<th>Reference</th>
</tr>
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<tr>
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<td>C</td>
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<td>(77)</td>
</tr>
<tr>
<td></td>
<td>IHC</td>
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<td>C</td>
<td>LNM</td>
<td>(78)</td>
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<td>Yes</td>
<td>C</td>
<td>Increased LI</td>
<td>(79)</td>
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<tr>
<td></td>
<td>IHC</td>
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<td>C</td>
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<td>(80)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Increased pelvic LNM and deep SI</td>
<td>(81)</td>
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<tr>
<td></td>
<td>IHC</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM and invasion depth</td>
<td>(82)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Decreased OS</td>
<td></td>
</tr>
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<td>Cervical</td>
<td>RT-PCR</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM, LI and depth of SI</td>
<td>(83)</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td>LNM, LI</td>
<td>(84)</td>
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<td></td>
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<td>LNM, LI, BVI, stage and liver metastasis</td>
<td>(85)</td>
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<td>IHC</td>
<td>Yes</td>
<td>C</td>
<td>LI, OS</td>
<td>(86)</td>
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<td></td>
<td>qPCR</td>
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<td>C</td>
<td>LNM, LI</td>
<td>(87)</td>
</tr>
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<td>Esophageal (SCC)</td>
<td>RT-PCR</td>
<td>Yes</td>
<td>C</td>
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<td>(88)</td>
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<td>Gallbladder</td>
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<td>C</td>
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<td>(89)</td>
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<td></td>
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<td>(90)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Decreased OS</td>
<td></td>
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<td>C</td>
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<td>(68)</td>
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<tr>
<td></td>
<td>IHC</td>
<td>Yes</td>
<td>C</td>
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<td>Head and neck (SCC)</td>
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<td></td>
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<td>(93)</td>
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<td>Laryngeal</td>
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<td>(94)</td>
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<td></td>
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<td>(95)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td>RT-PCR</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM</td>
<td>(96)</td>
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<tr>
<td>Tumor Type</td>
<td>Method</td>
<td>Positive</td>
<td>C and D</td>
<td>Combination high C &amp; low D: increased LNM and LI (NS for either alone)</td>
<td>Notes</td>
</tr>
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<td>--------</td>
<td>----------</td>
<td>---------</td>
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<td>------------------------------------------</td>
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<td>Lung NSC (stage I)</td>
<td>IHC</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM (associated with lower 3 &amp; 5 year OS)</td>
<td>(98)</td>
</tr>
<tr>
<td>Lung NSC</td>
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<td>Yes</td>
<td>C</td>
<td>Increased LNM and LI; decreases OS after surgery</td>
<td>(99)</td>
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<td>Lung NSC Neuroblastoma</td>
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<td>C</td>
<td>Decreased OS</td>
<td>(100)</td>
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<tr>
<td>Oral</td>
<td>IHC</td>
<td>Yes</td>
<td>C</td>
<td>Increased tumor stage</td>
<td>(101)</td>
</tr>
<tr>
<td>Oral (SCC)</td>
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<td>Yes</td>
<td>C</td>
<td>Increased LNM</td>
<td>(102)</td>
</tr>
<tr>
<td>Ovarian</td>
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<td>Yes</td>
<td>C, D</td>
<td>Increased LNM</td>
<td>(103)</td>
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<td>Pancreatic</td>
<td>IHC</td>
<td>Yes</td>
<td>C</td>
<td>Decreased OS</td>
<td>(104)</td>
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<td>Pleural mesothelioma</td>
<td>RT-PCR</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM</td>
<td>(105)</td>
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<tr>
<td>Prostate</td>
<td>RT-PCR</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM</td>
<td>(106)</td>
</tr>
<tr>
<td>Renal</td>
<td>qPCR</td>
<td>No</td>
<td>C</td>
<td>Expression in cancer</td>
<td>(107)</td>
</tr>
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<td>Testicular GCT (stage I)</td>
<td>RT-PCR</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM</td>
<td>(108)</td>
</tr>
<tr>
<td>Thyroid</td>
<td>PCR</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM</td>
<td>(109)</td>
</tr>
<tr>
<td>Uterine endometrial (PM)</td>
<td>IHC</td>
<td>Yes</td>
<td>C</td>
<td>Increased LNM; Decreased 5 and 10 year DFS (independent of LNM)</td>
<td>(110)</td>
</tr>
</tbody>
</table>

a SCC = squamous cell carcinoma, AC = adenocarcinoma, NSC = non-small cell, GCT = germ cell tumor, PM = post-menopausal

b IHC = immunohistochemistry, ISH = in situ hybridization, PCR = polymerase chain reaction, RT-PCR = reverse transcriptase/polymerase chain reaction, qPCR = quantitative or real-time PCR

c Statistical significance was determined between VEGF subtype expression and clinicopathological endpoint
d LNM = lymph node metastasis, IBC = inflammatory breast cancer, LI = lymphatic invasion, DFS = disease free survival, SI = stromal invasion, BVI = blood vessel invasion, OS = overall survival, NS = not significant, LVD = lymphatic vessel density

Tumors grown in the tail of mice are associated with lymphatic dilation in the peri-tumor region (113). In addition, when tumors overexpressing VEGF-C are grown in the tail, peri-tumor lymphatics are even larger than control (mock transfected) tumors (114) (Chapter 5). Looking at the interstitial pressure profile of a tumor, a large pressure drop can be seen at the periphery (16), indicating that fluid is flowing out of the tumor. Some of this fluid is collected by tumor margin lymphatics, and carried away. Since a variety of tumors produce VEGF-C, the intratumor fluid could have an elevated VEGF-C level, which is then drained by the tumor margin lymphatics. Since VEGF-C has a hyperplastic effects on lymphatics (63, 115), this could explain the lymphatic dilation seen by Leu et al. (113), a situation that is then amplified when VEGF-C is overexpressed in the tumor (115).

In addition to causing hyperplasia of tumor margin lymphatics, VEGF-C and VEGF-D overexpression increases lymph node metastasis in a variety of animal models. In mammary carcinomas grown in mice, VEGF-C overexpression correlates with an increase in lymphatic vessel density (identified by LYVE-1, see below) and lymph node metastasis (116, 117). Furthermore, VEGF-C overexpression in one study correlates with lung metastasis, which may suggest that VEGF-C may increase the malignancy of tumor cells independent of lymphatic vessels (116). Studies using VEGF-C overexpressing melanoma show similar results (118), even though studies using human specimens show
no lymphangiogenesis in dermal and uveal melanoma (119, 120). In a model of spontaneously arising pancreatic tumors, VEGF-C overexpression increases lymph node metastasis, even though no lymphatic vessels are found in the primary tumors (121). In the tumor margin, however, VEGF-C overexpression increases the number and size of lymphatic vessels. In an epithelioid tumor overexpressing VEGF-D, lymph node metastasis and lymphatic vessel density in the tumor are increased, which could be normalized by administering anti-VEGF-D antibodies (122). Tumors that overexpress insulin-like growth factor receptor (IGF-IR) increase the production of VEGF-C through IGF-IR signaling (123). These IGF-IR overexpressing tumors also become more metastatic. Molecules that breakdown extracellular matrix, such as matrix metalloproteinases (124), or increase cancer cell invasiveness, such as lysyl oxidase-related protein-1 (125), are also correlated to metastasis. Recently, attention has been given to the potential role that chemokines that direct the normal trafficking of lymphocytes may play in lymphatic metastasis. Data shows a correlation between CCR7, CXCR4 and lymph node metastasis in human tumors (126), suggesting that in some instances tumor cells may usurp mechanisms of normal lymphocytes to home to lymph nodes in order to metastasize.

**Lymphangiogenesis**

The first quantitative studies involving lymphangiogenesis were done by Clark and Clark using the rabbit ear chamber (127). In their model, lymphatics regrow into the wound after about 19 days, and are always preceded by blood vessels. Blood vessels are thought to help lead the lymphatics into the healing wound. Immunohistochemical staining for VEGFR-3 in the healing skin wounds of pigs shows vessels that are VEGFR-3 positive
and negative for vascular markers in the wound from days 5-9 after wounding. These vessels attach to similar vessels on the outside of the wounds, which are thought to be lymphatics (128). Cytokines released in inflammation are known to upregulate VEGF-C production, which may mediate this lymphangiogenesis associated with wound healing (129).

Recently, models have been developed to study mechanisms of lymphangiogenesis and its induction. In mice with a mutation in the VEGFR-3 receptor, VEGF-C gene therapy can induce lymphangiogenesis (130). Conversely, transgenic mice that produce soluble VEGFR-3 receptors do not form proper lymphatic vessels and develop lymphedema (131). These animal models also show that signaling through VEGFR-3 is sufficient to trigger lymphangiogenesis (132). In rabbits, wounds created at the base of the ear allow lymphedema to develop. Lymphangiogenesis is induced by VEGF-C gene therapy (133) or direct VEGF-C administration (134), which both lead to a subsequent reduction in auricular lymphedema. Wounds created in the mouse tail also induce lymphedema and can be relieved by a surgical skin flap (135). By allowing a tail wound to heal, lymphangiogenesis can be observed by fluorescent lymphangiography (136). The initial lymphatic network is guided by interstitial fluid flow which later remodels into the normal skin lymphatic network. Interstitial fluid flow may be the initial trigger to initiate cell migration and VEGF-C production leading to lymphangiogenesis (136, 137). Lymphangiogenesis has also been studied in the regenerating tail of lizards (138).

Somewhat surprisingly, animal models have shown that other angiogenic molecules can also induce lymphangiogenesis. Using the corneal pocket assay, low levels, but not high
levels of bFGF induce lymphangiogenesis without angiogenesis (139). However, blockade with anti-VEGFR-3 antibodies can eliminate the bFGF induced lymphangiogenesis (140). VEGF-C is upregulated by bFGF in this model. Additionally, in a mouse ear model, lymphangiogenesis occurs by adenovirus transduction of VEGF (141). The new lymphatic vessels persist long after the expression of VEGF returns to baseline and this happens in a VEGF-C independent manner. These data show that process of lymphangiogenesis may be as complex as angiogenesis.

Molecular Markers

The lack of specific lymphatic markers inhibits our understanding of the lymphatic system and lymphangiogenesis. Recently, a number of lymphatic markers have been identified, but to date none is absolutely specific for lymphatic vessels, and thus double staining and contextual clues are still very important in IHC applications. VEGFR-3 was identified as a potential lymphatic marker that is expressed in embryonic blood and lymphatic vessels (61). During development VEGFR-3 becomes restricted to adult lymphatic vessels (61). However, VEGFR-3 is found on blood vessels in tumors (62, 69, 75) as well as on adult angiogenic blood vessels (142), limiting it use as a lymphatic marker. VEGFR-3 may be useful as a clinically prognostic marker, as the presence of VEGFR-3 on tumor blood vessels occurs only when melanoma develops metastatic potential (143). Podoplanin was also identified as a lymphatic marker that shows better specificity than VEGFR-3, but it is not yet widely available (144).

Lymphatic Vessel Endothelial HA receptor (LYVE-1) is a molecule similar to CD44 that also binds hyaluronic acid (HA) and is expressed on lymphatic vessels (145-147). In many
tissues LYVE-1 is found only on lymphatic vessels, but in the liver (148), lung (Chapter 5) and in tumors (Chapter 5) LYVE-1 is expressed on blood vessels. So verification of LYVE-1 staining in tumors is needed. Prox 1 (a homeobox protein) (149) is expressed early in lymphatic development (34, 35, 37) and also has been used to identify lymphatic vessels in tumors (150). Although the specificity of Prox 1 seems to be preserved (151), it is found in the nucleus of cells, which leads to many false negatives on thin sections, as not every cell on the section will be cut through the nucleus. Thus combinations of LYVE-1 and Prox 1 (76) or CD31 and Prox 1 (151) seem to be the best currently available ways to identify lymphatic vessels.

A negative staining method has also been proposed for the identification of lymphatic vessels (119). The method identifies CD31+/PAL-E- cells in vessels without a smooth muscle layer as lymphatics. PAL-E is found on the endothelium of capillaries and veins but not on lymphatic vessels or arteries. The lack of expression of PAL-E is not ideal for identifying lymphatics, but this technique has been used to study the lymphatics associated with human melanomas (119, 120). Although, the field is progressing rapidly and tools are now available, no ideal lymphatic marker has been identified.

Using the available tools, no clear correlation between lymphatic markers, lymphatic metastasis, and clinical outcomes exists in clinical studies (Table 2.2) (152). However, many animal studies show a positive correlation between the density of lymphatic markers and lymphatic metastasis (114, 116, 121, 122, 153). Some studies have made claims that intratumor lymphatic vessel identified by LYVE-1 in clinical specimens are necessary for lymphatic metastasis (154, 155), but the data do not support these
conclusions. For instance in Maula et al. (155), 38 of 97 patients with head and neck squamous cell carcinoma had lymph node metastasis, yet only 9 of these patients had intratumor staining with LYVE-1. Thus, 29 patients had lymph node metastasis without intratumor LYVE-1 staining. Furthermore, intratumor LYVE-1 staining was not an independent predictor of disease-specific survival in these patients. Although in certain instances, LYVE-1 staining may correlate with lymph node metastasis (i.e. oropharyngeal, but not laryngeal or oral cavity tumors (154)), the majority of the data indicate that intratumor LYVE-1 staining is not a requirement for lymph node metastasis to occur. Furthermore, studies have found that staining for lymphatic vessels inside of the tumor or in the tumor margin increase overall survival (155-159). The improvement in survival may be due to increased lymphocyte recirculation and thus immune system control of the cancer.

Table 2.2. Association between Density of Putative Lymphatic Markers and Clinical Endpoints

<table>
<thead>
<tr>
<th>Cancer Type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Method of Analysis&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Significant Association</th>
<th>Putative Lymphatic Marker</th>
<th>Clinical Endpoint&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Ref.</th>
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<td>VEGFR-3</td>
<td>LNM, OS</td>
<td>(160)</td>
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<tr>
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<td>VEGFR-3</td>
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<tr>
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<td>RPA</td>
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<td>VEGFR-3</td>
<td>LNM&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(78)</td>
</tr>
<tr>
<td>Breast</td>
<td>IHC</td>
<td>Yes</td>
<td>Podoplanin&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Increased LI, LNM</td>
<td>(162)</td>
</tr>
<tr>
<td>Cervical</td>
<td>IHC</td>
<td>No</td>
<td>Podoplanin&lt;sup&gt;e&lt;/sup&gt;</td>
<td>OS</td>
<td>(156)</td>
</tr>
<tr>
<td>Cervical</td>
<td>IHC</td>
<td>Yes</td>
<td>Podoplanin&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Increased DFS, LNM</td>
<td>(156)</td>
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<tr>
<td>Cervical</td>
<td>IHC</td>
<td>No</td>
<td>Podoplanin</td>
<td>LNM&lt;sup&gt;157&lt;/sup&gt;</td>
<td>(157, 158)</td>
</tr>
<tr>
<td>Cervical</td>
<td>IHC</td>
<td>Yes</td>
<td>Podoplanin</td>
<td>Increased OS, LI, ISR</td>
<td>(157, 158)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>IHC</td>
<td>No</td>
<td>VEGFR-3</td>
<td>OS, DFS</td>
<td>(86)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>IHC</td>
<td>Yes</td>
<td>VEGFR-3 (on cancer cells)</td>
<td>Decreased OS</td>
<td>(163)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>qPCR, IHC</td>
<td>No</td>
<td>VEGFR-3</td>
<td>LNM, invasion depth</td>
<td>(87)</td>
</tr>
<tr>
<td>Endometrial</td>
<td>IHC</td>
<td>Yes</td>
<td>VEGFR-3</td>
<td>Decreased OS</td>
<td>(164)</td>
</tr>
<tr>
<td>Endometrial</td>
<td>IHC</td>
<td>Yes</td>
<td>VEGFR-3 (on</td>
<td>Increased LNM</td>
<td>(165)</td>
</tr>
<tr>
<td>Tumor Type</td>
<td>Method</td>
<td>IHC</td>
<td>RT-PCR</td>
<td>Decreased OS</td>
<td>Increased OS</td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
<td>-----</td>
<td>--------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Epithelial</td>
<td>IHC</td>
<td>No</td>
<td></td>
<td>PODoplanin</td>
<td>LNM, LI, BVI</td>
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<tr>
<td>Ovarian Gastric</td>
<td>RT-PCR</td>
<td>No</td>
<td></td>
<td>VEGFR-3</td>
<td></td>
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<tr>
<td>Gastric</td>
<td>IHC</td>
<td>Yes</td>
<td>VEGFR-3</td>
<td></td>
<td>Increased LNM, LI</td>
</tr>
<tr>
<td>Head and Neck SCC</td>
<td>IHC</td>
<td>Yes</td>
<td>LYVE-1</td>
<td></td>
<td>Increased OS</td>
</tr>
<tr>
<td>Laryngeal</td>
<td>IHC</td>
<td>No</td>
<td></td>
<td>LYVE-1</td>
<td>LNM</td>
</tr>
<tr>
<td>Lung</td>
<td>IHC</td>
<td>Yes</td>
<td>VEGFR-3</td>
<td></td>
<td>LNM</td>
</tr>
<tr>
<td>Lung (NSC)</td>
<td>IHC</td>
<td>No</td>
<td></td>
<td>LYVE-1</td>
<td>LNM, OS</td>
</tr>
<tr>
<td>Pleural</td>
<td>EHC</td>
<td>No</td>
<td></td>
<td>VEGFR-3</td>
<td>Decreased OS</td>
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<tr>
<td>Mesothelioma</td>
<td></td>
<td></td>
<td></td>
<td>5' Nucleotidase</td>
<td>LNM, OS</td>
</tr>
<tr>
<td>Melanoma</td>
<td>IHC</td>
<td>No</td>
<td></td>
<td>Lack of PAL-E</td>
<td>Invasion depth</td>
</tr>
<tr>
<td>Melanoma(IT)</td>
<td>IHC</td>
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<td></td>
<td>LYVE-1 and</td>
<td>Increased OS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PODoplanin</td>
<td>LNM, DFS</td>
</tr>
<tr>
<td>Melanoma(PT)</td>
<td>IHC</td>
<td>Yes</td>
<td>LYVE-1 and</td>
<td>Increased OS, DFS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PODoplanin</td>
<td></td>
<td>LNM</td>
</tr>
<tr>
<td>Oral Cavity</td>
<td>IHC</td>
<td>No</td>
<td></td>
<td>LYVE-1</td>
<td>LNM</td>
</tr>
<tr>
<td>Oral SCC</td>
<td>IHC</td>
<td>Yes</td>
<td>VEGFR-3</td>
<td></td>
<td>Increased LNM</td>
</tr>
<tr>
<td>Oral SCC</td>
<td>EHC</td>
<td>Yes</td>
<td>5' Nucleotidase</td>
<td></td>
<td>Increased LNM</td>
</tr>
<tr>
<td>Oropharyngeal</td>
<td>IHC</td>
<td>Yes</td>
<td>LYVE-1</td>
<td></td>
<td>Increased LNM</td>
</tr>
<tr>
<td>Ovarian</td>
<td>IHC</td>
<td>No</td>
<td></td>
<td>VEGFR-3</td>
<td>Increased LNM</td>
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<tr>
<td>Thyroid</td>
<td>RT-PCR</td>
<td>No</td>
<td></td>
<td>VEGFR-3</td>
<td>LNM</td>
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<tr>
<td>Tongue SCC</td>
<td>IHC</td>
<td>Yes</td>
<td>VEGFR-3</td>
<td></td>
<td>LNM</td>
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</table>

Notes:
- **a:** NSC: Non-small cell carcinoma; SSC: Squamous cell carcinoma; IT: Intratumor; Peritumor
- **b:** EHC: Enzyme-histochemistry; IHC: Immunohistochemistry; RPA: RNA protection assay; qPCR: quantitative or real-time PCR; RT-PCR: Reverse transcriptase-PCR
- **c:** BVI: blood-vessel invasion; DFS: disease-free survival; ISR: inflammatory stromal reaction; LI: lymphatic invasion; LNM: lymph node metastasis; OS: overall survival
- **d:** The reduced presence of the long form of VEGFR-3 mRNA was correlated to increased incidence of lymph node metastases
- **e:** Podoplanin was used to identify lymphatic invasion
- **f:** VEGFR-3 staining was shown to be mostly blood vessels in the tumor
- **g:** Correlation based on increased lymphatic diameter
Lymphatic Pathology

Clinical manifestations of lymphatic disease can occur as a result of either pathology in lymph nodes, or in the transport system of lymph. Although the former is of vast clinical importance, a detailed review of lymphoma is beyond the scope of this introduction. However, many early successes in the treatment of solid tumors were realized treating lymphomas. An introduction to lymphoma, its classification, presentation, and treatment can be found in Hematology (172, 173).

The primary result of pathologies in the transport of lymph is lymphedema, a specific type of edema. Lymphedema can be classified as either primary or secondary, with secondary being the most common. Primary lymphedema is most commonly hereditary in etiology with absent or hypoplastic lymphatics diagnosed at birth (congenital), with puberty (praecox), or around 35 years of age (tarda). Recently, mutations in FLT4 (5q), the gene for VEGFR-3, have been identified in families with congenital primary lymphedema. The mutations form a more stable VEGFR-3 with an inactive tyrosine kinase domain. The mutant receptors act in a dominant negative manner to cause the lymphedema phenotype (174). In animal models, this pathology can be somewhat reversed by local VEGF-C gene therapy, which induces lymphangiogenesis (130, 175, 176). Increased VEGF-C production increases the signaling through homogenous wild-type VEGFR-3 receptors and induces lymphangiogenesis (130). By using a modified VEGF-C (VEGFC-156S), which is specific only to VEGFR-3, the vascular side-effects (hyperpermeability) of VEGF-C production can be avoided (176).
Secondary lymphedema is generally acquired and is due to obstruction or destruction of lymphatics. Common etiologies are malignant obstruction, infections such as tuberculosis, streptococcal lymphangitis, and filariasis. Secondary lymphedema can also be iatrogenic as a result of surgery and radiation. Primary can be distinguished from secondary lymphedema through the use lymphoscintigraphy/lymphangiography and imaging to look for obstructive masses. The former are used to show absent or hypoplastic lymphatic vessels in primary lymphedema or dilated lymphatics in secondary lymphedema. This technique can help identify the level of obstruction or destruction of the lymphatic vessels (177-179).

The presentation of lymphedema is strikingly different in distribution and presentation from edema caused by congestive heart failure, hypoalbuminemia, nephrotic syndrome, cirrhosis, and venous obstruction, which are the most common causes of edema. Lymphedema usually presents as unilateral, non-pitting swelling of a limb and commonly includes its distal portion. The limb is usually painless, but patients often complain of a heavy, dull feeling, loss of contour of the limb, and some loss of range of motion. The swelling progresses from soft and pitting in the early stages to a woody, hard texture in the chronic stages. The woody texture forms from induration and fibrosis caused by chronic inflammation and fibroblast proliferation. The characteristic presentation of lymphedema allows it to be distinguished from other causes of edema, which usually present as softer (pitting), hyperpigmented, commonly with pain and not necessarily confined to the limbs (177-179).
It is currently unknown whether the lack of functional tumor lymphatics is due to a "primary" agenic cause or a "secondary" obstructive/destructive cause. The hypotheses presented in this thesis are designed in part to answer this question with Chapter 5 addressing a "primary" cause and Chapter 6 addressing a "secondary" cause.

**Tumor growth and solid stress**

As a tumor grows, the increase in cell number leads to an increase in solid stress in the tumor. Descriptively, this can be explained by the fact that more cells are occupying a limited space and each cell is pushing on its neighbors. In spheroids grown *in vitro*, this force was calculated to be between 45-120 mmHg(180). This force may then induce the collapse of vessels, both blood and lymphatic, which would alter the local microenvironment.

One way to lower the solid stress in tumors is to lower cell density by causing tumors cell specific apoptosis. Previous work has shown that taxane treated tumors have altered vascular morphology with a greater number of vessels with open lumen (181) (Figure 2.9). When the tumor regrew, vessels again appeared to be collapsed. However, taxane can also affect the host stromal and endothelial cells in the tumor, which slightly confounds the interpretation of these data. In *in vitro* models of spheroid growth, gels with greater stiffness, which is presumed to lead to higher solid stress in the spheroid, inhibited spheroid growth (182). This suggests a non-vascular component of the
response of tumors to solid stress. Solid stress induced cell signaling has not been well studied and the relationship between solid stress and cell proliferation is unknown.

Analytically, soft tissue has been modeled mechanically as an elastic or poroelastic material (183-187), accounting for both the solid, including cells and extracellular matrix, and fluid phases. In a growing tumor, the solid phase expands outward and is balanced by the forces of the surrounding tissue pushing inward. In addition, the density of the solid component relative to the fluid phase increases to accommodate the growing tissue. The reduction in fluid space further increase resistance to convection, making solid stress a potential direct barrier to drug delivery.

**Preliminary Data**

Prerequisites to test the proposed hypotheses are: i) the ability to establish appropriate animal and tumor models to monitor lymphatic structure and function, ii) the ability to modulate VEGF-C levels *in vivo*, and iii) the ability to modulate mechanical forces *in vivo*. Experiments and preliminary data, which lay the groundwork for the proposed hypotheses, are described below.

**Fluorescence Microlymphangiography**

The use of fluorescence microlymphangiography in the tail model has allowed lymphatic structure and function to be analyzed. Lymphatic structure in the tail can be seen by injecting fluorescently labeled dextran into the interstitium at the tip of the tail(188)
(Figure 2.4). Overall lymphatic function can then be analyzed through the use of residence time distribution (RTD) analysis (189), while local lymphatic velocity can be measured using fluorescence recovery after photobleaching (FRAP) (190). These techniques all use epifluorescence microscopy and are limited by poor depth of the imaging (~40 μm). Recent work using Multiphoton Laser Scanning Microscopy (MPLSM) has shown the ability to image lymphatics in the tail at depths up to 400 μm (191) (Chapter 4) (Figure 2.5). Lymphatic vessel diameter and density can be measured using computer-assisted image analysis.

Figure 2.4: A mouse tail injected with FITC-labeled 2 million MW dextran under constant pressure (60 cm H₂O) shows the typical hexagonal lymphatic network.
Figure 2.5: High-resolution image of normal tail lymphatic vessels taken with MPLSM.

**Epidermal VEGF-C overexpression**

Transgenic mice expressing VEGF-C driven by the human keratin 14 (K14) promoter were created in collaboration with Dr. Kari Alitalo. VEGF-C overexpression by the basal cells of the epidermis causes hyperplasia of dermal lymphatic vessels, but does not alter the linear density (63) (Figure 2.6). In addition, no effect is seen on blood vessels. This shows that altering the expression levels of VEGF-C can alter lymphatic physiology and morphology.
Figure 2.6: Overexpression of VEGF-C in the skin of transgenic mice leads to hyperplasia of superficial lymphatics when compared to wild-type control. The lymphatics are visualized using standard microlymphangiography. (Reprinted from Jeltsch, et al., Science, 276: 1423-5, 1997)

Development of VEGF-C overexpressing tumor lines

The T-241 (murine fibrosarcoma), B16-F10 (murine melanoma, selected for metastasis), and U87 (murine glioma) tumor cell lines were retrovirally transfected with a pMMP vector/murine VEGF-C construct (66). The stability of this transfection was verified by Northern Blotting samples from tumors grown in vivo (Figure 2.7a). VEGF-C mRNA is undetectable in T-241 MT tumor. The two bands of ~3.4 (top) and ~2.4 kb (bottom) between 18S and 28S ribosomal RNAs in T-241 VC+ tumors, correspond to the full-length transcript and a splice product lacking approximately 1 kb of viral non-LTR regions of the pMMP vector. The ability to detect protein expression is dependent on VEGF-C antibodies. Recently, Dr. Mouta-Carreira in our lab produced antibodies against the 21kDa dimer of VEGF-C (mature form). Western Blots (Figure 2.7b) performed using crude anti-sera with the anti-VEGF-C Ab detected a 38-kDa band in tumor and cell lysates. The 38 kDa band probably represents a partially processed propeptide. When recombinant human VEGF-C was tested, both a band at 38kDa and the expected 21kDa
were detected. Pre-immune sera did not detect VEGF-C, showing the anti-VEGF-C Ab is specific to these bands, and testing against VEGF and VEGF-B also did not show a band. Thus, this antibody appears to be specific to VEGF-C, but evidence is currently being gathered to concretely associate the specificity of the anti-VEGF-C Ab to VEGF-C.

![Image](image.jpg)

**Figure 2.7:** a) Northern analysis of T241 VC+ and MT tumor lysates. b) Western blot analysis of T-241 VC+ and MT cell and tumor lysates. Recombinant human VEGF-C is shown as a control (66). (This work was performed by Dr. Mouta-Carriera.)

**Peri-tumor microlymphangiography**

Standard fluorescence microlymphangiography uses constant pressure injections in order to label functional lymphatic vessels. In tumors, where there is significantly higher interstitial fluid pressure compared to normal tissue, this technique fails. Under high IFP
conditions, injection pressure must be raised in order to provide sufficient driving force to overcome the elevated IFP. High injection pressures deposit fluorescently labeled material in the tissue. This material is then visualized after the high-pressure injection is stopped using intravital microscopy. Using this technique, the superficial peri-tumor lymphatics can be visualized (115) (Figure 2.8).

![Figure 2.8: Peri-tumor microlymphangiography showing lymphatics in dermis overlying tumor.](image)

**Taxane-induced apoptosis decompresses blood vessels**

Neoplastic cells generate a solid stress of 40 – 125 mmHg during proliferation in agarose gels (180). This level of stress is hypothesized to compress both blood and lymphatic vessels that have a luminal hydrostatic pressure lower than this stress. In support of this hypothesis, taxane-induced tumor cell apoptosis around blood vessels doubles the diameter of these vessels (Figure 2.9). As cancer cells grow back, the vessels collapse again (181). A key limitation of this study is that taxane can also induce apoptosis of tumor associated stromal and endothelial cells, confounding the interpretation of the results.
Figure 2.9. A. Blood vessels in a tumor are compressed by cancer cells. B. Taxane-induced apoptosis in cancer cells can open up these vessels.

**Poroelastic Tumor Models**

Tissue has been mathematically modeled as a poroelastic continuum by many researchers. Recently this type of model has been applied to tumors. In one example, the effect of two different types of fluid transport resistance are compared (192). This work shows that the model fit experimental data well and that a majority of the resistance to fluid transport in tumors resides in the matrix. The resistance caused by the vessel wall is about two orders of magnitude smaller than that for the matrix, and thus only makes a small contribution. However, changes in microvascular pressure cause changes in the IFP due to the small resistance in the vessel wall. Thus, both microvascular pressure and vessel permeability contribute to the pathophysiology of tumors (192). In another example, the poroelastic model is applied to the mouse-tail and incorporates the function of lymphatics (137). When combined with experimental work, the tissue elasticity, hydraulic conductivity, and lymphatic conductance can be calculated. These parameters are also shown to change in different states of edema (137). Finally, a poroelastic model
has been used to calculate the mechanical forces due to both the fluid and solid components of a growing tumor (186). Thus, poroelastic models have been used to successfully model both tumor and tail tissue, and provide a basis for future modeling work.
Chapter 3: Material and Methods
This chapter is divided into three parts describing i) Animal Models, ii) Microscopy and Quantitative Measurements, and iii) \textit{ex vivo} methods.

3.1 Animal Models

All methods involving the experimental use of animals have been approved by the Massachusetts General Hospital Institutional Review Board Subcommittee on Research Animal Care (MGH SRAC protocol \#2000N000080 and \#2001N000139). The experiments were continuously monitored by the MGH veterinary staff.

\textbf{Harvesting Tumor Cells}: Tumor cells were harvested from nude mice with human or murine tumors growing in the subcutaneous space. The mice were euthanized with an intraperitoneal injection of sodium pentobarbital (200 mg/kg). Two incisions were made in the skin, from the left and right lateral, dorsal, distal torso to a point over the distal thoracic spine. This formed a skin flap containing the tumor. The connective tissue surrounding the tumor was resected and the tumor placed on a sterile plate. Each tumor was then minced until the tissue has a paste-like consistency. The cells were added to 100 ml of a 3:1 solution of Hanks' solution with trypsin (Sigma Chemicals). The cell/solution mixture was agitated vigorously until a uniform suspension was formed. The suspension was passed through a filter and aliquoted into centrifuge vials and centrifuged for 8 minutes. The supernatant was removed and the pellets from all of the vials were combined into a common vial. The combined pellets constitute the single cell suspension that was injected.
Injection of Single-cell Suspension: The mice were anesthetized by a subcutaneous injection of ketamine/xylazine (100/10mg/kg). A syringe with the single cell suspension and a 30-1/2gauge needle were used for the injection. The needle was used to puncture the skin with the tip of the needle remaining intradermal, not going subcutaneous. This procedure was used to inject cells in various anatomical sites including: i) 1 cm from the distal tip of the tail; ii) in the distal portion of the right ear; iii) in the skin above the distal portion of the gastrocnemius muscle of the right hindlimb; iv) in flank skin. Additionally, mammary carcinoma cells were injected into the right, distal, mammary fat pad to grow orthotopic breast tumors. Cells were also injected in the dorsal skinfold chamber (see below). Great care was taken to not disturb the vasculature in any of the injections. Approximately 20 μl of single cell suspension was injected to form tumors. The tumor lines that were injected include FSaII (murine fibrosarcoma), T-241 (murine fibrosarcoma), T-241-MT (mock transfected), T-241-VEGF-C (VEGF-C overexpressing), B16-F10 (murine melanoma), B16-F10-MT, B16-F10-VEGF-C, MDA-MB-435S (human mammary carcinoma), MDA-MB-231 (human mammary carcinoma), HSTS261 (human soft-tissue sarcoma), and BA-HAN-1C (human rhabdomyosarcoma).

After tumor cell injection, the mice were housed individually. Any mice showing signs of distress due to the tumor growth or metastasis were given Buprenex analgesia (0.1 mg/kg B.W. s.q.) to alleviate discomfort. Distress was monitored daily in the colony. Animals which had a tumor size greater than 13mm x 13mm were removed from the study and euthanized by an intraperitoneal injection of sodium pentobarbital (200 mg/kg).

Dorsal Skin-fold Chamber: The preparation of dorsal skin-fold chambers has been described previously (193) (Figure 4.1). Briefly, the back of a mouse was shaved,
depleted, and two mirror-image titanium frames were mounted so as to fix the extended double layer of skin between the frames. One 15 mm diameter layer of skin was excised leaving the striated muscle, subcutaneous tissue, and epidermis of the opposite side. The tissue was covered with a glass cover slip mounted into the frame. The surgeries were preformed in male SCID mice bred and maintained in our defined flora animal facility. Some chambers were implanted with T-241 fibrosarcoma to image tumor vasculature or BA-HAN-1C rhabdomyosarcoma to image leukocyte-endothelial interactions.

**Angiography:** Prior to imaging, the animal was anesthetized and given either a 0.1 ml intravenous injection of 2.5% 2x10⁶ MW FITC-dextran (Molecular Probes, Eugene, OR) by tail vein cannulation for vascular density measurement or a 0.05 ml intravenous injection of 0.1% rhodamine 6-G (Sigma Chemicals) for L-E interaction measurement. The animal was fixed to a metal plate designed to stabilize the dorsal skinfold chamber or intradermal tumor.

**Standard Microlymphangiography:** This technique was adapted from clinical medicine (194) for use in the mouse (188). The mouse was anesthetized by a subcutaneous injection of ketamine/xylazine (100/10mg/kg). The mouse was then laid on cover sponges and its tail taped to a metal board using double sided tape. A piece of tape was placed over the hips to secure the mouse to the metal board. The immobilization was necessary in order to obtain high quality in vivo images. A 30-gauge needle connected to a constant pressure source of tetramethylrhodamine (TRITC) fixable dextran (2 million MW) was used to inject the dextran into the interstitial space of the distal tail or at the base of the ear. Large molecular weight molecules are transported preferentially through lymphatics compared to blood vessels(195), a property which makes large molecules
ideal for lymphangiography. The tissue was visualized under a microscope using a mercury lamp and filters, which allowed visualization of TRITC fluorescence. The images were captured and digitized, and the intensities analyzed. A booster shot of 0.05 ml of ketamine/xylazine was administered every 30 minutes to ensure that the mouse remains deeply anesthetized.

**Peri-tumor Microlymphangiography:** The procedure is similar to “Standard Microlymphangiography” (see above). Instead of injecting tetramethylrhodamine fixable dextran (2 million MW) at the distal end of the tail, the dye was injected superficially just distal of the tumor. More pressure was needed to force fluid into the peri-tumor space. This was accomplished by using a syringe to create high pressures and push fluid into the tissue manually.

**Intratumor Microlymphangiography:** The procedure is similar to “Standard Microlymphangiography” (see above). Instead of injecting tetramethylrhodamine fixable dextran (2 million MW) at the distal end of the tail, the dye was injected approximately 1 mm inside the tumor. More pressure was needed to force fluid into the tumor. In order to maintain high signal-to-noise ratio of lymphatic vessels to tumor tissue, only about 3-5 μl of TRITC dextran (2 million MW) was injected.

**Ferritin Microlymphangiography:** To identify functional lymphatic vessels in histological sections, 5 μl of type I ferritin from horse spleen (MW 480 kDa, Sigma Chemical Co.) that has a concentration of 77 mg/ml was injected slowly into the tumor in three different locations. Ferritin was then detected in tissue sections through the
Prussian Blue reaction (see below). In order to distinguish lymphatic vessels from blood vessels, an i.v. injection of 100 µl of 2 mg/ml of biotinylated *L. esculentum* lectin (Vector Laboratories, Burlingame, CA) was given, which circulated for 5 minutes prior to perfusion fixation (see below).

**Spontaneous Metastasis Assay:**

**Tumor implantation:** Tumor cells (both VEGF-C overexpressing and mock transfected) were harvested and made into a single cell suspension (see above). 20 µl of single cell suspension was injected into the intradermal space above the distal gastrocnemius muscle of the right hindlimb. The animals were monitored and tumor growth measured.

**Primary Tumor Resection:** Tumors were resected when they reached a size of 12mm x 12mm. Following anesthesia using ketamine (90 mg/kg i.m.) and xylazine (9 mg/kg i.m.), the mouse was laid on a sterile drape on a heating pad. The tumor bearing leg was sterilized using betadine solution and alcohol. The skin was resected to access the significant vessels. These vessels were tied off and ligated to prevent blood loss and maintain homeostasis. The tumor bearing limb was then amputated. Any bleeding was controlled and then wound closed with interrupted sutures. Post-operative analgesia was supplied by administering Bupreorhine (0.1 mg/kg s.c. q12hrs for three days and as needed after 3 days if the animal shows any signs of discomfort, but not to exceed q12hrs). In our experience, mice undergoing such a procedure were fully ambulatory within two days of the procedure.

Detailed assessment of the extent of surgery required to achieve local control of FSA-II tumor growing in its orthotopic environment (mouse hind limb) has been done previously
(196). Local tumor control of a 5x5mm FSA-II tumor required at minimum radical resection of the posterior compartment of the hind leg. Larger tumors required amputation of the leg to achieve local control of the primary tumor. T-241 is a murine fibrosarcoma line similar to FSA-II, and B16-F10 is a very aggressive melanoma cell line, both of which invade the underlying gastrocnemius muscle. Therefore to achieve surgical local control, amputation of the leg was necessary.

**Autopsy:** Animals were euthanized by an intraperitoneal injection of sodium pentobarbital (200 mg/kg) 1 month after primary tumor resection. A full gross autopsy was performed including lymph node dissection, with select tissue harvested for histopathology and molecular analysis.

**Solid Stress reduction:** The solid stress inside tumors was reduced by causing the specific death of human tumor cells through the use of Diphtheria Toxin (DT) (197). DT catalyzes the ADP-ribosylation of eukaryotic aminoacyl transferase II (EF2) using NAD as a substrate, thereby inhibiting protein synthesis. This reaction forms the basis of its toxicity toward eukaryotic cells. The DT receptor (DTR) is the transmembrane form of the heparin-binding epidermal growth factor-like growth factor (HB-EGF). The DT binding site is in the EGF-like domain but alterations in several amino acids in this domain render the ability of DT to bind to murine HB-EGF substantially reduced as compared to human HB-EGF. Mice bearing tumors grown from HSTS26T cells were treated with either 1 µg DT or PBS intraperitoneal (i.p.) (197). Mice bearing tumors grown from murine T241 fibrosarcoma cells were also treated with 1 µg DT i.p. to serve
as negative controls. Analysis of tumor cell density, cell death, and vascular morphology were performed using intravital microscopy and immunohistochemistry.

3.2 Microscopy and Quantitative Measurements

*Epifluorescence Microscopy:* Light from a mercury lamp entered into the microscope and was deflected into the objective lens by a dichroic mirror appropriate for the excitation and emission wavelength of the fluorescence molecule to be imaged. Fluorescent light was collected by the same objective, passed through the dichroic mirror and an appropriate band pass filter, and was collected by a CCD camera. The CCD camera was controlled by an intensifier and the image was electronically amplified on-line. Images were recorded digitally or on SVHS videotape.

*Intravital multiphoton laser scanning microscopy:* The construction of the multiphoton laser scanning microscope (Figure 4.1) has been described previously (198, 199). Briefly, a tunable femtosecond mode-locked Ti:sapphire laser was directed into the microscope by a galvanometer-driven x-y scanner. A Glan-Thomson polarizer adjusted the power of the incident laser. The laser light entered the microscope (Zeiss Inc., Thornwood, NY) where it deflected into the objective by a custom-made dichroic mirror (Chroma Technology Inc., Brattleboro, VT). A Zeiss Achromplan 20×/0.5 NA/water objective and a Zeiss Achromplan 10×/0.3 NA/water objective were used. The axial position was set by a piezo-driven objective positioner, which was controlled by the data acquisition computer. The fluorescence emission was collected by the same objective lens, passed through the dichroic mirror and barrier filter, and focused on the photomultiplier tube (PMT) for detection. Unlike many commercial systems, the emission light was not descanned,
increasing the sensitivity of detection. The detector was a single photon counting R5600-P PMT (Hamamatsu, Bridgewater, NJ). The signal was pre-conditioned with a low noise pre-amplifier and a photon discriminator (Advanced Research Instrument Co., Boulder, CO). The digital signal was synchronized to the x-y scanner movement by custom software in order to reconstruct a 2-D image that was stored on the data acquisition computer.

**High-speed MPLSM:** For high-speed multiphoton imaging, the multiphoton laser scanning microscope described in the previous paragraph, was modified in two ways (200). First, a rotating polygonal mirror (Lincoln laser, Phoenix, AZ) was used to generate the line scan (along x-direction). The polygonal mirror has 50 facets around the perimeter and has a maximum rotation rate of 30,000 rpm. A scan line was generated at the image plane as a facet was swept across the excitation laser beam. This system has a maximum line scan rate of 25 kHz. For the slow scanning axis (along y-direction), a galvanometer-driven mirror (Cambridge Technology, Watertown, MA) was used. Second, since fewer fluorescent photons were generated over a shorter period of time, single photon counting detection was not used. Instead, the signal was acquired by a high sensitivity PMT (R3896, Hamamatsu, Bridgewater, NJ). A transimpedance amplifier (C1053-51, Hamamatsu) converted the current output from the PMT to an amplified voltage signal. The voltage signal was sampled with a 12-bit AD converter (AD9220EB, Analog Device, Norwood, MA) at approximately 11MHz and was transferred to the data acquisition computer.
Vascular volume calculation: Vascular volume fraction calculations were performed using the NIH Image software package (NIH, Bethesda, MD). Briefly, pixel counts in each optical section were binarized, using a threshold value of ~10% of the maximum pixel value for the section, and pixels were counted to determine the fraction of pixels inside the vessel. Vascular volume fraction was then calculated assuming the thickness of the optical section to be the distance between consecutive sections.

Interstitial Fluid Pressure Measurement: Interstitial Fluid Pressure (IFP) was measured using the wick-and needle technique (17). Briefly, a 23-gauge needle was filled with 3-5 suture threads and a small hole made in the distal side of the needle. The needle was attached with tubing to a pressure transducer that transmitted its output to a recording computer. The system was filled with heparinized water, with care taken to insure no air bubbles had entered the system. The system was calibrated using hydrostatic pressure heads. The needle was inserted into the tissue past the side hole and the fluid communication between tissue and transducer was verified by compressing the tubing and watching the recovery to equilibrium. IFP was then recorded once proper fluid communication was established. This technique was used to measure the IFP of both tumor and normal tissues.

Analysis of intravital microscopy images: Analysis of images taken with intravital microscopy was performed using the various measurement tools in NIH Image v 1.62 (NIH, Bethesda, MD). All microscopes were calibrated using a 100 μm grid micrometer.
NIH Image was used to measure the vessel density, vessel diameter, and depth of vessels in tumors.

*Analysis of immunohistochemical sections:* Analysis of images from immunohistochemical sections was performed using the various measurement tools in NIH Image v1.62 (NIH, Bethesda, MD). All microscopes were calibrated using a 100 μm grid micrometer. NIH Image was used to measure vessel diameter, luminal area, major axis, minor axis, vessel perimeter and the distance of a vessel from normal tissue. NIH Image was also used to calculate vessel density, count the number of cells, and count the number of TUNEL positive cells.

### 3.3 Ex vivo methods

*Tissue preparation and sectioning:* Animals were perfusion fixed in order to maintain vessel morphology. Briefly, animals were anesthetized and any other preparation performed (i.e. ferritin microlymphangiography, lectin circulation). Animals were then pinned to a dissection block, skin over the ventral thorax removed, and an incision made superior to the diaphragm (between the 3rd and 4th rib). The incision was extended between the axillary midline of each side. The rib cage was pulled up and held using hemostats to expose the beating heart and lungs. The heart was held between forceps, while 2 mm incision was made in the apex of the heart. A cannula was inserted through this incision into the proximal aorta and held. 4% paraformaldehyde was perfused through the cannula into the systemic vasculature of the mouse under a pressure of 80-100 mmHg. The left and right atrial appendages were nicked in order for the circulating fixative to exit the circulation. The fixative circulated for 5 minutes. The tissues of
interest were then prepared for paraffin embedding by 3 more hours in 4% paraformaldehyde. The tissue was then embedded in paraffin using the standard procedures of the MGH Pathology Department. 5 μm sections were cut from the blocks in serial, and placed on microscope slides for immunohistochemistry (IHC).

*IHC Protocol for LYVE-1 and Ferritin:*

1. Deparaфинize: Xylene/EtOH series
   - 3x5’ Xylene
   - 2x5’ 100% EtOH
   - 2x3’ 96% EtOH
   - 2x3’ 70% EtOH
   - 2x5’ H₂O

2. Block of Endogenous Peroxidase
   - 20 minutes @ RT in 3% Hydrogen Peroxide in Methanol
     - 30 ml 30% Hydrogen Peroxide
     - 270 ml 100% Methanol

3. Rinse: Wash TBS-T 3x5’

*TBS-T Recipe*

0.05 M Tris
0.3 M NaCl
0.1% Tween-20

*For 5 Liters:*

250mL 1M Tris-HCl pH 7.4
300mL 5M NaCl
5.0mL Tween-20
4445mL of dH₂O

4. Antigen Retrieval: Acid/low pH Target retrieval solution DAKO, dilute in H₂O
   200ml in 20 slide bath
   In microwave: 4 minutes on High
   6 minutes on 10% Power
   Check so that solution doesn’t boil over.
   Let cool for 20 minutes @ RT w/ partial cover.

5. Rinse: TBS-T 3x5’

6. Block for 1 hour with 3% BSA solution in TBS-T @ RT (dry slide, PAP pen, cover).

7. Incubate for 2 hours with Rabbit anti-mouse-LYVE-1 (from E. Rousalhti) (1:500)
   dilution in TBS-T/1% BSA @ RT.
   Take out DAKO Envision Plus anti-rabbit polymer from 4 C to RT.

8. Rinse: TBS-T 3x5’.

9. Incubate for 30 minutes in anti-rabbit labeled polymer (DAKO Envision Plus) @ RT.

10. Rinse: TBS-T 3x5’.

11. Develop for 5’-10’ with liquid DAB from DAKO Envision Plus kit. Stop with H₂O.

12. Rinse: H₂O 3x3’.

13. Incubate with 50:50 solution of 20% HCl and 10% Potassium Ferrocynate for 40
    minutes in humidified chamber @RT for Prussian Blue stain.

14. Rinse: H₂O 3x3’.

15. Stain with Hematoxylin (Fisher Gill’s #2) (4 dips)/Rinse H₂O/Scott’s Water (4
    dips)/Rinse H₂O.

**IHC Protocol for Prox 1 and Ferritin:**

1. Deparaffinize: Xylene/EtOH series
   - 3x5’ Xylene
   - 2x5’ 100% EtOH
   - 2x3’ 96% EtOH
   - 2x3’ 70% EtOH
   - 2x5’ H₂O

2. Block of Endogenous Peroxidase
   - 20 minutes @ RT in 3% Hydrogen Peroxide in Methanol
   - 30 ml 30% Hydrogen Peroxide
   - 270 ml 100% Methanol

3. Rinse: Wash PBS 3x5’

4. Antigen Retrieval: Acid/low pH Target retrieval solution DAKO, dilute in H₂O
   - 200ml in 20 slide bath
   - In microwave: 4 minutes on High
   - 6 minutes on 10% Power
   - Check so that solution doesn’t boil over.
   - Let cool for 20 minutes @ RT w/ partial cover.

5. Rinse: PBS 3x5’

6. Block for 1 hour with PBS, 0.3% Tritonx100, 1% BSA @ RT (dry slide, PAP pen, cover).

7. Incubate with antibody. Use 1:500 dilution of purified IgG rabbit anti-mouse-Prox-1 (from S. Tomarev) for 1 hour @ RT.
   - Take out DAKO Envision Plus anti-rabbit polymer from 4 C to RT.

8. Rinse: PBS 3x5’.

9. Incubate for 30 minutes in anti-rabbit labeled polymer (DAKO Envision Plus) @ RT.

10. Rinse: PBS 3x5’.
11. Develop for 5'-10' with liquid DAB from DAKO Envision Plus kit. Stop with H₂O.

12. Rinse: H₂O 3x3’.

13. Incubate with 50:50 solution or 20% HCl and 10% Potassium Ferrocyanate for 40 minutes in humidified chamber @ RT for Prussian Blue stain.

14. Rinse: H₂O 3x3’

15. Stain with Hematoxylin (4 dips)/Rinse H₂O/Scott’s Water (4 dips)/Rinse H₂O.


*IHC Protocol for MECA-32 and Ferritin*

1. Deparafinize: Xylene/EtOH series
   3x5’ Xylene
   2x5’ 100% EtOH
   2x3’ 96% EtOH
   2x3’ 70% EtOH
   2x5’ H₂O

2. Block of Endogenous Peroxidase
   20 minutes @ RT in 3% Hydrogen Peroxide in Methanol
   30 ml 30% Hydrogen Peroxide
   270 ml 100% Methanol

3. Rinse: Wash PBS 3x5’

4. Antigen Retrieval: Acid/low pH Target retrieval solution DAKO, dilute in H₂O
   200ml in 20 slide bath
   In microwave: 4 minutes on High
   6 minutes on 10% Power
   Check so that solution doesn’t boil over.
   Let cool for 20 minutes @ RT w/ partial cover.

5. Rinse: PBS 3x5’
6. Block for 1 hour with PBS, 0.3% Tritonx100, 3% BSA @ RT (dry slide, PAP pen, cover).

7. Incubate with 1 antibody. Use 1:200 dilution of purified IgG rat anti-mouse MECA-32 (BD Biosciences Pharmingen, San Diego, CA) in PBS, 0.3% Tritonx100, 3% BSA overnight at 4°C.

8. Rinse: PBS 3x5'

9. Incubate with 1:200 dilution of purified IgG rabbit anti-rat antibody (Vector Laboratories, Burlingame, CA) in PBS, 0.3% Tritonx100, 3% BSA for 1 hours at RT.

Take out DAKO Envision Plus anti-rabbit polymer from 4°C to RT.

10. Rinse: PBS 3x5'.

11. Incubate for 30 minutes in anti-rabbit labeled polymer (DAKO Envision Plus) @ RT.

12. Rinse: PBS 3x5'.

13. Develop for 5'-10' with liquid DAB from DAKO Envision Plus kit. Stop with H2O.

14. Rinse: H2O 3x3'.

15. Incubate with 50:50 solution of 20% HCl and 10% Potassium Ferrocyanate for 40 minutes in humidified chamber @RT for Prussian Blue stain.

16. Rinse: H2O 3x3'.

17. Stain with Hematoxylin (Fisher Gill’s #2) (4 dips)/Rinse H2O/Scott’s Water (4 dips)/Rinse H2O.

18. Mount sections in DAKO Faramount Aqueous.

IHC Protocol for Lectin and Ferritin
1. Deparafinize: Xylene/EtOH series
   3x5' Xylene
2x5’ 100% EtOH
2x3’ 96% EtOH
2x3’ 70% EtOH
2x5’ H₂O

2. Block of Endogenous Peroxidase
   20 minutes @ RT in 3% Hydrogen Peroxide in Methanol
   30 ml 30% Hydrogen Peroxide
   270 ml 100% Methanol

3. Rinse: Wash PBS 3x5’

4. Antigen Retrieval: Acid/low pH Target retrieval solution DAKO, dilute in H₂O
   200ml in 20 slide bath
   In microwave: 4 minutes on High
   6 minutes on 10% Power
   Check so that solution doesn’t boil over.
   Let cool for 20 minutes @ RT w/ partial cover.

5. Rinse: PBS 3x5’

6. Incubate slide with ABC reagent (VectaStain ABC kit, Vector Laboratories,
   Burlingame, CA) for 1 hour at RT (dry slide, PAP pen, cover).

7. Rinse: PBS 3x5’

8. Develop for 5’-10’ with liquid DAB from DAKO Envision Plus kit. Stop with H₂O.

9. Rinse: H₂O 3x3’.

10. Incubate with 50:50 solution or 20% HCl and 10% Potassium Ferrocyanate for 40
    minutes in humidified chamber @ RT for Prussian Blue stain.

11. Rinse: H₂O 3x3’

12. Stain with Hematoxylin (4 dips)/Rinse H₂O/Scott’s Water (4 dips)/Rinse H₂O.

IHC Protocol for TUNEL

This procedure is done utilizing the ApopTag Peroxidase In Situ Apoptosis Detection Kit by Intergen®.

1. Deparaffinize: Xylene/EtOH series
   
   3x5' Xylene
   2x5' 100% EtOH
   2x3' 96% EtOH
   2x3' 70% EtOH
   2x5' H₂O

2. Treat the sections with Proteinase-K (20µg/ml) for 15 min at RT. Figure on ~60µl/5cm². After, wash slides in PBS, 3x3 min.

3. Block endogenous peroxidase by washing the slides in 3% H₂O₂/dH₂O for 5 min at RT, then rinse in PBS, 3x3 min.

4. Apply ~75µl of Equilibration buffer for 10-20 sec, then...

5. Blot and apply the TdT dilution (30% TdT enzyme/70% Reaction buffer; figure on 55µl/5cm²) for 1 hour at 37 C in a humidified chamber. Cover with plastic strips.

6. Place slides in Stop/Wash buffer. Agitate for 15 sec, then incubate for 10 min at RT (Stop/Wash buffer dilution: 1mL of Stop/Wash buffer + 34ml of dH₂O). Then rinse in PBS, 3x3 min. While washing, remove the appropriate aliquot of Anti-Digoxigenin Peroxidase Conjugate (figure on ~65µl/5cm²) so it can reach room temperature.

7. Apply Anti-Digoxigenin Peroxidase Conjugate for 30 min at RT; cover with plastic strips.

8. Wash the slides in PBS, 3x3 min.
9. Apply DAB (~75μl/5cm²) to the sections and monitor the reaction under the microscope to avoid over-development. If color change is very slow or doesn’t happen at all, allow to sit for 10-15 min.

10. Terminate DAB reaction by placing slides in dH₂O.

11. Counterstain with hematoxylin, dehydrate and coverslip.

**IHC Protocol for PCNA**

*Uses DAKO ready to use PCNA (N1529) in conjunction with DAKO anti-mouse EnVision kit*

1. Deparafinize: Xylene/EtOH series
   - 3x5’ Xylene
   - 2x5’ 100% EtOH
   - 2x3’ 96% EtOH
   - 2x3’ 70% EtOH
   - 2x5’ H₂O

2. Antigen Retrieval: Acid/low pH Target retrieval solution DAKO, dilute in H₂O
   - 200ml in 20 slide bath
   - In microwave: 4 minutes on High
   - 6 minutes on 10% Power
   - Check so that solution doesn’t boil over.
   - Let cool for 20 minutes @ RT w/ partial cover.

3. Rinse: PBS 3x5’

4. Block of Endogenous Peroxidase
   - 20 minutes @ RT in 3% Hydrogen Peroxide in Methanol
     - 30 ml 30% Hydrogen Peroxide
     - 270 ml 100% Methanol

5. Rinse: Wash PBS 3x5’
6. Apply primary antibody (dilute 1:2 with BSA if background staining is too strong).
   Incubate for 1 hour @ RT.

7. Rinse with PBS 3x3 min.

8. Apply labeled polymer (Bottle 2 from kit). Incubate for 30 min @ RT.

9. Rinse with PBS 3x3 min.

10. Develop stain with DAB
    a. 1 ml of buffer (bottle 3A) and add,
    b. 1 drop of substrate (bottle 3B).

11. Terminate reaction in water.

12. Counterstain with hematoxylin, dehydrate and coverslip.

**Northern Blot Analysis:** Total cellular RNA was isolated from tumor tissue using the guanidium thiocyanate method. A mouse VEGF-C cDNA probe was amplified by PCR. A cDNA for β-actin was used as a probe to control for equal RNA loading, blotting, and hybridization. Hybridization probes were prepared with a random-primed synthesis kit (MultiPrime; Amersham, Arlington Heights, IL). Blots were washed at high stringency (0.1 X SSC/1% SDS at 65 °C) and exposed on Kodak X-OMAT film.

**Western Blot Analysis:** Total protein from portions of tumors was prepared by macerating the tumors with a liquid nitrogen cooled mortar and pestle. The powder was suspended in Lameli sample buffer and 2-ME, passed through a syringe, centrifuged with the supernatant then heated to 95 °C before loading onto the gel. Protein amount was quantified using the Lowry assay (BioRad DC protein assay instruction manual). 12.5%SDS-PAGE gel was run and the gel blotted on to ECL nitrocellulose membranes.
Transfer was confirmed and lanes marked with Ponceau stain. The membrane was blocked with 3% bovine serum albumin solution and then incubated with antibody for 2 hours at room temperature. The membrane was washed, incubated with the secondary antibody, washed again and exposed to the ECL reagents for 1 minute. The membrane was then exposed and developed.
Chapter 4: Multiphoton Laser Scanning Microscopy of the Lymphatic System

Portions of this chapter have been taken from:

Over the past 75 years many chronic animal models have been developed to investigate physiology and pathophysiology using intravital microscopy(201). These models overcome limitations associated with more traditional histological techniques. More specifically, they allow for the study of biological events without loss of temporal dynamics or in vivo microenvironment. Intravital epifluorescence microscopy has been used in combination with these models to measure gene expression(202), permeability of blood vessels(203), cell interactions(66, 204), and cell transport(205, 206), as well as diffusion (28), convection(26), and binding(30), pO₂, and pH(207). The information gained from these measurements is crucial to understanding molecular and cellular processes underlying physiology and pathophysiology and to the design and delivery of molecular therapy.

In the post-genomic era, many challenges remain and intravital microscopy is well tailored to address two of these in particular. First, the function and regulation of many newly described genes remains unknown(11). Novel approaches to determine the regulation and function of these genes in vivo are critical to understanding their role(202). Second, the ability to alter gene expression and potentially repair mutated genes will require effective delivery of macromolecules to the target tissue(208). This requires studying the transport of these macromolecules in blood, the lymphatic system, and the interstitial compartment of the tissue to understand and overcome the barriers that impede their delivery and efficacy. These two challenges are particularly pertinent in a solid tumor, a genetically unstable target that presents substantial obstacles to the delivery and effectiveness of macromolecular therapy, e.g., gene therapy. However, three-dimensional resolution and greater depth of imaging are needed to enable high resolution intravital
imaging to reach its full potential. Confocal microscopy, used extensively in bioimaging applications, permits acquisition of three-dimensional information by eliminating out-of-focus light through use of a pinhole. However, the energy delivered by the excitation light causes extensive photo-damage to living tissues (209) and the use of a pinhole, along with descanning and short wavelength light, limits the depth of signal detection to ~100 μm.

Recently, multiphoton laser scanning microscopy (MPLSM) (210) has been applied to in vivo systems (199, 209, 211-215). MPLSM represents a significant improvement over traditional epifluorescence microscopy, which has the following limitations in vivo: i) scattering of excitation and emission light limits imaging depth, leaving tissue structure and function below approximately 10-40 μm concealed; and ii) out-of-focus light from three-dimensional structures is projected onto a two-dimensional plane, yielding surface-weighted images and measurements. MPLSM overcomes these limitations by using longer wavelength light, which penetrates deeper into tissue. The excitation of a fluorophore using infrared light requires the simultaneous absorption of two photons (216). As a consequence, excitation in MPLSM occurs only at the focal point of the objective, giving high x-y-z spatial resolution while eliminating the need for a pinhole to block out-of-focus light, and resulting in less tissue phototoxicity.

One disadvantage of conventional MPLSM is the slow imaging speed (~ 1-2 frames/sec for high resolution). This is particularly problematic when studying blood flow and leukocyte-endothelial interactions. Although a technique has been developed to measure steady blood velocity (199, 214), imaging of more complicated or sporadic short time-
scale phenomena in vivo has, until now, not been demonstrated. The speed at which an image can be acquired is dependent on the ability of two galvanometer-driven mirrors to move the laser across the x-y plane of the image. Recently, a high speed multiphoton laser scanning microscope has been developed (200) that replaces one of the slow galvanometer driven mirrors with a high speed, rotating polygonal mirror allowing acquisition of up to 100 frames per second.

The current work builds upon and extends our recently published study (199). We apply conventional as well as high speed MPLSM to study the pathophysiology of solid tumors. First, we imaged lymphatics for the first time with MPLSM, which play a role in the spread of cancer to distant sites as well as in the development of elevated interstitial fluid pressure inside tumors. Second, we imaged angiogenic blood vessels, which supply a growing tumor with oxygen, provide a pathway for the hematogenous dissemination of cancer to distant sites, and serve as a conduit for the delivery of systemic therapies. These vessels are also the target of potential anti-angiogenic treatments. Finally, we used high speed MPLSM to image the interaction of leukocytes with the endothelium (L-E interactions). L-E interactions are important in monitoring the ability of the immune system to control tumor spread and also serve as a marker of stimulated endothelium.

**Materials and Methods**

**Intravital multiphoton laser scanning microscopy**

The construction of the multiphoton laser scanning microscope (Fig. 4.1) has been described previously (198). Briefly, a femtosecond mode-locked Ti-sapphire laser (Mira 900; Coherent Inc., Palo Alto, CA) tuned to a wavelength of 780 nm was directed into the
microscope by a galvanometer-driven x-y scanner (Cambridge Technology, Watertown, MA). A Glan-Thomson polarizer adjusted the power of the incident laser. The laser light entered a Zeiss Axiovert 110 microscope (Zeiss Inc., Thornwood, NY) where it deflected into the objective by a custom-made dichroic mirror (Chroma Technology Inc., Brattleboro, VT). A Zeiss Achromplan 20×/0.5 NA/water objective and a Zeiss Achromplan 10×/0.3 NA/water objective were used. The axial position was set by a piezo-driven objective positioner, which was controlled by the data acquisition computer. The fluorescence emission was collected by the same objective, passed through the dichroic mirror and barrier filter (short pass 2 mm BG-39 Schott glass filter; CVI Laser, Livermore, CA), and focused on the photomultiplier tube (PMT) for detection. Unlike many commercial systems, the emission light was not descanned, increasing the sensitivity of detection. The detector was a single photon counting R5600-P PMT (Hamamatsu, Bridgewater, NJ). The signal was pre-conditioned with a low noise pre-amplifier and a photon discriminator (Advanced Research Instrument Co., Boulder, CO). The digital signal was synchronized to the x-y scanner movement by custom software in order to reconstruct a 2-D image that was stored on the data acquisition computer. Three-dimensional reconstructions were performed offline by stacking images using the Slicer software package (Fortner Research, Sterling, VA). Vascular volume fraction calculations were performed using the NIH Image software package (NIH, Bethesda, MD). Briefly, pixel counts in each optical section were binarized, using a threshold value of ~10% of the maximum pixel value for the section, and pixels were counted to determine the fraction of pixels inside the vessel. Vascular volume fraction was then
calculated assuming the thickness of the optical section to be the distance between consecutive sections.

Figure 4.1. Schematic diagram of the multiphoton laser scanning microscopy apparatus used for experiments. Mice bearing dorsal skin-fold preparations were restrained on the stage of the inverted microscope and normal and tumor microvasculature was visualized through a window in the chamber. The same setup was used in the mouse tail microlymphangiography experiments. For high speed MPLSM, one of the galvanometer-driven mirrors in the X-Y scanner was replaced by a rotating polygonal mirror to produce faster scans in the x-direction. Additionally, the single-photon counting PMT was replaced by a high-sensitivity PMT in order to detect the fewer fluorescent photons generated over a shorter period of time in video-rate imaging.
High-speed intravital multiphoton laser scanning microscopy

For high-speed multiphoton imaging, the multiphoton laser scanning microscope described in the previous paragraph, was modified in two ways (200). First, a rotating polygonal mirror (Lincoln laser, Phoenix, AZ) was used to generate the line scan (along x-direction). The polygonal mirror has 50 facets around the perimeter and has a maximum rotation rate of 30,000 rpm. A scan line was generated at the image plane as a facet is swept across the excitation laser beam. This system had a maximum line scan rate of 25 kHz. For the slow scanning axis (along y-direction), a galvanometer-driven mirror (Cambridge Technology, Watertown, MA) was used. Second, since fewer fluorescent photons were generated over a shorter period of time in video-rate imaging, single photon counting detection cannot be used. Instead, the signal was acquired by a high sensitivity PMT (R3896, Hamamatsu, Bridgewater, NJ). A transimpedance amplifier (C1053-51, Hamamatsu) converted the current output from the PMT to an amplified voltage signal. The voltage signal was sampled with a 12-bit AD converter (AD9220EB, Analog Device, Norwood, MA) at approximately 11MHz and was transferred to the data acquisition computer.

Animal models

Dorsal Skin-fold Chamber. The preparation of dorsal skin-fold chambers (Fig. 4.1) has been described previously (193) (Chapter 3). Some chambers were implanted with T-241 fibrosarcoma to image tumor vasculature or BA-HAN-1C rhabdomyosarcoma to image leukocyte-endothelial interactions. Prior to imaging, the animal was anesthetized and given either a 0.1 ml intravenous injection of 2.5% 2×10^6 MW FITC-dextran (Molecular Probes, Eugene, OR) by tail vein cannulation for vascular density measurement or a 0.05
ml intravenous injection of 0.1% rhodamine 6-G (Sigma Chemicals) for L-E interaction measurement. The animal was fixed to a metal plate designed to stabilize the chamber. The FITC-dextran was excited by multiphoton absorption allowing visualization of microvasculature. Images 100-300 μm deep were obtained in both non-tumor and tumor-bearing dorsal windows. Tumors imaged were ~1-2 mm thick.

**Mouse Tail Model.** The experimental procedure used for microlymphangiography in the mouse tail has been described previously (188) (Chapter 3). Images up to 450 μm deep were obtained.

**Statistical analysis.** Results are presented as mean±SEM. Two sample Student’s t-tests for independent samples of equal variances were performed to compare sample means. Statistical significance was based on p values smaller than 5%

**Results and Discussion**

**MPLSM of lymphatics show first high resolution images of microanatomy in vivo.** Microlymphangiography has been used to evaluate the structure and function of lymphatic vessels in the mouse tail (113, 188-190). Under epifluorescence the regular hexagonal mesh pattern of the collecting lymphatics is readily apparent (Fig. 4.2A), but much of the anatomical detail is obscured. A detailed anatomical study of collecting lymphatics has yet to be performed due to the limitations of epifluorescence microscopy. We performed microlymphangiography in the tails of nude mice with MPLSM (Figs. 4.2B,C,D,E). The MPLSM images reveal morphological features not discernible using epifluorescence microscopy. The vertices of the hexagonal mesh along with some of the vessels on the sides are made up of small vessels merging together; these appear to be
single vessels in epifluorescence images. As a result, measurements of lymphatic vessels in the tails of nude mice taken using MPLSM (mean diameter = 40.6 ±1.4 μm; n = 141 vessels from 19 animals) are smaller (p < 0.005) than lymphatics imaged using epifluorescence microscopy (Mean diameter = 54.1 ±2.1 μm; n = 55 vessels from 8 animals). In addition, the deeper, draining vessels, which have not been imaged previously, can also now be seen due to the inherent z-resolution and increased depth of imaging of MPLSM (Fig. 4.2C). Such detailed images play an important role in understanding the creation of new lymphatic vessels (lymphangiogenesis), as the temporal dynamics of microanatomical development can now be studied in a non-invasive manner. As growth factors with putative lymphangiogenic or anti-lymphangiogenic activity are discovered, MPLSM will play a crucial role in investigating their effect in vivo on the growth of new lymphatics or the inhibition of this process.

![Image of microlymphangiography in the tail of nude mice.](image)

**Figure 4.2.** Microlymphangiography in the tail of nude mice. (A) Hexagonal, superficial lymphatic pattern in the tail visualized using epifluorescence microscopy. With this technique it appears as if the hexagonal mesh is composed of single vessels. (B) MPLSM image taken using 10x water immersion objective at a depth of 100 μm below the surface
of the skin. Arrowheads point to the multiple small vessels that make up the hexagonal mesh. This fine detail is not distinguishable at the same magnification using epifluorescence. (C) MPLSM image taken using 10x water immersion objective at a depth of 225 μm below the surface of the skin. Arrowheads point to deeper draining lymphatics not visible using traditional epifluorescence microscopy. (D) MPLSM image taken using 20x water immersion objective at a depth of 100 μm below the surface of the skin. Arrowhead points to a cell (presumably a leukocyte) in the lymphatic vessel. (E) MPLSM image taken using 20x water immersion objective at a depth of 150 μm below the surface of the skin. Due to the curvature of the surface of the mouse tail, all cited depths are estimates of the average depth below the surface of the skin in the image. The scale bars represent 500 μm (A), 100 μm (B and C), and 50 μm (D and E).

The development of new tumor models in combination with MPLSM, presents opportunities to answer some fundamental questions of tumor biology. For instance, elevated interstitial fluid pressure in solid tumors(217-221) suggests the lack of a functional lymphatic system. However, the existence of functional lymphatics in tumors is still a subject of debate due to the lack of a conclusive study. With ten-fold greater depth of imaging, a definitive answer to this question may begin to emerge. As another example, the details underlying the mechanism(s) of lymphatic metastasis are not well understood. MPLSM has the resolution to image single cells in lymphatic vessels (Fig. 4.2D), a capability that could be enhanced by the use of reporter genes such as green fluorescent protein (GFP)(222, 223). Insight into how lymphatic metastasis occurs may suggest new therapeutic targets for future treatments. As more is learned about the
molecular differences between vascular and lymphatic endothelium, cell specific markers will become available, allowing for the study of angiogenesis and lymphangiogenesis on the molecular level. Additionally, cell specific markers will allow monitoring of treatments aimed at enhancing or inhibiting blood vessel and lymphatic growth.

*High-resolution images show heterogeneous, tortuous tumor vasculature.*

Images of blood vessels in non-tumor bearing dorsal windows obtained using MPLSM technology (Figs. 4.3B,C) add deeper, three-dimensional structure to the planar projections provided by epifluorescence (Fig. 4.3A). These vessels exhibited normal density and tortuosity. In contrast, the vasculature of the T-241 fibrosarcoma was highly tortuous and had increased vascular density both at the surface (~40-50 mm) seen using epifluorescence (Fig. 4.3D) and deeper (~200 mm) in the tumor imaged using MPLSM (Figs. 4.3E,F). The images obtained using MPLSM were used to calculate vascular volume fraction. The ratio of vessel volume to tissue volume in tumor tissue (Figs. 4.3E,F) was 0.199 compared to 0.098 in normal tissue (Figs. 4.3B,C). This quantification demonstrates the potential for performing an improved angiogenic assay using MPLSM. Such an approach may prove invaluable for studying and accurately assessing the effectiveness of various anti-angiogenic agents as potential cancer treatments.
Figure 4.3. Images of non-tumor- (A,B,C) and tumor-bearing (D,E,F) mouse dorsal skin obtained using the dorsal skin-fold preparation. (A) Epifluorescence image of non-tumor-bearing dorsal skin shows normal vessel density and tortuosity. (B,C) MPLSM images of non-tumor associated mouse vasculature also show normal vessel density and tortuosity but at considerably greater depth (~200 mm). These images illustrate the three-dimensional reconstructive capability of MPLSM. The image shown in (B) represents the view looking down into the dorsal chamber from the surface of the chamber. (The z-axis in the lower left points into the page.) The image in (C) is a three-dimensional representation obtained by rotating the image in (B) as indicated by the axes in the lower left. The origin of the axes is fixed to the same corner of the cube (indicated by the red
(D) Epifluorescence image of tumor grown in dorsal skin-fold chamber illustrates the increased vessel density and tortuosity characteristic of neoplastic tissue, but yields only a surface-weighted picture of the microvascular architecture. (E,F) MPLSM images of tumors grown in the dorsal skin-fold preparation illustrate the chaotic vascular network in greater detail and demonstrate that increased vessel density and tortuosity persist even at greater depths. The ability to image deeper provides a more accurate picture of the microvascular architecture and allows for more precise quantification of vessel density. As in (B) and (C), the image shown in (E) represents the view looking down into the dorsal chamber from the surface of the chamber. (Again, the z-axis in the lower left points into the page.) The image in (F) is a three-dimensional representation obtained by rotating the image in (E) as indicated by the axes in the lower left. As before, the origin of the axes is fixed to the same corner of the cube (indicated by the red dot) in both (E) and (F) and the face of the cube indicated by the darker lines in (F) is the face closest to the reader in (E). The images in (E) and (F) further illustrate the utility of MPLSM for viewing spatial relationships between anatomic structures in three dimensions. The images were obtained using a 20× objective and span a depth of ~200-300 mm. All scale bars represent 100 mm. Note that the reconstructions shown require thresholding of the data collected in order to make the tissue appear transparent. As a result, some small vessels are only partially visible or absent. These renderings are intended to illustrate the three-dimensional representations that can be obtained and were not used for calculation of vascular volume fraction.
High Speed MPLSM allows study of short time scale phenomena.

Video-rate imaging is necessary to study physiologic phenomena occurring on time scales shorter than a second. We have used high-speed MPLSM to measure the L-E interactions in normal and tumor blood vessels as deep as 350 μm in tissue (Figs. 4.4A-H). In the BA-HAN-1C rhabdomyosarcoma (Figs. 4.4F-H) there is no difference (p>0.05) in leukocyte flux (16.4 ± 1.8 cells/30s) or percentage of rolling cells (30.0 ± 3.7%; n = 10 vessels from 2 animals) when compared to normal vessels (Figs. 4.4A-E) in the skin (16.4 ± 1.8 cells/30s and 20.5 ± 6.5%; n = 6 vessels from 2 animals). This suggests that the rhabdomyosarcoma is not producing cytokines that stimulate the endothelium. The absence of an increased leukocyte response deep in this tumor tissue explains, at least in part, the inability of the host immune system to clear all tumor cells. MPLSM will help to identify conditions in which L-E interactions are increased, which, in turn, could lead to novel immunotherapies for cancer.
Figure 4.4: Single frames extracted from movies acquired using high-speed MPLSM of leukocyte-endothelial interaction in the dorsal skinfold chamber. (A-E) Vessel in a non-tumor bearing chamber imaged at a depth of 150 \( \mu \text{m} \) below chamber window. The elapsed times are shown. The arrowhead follows a single leukocyte that had adhered to the vessel wall (A,B), released (C), and rolled along the vessel wall (D,E). Leukocytes and platelets can be seen in the blood flow, which has an average velocity of 80 mm/s. (F-H) Vessel in BA-HAN-1C rhabdomyosarcoma growing in a dorsal skinfold chamber imaged at a depth of 200 \( \mu \text{m} \) below the chamber window. The elapsed times are shown. The arrowhead follows a single leukocyte traveling with the blood flow, not interacting with the vessel wall. The blood flow has an average velocity of 220 mm/s. All scale bars represent 40 mm.

With the development of \textit{in vivo} fluorescent reporters such as GFP(202, 204, 206, 222-225), high-speed MPLSM will permit the direct observation of various steps in metastasis. Tumor cell extravasation at the primary site, circulation, and intravasation at the metastatic site are not understood and very difficult to observe directly due to the inability to image at depth with high resolution. High-speed MPLSM will be able to capture these phenomena. As new molecular markers are developed, the power of high-speed MPLSM can be used to monitor many phases of cell response to treatment, including cell proliferation and apoptosis. In addition, although steady blood velocity has been measured with MPLSM(199, 214), unsteady, heterogeneous, and/or reversing flow, common phenomena in tumors(5), can also be analyzed with high speed MPLSM.
Conclusion

We have demonstrated the utility of MPLSM to study biological events in vivo and, for the first time, described the use of high-speed MPLSM in vivo. The inherent three-dimensional resolution deep in tissue and low phototoxicity, make MPLSM ideal for these applications. The ability to image at video-rate, further increases the utility of this technique, expanding the range of problems that can be addressed to include phenomena that occur on short time scales. We have illustrated the use of MPLSM and high-speed MPLSM to address questions relevant to tumor biology; high-resolution imaging of functional lymphatics, tumor blood vessels, and the interaction of leukocytes with tumor endothelium. Future studies based on these techniques may finally resolve long-standing, unanswered questions about the molecular, cellular, and integrative biology of solid tumors. MPLSM will allow the deeper, often poorly vascularized and treatment-resistant parts of the tumor to be studied, which cannot be done with epifluorescence microscopy. The continued application and development of in vivo MPLSM techniques for measuring biological parameters (e.g., pH, ligand-receptor interactions, promoter activity), combined with fluorescent molecular markers, can determine the regulation and function of genes (Fig. 4.5) and give novel insight into the recently sequenced genome.
Figure 4.5. MPSLM images of tumors grown in the dorsal skinfold chamber of VEGF-GFP transgenic mice. The green cells are host fibroblasts that are expressing GFP due to the activation of the VEGF promoter in these cells. Red vessels are highlighted by TRITC-dextran. A) Host fibroblasts are abundant at the edge of the tumor. B,C) Inside the tumor, fibroblasts producing VEGF associate with blood vessels, implying that these host cells play an integral role in tumor angiogenesis.
Chapter 5: VEGF-C in the Lymphatic Pathophysiology of Tumors

Portions of this chapter have been taken from:


Cancer cells metastasize to distant sites via both the vascular and lymphatic system. Our insight, however, into the lymphatic system lags far behind that of the vascular system, due largely to the limitations of available techniques. The debate over the presence or absence of functional lymphatics in tumors has been ongoing for over 100 years. However to date, studies investigating lymphatic vessels in tumors have relied exclusively on either molecular markers (76, 226) or functional techniques (18, 19, 113). The former studies have identified lymphatic markers in tumors (116, 118, 122, 150), whereas the latter have failed to demonstrate functional intratumor lymphatics (18, 19, 76, 113, 226-231). Early functional studies by Krause (232) and Evans (233) stand in contrast to the majority of data, but the conclusions in these papers have been criticized because they do not show the relative position of functional lymphatic vessels and cancer cells (18). Functional lymphatics, however, do exist in the tumor margin (defined as < 100 microns from the tumor edge) (113). These disparate observations call into question the relationship between functional lymphatic vessels and widely accepted lymphatic molecular markers (Table 2.2), and the role they play in lymphatic metastasis. Furthermore, these observations highlight an unresolved paradox: How can tumor cells metastasize through the lymphatic system if there is no functional lymphatic network in tumors? In this chapter, I will test the hypothesis that VEGF-C overexpression in tumors can induce functional intratumor lymphatic vessels, alter tumor margin lymphatic vessels, and increase the rate of lymph node metastasis from solid tumors (Specific Aim 1; Chapter 2).

*VEGF-C overexpressing tumors are more metastatic to lymph nodes.*
To begin, we created two stable cell lines from T-241 murine fibrosarcoma and B16-F10 murine melanoma that overexpress VEGF-C, a known lymphangiogenic molecule (66, 234)(Fig. 5.1A). We used a metastasis model described previously (196) (Chapter 3). Briefly, tumors were implanted orthotopically in the hindlimb of nude mice from a single cell suspension of excised tumor. When the tumor reached 1150 mm³ in size the tumor was resected by hindlimb amputation. After 5 or 30 days, animals were sacrificed and autopsies were performed including lymph node dissection. VEGF-C overexpressing tumors grow more slowly than mock-transduced control tumors (p < 0.05; Fig. 5.1B). We had previously shown VEGF-C overexpression increased the rate of tumor growth in a different tumor site (66), while in another tumor model, others have reported VEGF-C slowed tumor growth (118). Thus, the effect of VEGF-C on tumor growth appears to be dependent on the host microenvironment (235). VEGF-C overexpressing melanomas and fibrosarcomas exhibit an increase in lymphatic metastasis (Fig. 5.1C), but no increase in hematogenous lung metastasis (Table 5.1). Interestingly, VEGF-C overexpressing melanomas also show increased local invasion (Table 5.1). Additionally, VEGF-C overexpression in B16-F10 melanomas induces lower-body edema in ~30% of animals consistent with lymphatic blockage (Fig 5.1C) and/or the permeability enhancing effects of VEGF-C (66). In vitro analysis shows that VEGF-C overexpression does not enhance cell migration on fibronectin-coated surfaces, suggesting that increases in cell motility are not responsible for increased invasion (Fig. 5.1D).
Figure 5.1. Metastasis associated with VEGF-C overexpressing tumors. (A) RT-PCR (left) confirming overexpression of VEGF-C (66). Only transduced VEGF-C, not endogenous VEGF-C, is detected. Western Blot analysis (right) shows mostly 37kDa form of VEGF-C is produced. (B) Growth rate of VEGF-C overexpressing tumors is slower than control (mock-transduced) (based on time in days to reach external tumor size of 13mm x 13mm, ~1150 mm³). *p < 0.05; (C) Melanoma-filled lymph node (arrow) near iliac bifurcation of aorta on tumor bearing side. Contralateral lymph node is grossly free of tumor (arrowhead). Scale-bar: 5mm. (D) VEGF-C overexpression does not increase cell migration. Error bars show SEM.
Table 5.1: Invasion and metastasis from mock-transduced (MT) and VEGF-C overexpressing tumors.

<table>
<thead>
<tr>
<th>Property</th>
<th>B16F10-MT</th>
<th>B16F10-VEGF-C</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphatic Metastasis</td>
<td>6/21</td>
<td>15/19</td>
<td>0.002</td>
</tr>
<tr>
<td>Local Invasion</td>
<td>3/21</td>
<td>12/18</td>
<td>0.001</td>
</tr>
<tr>
<td>Lung Metastasis†</td>
<td>5/22</td>
<td>2/19</td>
<td>N.S.</td>
</tr>
<tr>
<td>T-241-MT</td>
<td></td>
<td>T-241-VEGF-C</td>
<td></td>
</tr>
<tr>
<td>Lymphatic Metastasis</td>
<td>2/14</td>
<td>8/14</td>
<td>0.046</td>
</tr>
<tr>
<td>Local Invasion</td>
<td>4/14</td>
<td>5/14</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lung Metastasis†</td>
<td>5/14</td>
<td>6/14</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Numerator is the number of mice that exhibited the property; denominator is the total number of mice.
*
*: P-value based on Fisher’s Exact Test. N.S.: not significant
†: Hematogenous metastasis was analyzed microscopically in 10-20 sections of lung per animal.

T-241 fibrosarcomas and B16-F10 melanomas contain LYVE-1 positive structures

To determine whether intratumor lymphatics are present, we stained both tumor types for the putative lymphatic markers LYVE-1 (147) and Prox 1 (34), along with the murine pan-endothelial marker MECA-32 (148, 236) (Figs. 5.2A-C). To identify functional blood vessels, biotinylated Lycopersicon esculentum lectin (Vector Laboratories, Inc., Burlingame, CA) was injected intravenously (10 µg/ gram body weight). Five minutes after i.v. injection, the animal was sacrificed by perfusion fixation (148). Vessel density was quantified by counting total number of vessels in 10 randomly selected 480x720 micron fields. Immunohistochemistry for LYVE-1, Prox 1, MECA-32, and Biotinylated L. esculentum lectin was performed according to published methods (148). Lymphatic vessels are classified as follows: normal tissue lymphatics (NTL); tumor margin lymphatics (TML), found within 150 µm of the surface of the skin overlying the tumor;
and intratumor lymphatics (ITL), found further than 150 μm from the surface of the skin overlying the tumor. The 150 μm delineation comes from the addition of ~50 μm for the thickness of the skin and 100 μm designated as the tumor margin in previous studies(113).

*VEGF-C overexpression increases the number of LYVE-1 and MECA-32 positive structures.*

VEGF-C overexpressing tumors exhibit more extensive LYVE-1 and MECA-32 staining compared to mock-transduced tumors (Fig. 5.2D), especially in the tumor margin (Fig. 5.2E). Morphologically, LYVE-1 positive structures in the tumor appear to be vessels that collapsed or became filled with tumor cells that occlude the lumen, with very few structures with an open lumen (Fig. 5.2F). In contrast, LYVE-1 positive structures exhibited a more normal lymphatic morphology in the tumor margin.

We suggest that the increase in LYVE-1 staining in tumors overexpressing VEGF-C is due to VEGF-C-aided survival of lymphatic endothelial cells from lymphatic vessels compressed or destroyed by the growing tumor (18, 19, 113, 180) (Fig. 5.2F). This implies that the increase in LYVE-1 positive structures within VEGF-C overexpressing tumors may not be due to intratumor lymphangiogenesis, but rather incorporation of lymphatic vessels at the edge of the growing tumor. Using PCNA to identify proliferating endothelial cells, 51% of LYVE-1 positive structures in the tumor margin of VEGF-C overexpressing fibrosarcomas are PCNA positive, compared to 23% in control tumors (P value = 0.0012 based on Chi-Square analysis). No difference in proliferation of LYVE-1
positive structures is seen inside of the tumor when VEGF-C overexpressing tumors are compared to control. Similarly, there is no difference in apoptosis, as determined by the TUNEL assay, of LYVE-1 positive structures in VEGF-C overexpressing and control fibrosarcomas. Thus the increase in lymphangiogenesis at the tumor margin in VEGF-C overexpressing tumors could account for the greater incorporation of LYVE-1 staining in these tumors, without the need for intratumor LEC proliferation.
Figure 5.2: Immunohistochemical characterization of lymphatic markers. (A) LYVE-1 stain (brown) of ferritin-filled lymphatic vessel (Ly) at the edge of a T-241 fibrosarcoma. (B) Lectin stain (brown) showing neighboring blood vessel (BV). (C) Prox 1 stain (nuclear, brown) of same lymphatic vessel. (D) VEGF-C overexpressing tumors exhibit higher staining densities compared to control tumors. (E) VEGF-C overexpressing tumors exhibit higher LYVE-1 staining densities in tumor margin (referred to as TML) compared to MT tumors. (F) Structures stained with LYVE-1 (brown) are compressed or filled with cancer cells in B16-F10 melanoma. Error bars show SEM. Scale-bars: A-C, F: 100µm.

LYVE-1 is expressed on a fraction of blood vessels in tumors.

Surprisingly, ~10% of all tumor-associated LYVE-1 positive structures either colocalizes with the intravenous lectin, a marker of perfused blood vessels, or contains RBCs (Figs. 5.3A, B). This strongly suggests that a fraction of LYVE-1 stained structures in our B16-F10 melanoma and T-241 fibrosarcoma models are blood vessels. In the dermis of normal tail, however, LYVE-1 positive structures do not co-localize with lectin-perfused blood vessels. Inside both tumor types, there is a ~50% decrease in co-localization of MECA-32 or Prox 1 with LYVE-1 positive structures when compared to normal tissue and tumor margin (Table 5.2). It is important to note that MECA-32 may not stain 100% of vascular and lymphatic endothelium. However, the decrease in the co-localization with LYVE-1 in the tumor, when compared to normal tissue or the tumor margin is striking and raises questions about the interpretation of these stains in tumors. Furthermore, LYVE-1 co-localization with Prox 1 is less than co-localization with
MECA-32 (Table 5.2). The surprisingly poor co-localization of Prox 1 with LYVE-1 is partially due to the nuclear nature of the Prox 1 stain, which is difficult to quantify when using 5-micron sections. Any individual section may not have cut through the nucleus of the cell of interest, thus giving a false negative and underestimating the staining density or co-localization with other markers. This limits the use of Prox 1 as an independent lymphatic marker. Nonetheless, the Prox 1 data, along with data showing LYVE-1 staining of blood vessels (Fig. 5.3A, B), suggest that LYVE-1 may stain structures other than lymphatic vessels.

![Image](image.png)

Figure 5.3. Blood vessel stained with LYVE-1 (A) not Prox 1 (B). This vessel was not well perfused at the time of i.v. lectin injection and subsequent perfusion fixation, and thus remains packed with RBCs. Scale-bar: 30mm.

Table 5.2. Co-localization of various vascular and lymphatic markers

<table>
<thead>
<tr>
<th>Markers</th>
<th>Total</th>
<th>Tumor margin</th>
<th>Tumor tissue</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYVE-1 + MECA-32</td>
<td>181/304</td>
<td>125/170</td>
<td>46/110</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LYVE-1 + Prox 1</td>
<td>70/317</td>
<td>53/188</td>
<td>14/105</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>LYVE-1 + Lectin</td>
<td>30/200</td>
<td>19/170</td>
<td>6/67</td>
<td>NS</td>
</tr>
<tr>
<td>Ferritin + LYVE-1</td>
<td>6/28</td>
<td>6/7</td>
<td>0/21</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ferritin + MECA-32</td>
<td>8/23</td>
<td>6/7</td>
<td>2/16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ferritin + Prox 1</td>
<td>4/23</td>
<td>4/7</td>
<td>0/16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ferritin + Lectin</td>
<td>1/22</td>
<td>1/2</td>
<td>0/15</td>
<td>NS</td>
</tr>
</tbody>
</table>

Numerator is the number of vessels with co-localization of the indicated markers; denominator is the total number of vessels analyzed in that region (margin vs. tumor).

* P-value based on Chi-Square test comparing tumor tissue to the tumor margin. NS: not significant.
**VEGF-C overexpression does not reduce IFP.**

To correlate structure with function, we performed four functional assays. First, we measured interstitial fluid pressure (IFP) in VEGF-C overexpressing and control T-241 fibrosarcomas. There is no difference in IFP in VEGF-C overexpressing and MT tumors, although the tumors exhibit higher IFP than normal tail tissue (Fig. 5.4A). The universal finding that tumors have elevated IFP suggests that tumors lack functional lymphatics (237). IFP, however, like molecular clearance rate (118), depends on many parameters besides lymphatic function, including blood vessel permeability, microvascular pressure, and tissue hydraulic conductivity (21, 237). Thus, to analyze functional lymphatics directly, we performed three different microlymphangiography assays. Lymphangiography involves injecting labeled macromolecules into the interstitium for uptake into lymphatics and provides microscopic demarcation of functional draining lymphatics, as demonstrated in normal tail (Fig. 5.4B) (191). Specifically, fluorescence microlymphangiography was performed by injecting tetramethylrhodamine dextran (2M MW; red) into the tumor (MPLSM) or dermis distal to tumor (epifluorescence microscopy) at low pressure. Ferritin microlymphangiography was performed according to published methods (113), and angiography was performed via intravenous injection of FITC dextran (2M MW; green) (191) (Chapters 3 and 4).

*Functional intratumor lymphatic vessels are not found in VEGF-C overexpressing tumors imaged by MPLSM.*

Multiphoton laser-scanning microscopy (MPLSM) allows three-dimensional, high-resolution imaging of blood and lymphatic vessels at depths extending 400 \( \mu \text{m} \) below the
surface of the skin (191, 199) (Chapter 4) (Figs. 5.4C,D). VEGF-C increases the diameter of functional lymphatics in the tumor margin by 26% compared to mock-transduced controls (Fig. 5.4E) in a tumor size-independent manner (p>0.05). VEGF-C was previously shown to qualitatively increase the size of peri-tumor LYVE-1 stained vessels (118). No intratumor lymphatics are observed below 260 μm from the surface of the skin, in accord with our previous report (113). In fact only three intratumor lymphatics (ITLs) are found at all. One ITL per tumor is found in 3 of 14 tumors at depths of 160 microns (MT), 200 microns (VEGF-C), and 260 microns (VEGF-C). Blood vessels are clearly visible at these depths using MPLSM. Importantly, VEGF-C overexpression does not induce functional intratumor lymphatic vessels. The virtual absence of lymphatic function between 100 and 400 μm into these fibrosarcomas indicates that any deeper structures would not connect to the functional lymphatic network outside the tumor.

Tumor margin lymphatics are pre-existing. Next, using epifluorescence microscopy, we discerned the architecture of the functional lymphatic network in the tumor margin of T-241 fibrosarcomas. Strikingly, superficial lymphatics maintain the approximate hexagonal pattern observed in normal dermis (Figs. 5.4B, F), suggesting that they are pre-existing lymphatic vessels of the normal tissue overlying the tumor. However, tumor VEGF-C overexpression increases the diameter of lymphatics in the tumor margin (Fig. 5.4G), consistent with the MPLSM results (Fig. 5.4E) and studies of VEGF-C overexpression in the skin (63).
Figure 5.4: Evaluation of tumor lymphatic function. (A) Interstitial fluid pressure (IFP) is elevated in T-241 fibrosarcoma tissue compared to normal tail, but VEGF-C overexpression does not alter IFP. (B) Epifluorescence lymphangiography of normal tail showing hexagonal pattern of lymphatic network. (C, D) Maximum intensity projection of MPLSM images from combined angiography and lymphangiography in T-241 fibrosarcoma. Functional lymphatics (red, Ly) and nearby blood vessels (green, BV) are shown at tumor margin. (E) Functional tumor margin lymphatics (TMLs) in VEGF-C
overexpressing T-241 fibrosarcomas have larger diameters compared to those in control tumors as determined using MPLSM. (F) Epifluorescence lymphangiography shows functional lymphatic vessels in the margin of T-241 fibrosarcoma with a distorted, yet somewhat normal hexagonal network, suggesting that they are pre-existing vessels, not newly formed. (G) Functional TMLs in VEGF-C overexpressing T-241 fibrosarcomas have larger diameters compared to control tumors as determined using epifluorescence microscopy. Error bars show SEM. Scale-bars: B: 250µm; C, D: 100µm; F: 500µm. (See Chapter 4 for explanation of the discrepancy between the values of lymphatic diameters shown in (E) and (G).)

*Functional intratumor lymphatic vessels are not found in VEGF-C overexpressing tumors detected by ferritin microlymphangiography.*

Lastly, we performed ferritin microlymphangiography (113) to probe deeper than 400 µm into the tumor, and subsequently stained for LYVE-1, Prox 1, MECA-32, and intravenous lectin (Figs. 5.2A-C), the first time these techniques were combined. Given that only lymphatics near the injection site and subsequent drainage areas can be identified by this method, we focused our experiments to detect potential intratumor lymphatics by injecting deeply into the tumor. Ferritin staining in VEGF-C overexpressing and mock-transduced tumors does not co-localize with LYVE-1 in any of the 14 fibrosarcomas stained, again suggesting that LYVE-1 positive structures within these tumors are not functional lymphatics (Table 5.2, Fig. 5.5A). In contrast, ~85% of ferritin staining co-localizes with LYVE-1 and MECA-32 in both the tumor margin and in normal tissue, showing that lymphatics in these areas are functional. Ferritin appears
to travel through non-endothelialized channels and accumulates in the skin, distant from the injection site, where it is absorbed by functional tumor margin lymphatics (Fig. 5.5B) and transported through the lymphatic system. Similarly, the pattern of fluorescent dextran in the MPLSM experiments shows that the dextran was mostly moving through tissue matrix, not through vessels. Individual tumor cells are outlined by fluorescence, suggesting that drainage of the tumor occurs through the matrix and that the dextran is available to be absorbed by lymphatics if present. It is possible that the scant numbers of ITLs observed by MPLSM are not lined by endothelium, but are preferential fluid channels instead. From our MPLSM data alone, we cannot make any conclusions about the presence or absence of endothelium in the ITLs. As expected, a fraction of the interstitial ferritin is absorbed by blood vessels, represented by 12.5% of ferritin co-localizing with MECA-32 without LYVE-1. Taken together, these data suggest that i) intratumor transport of injected contrast agent does not occur through lymphatic vessels, but rather through preferential pathways in the interstitium lacking endothelial lining (19); and ii) LYVE-1 alone cannot be used to detect functional lymphatics.

Figure 5.5. (A) Ferritin (deep blue) travels through non-endothelialized preferential channels within the tumor, not through functional lymphatic vessels. MECA-32 co-stain is not present. (B) Ferritin dye (deep blue) accumulates in surrounding dermis after
intratumor injection of ferritin. Some TML vessels (brown LYVE-1 stain) have absorbed ferritin. Scale-bars: A: 30mm, B: 300mm.

Spontaneously arising tumors also lack intratumor functional lymphatics.

We also performed ferritin lymphangiography and immunohistochemistry on five tumors (4 adenocarcinomas and 1 fibrosarcoma) arising spontaneously in aged C3H/Sed mice to compare our results from tumor xenografts. In these tumors the density of LYVE-1 staining is $5.2 \pm 2.8$ structures/mm$^2$ and the functional vascular density is $317 \pm 107$ structures/mm$^2$ based on lectin staining. The tumors consist of multiple lobules separated by large connective tissue tracts containing vessels. LYVE-1 staining is commonly observed in the connective tissue tracts inside of the tumor, but these structures do not take up ferritin after intratumor microlymphangiography (Fig. 5.6A,B). The IFP in these tumors is elevated (10.5±1.7 mmHg), corroborating the lack of functional lymphatic drainage from the tumor as determined by ferritin microlymphangiography. Furthermore, LYVE-1/Prox 1- positive structures in two human breast cancer cell lines (MDA-MB-231 and MDA-MB-435S) (Fig 5.7) grown orthotopically in mice also do not co-localize with ferritin within the tumor, however they do in the tumor margin (152).
Figure 5.6: Evaluation of spontaneous tumors arising in aging mice. A) LYVE-1 stain of tumor showing LYVE-1 positive structures in fibrous tissue between tumor lobule with no LYVE-1 stain within the tumor lobule. No co-localization with intratumorally injected ferritin is seen in any similar structures, suggesting that these structures do not collect fluid or macromolecules from the tumor lobules. B) Adjacent section stained for lectin, showing significant perfusion of both the tumor lobules as well as the interlobular connective tissue. There is no co-localization of lectin and LYVE-1 seen in this section. Scale-bar: 300 μm.
Figure 5.7. Ferritin (highlighted in red), was used to trace the drainage patterns (113) of MDA-MB-231 mammary carcinoma grown orthotopically. Although LYVE-1 (black arrows) stains various structures within these tumors, ferritin is not present in these structures. Similar results are found within MDA-MB-435S mammary carcinomas. However, ferritin is found in LYVE-1 stained structures in the margin and normal tissue surrounding these tumors (data not shown). Scale bar: 100 μm.

LYVE-1, IFP, lymph node metastasis, and survival do not correlate in human lung tumors.

Finally, we measured IFP in lung tumors in a cohort of patients between 1992 and 1993 prior to surgical resections with the approval of the Massachusetts General Hospital Institutional Review Board. The surgical specimens were stained for LYVE-1, Prox 1, and CD31. The patients had a variety of pre- and post-surgical treatments, tumor stages, and clinical outcomes (Table 5.3). Human lung tumors are known to metastasize to lymph nodes, but the alveolar walls of the lung are devoid of lymphatics. This suggests that the draining lymphatics associated with bronchial vascular bundles, pleura, and lobular septa are responsible for metastatic dissemination. Twelve of these 29 patients (14 adenocarcinomas, 10 squamous cell carcinomas, 3 bronchioloalveolar carcinomas, 1 large cell carcinoma, 1 small cell carcinoma), had lymph node metastasis. The average post-surgical survival was 4.4 ± 0.7 years (Table 5.3). Only 22 of 29 total patients in the study had valid IFP measurements. The average tumor IFP in these patients is elevated (9.5 ± 1.6 mmHg), with squamous cell carcinomas (12.8 ± 2.8 mmHg) having a higher IFP than adenocarcinomas (6.4 ± 0.9 mmHg; p < 0.05). IFP is elevated in each tumor
measured, but there is no difference in IFP in patients that have lymph node metastasis compared to those that do not (Fig. 5.8A) in contrast to a recent animal study (238). There is no correlation between IFP and patient overall survival (P = 0.663). In tissue sections that were subsequently stained with LYVE-1, there are no positively stained structures beyond 500 microns from the tumor margin in any tumor. Only 5 tumors exhibit LYVE-1 positive structures in the tumor margin (defined as ~500 μm from the tumor edge due to difficulties in exactly identifying the tumor edge), and none exhibit staining in the bulk of the tumor. Additionally, in a few cases LYVE-1 marks capillaries in the alveolar walls, which do not possess lymphatics, as well as endothelial cells of an occasional artery and vein, confounding the interpretation of this stain (Fig. 5.8B). We therefore cannot conclude with any confidence that LYVE-1 positive structures in the tumor margin are indeed lymphatics. CD31 staining, however, shows preservation of alveolar capillaries deep within these tumors, particularly those with bronchioloalveolar growth patterns (Fig. 5.8C). The salient feature of the bronchioloalveolar pattern is the maintenance of the alveolar organization as the tumor grows along the alveolar scaffolding. There is no correlation with the LYVE-1 staining and IFP, lymph node metastasis, or patient overall survival (Table 5.3). Patient overall survival was compared based on log-rank test of the Kaplan-Meier estimator survival analysis. Only patients who had surgery alone are included in the analysis. Prox 1 stain is not observed in any of the tumors. The ability of these tumors to metastasize to lymph nodes without LYVE-1 positive structures in the tumor draws into question the utility of LYVE-1 as a clinical marker in human lung tumors. LYVE-1 staining, however, has been correlated to metastasis in murine models (116, 122), and in oropharyngeal cancers in humans, but not in other head and neck cancers (154) (Table 2.2). LYVE-1, therefore, may have some clinical utility in specific circumstances. However, these data suggest that human lung
tumors do not contain functional lymphatics and that LYVE-1 staining is not clinically predictive in this setting.

![Graph showing IFP and lymph nodes](image)

Figure 5.8. A) Interstitial fluid pressure (IFP) in tumors is not significantly different in patients with disease positive lymph nodes compared to patients with disease negative lymph nodes. These patients did not receive treatment prior to IFP measurement. B) LYVE-1 IHC of lung showing capillaries of the alveolar wall staining with LYVE-1. This is only seen in a few of the cases studied. Arrow shows LYVE-1 marking a lymphatic in the wall of a bronchus. Arrowheads show LYVE-1 marking alveolar capillaries in normal lung. C) These alveolar capillaries also stain for CD31, as expected.
Table 5.3. Patient Characteristics

<table>
<thead>
<tr>
<th>TNM stage</th>
<th>Histology</th>
<th>EBP (mmHg)</th>
<th>Survival (months)</th>
<th>Treatment*</th>
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<td>8</td>
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*: Sx=surgical resection, RT=radiation therapy, Cx=chemotherapy
1: These tumors have LYVE-1 staining within 500 microns of normal lung. They were classified as T1 by Masson's method. These structures were Prox 1 negative.
2: Tumor was alive as of February, 2002.
3: Bronchioloalveolar cell carcinoma
4: Patient was lost during follow-up period.

The mechanisms underlying the lack of lymphatic function in tumors are not understood. It is possible that i) the primary valve structure (24) is lacking in the LYVE-1 positive structures in tumors, preventing effective fluid uptake and lymphatic function; ii) the mechanical forces generated by the growing tumor cells collapse lymphatics rendering them non-functional (180) (Chapter 6); or iii) invading tumor cells destroy the lymphatic network leaving only remnant endothelia inside the tumor.
Regardless of the etiology for the lack of functional lymphatics in tumors, we propose that functional lymphatics in the tumor margin are sufficient for lymphatic metastasis, since i) increases in functional lymphatic surface area (and thus more opportunity for cancer cell intravasation) are accompanied by increases in lymphatic metastasis in VEGF-C overexpressing tumors (Fig. 5.4E, Table 5.1) (121); and ii) tumors that lack intratumor LYVE-1 staining still metastasize (Table 5.3) (121). The increase in LYVE-1 staining in VEGF-C overexpressing tumors (Fig. 5.2D, E) (116-118) suggests that VEGF-C may act as a survival factor (239) for the endothelial cells of lymphatic vessels compressed or destroyed by the growing tumor (18, 19, 113, 180) (Fig. 5.2F) and as a mitogen for lymphatic endothelial cells in the tumor margin. The ability of VEGF-C to i) serve as a survival factor for lymphatic endothelial cells; ii) induce lymphatic metastases by increasing the functional lymphatic surface area in the tumor margin; and iii) induce angiogenesis in some tumors (66, 118), makes VEGF-C an ideal target for controlling both tumor growth and metastasis.

Since cancer cells escape a tumor via two primary routes—blood vessels and lymphatic vessels—to establish distant metastases, it seems reasonable to hypothesize that blocking the growth of new blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis) will inhibit hematogenic and lymphogenic metastases, respectively. An impressive array of pre-clinical studies has demonstrated prevention as well as suppression of hematogenic metastases by anti-angiogenic and anti-vascular approaches. Whether anti-lymphangiogenic and anti-lymphatic approaches will yield similar results for lymphogenic metastases remains to be seen. Both vascular endothelial growth factor
(VEGF) -C and -D are known to induce angiogenesis(66, 122) and lymphangiogenesis(36, 114, 121, 122) in tumors and to be associated with lymphogenic metastasis in a variety of human tumors(76). Stacker et al. (122) present the first direct evidence for the prevention of lymphatic metastasis from a tumor grown in the mammary fat pad by blocking VEGF-D. Similarly, He et al. (153) report similar findings in subcutaneously grown tumors that lack VEGF-D by blocking VEGF-C. While Stacker et al. (122) use a blocking antibody against VEGF-D, He et al. (153) use a receptor-body (VEGFR-3-Ig fusion protein) that can trap both VEGF-C and -D. The receptor-body is generated in vivo either by cancer cells engineered to secrete soluble VEGFR-3-Ig or by the liver infected by an adenovirus expressing VEGFR-3-Ig. In both studies, treatment is initiated within a day after tumor implantation, before the primary tumor becomes established, and lymph nodes were examined for metastatic lesions ~4-6 weeks later. Blocking VEGF-C or -D suppresses lymphangiogenesis associated with the primary tumor as well as regional lymph node metastasis. These findings raise many important questions about the biology and potential treatment of lymphatic metastases.

First, how do VEGF-C and -D alter tumor biology to promote lymphatic metastasis? The work of He et al. (153) employs an ectopic (subcutaneous) model of a lung tumor and found an association between lymphatic vessel density (measured as LYVE-1 positive structures) and lymph node metastases, in one of two tumor lines. The data presented in this chapter (114) employ orthotopically grown tumors engineered to overexpress VEGF-C and find an increased diameter of functional lymphatic vessels in the tumor margin and a greater number of lymph node metastases when compared to mock transduced controls.
Thus, by increasing the surface area of lymphatic vessels, VEGF-C and -D increase the opportunity for metastatic spread through lymphatics. VEGF-C and -D may also serve as a survival factor for endothelial cells of newly formed or co-opted lymphatic vessels in a tumor (36). Furthermore, He et al. (153) show that targeting VEGF-C only inhibits lymphatic metastasis without affecting hematogenic lung metastasis. Some investigators have recently proposed that lung metastases from VEGF-C overexpressing breast tumors could be secondary to lymph node metastases (116). These data do not support this hypothesis. The differential regulation of lymphogenic versus hematogenic metastases by VEGF-C and -D may be a function of the proteolytic processing of these molecules (36), which in turn may be governed by host-tumor interactions (76).

Second, what is the clinical evidence to support the use of lymphatic markers to predict lymphatic metastasis? Both VEGF-C and VEGF-D are associated with increased lymphatic metastasis in some human tumors (76). On the other hand, there is a lack of correlation between lymphatic metastasis and the density of a variety of lymphatic markers for a number of human malignancies (Table 2.2), including human lung cancer presented in this chapter. However, many animal studies show a positive correlation between the density of lymphatic markers and lymphatic metastasis (114, 116, 121, 122, 153). Some studies have made claims that intratumor lymphatic vessel identified by LYVE-1 in clinical specimens are necessary for lymphatic metastasis (154, 155), but the data do not support these conclusions (discussed in Chapter 2). Although in certain instances, LYVE-1 staining may correlate with lymph node metastasis, the majority of
the data indicate that intratumor LYVE-1 staining is not a requirement for lymph node metastasis to occur.

Is this discordance between pre-clinical and clinical findings a result of the limitations of the animal models used or non-specificity of current lymphatic markers? Is it possible that the currently available markers are unable to distinguish between functional and non-functional lymphatics (Fig 5.7) (114) leading to these contradictory results? The work presented in this chapter on human lung cancer show that lymphatic metastasis can occur in tumors that lack intratumor functional lymphatics, suggesting that functional lymphatics in the tumor margin are responsible for lymphatic dissemination (114). Therefore, microlymphangiography in patients coupled with lymphatic identification by molecular markers is needed to provide clearer insight into lymphatic metastasis and prevention.

Third, how can anti-lymphangiogenic therapy be translated to the clinic? The work presented by He et al. (153), as well as the work of Stacker et al. (122) clearly show that lymphatic metastasis can be prevented by anti-VEGF-C and -D therapy given prior to the establishment of the primary tumor. However, by the time most tumors are detected clinically, they are already established and metastatic cells may have already spread to the nearby lymph nodes or distant organs. Therefore, pre-clinical studies that initiate treatment after the primary tumor is established and lymphatic spread has occurred are now needed to build the basis for clinical trials of anti-lymphangiogenic therapy. Moreover, additional molecular players, other than VEGF-C and -D, may be involved in
determining the clinical end-points. After all, metastasis is a multi-step process (235), in which cancer cells must detach from their neighbors, invade the surrounding extracellular matrix, enter the blood/lymphatic vessels, survive in the lumen, attach at a distant site, extravasate, migrate, proliferate and recruit new vessels. It is likely that VEGF-C and -D are key players for some tumors and not others. As the techniques for detection of various proteolytically processed forms of VEGF-C and -D become widely available, we may be able to pre-select tumors that are VEGF-C or -D dependent and design appropriate interventions. The future of cancer treatment may involve a cocktail of agents that block the different steps of metastasis and are tailored to individual patients based on their proteomic profile.
Chapter 6: Solid Stress in the Lymphatic

Pathophysiology of Tumors

Data in this chapter have been collected in collaboration with Brian Stoll.
**Introduction**

Although recent discoveries of molecular regulators and markers of lymphatic vessels have advanced the field (Chapter 2), the etiology of the lack of functional lymphatic vessels in tumors has not yet been determined. In experiments that combined both functional and molecular assays for lymphatic vessels in tumors that overexpress VEGF-C, no functional lymphatic vessels are found in the tumor (Chapter 5) (114). This finding suggests a non-molecular cause for the absence of functional lymphatics in tumors. Many hypotheses can be generated to explain the etiology of the lack of functional lymphatics in tumors including the dysfunction of lymphatic endothelial microvalves, the absence of lymphatic endothelial cell anchoring filaments, and the collapse of lymphatic vessels caused by compressive mechanical forces.

In superior vena cava (SVC) syndrome, tumor growth compresses the SVC causing vast edema of the head and neck(240, 241). This condition becomes life threatening when venous return from the head and neck is completely obstructed. The ability of a tumor to exert enough force to collapse the SVC suggests that a large amount of solid stress is generated in a growing tumor. The generation of solid stress by growing tumors has also been predicted by various mathematical models (183-187). We hypothesize that the solid stress may also act on the microvasculature of tumors, causing the tumor associated lymphatic vessels to collapse and become non-functional (113, 114) (Specific Aim 2; Chapter 2).
Tumor growth depends on the balance between tumor cell proliferation and death (242). As the balance shifts toward tumor cell proliferation, the tumor increases in size. The increase in cell number, when confined to a limited space, leads to an increase in solid stress in the tumor. Descriptively, this can be explained by the fact that more cells are occupying a limited space and each cell is pushing on its neighbors. In spheroids grown in vitro, this force is calculated to be between 45-120 mmHg (180). This compressive force may then induce the collapse of vessels, both blood and lymphatic, which would alter the local microenvironment. Blood vessel collapse would lead to reduced perfusion causing reduced drug delivery and ischemia. Lymphatic collapse would lead to increased IFP, which also would limit drug delivery. These pathophysiologies are observed in solid tumors, lending support to this hypothesis.

Solid stress is the force transmitted by the solid components of a tissue, mostly the cells and extracellular matrix. Analytically, soft tissue is modeled mechanically as an elastic or poroelastic material (183-186, 243), allowing for force generation in both the solid (stress), including cells and extracellular matrix, and fluid (pressure) phases. In order to model a growing tumor, a balance is made between the solid forces expanding outward and the surrounding tissue pushing inward. In addition, the density of the solid component relative to the fluid phase increases to accommodate the growing tissue. The reduction in fluid space further increases resistance to convection, making solid stress a potential direct barrier to drug delivery and contributor to the flat radial pressure profiles predicted and measured in tumors (15-17).
Most studies involving solid stress have been carried out in the orthopedic setting, where the forces generated have been shown to alter tissue remodeling (244-246) and cellular mechanotransduction (180, 182, 247-249). However the effect of solid stress on soft organs has been largely uninvestigated, leaving a potentially important signaling and physiological parameter unexplored. Tools with which to manipulate and measure solid stress are also lacking. In in vitro models of spheroid growth, gels with greater stiffness, which was presumed to lead to higher solid stress in the spheroid, inhibited spheroid growth (182). This suggests a non-vascular component of the response of tumors to solid stress. Solid stress induced cell signaling has not been well studied and the relationship between solid stress and cell proliferation is unknown. In an experiment in which mammary carcinomas were treated with taxane, tumor cell apoptosis is observed, lowering the cell density (181). This was accompanied by an altered vascular morphology with a greater number of vessels with open lumen (181). When the tumor regrew, vessels again appear to be collapsed. However, taxane can also kill host stromal and endothelial cells in the tumor, which slightly confounds the interpretation of these data.

In order to selectively kill only tumor cells without affecting host stromal or endothelial cells, we treated mice growing tumors of human origin with Diphtheria toxin (DT). DT has been previously used to selectively kill cancer cells of human origin (197). DT catalyzes the ADP-ribosylation of eukaryotic aminoacyl transferase II (EF2) using NAD as a substrate, thereby inhibiting protein synthesis. This reaction forms the basis of its toxicity toward eukaryotic cells. The DT receptor (DTR) is the transmembrane form of
the heparin-binding epidermal growth factor-like growth factor (HB-EGF). The DT binding site is in the EGF-like domain but alterations in several amino acids in this domain render the ability of DT to bind to murine HB-EGF substantially reduced as compared to human HB-EGF. Therefore cells of murine origin show no toxicity to DT, whereas DT causes apoptosis in cells of human origin. In this chapter, DT is used to reduce solid stress in experimental tumors of human origin grown in mice and the subsequent changes in lymphatic vessel morphology are quantified using intravital and histological microscopy.

Material and Methods

*Tumor cell lines:* HSTS26T human soft tissue sarcoma (250, 251), and T-241 murine fibrosarcoma (114) were grown subcutaneously in nude and SCID mice according to standard techniques (Chapter 3). Tumors for immunohistochemistry were grown in the flank. Tumors for intravital imaging were grown in the hindlimb and tail. Since HSTS26T is of human origin, DT caused apoptosis of these cells. Since T-241 is of murine origin, DT did not cause apoptosis of these cells and was used as a negative control.

*Diphtheria Toxin administration:* 1 μg of DT was given in 0.30 μl of PBS intraperitoneally (197). For intravital imaging, DT was administered after the first imaging session. For IHC analysis, DT was administered 1, 3, 5, or 7 days prior to perfusion fixation and tumor resection. Perfusion fixation with 4% paraformaldehyde was performed according to previously described techniques (Chapter 3). 0.30 μl of PBS was
given intraperitoneally as control. Animals were randomly assigned to DT treatment or control groups.

*Immunohistochemistry*: IHC for LYVE-1 (antibody a generous gift from E. Rouslahti), MECA-32 (BD Biosciences Pharmingen, San Diego, CA), apoptosis (TUNEL; ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, Intergen, Purchase, NY), proliferation (PCNA; N1529, DAKO corporation, Carpinteria, CA), functional lymphatics (ferritin lymphangiography) and functional blood vessels (biotinylated *L. esculentum* lectin; Vector Laboratories, Burlingame, CA) was performed according to published methods (Chapter 3) (114).

*Intravital Multiphoton Laser Scanning Microscopy (MPLSM)*: Intratumor lymphangiography was performed using tetramethylrhodamine fixable dextran (2 million MW) according to described methods (Chapter 3). MPLSM was used to image lymphatic vessels at depth in tumor tissue according to previously described techniques (Chapter 3) (114, 191).

*Image analysis*: Images were opened in NIH image and calibration performed. For MPLSM images, the diameter of lymphatic vessels was measured in each segment after a junction and approximately every 200 microns in longer vessels. For IHC images, the cross-sectional area, perimeter, major axis and minor axis of LYVE-1 positive structures were measured in randomly selected areas that contained LYVE-1 positive structures. Of the vessels measured, a distinction was made whether the vessel had an open lumen or closed (compressed) lumen. Regions of interest (ROI) (oval 150 by 100 μm oriented
appropriately for the LYVE-1 positive structure) were drawn around randomly selected vessels and the number of cells in the ROI was counted. TUNEL positive nuclei were counted as a percentage of total nuclei in non-necrotic tissue. PCNA positive/MECA-32 positive nuclei were counted in selected vessels. In all cases, the investigator performing the quantification was blinded to the treatment group.

Statistical Analysis: A Student t-test was performed on all comparisons. Satterthwaite’s correction was used where appropriate as determined by F-tests or ANOVA. Statistical significance was assigned when $p < 0.05$. All values are reported as mean ± SEM. $N =$ the number of animals per treatment and/or time. $n =$ the number of vessels per treatment and time.

Results

HSTS26T tumors regress in response to DT treatment

T-241 tumors used for intravital MPLSM were treated with DT at an average size of 258 ± 44 mm$^3$ and continued to grow (Figure 6.1a). Similarly, T-241 tumors treated with DT used in IHC analysis continued to grow (data not shown). In contrast, HSTS26T tumors were treated with DT at an average size of (290 ±84 mm$^3$) and each tumor was smaller 2 and 5 days after treatment (Figure 6.1b). However the tumors appeared to regrow 7 days after DT treatment. HSTS26T tumors treated with DT for IHC analysis also regressed (data not shown).

HSTS26T tumors treated with DT show more TUNEL positive nuclei
To assess the affect of DT on tumor cells of human origin, the fraction of apoptotic nuclei to total nuclei was assessed by TUNEL. The TUNEL stain identifies potential apoptotic nuclei, which are then considered apoptotic if the nucleus showed characteristics of apoptotic morphology, such as nuclear condensation and blebbing. The percentage of apoptotic nuclei (0.24±0.048% apoptotic) is not different in the liver of DT and PBS treated mice (N=3, n=5; p > 0.05). Similarly, the number of apoptotic nuclei (1.06±0.10% apoptotic) is not different in T-241 tumors 5 days after treatment with DT or PBS control (N=5, n=2; p > 0.05). In HSTS26T tumors, the number of TUNEL positive nuclei is greater in tumors treated with DT compared to PBS control on days 3, 5, and 7 (Figure 6.1c).

![Graphs showing tumor size and apoptotic index](image)

Figure 6.1. Growth response to Diphtheria Toxin. A) T-241 murine fibrosarcoma has no growth response to DT (N=7). B) HSTS26T human sarcoma regresses slightly during 5 days after DT treatment (N=7). C) Apoptotic index is increased in DT treated HSTS26T human sarcomas (N=5, n = 8-10).

*Functional lymphatics in the margin of HSTS26T tumors increased in diameter*
MPLSM with intratumor fluorescent lymphangiography was used to image functional lymphatic vessels in the margin of tumors (Figure 6.2). There is no change in the average diameter or distribution of diameters of functional lymphatics in the tumor margin of T-241 fibrosarcomas (Figure 6.3a,c). HSTS26T tumors treated with DT have a larger average diameter 2 days after treatment (57.3 ± 1.3 μm) compared to the average diameter just prior to treatment (52.1 ± 1.6 μm) (p < 0.05) (Figure 6.3b). The average diameter of tumor margin lymphatics (TML) on day 5 and day 7 fall in between the day 0 and day 2 average diameter and are not statistically different from either (p > 0.05). The distribution of TML diameters after DT treatment is shifted to the right with a reduction in the number of small diameter TMLs (Figure 6.3d).
Figure 6.2. Functional tumor margin lymphatics of a HSTS26T imaged with intravital MPLSM. The functional lymphatic vessels retain the somewhat hexagonal architecture seen in normal dermal lymphatics. Scale bar = 100 μm.

Figure 6.3. Quantification of functional lymphatics in the margin of T-241 or HSTS26T tumors imaged with MPLSM. A) No change in the average diameter of TML of T-241
tumors (N=7). B) On day 2 after DT treatment, the average diameter of TML of HSTS26T tumors is smaller (N=7). Average diameters on day 5 and 7 are not statistically different from day 0 or day 2. C) Histogram of average diameter of TML of T-241 tumors showing no change after DT treatment. D) Histogram of average diameter of TML of HSTS26T tumors showing a reduction in the number of smaller lymphatic vessels after DT treatment.

*LYVE-1 positive structures inside HSTS26T tumors treated with DT have a more circular cross-section and have an increased fraction with open lumen*

Sections of HSTS26T tumors resected 1, 3, 5, or 7 days after treatment with either DT or PBS were stained anti-LYVE-1 Ab. A greater percentage of LYVE-1 positive structures inside of HSTS26T tumors (defined as greater than 50 microns from the edge of the tumor) have open lumen after treatment with DT when compared to PBS control on days 1, 5 and 7 (Figure 6.4a,b,c). Similar data looking at blood vessels show more blood vessels with open lumen on days 5 and 7, but not on days 1 and 3 (personal communication with Brian R. Stoll). The results of LYVE-1 positive structures on day 1 are not expected, particularly in light of the day 3 data from LYVE-1 positive structures and the data from blood vessels. There is also no significant difference in tumor cell apoptosis on day 1 (Figure 6.1c). Thus there is no clear explanation for the finding of a significant difference in the fraction of LYVE-1 positive structures with open lumen on day 1 when DT and PBS treated tumors were compared.
Each LYVE-1 positive structure was outlined and the cross-sectional area, major axis and minor axis measured. The ratio of major axis to minor axis was calculated to quantify the shape of the cross-sections. A major/minor ratio of 1 equates to the measured structure being a perfect circle. The larger the major/minor ratio, the more compression of the structure occurs. The average major/minor ratio of LYVE-1 positive structures in HSTS26T tumors treated with DT is smaller than time matched HSTS26T tumors treated with PBS (Figure 6.4d). Endothelial proliferation assessed by the co-localization of PCNA and MECA-32 staining is not different between the DT treated and PBS control group (personal communication with Brian Stoll). There is no change in the percentage with open lumen or major/minor ratio of LYVE-1 positive structures in T-241 tumors 5 days after treatment with DT when compared to PBS control treatment.
Figure 6.4. DT treatment of HSTS26T reverses lymphatic collapse. A) HSTS26T tumor treated with PBS stained with anti-LYVE-1 Ab. B) HSTS26T tumor treated with DT stained with anti-LYVE-1 Ab. C) In HSTS26T tumors treated with DT, more LYVE-1 positive structures have open lumen compared to PBS treated controls. D) In HSTS26T tumors treated with DT, the cross-sections of LYVE-1 positive structures are more circular compared to PBS treated controls. Scale bar = 50 μm (A, B) (N=4-5).

LYVE-1 positive structures in the margin of HSTS26T tumors do not morphologically change with DT treatment

In HSTS26T tumors treated with DT, there is no change in the fraction of LYVE-1 positive structures with open lumen when compared to PBS treated control (Figure 6.5a).
Similarly, there is no difference in the major/minor ratio of LYVE-1 positive structures in the tumor margin based on DT or PBS treatment (Figure 6.5b). However, the fraction of LYVE-1 positive structures with open lumen in the tumor margin (0.65 ± 0.015) is significantly greater than in the tumor itself (0.33 ± 0.009; p < 0.05). The major/minor ratio is also significantly smaller in LYVE-1 positive structures in the tumor margin (5.9 ± 0.17) compared to inside of the tumor (6.9 ± 0.11; p < 0.05). This analysis combines LYVE-1 positive structures from all tumors studied, regardless of treatment type or time after treatment in order to show that the large decrease in solid stress in the tumor margin (186) dominates the decrease in solid stress due to DT treatment.

Figure 5.5. LYVE-1 positive structures in the tumor margin are not affected by DT treatment. A) There is no change in the fraction of LYVE-1 positive structures with open lumen in the margin of HSTS26T tumors when treated with DT. B) There is no change in the major/minor axis ratio in the margin of HSTS26T tumors when treated with DT (N=4-5).

*Cell density is reduced around LYVE-1 positive structures with an open lumen*
Regions of interest were drawn around randomly selected LYVE-1 positive structures in HSTS26T tumors treated with DT or PBS control. The number of cells in the ROI was counted and a comparison was made based on whether the LYVE-1 positive structure had an open lumen or not, regardless of the treatment received. LYVE-1 positive structures with an open lumen have a lower surrounding cell density compared to structures with a closed lumen (Figure 6.6a,b, c).

Figure 6.6. LYVE-1 positive structures in HSTS26T tumors with open lumen are surrounded by lower cellular density. A) LYVE-1 positive structure with open lumen and low surrounding cell density. B) LYVE-1 positive structure with collapsed morphology and high surrounding cell density. C) The cell density in HSTS26T tumors surrounding LYVE-1 positive structures with open lumen is lower than the cell density surrounding collapsed LYVE-1 positive structures. Scale bar = 25 μm (A, B) (N=34).
Functional lymphatics are not found in DT treated HSTS26T tumors

As previously reported, functional lymphatic are not found in T-241 tumors, but are found in the tumor margin (114). In this study, ferritin microlymphangiography and intravital fluorescence lymphangiography were used to detect the presence of intratumor functional lymphatics (Chapter 3). As expected, intratumor functional lymphatics are not found in T-241 tumors, even after DT treatment. Surprisingly, functional lymphatics are also not found in HSTS26T tumors after DT treatment in either assay, even though more LYVE-1 positive structures have open lumen.

Discussion

The inability to observe functional lymphatics in tumors, both in patients and animal models, has been documented for the better part of a century (18, 19, 113, 114). To date, there are no known explanations for this phenomenon. Many hypotheses for the inability of functional lymphatics to exist in tumors involve molecular mechanisms (114, 252) (Chapter 5). However, tumors are heterogeneous and genetically unstable, with each tumor being driven by different molecular pathways. Thus the universal finding that tumors lack functional lymphatic vessels and have elevated IFP (12, 17) make single molecular alterations leading to this phenomenon unlikely. Therefore, we looked for properties of tumors that are found across all tumor types and genetic backgrounds.

Clinically, there are indications that tumors generate mechanical forces. In SVC syndrome, a tumor collapses a large vein(240, 241) initially causing edema of the head
and neck, but eventually compromising the cardiovascular system. Spinal cord compressions occur when a tumor pushes into the spinal cord and compromises neurologic function (253-255). Both of these clinical scenarios are poignant and life-threatening examples of the capacity of tumors to generate compressive mechanical forces. We hypothesize that the solid stress generated by growing tumor cells can act on the microvasculature as well, causing the collapse of lymphatic vessels and rendering them non-functional.

The data presented in this chapter provide insight into this possibility. Previous data show that by killing tumors cells, as well as host stromal and endothelial cells, with taxane, blood vessels increase their diameter (181). To avoid the death of stromal and endothelial cells, we used a novel approach which relied on the relative potency of DT on human versus mouse cells. By growing tumors of human origin in mice, tumor cells can be killed specifically, while not affecting host cells (197). The loss of tumor cells reduces the tumor solid stress, while leaving the host vessels intact to respond to the reduction in stress. However, it is possible that by killing tumor cells, there will be a local reduction in the production of endothelial cell growth and survival factors, such as VEGF and VEGF-C. This may destabilize and potentially destroy the tumor vasculature, and needs to be considered in this analysis.

After DT treatment of HSTS26T tumors, more vessels with open lumen are observed when compared to PBS control treatment (Figure 6.4). The ability of these structures to open suggests that they are still tethered to the surrounding tumor tissue, possibly with
anchoring filaments intact (20, 21) (Figure 2.2). Thus, the collapsed lymphatic vessels can be pulled open as solid stress is released and the tissue relaxes. This idea is supported by the local decrease in cell density that surrounds open vessels when compared to collapsed structures (Figure 6.5).

As lymphatic vessels open, one would expect these vessels to begin functioning. However, functional lymphatic vessels are not observed inside DT treated tumors. One explanation is that the entire length of the vessel may not be patent. Although in local regions, the vessels may be surrounded by a lower cell density and become patent, in other regions, the cell density may remain high and keep the same vessels compressed. The reduction in cell density is heterogeneous throughout the tumors based on the dose of DT used, even though greater levels of cell death are seen in DT treated tumors (Figure 6.1e). This may be due to heterogeneous delivery of DT to the tumor or heterogeneous potency of DT on tumor cells. Other explanations for the lack of function could be the loss of normal lymphatic endothelial microvalves (24) and the lack of pulsatile motion from arterioles or muscle in tumors.

There are mathematical models predicting that mechanical forces can build up inside growing tumors. The models are based on an elastic model of tissue that takes into account the expansion of the proliferating tumor cells and the material properties of the tumor and surrounding tissue (183-185). These studies predict a uniform stress profile throughout the tumor when there is homogenous proliferation. In heterogeneous growth patterns, the solid stress is higher in regions undergoing cellular proliferation. These
models, however, do not account for the role of fluid pressure on solid stress. Recent work uses a poroelastic model to account for the contribution of IFP to solid stress (186). These papers demonstrate that solid stress has a compressive radial and circumferential solid stress component in the tumor, and a compressive radial and tensile circumferential stress in the surrounding normal tissue.

Based on the mathematical models, the magnitude of the compressive forces decreases at the tumor edge (186). Thus one would expect fewer collapsed vessels, and vessels with a more circular cross-section near the tumor edge. Data from HSTS26T tumors confirm this prediction, irrespective of treatment with DT or PBS, showing a two-fold increase in the fraction of LYVE-1 positive structures with open lumen in the tumor margin. Furthermore, LYVE-1 positive structures in the tumor margin are not morphologically different when treated with DT compared to PBS treatment. This is explained by the lower solid stress in the tumor margin, which means only a small reduction in solid stress in the tumor margin can occur with DT treatment when compared to the central portions. Thus DT treatment does not produce significant alteration of LYVE-1 positive structure morphology in the tumor margin. A slight difference, however, is seen in the diameter of functional lymphatic vessels in the tumor margin imaged with MPLSM. Intravital MPLSM imaging selects only functional vessels, not all LYVE-1 positive structures, and the DT induced reduction in solid stress allows the functional lymphatic vessels to dilate.

Since measurements of solid stress have not yet been made in tumors in vivo, data from in vitro measurements can be used to estimate the magnitude of solid stress expected in
tumors. One mode of solid stress generation in tumors is the packing of proliferating
cancer cells in a confined space. Cells, when unconfined, have a small elastic modulus,
but as a cell volume shrinks its deformability becomes limited by its cytoskeleton (256),
its maintenance of osmolality and the incompressibility of water. In vitro, an estimate of
the force of cells proliferating in a confined matrix is calculated as 45-120 mmHg (180),
based on the deformation of the stiff matrix. Furthermore, the forces generated during
unconfined cell division are on the order of 1 nN/cell – 1 µN/cell (257), which equates to
\(~0.02 – 23.8\) mmHg when normalized over the surface area of an average cell (\(~20 \) µm
diameter). Another mode of stress generation is from contractile forces of cells pulling on
the extracellular matrix. From in vitro data, the range of contraction forces of fibroblasts
and myofibroblasts is 1 nN/cell – 1 µN/cell (258-264), which again equates to \(~0.02 –
23.8\) mmHg when normalized over the surface area of an average cell. This contractile
stress in the tumor may be released upon tumor cell death. Based on these data, one
would expect an internal stress in the tumor on the order of 10 – 100 mmHg.

The critical stress needed to start collapsing a blood vessel is governed by the following
equation (265):

\[ \Delta \sigma_{\text{crit}} = \frac{Eh^3}{4(1-\nu^2)R^3} \]  \hspace{1cm} (6.1)

where \( \Delta \sigma_{\text{crit}} \) is the critical stress necessary to start collapsing a blood vessel, \( E \) is the
elastic modulus of the vessel wall, \( h \) is the thickness of the vessel wall, \( \nu \) is the Poisson’s
ratio, and \( R \) is the radius of the open vessel. The range of the critical stresses needed to
start collapsing a blood vessel is \( 0 – 100 \) mmHg (see Ph.D. Thesis of Brian R. Stoll). This
estimate is in the same range as the tumor solid stress predicted from in vitro data, supporting the mechanism of solid stress induced vessel collapse.

From equation (6.1), vessels that have thin walls and/or weaker vessel walls would be more prone to collapse. Also, vessels that become dilated are also prone to collapse. Lymphatic capillaries have thin and fenestrated basement membranes, suggesting that the critical stress for lymphatic vessels is smaller than that for blood vessels. Furthermore, lymphatics in the tumor margin are dilated (113), which further primes these lymphatics for collapse. In a measurement of the strength of the vessel wall, muscular bovine mesenteric lymphatic vessels were shown to withstand only about 24 mmHg before bursting (266), also in the lower end of the range of critical stresses for blood vessels. Furthermore measurements made on bovine mesenteric lymphatic vessels (\(R=2.8\) mm, \(h=0.1\) mm, \(E=4\times10^4\) dynes/cm\(^2\)) and canine thoracic ducts (\(R=3.0\) mm, \(h=0.05\) mm, \(E=2\times10^5\) dynes/cm\(^2\)), allow the calculation of the critical stress for lymphatic vessels (266). Based on these measurements, the range of critical stress for large lymphatic vessels is \(2.3\times10^4\) to \(4.5\times10^4\) mmHg. One would expect the critical stress of smaller lymphatic capillaries to be even smaller. Thus, based on the estimates of tumor solid stress and the critical stress for lymphatic collapse, it is very possible that mechanical forces generated by the tumor collapse lymphatic vessels.

Solid stress may also collapse blood vessels (see Ph.D. Thesis of Brian R. Stoll) (180, 186). The collapse of tumor blood vessels can lead to reduced perfusion, and with it, reduced drug and oxygen delivery, making treatment with standard therapies more
difficult. Furthermore, if the vasculature of an entire region were collapsed, an area of ischemia followed by necrosis would ensue, a common finding in tumors in cancer patients regardless of molecular background or origin of the tumor.

In treatment of a tumor, the goal is to selectively kill cancer cells without killing host tissues. It is therefore likely that during the course of therapy the solid stress in tumors in patients is also reduced. This reduction in stress may increase both blood flow and lymphatic function, and paradoxically lead to an increase in tumor dissemination. This can be demonstrated in patients who receive radiation therapy for non-disseminated disease and have a local tumor response, but later fail with distant disease (267, 268). Although, there are many hypotheses to explain this phenomenon, such as the reduction of tumor produced angiogenesis inhibitors after treatment (242), solid stress reduction may also play a role.

The role of solid stress in tumor pathophysiology has been largely unexplored. There are data showing that the growth rate of tumor cells is slowed by mechanical forces (180, 182), which suggests that solid stress may affect cellular signaling. Furthermore, solid stress in tumors may induce collapse of both blood and lymphatic vessels, which can lead to underperfused regions and interstitial hypertension, both of which reduce the ability to homogeneously deliver drugs to all parts of the tumor. Thus solid stress may be a major factor in tumor biology, and ultimately clinical outcomes, that should continue to be explored.
Chapter 7: Discussion
Lymph node metastases are a negative survival factor in many cancers, including breast cancer (269-272). The importance of this is emphasized by the fact that breast cancer patients, who have no detectable axillary node disease at the time of tumor resection, have better disease specific survival when more than 20 ipsilateral axillary lymph nodes are removed (269, 271). Furthermore, node negative patients who do not have lymph nodes removed, show high incidences of axillary lymph node recurrence (270). Thus, axillary lymph node dissection, which is used for diagnosis and treatment decisions, has itself proven to be therapeutic (270). The dissection of the axillary lymph nodes however comes with a price, with lymphedema formation being common (133, 134, 177-179). Can the results presented in this thesis shed any light on appropriate means to combat these problems seen in the clinic?

Problems with cancer treatment begin with achieving local tumor control. One key determinant in achieving local control is the ability to deliver therapeutic agents to the tumor in sufficient quantities. In solid tumors, drug delivery is poor due to heterogeneities in the blood supply (5) and interstitial hypertension (17), a result, at least in part, of the absence of functional lymphatics in tumors. The results presented here show that mechanical compression of lymphatic vessels plays a major role in the deficiencies in interstitial fluid drainage in tumors (Chapter 6). Furthermore, efforts to correct the lymphatic dysfunction with VEGF-C are not sufficient (Chapter 5). In light of the mechanical etiology of the lymphatic dysfunction and the similar compression of blood vessels (see Ph.D. Thesis of Brian R. Stoll), one may predict a paradoxical increase in both drug delivery and metastasis when cancer therapy kills tumor cells and reduces
the compressive forces. Evidence for this again comes from the clinic, where some patients with node-negative disease and without distant metastasis at the time of radiation treatment develop metastasis sometime later (267, 268). Others hypothesize that this appearance of metastasis is a result of a shift in the balance of circulating angiogenic and anti-angiogenic molecules after the tumor is treated (242). We offer a new hypothesis, which is that the appearance of new metastasis could be due to the increase in both lymphatic and blood vessel function after the compressive forces are relieved by radiation therapy, which gives more opportunities for metastasis to occur. These potential increases in both metastasis and drug delivery after therapy need to be further investigated.

Another problem that arises from the lack of lymphatic function in the primary tumor is the reduction of lymphocyte recirculation in the tumor. The immune system is important in combating the growth of tumors (273-275). By preventing the effective recirculation of T-cells in the primary tumor, the immune response to the tumor is blunted. This, in combination with rapid proliferation of cancer cells, can lead to tumor growth without a potent immune response. The immune system may still be able to combat smaller, metastatic lesions, but the primary tumor may never be cleared. The circulation of lymphocytes is most important in initiating an immune response, but less important for the actual clearance of foreign antigens or cells. Therefore, approaches to therapy that exogenously initiate the immune response may still be effective (273-275).

Even with the elimination of the primary tumor, patient outcomes are still dependent on the ability to treat metastasis (235, 276). The work presented here helps elucidate the
origin of lymph node metastasis (Chapter 5). These data indicate that lymphatic metastases originate from lymphatic vessels in the tumor margin that are either engulfed by the growing tumor or invaded by migrating cancer cells. Thus removal of the tumor does not necessarily lead to the removal of the routes of metastasis. This presents an opportunity to directly treat lymph node metastasis in a site-specific manner. By placing chemotherapeutics in the tumor bed after surgical removal, one may be able to adjuvantly treat the draining lymph nodes. This process can be enhanced with clever packaging of the drug into large molecular vehicles to bias uptake into the lymphatic system. Lymphatic drug delivery may be able to locally treat lymph nodes with very high concentrations of chemotherapeutics, while avoiding side effects of systemic therapy. Furthermore, the patency of lymphatic vessels may remain intact since the endothelium is slowly dividing and is less affected by a chemotherapeutic. This would eliminate the lymphedema that follows surgical lymph node removal, currently the most definitive treatment. Lymphatic drug delivery is used mostly in an effort to avoid the first pass effect associated with biotransformation or inactivation of the drug in the liver (195, 277-279). But the concept of directly targeting lymph nodes in this manner is novel and may be beneficial.

Although the origin of the lymphatic metastasis is now clearer, how cancer cells find the lymphatics in the tumor margin in order to invade and metastasize is still unknown. Lymphatic vessels produce chemokines that bind to receptors on lymphocytes, which help lymphocytes home to lymph nodes (280). Once again looking to clinical experience for answers, recent evidence links these same chemokines to lymph node metastasis in
patients (126). Is it possible that tumor cells hijack the mechanism of lymphocyte homing in order to find lymphatic vessels and spread to lymph nodes? This concept may lead to many opportunities for the novel therapy of lymphatic metastasis. For example, the lymphocyte homing mechanisms may be used to direct cellular based therapies to sites of lymphatic metastasis.

The role of VEGF-C and VEGF-D in advancing lymph node metastasis is also generating interest as a target to combat metastasis. Blocking VEGF-C or VEGF-D signaling leads to a reduction in lymph node metastasis (122, 153). However these pre-clinical studies treat the tumor during the early stages of tumor growth, which is not necessarily a clinically relevant scenario. Patients who present with a tumor, either already have metastasis or not. Thus it is not possible to prophylactically treat patients to prevent nodal metastasis. Thus further thought needs to go into this approach for a clinically useful therapy to emerge.

VEGF-C therapy is used to induce lymphangiogenesis and reduce secondary lymphedema in animal models (133, 134). However, there is a danger in using this therapy in the setting of post-operative cancer patients. VEGF-C is associated with an increase in lymph node metastasis and poorer overall survival (76) (Table 2.2). So by increasing the level of VEGF-C, the treatment intended to reduce lymphedema may lead to the lethal spread of the cancer (281). Again, further thought needs to go into this approach for a clinically useful therapy to emerge.
The work presented in this thesis helps to answer unresolved questions in the field of lymphatics and cancer. In doing so, we gain insight into appropriate therapeutic strategies. Hopefully, actions taken to combat lymphatic metastasis will bear the lessons learned from this thesis in mind and improve cancer therapy. In addition, the techniques employed here will serve the lymphatic field well, as now both molecular and functional aspects of lymphatic physiology and biology can be studied simultaneously. These small steps forward in an oft-neglected field aspire to drive lymphatic research in the coming years and help to overcome the problems currently facing cancer therapy.
References


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