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TITLE: Lymphatic Metastasis of Breast Cancer Cells: Development
of In Vivo Video Microscopy to Study Mechanisms of
Lymphatic Spread

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13. ABSTRACT (Maximum 200 Words) Introduction: Metastasis via the lymphatic system is considered a major factor in mortality from breast cancer. At present there are no models for direct measurement of tumor cell spread by lymphatics. This is needed for understanding basic biology of lymphatic involvement, developing treatments and assessing their effectiveness. Purpose: To develop and assess IVVM for imaging the morphology and fluid dynamics of lymphatic vessels in mouse models during tumor development. Scope: Assessment of the concept that in vivo video microscopy (IVVM) can be used for studying experimental cancer spread via the lymphatic system. Methods: Primary tumors were formed by intradermal injection of cancer cells (ventral midline between inguinal lymph nodes). IVVM was used to examine lymphatics on one side (skinflap under anesthesia, 1-25 days post injection); conventional histology used to examine other side. Results: Tumor formation was accompanied by a well developed lymphatic network adjacent to the tumor and enlarged lymphatic vessels with valves in tissues between the tumor and lymph nodes as seen by histology and IVVM. By IVVM, lymphatic endothelium and cells carried by lymph flow could be clearly seen. Conclusion: IVVM is a powerful tool for studying cancer cells as they interact with the lymphatic system.

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INTRODUCTION

Metastasis is responsible for most cancer related deaths. The American Cancer Society states that breast cancer is one of the most widespread cancers affecting women in the United States, with over 200,000 new cases to be diagnosed in 2002, and an estimated 40,000 deaths resulting from metastatic spread of the disease. Clinical data suggest that by providing a pathway for tumor cell dissemination, tumor associated lymphatics are a key component of metastatic spread (1). The movement of tumor cells from the primary site to the lymph nodes is thought to occur either via lymphatic capillaries and channels directly to lymph nodes, or via the blood circulation with tumor cells seeding out in the hilum of the nodes. Although the latter hypothesis is supported by a correlation between the number of tumor cells in the circulation and lymph node involvement, those cells must pass first to the liver or lung before returning to the nodal microcirculation. Since it is now known that the majority of blood borne tumor cells are trapped in the first microvascular bed they encounter (2), the return of those cells via the blood circulation specifically to the lymph nodes draining the region of the primary tumor is highly unlikely. Therefore, the movement of tumor cells to the lymph nodes must be highly dependent on the afferent lymphatic vessels leading to them. The biology of this process is poorly understood, and at present there are no good in vivo models for assessing changes in lymphatic morphology and fluid dynamics resulting from primary tumor development, or for directly observing tumor cell trafficking through lymphatic vessels. Because breast cancer is a major killer, the development of a model for lymphatic metastasis is critical: for understanding the basic biology of the process, for identifying therapeutic targets within the process and for assessing the effectiveness of such therapies. The goal of this research was to develop in vivo videomicroscopy (IVVM) procedures that can be applied to mouse models for quantitative analysis of the early stages of the metastatic process involving lymphatic vessels. The scope of this research included the technical aspects of image formation by IVVM of cutaneous lymph vessels as well as the murine model used for studying metastasis via lymph vessels. Technical aspects included surgical preparation, tissue stabilization, illumination, optics, and camera characteristics. This in turn depended upon developing a suitable animal model for viewing lymphatics via IVVM. Model development included consideration of the progressive effects of primary tumor growth on the morphology and fluid dynamic characteristics of the lymph vessels.

BODY:

The overall objective of the research, as stated in the concept proposal submission, is the development of in vivo videomicroscopy (IVVM) procedures for studying the experimental spread of cancer via the lymphatic system in mice. The strategy was to draw from our experience using in vivo videomicroscopy of hematogenous metastasis (3).

MODEL DEVELOPMENT

The mouse model for this study was designed to provide IVVM views of primary tumors, lymph nodes and the lymphatic channels connecting them. All animals were treated in accordance with the requirements of experimental protocol approved by the University of Western Ontario. Primary tumors were formed by intradermal injection of cancer cells grown in culture.

Cell types:

The goal of this study was the development of a model for studying lymphatic metastasis of breast cancer cells. As a starting point, we chose initially to establish a murine lymphatic metastasis model based on B16F10 murine melanoma cells. There were a number of reasons for this decision: 1. The metastatic processes of both breast cancer and melanoma involve lymphatic spread. 2. Melanin provides a distinct optical marker for positive identification of cancer cells. 3. Syngeneic (C57 black) mice can be used, avoiding the complications of using immune deficient mice during the development phase of the project. 4. Restrictions required for handling breast cancer cell lines of human origin are avoided. 5. B16F10 tumors develop rapidly and consistently. Previous studies also indicate that B16F10 models are appropriate for IVVM studies. (4).

In addition to the murine melanoma model, experiments were performed on a limited number of Nude and SCID mice to examine the characteristics of other cell lines and for assessing methods for transcutaneous IVVM observations.

Injection site:

In preliminary experiments, mice were injected with a B16F10 melanoma cell suspension intradermally along a line in the region of the lower mammary fat pad which has lymphatic drainage to the inguinal lymph node. In later experiments, intradermal injections of both B16F10 cells and saline controls were located on the abdominal midline between the inguinal lymph nodes. This provided lymphatic involvement of both sides for paired measurements by IVVM and conventional histology.

Timing of observations:

Because most metastasis models are based on end point measurements, little information is available on the progression of tumor and lymphatic changes during the initial stages of the metastatic process. In this study, 3-4 mice were used for histological assessment each day for 25 days following injection. IVVM experiments to develop imaging techniques were performed on

a subset of these animals before tissue samples for histology were taken from undamaged tissues on the contralateral side. Post mortem measurements of the tumor and nodal dimensions were used to estimate structure volumes.

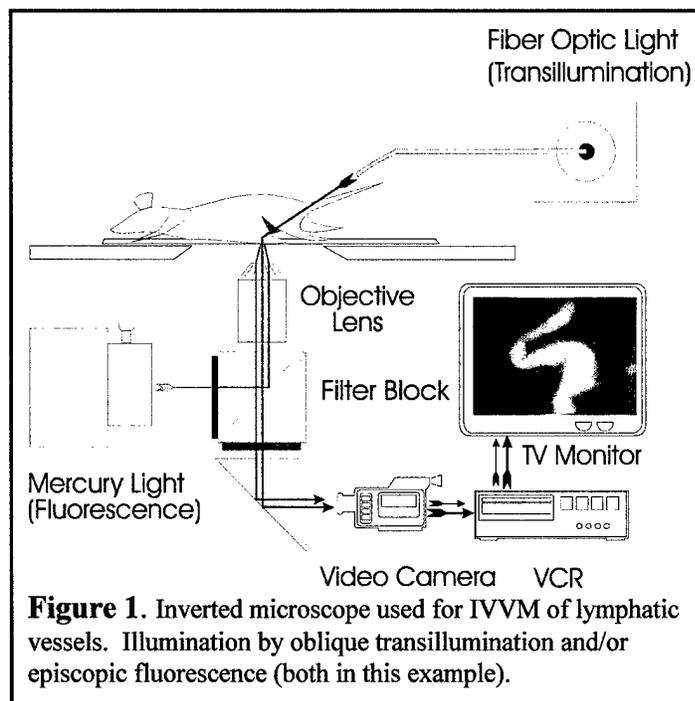
IMAGING:

Image formation by IVVM:

For IVVM, mice were anesthetized in accordance with the approved protocol, usually with sodium phenobarbital with supplemental doses when necessary for prolonged experiments or Ketamine/Xylazine for induction or short experiments.

Tissue being examined was positioned over a coverglass window in an acrylic platform on the stage of an inverted microscope (Nikon Optiphot, Fig. 1).

Tissue temperatures could be monitored with an electronic thermistor thermometer and maintained using an adjustable power heat lamp. Saline was used to keep the tissues moist and maintain contact with the coverglass window. A thin flexible transparent plastic film (Saran) was used to cover the tissue to keep it from drying, and to prevent lateral motion on the coverglass. Tissue could also be stabilized by placing a small piece of paper between the tissue and the coverglass in an area adjacent to the desired field of view. Objective lenses up to 100x oil immersion were used to view the tissues.



Images could be seen using the binocular eyepieces, captured using a 35mm still film camera, or recorded as moving images on video tape or as digital files. Video cameras used included a black and white Panasonic WV1550 analog camera with a 2/3 inch Newvicon tube, a Panasonic

CL350 1/2 inch CCD color camera with an analog (NTSC) output, and a Sencicam black and white high sensitivity cooled CCD camera with direct computer capture for digital processing. Images could be viewed in real time on a video monitor, or recorded for subsequent viewing, image processing and analysis.

Regions of interest were selected at low magnification using the eyepieces and then viewed at high magnification using the video monitor. Depth of field depended on objective magnification and corresponding numerical aperture of the lens. At low power, the greater depth of field made it easier to see larger structures such as arteries, but the overlapping images of small structures made them hard to resolve. At high magnification, the shallow depth of field meant that the plane of focus could be less than 1 μm so structures above and below that plane would be out of focus and not interfere with the image. This technique could be used to optically section the intact living tissue to a depth of 50 μm or more. When viewing at such high magnifications, image stability becomes very important, and the contact of the tissue with the stationary coverglass suppresses motion there despite the fact that the rest of the animal is in constant motion from respiration and heart beat.

Contrast to form images by IVVM can be provided in several ways:

Absorption of transilluminated light by melanin within the B16F10 cells shows well at all magnifications, particularly if there are only a few cells against a translucent background. Within highly melanotic larger tumors, however, absorption of light would reduce illumination to levels too low for image formation. Additionally, structures such as endothelial cells or macrophages that do not absorb light could not be viewed by this method.

Refraction of transilluminated light provides contrast where cells or organelles within them have a different refractive index from the surrounding medium. By using a fiber optic light guide to supply light at an angle through the tissue, regions of higher refractive index redirect the light



Figure 2. IVVM view of red blood cells and leukocytes in mouse spleen. From video taken using oblique transillumination and 100x oil immersion objective lens. Figure width 15 μm .

more towards the lens, so the near edges appear brighter while the far edges are shadowed. (Fig 2). This technique works particularly well when cells such as endothelium, macrophages or red blood cells are suspended in or adjacent to lymph fluid or plasma. At high magnifications, nuclei, granules and vesicles within the cytoplasm of cells can also be seen with this technique. To take advantage of the high numerical aperture of the lens, light must be provided to it at a wide angle. In thick tissues, this is provided by scattering of the light as it passes through, but in thin samples, the highly aligned light from the fiber optic source results in strong diffraction bands that interfere with the image. This can be avoided by applying a layer of translucent material such as wet filter paper over the tissue to scatter the light and increase the effective numerical aperture.

Fluorescent labeling of cells can be used to positively identify them within the tissue. This can be due to naturally occurring fluorescence, addition of a fluorescent label to cells in culture

before injection into the animal, transfection of cells to express fluorescent proteins (GFP) or introduction of a fluorescent material that is preferentially taken up by cells within the living tissues (e.g., high MW FITC-dextran). This works well for identifying specific cells, tracking the movement of individual cells and, in conjunction with oblique transillumination, observing the interaction of cells with the surrounding tissues.

Fluorescent labeling of fluids such as plasma or lymph can be used to identify flow pathways and the lumens of vessels containing the fluid. FITC-dextran injected i.v. circulates with the plasma, identifying the blood vasculature. At high molecular weights, dextran remains within the vasculature as long as endothelial integrity is maintained. Low molecular weight dextran will leak into the interstitial spaces and be taken up by the lymphatic drainage. If leakage occurs everywhere, only diffuse fluorescence is seen throughout the tissue, but if the fluorescent label is injected locally, e.g., adjacent to a tumor, lymphatic drainage from that region will become fluorescent, providing contrast for those vessels.

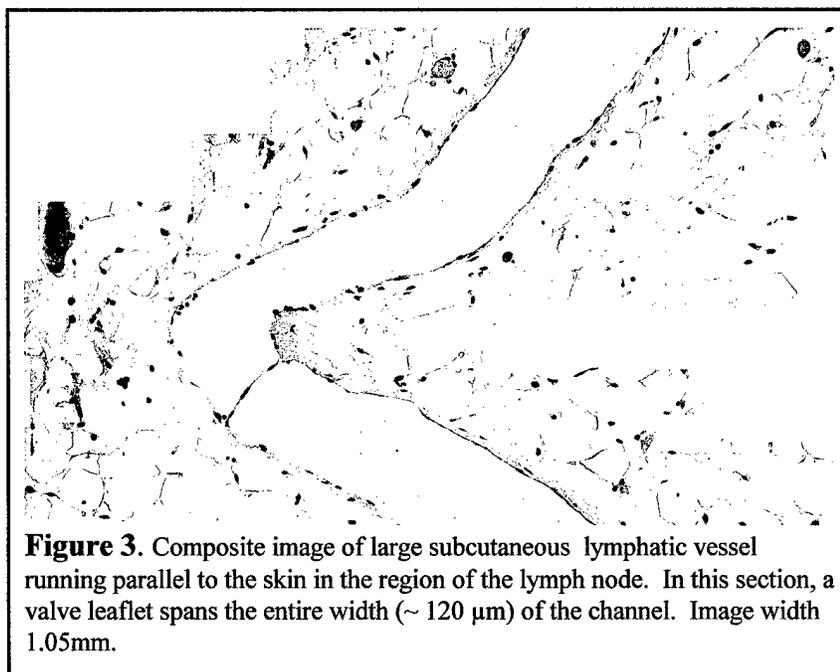
Motion analysis from video sequences was done for this study by measuring the position of cells during replay of sequential video fields.

Preliminary experiments included transcutaneous IVVM in nude mice. In areas such as the ear where the skin is thin, capillary loops approaching the surface can be clearly seen with red and white blood cells passing through them. Resolution of cellular structures associated with subcutaneous lymphatic vessels in the region of the tumor where the skin was thicker was not possible by transcutaneous IVVM however, so the tissue was dissected for direct observation. For observation of tissues overlying a tumor or lymph node, the epidermis was removed and the exposed tissue placed directly on the coverglass window of the microscope stage. Because the thickness of the preparation limited transillumination, fluorescence imaging was generally used for these observations. To observe the subcutaneous lymphatic vessels between the tumor and the lymph node, a skin incision was made inferior to the region of interest and a skin flap was dissected from the underlying muscle layers of the abdominal wall, leaving the cutaneous blood supply intact.

Images from histological sections of tissue samples:

Histological sections were taken for two reasons: 1. to assess changes that occurred in the morphology of the cutaneous lymphatics during tumor development and 2. to determine the position of the lymph vessels with respect to the surface and other structures as a guide to locating them by IVVM. Skin and underlying tissues in the region of the tumor and lymph nodes were preserved in formalin for histological analysis. Initially, wax embedded samples were sectioned in the plane of the skin to increase the probability of observing the lymphatic vessels which are preferentially oriented in that plane. Although this provided good views of the tissue, it was impossible to measure how far from the surface the vessels were. Subsequent sections were cut at right angles to the surface in a line running from the tumor to the lymph node. Using this technique, distances of vessels to the surface could be measured, but identification of the vessels was much more difficult.

In histological sections, large lymphatic vessels could be identified by their thin, intact endothelium, absence of red blood cells, occasional clusters of lymphocytes and the presence of valves (Fig. 3). These larger lymph vessels were seldom seen in untreated mice. This is perhaps



not surprising as inflammation from the injection and fluid leakage from the developing tumor would increase lymph production, opening those vessels that were already in the tissue and promoting the development of new ones. Although lymphangiogenesis has been attributed to molecular factors such as VEGF expressed by growing tumor cells, the effect could be induced by purely physical mechanisms. As changes in the lymphatic vasculature could be important for the movement of cancer cells via this route in murine models, an assessment of such changes during the early stages of tumor development is important so tissue samples have been preserved for further analysis.

Whereas larger lymphatic vessels were relatively easy to detect when present, smaller vessels throughout the interstitial spaces had incomplete walls that were often hard to distinguish from connective tissue cells (Fig. 4). Transverse sections of fat cells also resembled the cross section of small lymphatic vessels, particularly when no nuclei were present. Thus, from the histological sections, positive identification of small lymphatic channels was often not possible, and because

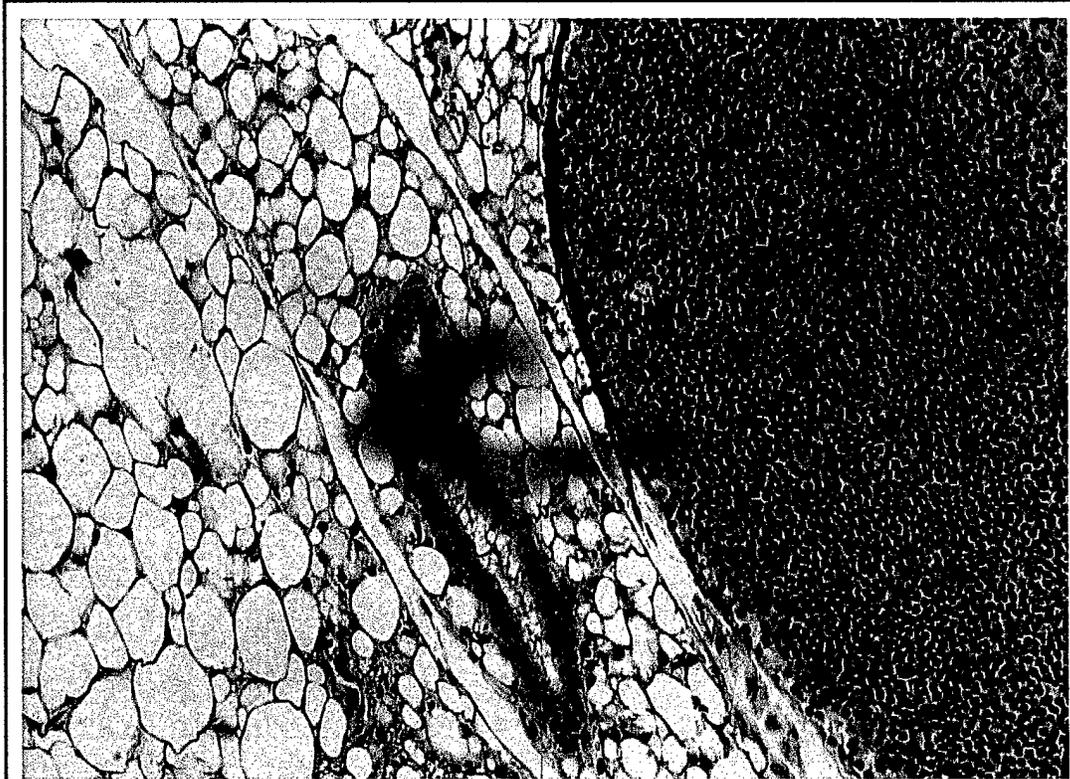


Figure 4. Histological section of subcutaneous connective tissue adjacent to inguinal lymph node. Long open structures are lymph vessels and circular openings (up to $\sim 50 \mu\text{m}$) are fat cells. Large irregular open space could be either. Image width $750 \mu\text{m}$.

of the loose open structure of the subcutaneous connective tissue, well registered serial sections were not available for 3D reconstruction. Tissue samples that have been kept for thin ($4 \mu\text{m}$) sections will also be used for further studies using optical sectioning on thick ($100 \mu\text{m}$) sections.

Observations from IVVM:

1. In untreated animals, blood vessels were clearly seen by oblique transillumination, but there was little evidence of patent lymphatic vessels. Intradermal injection of Evans' blue resulted in staining of cells in the connective tissue and blood vessel endothelium (Fig.5). Intradermal injections of FITC dextran resulted in a diffuse fluorescence locally in the tissue, which gradually faded over approximately 20 minutes and was taken up by connective tissue cells (Fig.6). In both cases, there was no obvious preferential filling of small lymphatic capillaries by injected contrast agents.



Figure 5. IVVM of microvascular blood vessel endothelium, pericytes and surrounding connective tissue cells stained with Evans' blue. Rapid blood flow was seen in the vessels. Image width 170 μ m.

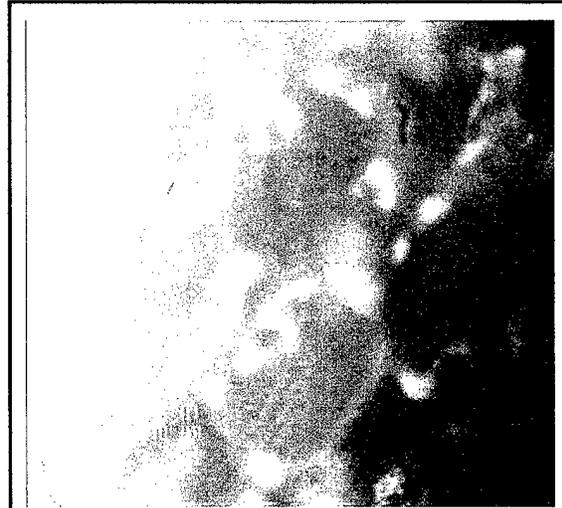


Figure 6. IVVM of subcutaneous connective tissue following intradermal injection of FITC-dextran MW 70000. By 20 minutes, fluorescent dye is concentrated in cells in the connective tissue. Image width, 170 μ m.

Lymphatic endothelium was visible, however, by oblique transillumination (Fig.7) and fluorescent cells could be seen in relation to it. In these views, the endothelial walls and fat cells are unmistakable (as compared with figure 4).



Figure 7. IVVM view of small lymphatic vessel in subcutaneous tissue. (a) Epifluorescence showing macrophages containing FITC-dextran. (b-d) Progressive increase in oblique transillumination to reveal non-fluorescent large spherical fat containing cells and fine lymphatic endothelial cells. This technique allows positive identification of fluorescent cells within detailed views of non-fluorescent tissue.

In mice with well developed tumors, intradermal injection of FITC-dextran in the peritumoral region resulted in transient filling of larger lymphatic vessels draining the area. These vessels could be identified at that time by their fluorescent content and then examined in detail by oblique transillumination (figs. 8,9).

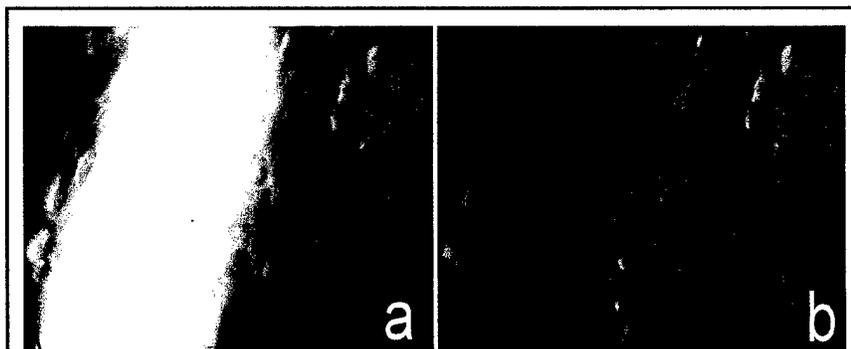


Figure 8. Large lymphatic vessel draining tumor region. (a) Viewed by epifluorescence together with oblique transillumination to locate lumen of vessel, (b) by transillumination to view details of cells within the vessel. Image width 170 μ m.

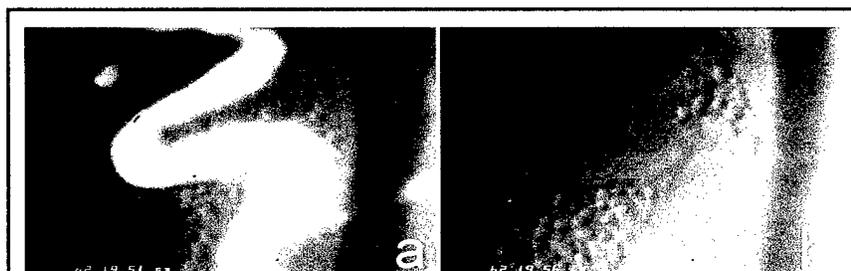


Figure 9. Lymphatic vessel passing through inflamed tissue. (a) Tortuous configuration revealed by FITC-dextran filling. Note folding of membrane at bend and small branch at right. (b) Transillumination alone showing inflammatory cells surrounding vessel and relatively clear lymph fluid inside. Image width 170 μ m.

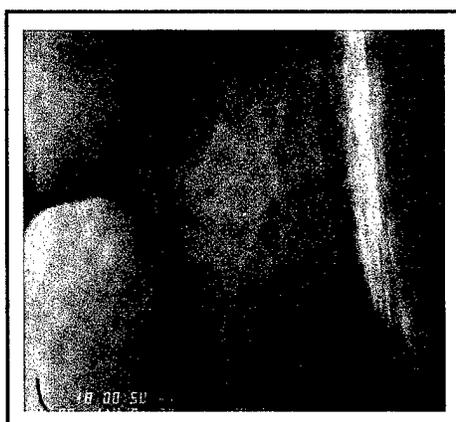
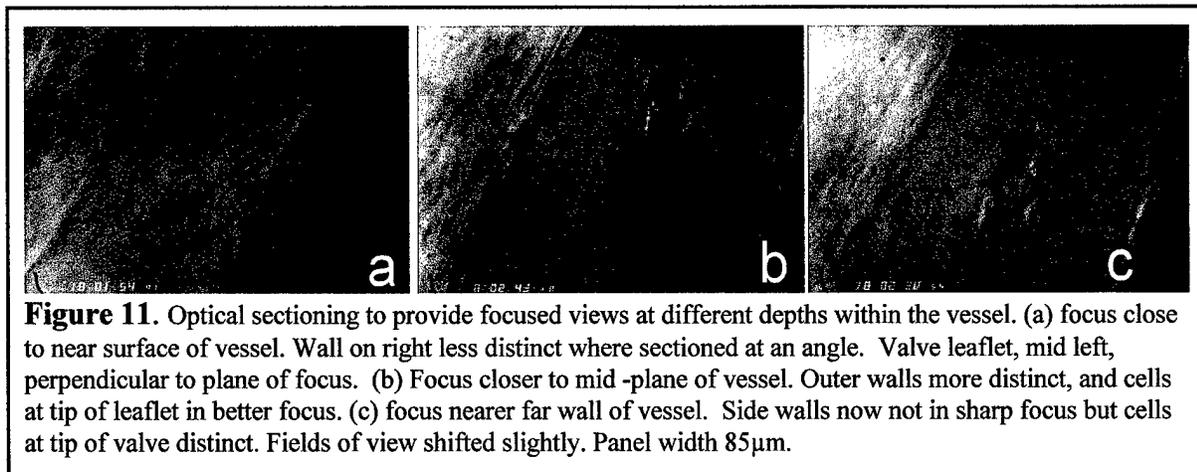


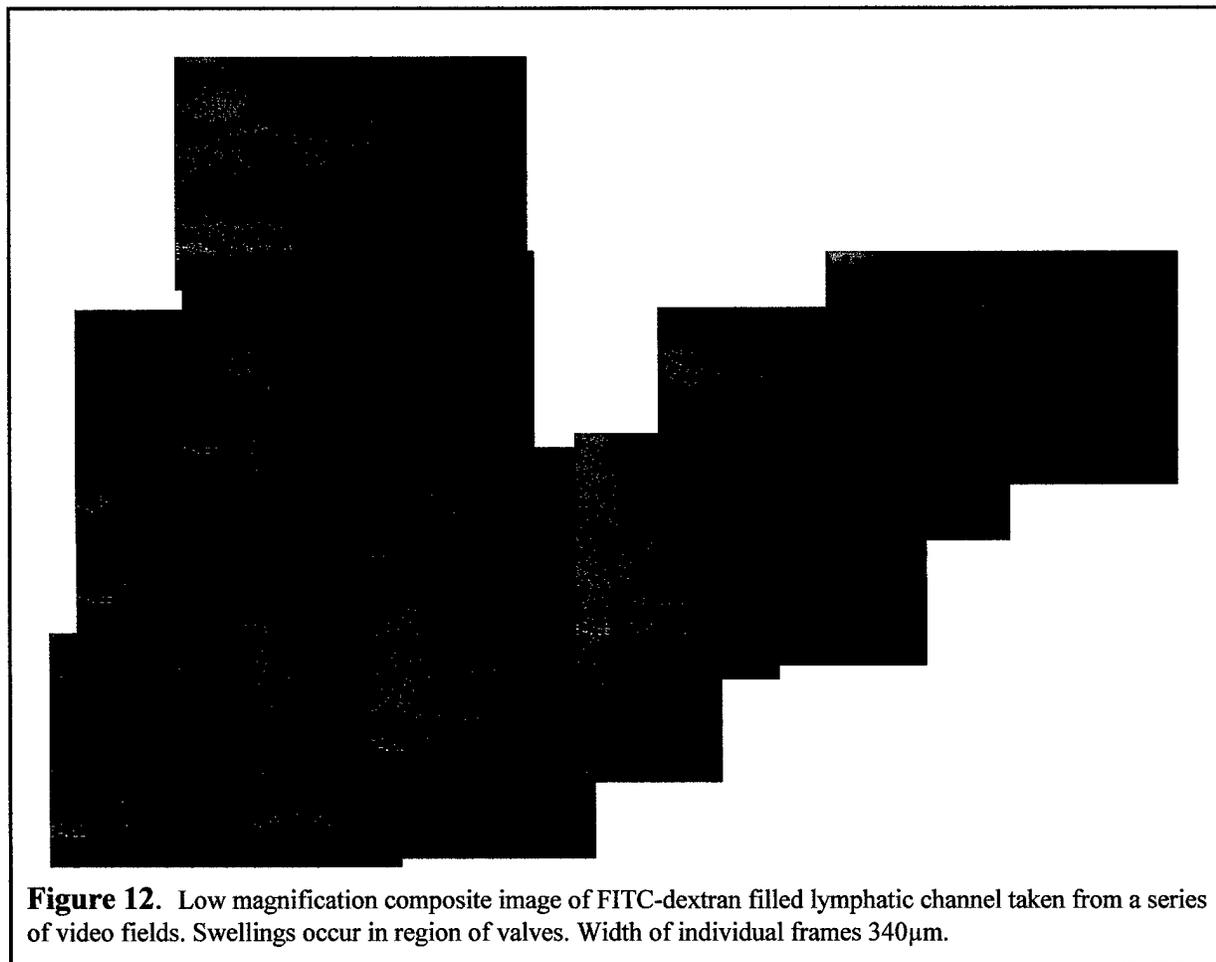
Figure 10. Lymphatic vessel in subcutaneous tissue clearly outlined by endothelium when focus is at the mid-plane of the vessel. Image width 85 μ m.

At higher magnifications, optical sectioning provides sufficient resolution and contrast to locate endothelial walls of lymphatic vessels (fig. 10).

By focusing up and down, details at different depths can be separated so the relationship between features and the surrounding structures can be determined (fig).

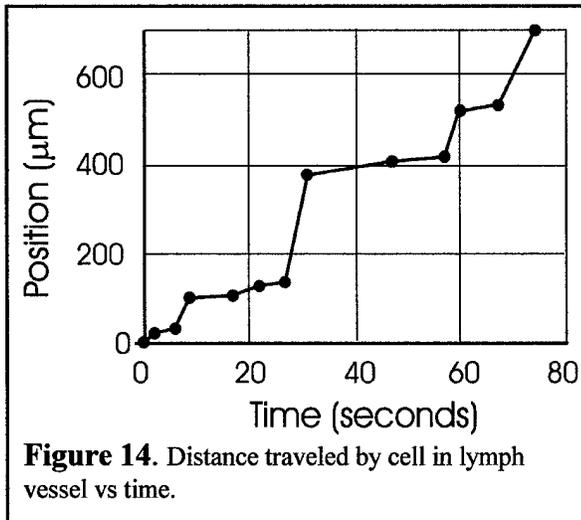
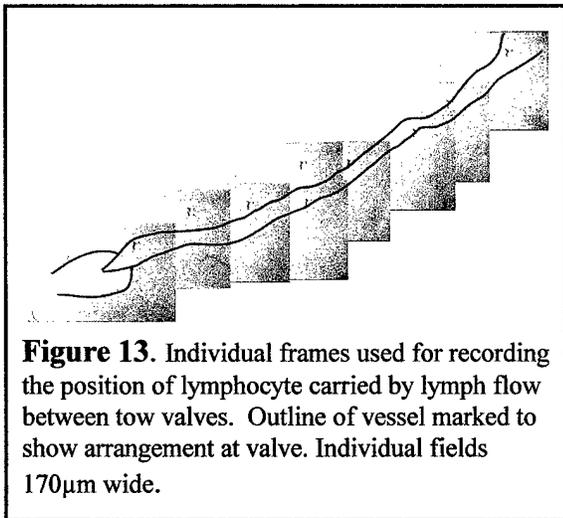


Tracking motion within lymphatic vessels:



Although the morphology of the lymphatic system could be defined from serial reconstruction of appropriately stained histological sections, the dynamics of lymphatic function can only be assessed in vivo. The fact that the lymph channels will fill with a soluble fluorescent label against a background of non-fluorescent tissue means that there must be flow in them.

Figure 12 shows subcutaneous blood vessels (dark against transilluminated background) and lymphatic vessel (light due to FITC fluorescence against background). Swellings in lymph vessel occur at intervals of about $300\mu\text{m}$ at the location of valves. Although the fluorescent marker was eventually flushed from this vessel, its position had been identified (Fig 13), and cells moving within it were tracked at higher magnification using oblique transillumination and recorded for further analysis. During replay, position and time were recorded and graphed (Fig 14).



Suspended cells moved freely with the lymph and did not appear to adhere to the vessel wall. Upon reaching the valve however, forward progress was impeded, either because the valve opening was not large enough or because the cell had adhered to the leaflet.

Lymph movement was not steady as indicated by the movement of the suspended cell (Fig 14). Flow was stationary for periods of about 20 seconds and then advanced quickly for several seconds. Mean forward flow was about $10\mu\text{m}$ per second. If cells continue to move at that rate they could pass from the tumor to the lymph node in about half an hour.

KEY RESEARCH ACCOMPLISHMENTS:

Positive findings:

- IVVM is a powerful tool for studying the interactions of cells and lymphatic vessels.
- The murine melanoma model was successful for providing a system for studying lymph function in inflamed or tumor challenged tissue.
- The endothelium of large and small lymphatic vessels was clearly visualized by IVVM using oblique transillumination.
- In the tumor model, afferent lymphatic vessels draining the peritumoral region were clearly identified using fluorescent FITC-dextran to label the lymph fluid.
- Injected FITC-dextran is picked up by large afferent lymph vessels within about 20 minutes, corresponding well with the rates of flow observed in them, and is cleared in about 1 hour.
- Macrophages or other cells in the subcutaneous connective tissue picked up the fluorescent label in about 1.5 hours. These cells were found in high concentrations throughout the tissues.
- Labeled cells were identified by their fluorescence and then studied in detail by oblique transillumination.
- Optical sectioning was used to examine details of lymphatic morphology at different depths within the tissue.
- Lymphatic vessels changed their structure and function in response to tumor development.
- Cell and lymph fluid movement was detected and measured by IVVM.
- Cells did not appear to adhere to the walls of the lymph vessels.
- Lymph flow was as high as 10 μm per second on average and peaked at about 10x that rate.
- IVVM views of lymphatic vessels correspond well with those revealed by conventional histology, are easier to obtain and provide dynamic 3D information.

Negative findings:

- Lymphatic vessels that were easily viewed by IVVM were not found in normal mice.
- Transcutaneous imaging was not successful. Skin dissection was required to expose lymphatic vessels.
- Background fluorescence at the site of FITC-dextran injection masked any filling of lymph vessels there.
- In the melanoma model, cancer cells were occasionally seen in the subcutaneous tissues between the tumor and the lymph node, but none were seen within the afferent lymphatic vessels.
- Because lymphatic vessels were not developed in untreated mice, direct injection of cells was not possible. Timing and location of vessel development awaits the results of further analysis of tissue samples collected in this study.

REPORTABLE OUTCOMES:

a) *Abstract submissions:*

LYMPHATIC CHANGES DURING EARLY TUMOR DEVELOPMENT

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Departments of Medical Biophysics¹ and Oncology², University of Western Ontario
Third Era of Hope meeting for the DOD BCRP
September 2002, Orlando, Florida
(see Appendix I)

LYMPHATIC CHANGES DURING EARLY TUMOR DEVELOPMENT, ASSESSED BY
INTRAVITAL VIDEOMICROSCOPY

Ian C. MacDonald^{1,2}, Hemanth J. Varghese¹, Alan C. Groom¹, Dolores A¹. Steinman and Ann F.
Chambers^{1,2}
Departments of Medical Biophysics¹ and Oncology², University of Western Ontario
IXth International Congress of the Metastasis Research Society
September 2002, Chicago, Illinois
(see Appendix II)

b) *employment or research opportunities applied for and/or received based on
experience/training supported by this award*

Application to the Cancer Research Society (Canada) for Postdoctoral Research Fellowship
Dolores A. Steinman.

THE DYNAMICS OF LYMPHATIC SYSTEM CHANGES TO ACCOMMODATE TUMOUR
CELL TRAFFICKING

c) *Funding applied for and awarded based partially on support from this award*

Canada Foundation for Innovation
Project 5055
Ian MacDonald
University of Western Ontario

MULTIDISCIPLINARY FACILITY FOR COMPUTATIONAL MICROSCOPY OF LIVING
TISSUES

Funding towards equipment provided by the US DOD has generated an additional 4x
contribution from the Canadian and Ontario Governments. (~\$80,000CA, total award
~\$2,100,000CA)

CONCLUSIONS:

Breast cancer is a major killer due to metastatic spread. Although the lymphatic system is closely associated with this problem, there are no models for directly observing the interaction of cancer cells with the lymphatic system in vivo. This study was designed to explore the concept that IVVM could be used to obtain real time images of the intact lymphatic vasculature and any cancer cells that interact with it.

A model based on IVVM as used for hematogenous metastasis was developed that could clearly define large lymphatic vessels and small endothelial lined channels in skin using oblique transillumination on an inverted microscope. Fluorescent dyes could be used to locate large vessels ($>30 \mu\text{m}$ diameter), but not small ones. Cells associated with lymphatic function could be seen by fluorescence or by oblique transillumination.

The development of primary tumors was accompanied by increases in the density of visible lymphatic vessels and fluid flow within them. Lymph flow was intermittent with surges of up to $100 \mu\text{m}$ per second, several times per minute.

In vivo videomicroscopy can be used to image the intact lymphatic system and follow cells as they move through it. It can be used in experimental live models to study the movement of cancer cells from a primary tumor via the afferent lymphatic vessels to the lymph nodes, and assess the effects of therapies designed to block such movement.

REFERENCES:

1. Swarz MA, Skobe M. Lymphatic function, lymphangiogenesis, and cancer metastasis. *Microsc Res Tech* 2001;55:92-97
2. Chambers AF, Naumov GN, Varghese HJ, Nadkarni KV, MacDonald IC, Groom, AC. Critical steps in hematogenous metastasis: an overview. *Surg Oncol Clin N Am* 2001;10:243-255
3. MacDonald IC, Groom AC, Chambers AF. Cancer spread and micrometastasis development: Quantitative approaches for in vivo models. *BioEssays* (in press)
4. Trites J, Yoo J, Taylor M, Schmidt E, Morris V, MacDonald I, Chambers A, Groom A. Lymph node metastasis in malignant melanoma: an in vivo model. *Otolaryngology* 2000;29:322-238

PERSONNEL RECEIVING PAY:

Dolores A. Steinman, Ph.D., Post Doctoral Fellow

Appendix I

Abstract: Third Era of Hope meeting for the DOD BCRP
September 2002, Orlando, Florida

LYMPHATIC CHANGES DURING EARLY TUMOR DEVELOPMENT

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Death from breast cancer is usually due to the spread of tumor cells from the primary site, which can be effectively treated, to distant inaccessible or untreatable sites where metastases form. The movement of tumor cells to typical metastatic sites such as bone or brain, must ultimately be via the blood circulation, but entry to the blood stream may occur indirectly via lymphatic channels draining the primary site or surrounding tissues. Spread via the lymphatic system is especially implicated in breast cancer, so *in vivo* models must be developed for studying the basic biology of the process and assessing therapies targeted to block it.

Intravital videomicroscopy (IVVM), based on established methods for studying hematogenous metastasis (Chambers et al, *Adv. Cancer Res.* 2000;79:91-121), was used to visualize subcutaneous lymphatic channels in mice. At high magnifications, oblique illumination clearly revealed endothelium and valves in dilated lymphatic vessels. Tumor cells (B16F1 murine melanoma, 10^5) were injected intradermally to form midline tumors between the inguinal lymph nodes. Tissue on one side was used for IVVM and on the other for histology.

In untreated animals, afferent lymphatic channels were collapsed as seen by histology and were rarely evident by IVVM. Tumor formation, however, was accompanied by dilated afferent lymphatic vessels, seen by histology and IVVM. Lymphatic capillaries originating in the region of the tumors had distinct valves at intervals ($\sim 500\mu\text{m}$) and were much wider ($\sim 40\mu\text{m}$) than blood capillaries. Lymphocytes were seen moving intermittently within these channels, advancing ($\sim 150\mu\text{m}$) during 3-5 s periods at 10-20 s intervals. Although tumor cells were seen by histology within subcutaneous interstitial spaces, none were seen within the lymphatic vessels themselves, so any such transport must be occasional but rapid. Inflamed tissues immediately overlying the tumor contained closely spaced, highly anastomotic lymphatic channels with no valves. Many lymphocytes were seen within these vessels moving at a steady rate ($\sim 30\mu\text{m/s}$). Although lymphangiogenesis has been attributed to growth factors produced by tumor cells, sham injections (saline alone) also result in enlarged lymphatic channels. Thus, lymphatic changes accompanying tumor development may also be due to the physical effects of local damage or increased volume loading of the tissues. Our studies indicate that although lymphatic transport is increased during tumor development, lymphatic intravasation of tumor cells may be an inefficient process and thus may be an effective target for blocking lymphatic metastasis.

Support: U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0668.

Appendix II

Abstract: IXth International Congress of the Metastasis Research Society
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Lymphatic Changes During Early Tumor Development, Assessed by Intravital Videomicroscopy

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Death from breast cancer is usually due to the metastatic spread of tumor cells from the primary site. The movement of tumor cells to metastatic sites such as bone or brain, must ultimately be via the blood circulation, but entry to the blood stream may occur either directly into vasculature of the primary tumor, or indirectly via the lymphatic system. If lymphatic spread is a major contributor to metastasis, then in vivo models must be developed to directly observe the basic biology of lymphatic responses to tumor development.

Intravital videomicroscopy (IVVM), based on well established methods for studying hematogenous metastasis (Chambers et al, *Adv Cancer Res* 2000;79:91-121), was used to visualize subcutaneous lymphatic channels in mice. Tumor cells (B16F1 murine melanoma, 105) were injected interdermally to form midline tumors between the inguinal lymph nodes. One side was used for IVVM, the other for histological examination.

Preliminary results showed that in untreated animals, afferent lymphatic channels were collapsed as seen by histology and were not evident by IVVM. Intradermal injection of FITC-dextran (70 kDa) resulted in diffuse subcutaneous fluorescence with no distinct lymphatic filling. In the treated mice, tumor formation gave rise to dilated afferent lymphatic vessels as seen by histology and IVVM. At high magnification, oblique illumination clearly revealed endothelium and valves in dilated lymphatic vessels as well as individual cells carried by the lymph flow. Vessels originating in the vicinity of the tumor had distinct valves at intervals (~250 μ m), were much larger (~50 μ m) than blood capillaries, and became filled with FITC-dextran. Lymphocytes within lymphatic channels advanced about 50 μ m during a 2-4s period every 10-20s.

These studies indicate that lymphatic transport is greatly increased in response to tumor development, and that IVVM can be used to quantify the numbers and movement of cells within lymphatic vessels.

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