New Approaches to Lymphatic Imaging

Richard T. Lucarelli, B.Sc., Mikako Ogawa, Ph.D., Nobuyuki Kosaka, M.D., Ph.D.,
Baris Turkbey, M.D., Hisataka Kobayashi, M.D., Ph.D., and Peter L. Choyke, M.D.

Abstract

Accurate imaging of the lymphatic system can aid in cancer staging, optimize surgical procedures to reduce lymphedema, and may one day be a means of delivering intralymphatic therapy. The Sentinel Lymph Node (SLN) concept has been pivotal in driving new imaging techniques. Metastasis to a SLN is a critical indicator of advanced disease. However, presently, few tools are available for imaging the lymphatics, and even fewer are available for locating the SLN for biopsy. Recently, new macromolecular agents, including gadolinium-labeled dendrimers, fluorescent quantum dots, and fluorescently-labeled immunoglobins, have been used to image the lymphatics and SLN with MRI and optical techniques, and new fluorescent nanoparticles such as upconverting nanocrystals are potential future agents. Additionally, multi-modality probes combining two modalities such as optical/MR dendrimers have been designed to provide both preoperative imaging, and intraoperative guidance during lymph node resections. These probes can map the lymphatic system for maximal therapeutic benefit while minimizing complications such as lymphedema. Advances in the understanding of the molecular mechanisms of lymphangiogenesis and lymphatic spread of tumors offer the opportunity for more targeted imaging of the lymphatic system. Additionally, these imaging agents could be used as powerful research tools for tracking immunological cells and monitoring the immune response as well as providing the means to deliver lymphatic-targeted therapies. The future holds great promise for the translation of these methods to the clinic.

Introduction

ACCURATE IMAGING OF THE LYMPHATIC SYSTEM can aid in cancer staging, optimize surgical procedures to reduce lymphedema, and may guide delivery of intralymphatic therapy. The major physiologic functions of the lymphatic system are the absorption of fat and fat-soluble vitamins, drainage of peripheral tissue fluid, and the route for migration of activated dendritic cells for processing acquired immunity or transportation of antigens to lymph node dendritic cells for initiating immune response. However, the lymphatics can serve as a conduit for cancers to metastasize through the lymphatic system to distant tissues and organs. Cancer cells enter the lymphatic system through lymphatic capillaries and travel to progressively larger vessels until they reach the primary draining lymph node of the cancer site, known as the sentinel lymph node (SLN). The presence of cancer in the SLN indicates metastatic disease and implies that the cancer may have spread to distant lymph nodes and other organs. SLN biopsy has become part of the standard of care for breast cancer and is commonly used in melanoma, but is being considered for other cancers as well.

Unlike the cardiovascular system, which is a closed loop, the lymphatic system is a unidirectional, open-ended system that moves lymph to lymph nodes and eventually back into the circulation. The motive force for lymphatic flow is provided by skeletal and smooth muscle contractions with backflow of lymph prevented by intralymphatic valves. In the skin, lymph first enters the lymphatic system through lymphatic capillaries that demonstrate gaps between the lymphatic endothelial cells and have no basement membrane under certain conditions, facilitating the uptake of cells and macromolecules.¹ These lymphatic capillary endothelial cells are fixed to the surrounding extracellular matrix by anchoring filaments that serve to expand the lymphatic capillary lumen when interstitial pressure increases. From the lymphatic capillaries, lymph flows to progressively larger lymphatic vessels, or collecting vessels, which are increasingly impermeable with fully formed basement membranes and encasing smooth muscle cells. The smooth muscle cells, along with skeletal muscle contractions, assist in propelling the lymph rapidly through the afferent lymphatics to lymph nodes.¹ A single lymph node receives input from multiple afferent lymphatics while lymph flows out of nodes via efferent

lymphatic vessels. The efferent lymphatics network with other distal lymph nodes and eventually the lymph empties into lymphatic trunks, and then into the left subclavian vein via the thoracic duct. Through this network cancer cells can spread to lymph nodes, blood, and eventually distant organs.²

Imaging of the lymphatics can be roughly categorized into one of three types: anatomic, functional, and molecular. Anatomic imaging provides direct visualization of the lymphatic channels, lymph nodes, and deep lymphatic system. Functional imaging is able to detect dynamic physiologic processes such as lymphatic flow or metabolism. Finally, molecular imaging targets specific markers or receptors on lymphatic endothelial cells that can differentiate cell type and also molecular pathways that signal lymphangiogenesis. Conventional methods such as CT and MRI are principally anatomic and rely only on the size of lymph nodes to determine their status (malignant vs. benign) and are thus, prone to both false negatives and false positives. Furthermore, these techniques do not provide information on the location or metastatic status of the SLN.

Currently, only two methods are used in the United States to detect SLNs. One method involves the injection and surgical localization of a blue dye, isosulfan blue or Lymphazurin, which is injected into the breast or near the tumor. Another SLN imaging method involves lymphoscintigraphy, where a radioactive tracer, Technetium 99m-sulfur colloid, is injected in the breast or around the tumor to locate the SLN. However, these methods are cumbersome and have a number of drawbacks. For instance, blue dye is difficult to see unless the SLN is actually dissected. Lymphoscintigraphy relies on the use of a gamma counter during the surgical procedure and is dependent on the experience of the surgeon. Experimental methods, such as macromolecular magnetic resonance (MR) and optical agents, as well as targeted optical, nuclear, and MR probes hold promise in overcoming the drawbacks of the present methods. Furthermore, methods are being developed to determine whether lymphangiogenesis is present in a lymphatic bed, an indicator that the tumor is preparing to spread intralymphatically, if it has not already done so. It is thus possible that lymphangiogenesis imaging could predict the likelihood of metastases even before they occur. Moreover, to the extent that lymphatic imaging provides insight

into the anatomic interconnections of the lymphatic system, it may be possible for surgeons to avoid lymphedema when removing a collection of lymph nodes. Further development of these new methods will yield a better understanding of the lymphatic system and provide important clinical tools in the diagnosis and treatment of cancer and lymphedema.

Conventional Imaging of Lymphadenopathy

For many years conventional imaging modalities including CT and MRI have been utilized to diagnose metastases in lymph nodes (Fig. 1). These methods rely on the enlarged size of lymph nodes to diagnose lymphadenopathy and infer metastases and are thus prone to error. There are many non-cancerous causes of lymph node enlargement that can result in false-positives including acute and chronic infections and autoimmune disease. Additionally, small metastases can exist in lymph nodes without enlarging them and thus, go undetected.³ Generally, there is a tradeoff between the size of the lymph node, and the sensitivity/specificity.⁴ Any size threshold is therefore somewhat arbitrary. If one were to lower the threshold to 0.5 cm, conventional imaging would be more sensitive but would lose specificity. Likewise, if the criteria were changed from 1 cm to 4 cm, the test would be highly specific but would be insensitive to metastases. Generally, 1 cm is used as a cutoff for intra-abdominal nodes, but this results in a poor sensitivity and moderate specificity that is dependent on the tumor being staged. For instance, with a slow growing prostate cancer, CT and MR have very low sensitivity (approximately 30%) using the standard 1 cm diameter size criterion. In addition to the inherent problems of relying on size to determine metastatic status, these methods are unable to locate the SLN and do not provide any information on the flow of lymphatics. Nevertheless, CT and MRI are still the most common methods used to diagnose lymph node metastases because they are easy to perform and widely available.

A more sensitive and specific method of identifying metastases in lymph nodes involves using magnetic nanoparticles as MRI contrast agents. In this method ultrasmall super-paramagnetic iron oxide (USPIO) nanoparticles, such as ferumoxtran-10 (commercial name: Combidex™ Advanced

FIG. 1. Conventional imaging, MR, and CT, rely on size to determine lymphadenopathy and thus metastasis. Size alone is an insufficient criterion for accurate metastatic diagnosis and is thus prone to both false positives and false negatives. (a) Prostate cancer patient, 60 year-old: axial CT showing retroperitoneal enlarged lymph nodes (*arrows*); (b) also seen on the axial MRI (contrast enhanced T1 weighted gradient echo image).

Magnetics or Sinerem™ Laboratoire Guerbet, Paris, France) are injected intravenously.⁵ The particles are readily taken up by the lymphatics and transported via the afferent lymphatics to the lymph nodes where they are internalized by resident macrophages. This results in the opacification of the normal portion of the lymph nodes containing iron, resulting in their turning dark on T2 weighted MRI while tumor-harboring portions of the node, devoid of macrophages, remain high in signal. Despite its sensitivity, this agent is not approved in the US but is available in some centers in Europe. However, another USPIO nanoparticle, ferumoxytol (Feraheme™ AMAG Pharmaceuticals, Lexington, MA) has very recently received FDA approval for iron replacement therapy in anemia. This particle functions similarly to ferumoxtran-10 as an MR contrast agent at already approved doses and will likely find its way into the clinic in the near future.⁶

Other conventional methods to diagnose lymph node metastasis include positron emission tomography (PET) using F-18 Fluoro-deoxy glucose (FDG), dynamic contrast-enhanced MRI (DCE-MRI), and color Doppler ultrasound (CDUS). These methods offer advantages over standard CT and MRI but a detailed discussion is beyond the scope of this review.

Present Methods for Sentinel Lymph Node Imaging

The SLN is the first lymph node to which a primary tumor drains. The metastatic status of the SLN is a strong predictor for the nodal status of the tumor in general. If the SLN biopsy is positive for metastases, then it is likely the cancer has spread to distal nodes and possibly other organs. However, if it is negative, there is no need for lymphadenectomy, thus reducing disfigurement and complications such as lymphedema.⁷ Sentinel node imaging is considered a standard of care in breast cancer surgery.

In one method, the dye isosulfan blue (tradename: Lymphazurin™ Covidien Inc, Hazelwood, MO) is used to stain the SLN. After subcutaneous injection proximal to the site of a primary superficial tumor, the skin is incised and injected with the dye; the SLN eventually stains blue. The SLN can then be excised and evaluated. The primary disadvantage to this method is its lack of preoperative localization. An incision must be made and the incision must be close enough to the dye, which has very little penetration through tissue, so that it can be seen. This requires surgical skill and experience and

often requires the prelocalization of the SLN with another imaging method such as lymphoscintigraphy. Furthermore, the dye leaves an unsightly stain on the skin that can last for months.

Lymphoscintigraphy is a more versatile method that utilizes ^{99m}Tc-sulfur colloid injected peritumorally or intratumorally to localize the SLN. After injection, the colloid is taken up by the lymphatic vessels and transported to the lymph node. The SLN is preoperatively visualized with a gamma camera (Fig. 2). Then intraoperatively, a hand-held gamma probe is used to more precisely locate the node for excision. There are a number of drawbacks to this method. First, and perhaps most importantly, it has very low resolution and it is easy for the SLN to be masked by background signal around the injection site, or to confuse a secondary lymph node for a SLN. Furthermore, operating the gamma probe is awkward and requires both skill and experience to manipulate the probe and differentiate the small nodal signal from the injection site. Finally, while it exposes the patient to a small dose of radiation, the fingers of the surgical and pathology team are repeatedly exposed each time such a procedure is performed.⁸

Given the significant drawbacks of the above methods, new approaches are needed for SLN imaging.

Indirect Lymphatic Imaging

Indirect peritumoral injection of contrast agents is preferential to the difficult and potentially life-threatening procedure of direct cannulation of lymphatic vessels. Lymphography is a now abandoned technique in which an oily iodinated dye was injected into a cannulated lymphatic vessel on the dorsum of the foot. Occasionally, overinjection led to pulmonary emboli from the oily dye. Far more practical is the injection of a tiny volume of contrast agent wherein the interstitial fluid pressure is increased, thus creating tension on lymphatic capillary anchoring filaments that results in the opening of the otherwise collapsed lymphatic capillaries and subsequent uptake of the agent into the lymphatics.^{2,9} Macromolecules larger than approximately 6–10 nm preferentially enter the lymphatic capillaries and are retained throughout the length of the afferent vessel, resulting in efficient labeling of the vessels and the SLN. Contrarily, macromolecules smaller than approximately 6 nm enter the lymphatic

FIG. 2. Lymphoscintigraphy with Technetium-99m-labeled sulfur colloid can locate the Sentinel Lymph Node (SLN) prior to incision. Tc-99m-labeled sulfur colloid is injected into the breast (*) and the SLN appears in about 1–2 h (arrow). Presurgical localization anteriorly (a) and laterally (b) with a gamma camera provides a low resolution image of the SLN. Localizing the SLN during the operation requires considerable experience.

FIG. 3. Many macromolecular agents are available for imaging the lymphatics and SLN. The macromolecular agents shown are of an ideal size, greater than 6 nm for efficient lymphatic uptake and retention, and may be modified in numerous ways. *Top panel:* MRI with gadolinium (Gd)-dendrimers may be conjugated with a NIR fluorophore and/or radionuclide for dual or tri-modality lymphography. IgG can be used as a carrier for fluorophores or radionuclides. Quantum dots are very bright fluorescent nanoparticles that can be conjugated to radionuclides or Gd for dual or tri-modality lymphography. Ultrasmall particles of iron oxide (USPIO) function as a negative-contrast agent and can be used to locate metastases in lymph nodes on MRI with higher sensitivity and specificity than conventional MRI. USPIO can also be modified with additional probes, not pictured. Nonspecific proteins such as albumin can also be conjugated to imaging agents or in the case of the NIR fluorophore, ICG, act as a nonconjugate carrier molecule. *Bottom panel:* Agents are injected into the interstitial space peritumorally. The increase in interstitial pressure opens the capillary lumen via the action of the anchoring filaments on the loose endothelial cell layer allowing fluid and macromolecules to enter. Along the route towards the SLN, the lymphatic capillary vessels merge into the collecting vessels that are increasingly impermeable and contain valves to ensure one-way lymph flow. The afferent lymphatics finally converge at the SLN where the imaging agent accumulates.

capillaries but quickly diffuse across the afferent endothelium, resulting in increased background and reduced labeling of the SLN.¹⁰ Larger particles, greater than 12 nm, get into the lymphatics as well, but at a much slower rate. Molecules with ~10 nm are ideal for visualizing the lymphatic drainage. Many macromolecules are available for indirect lymphatic imaging including gadolinium-labeled dendrimers for MRI, fluorescent water soluble quantum dots for optical imaging, and radiolabeled immunoglobins or peptides for radionuclide imaging (Fig. 3). These materials will be discussed in detail below. Additionally, ultrasound using microbubbles have been used to locate the SLN, especially in superficial nodes as are found surrounding melanoma lesions.

Magnetic Resonance Lymphangiography

Dendrimers are polymerized nanoparticles that when chelated with gadolinium (Gd) are promising contrast agents for MR lymphangiography. Dendrimers consist of a central core followed by multiple polymerized layers, or generations of shells, which are surrounded with an outer functional surface that provides excellent conjugate chemistry scaffold-

ing for Gd-chelates, fluorophores, or radionuclides.¹¹ As discussed above, the size of a macromolecule is critical for its efficient uptake and retention by the lymphatic system. By increasing or decreasing the number of generations during synthesis, the dendrimer size can be controlled for favorable lymphatic uptake and retention as well as improved excretion and elimination characteristics.^{11,12} It has been reported that the approximately 10 nm diameter Gd-chelated PAMAM G6 dendrimer is efficiently taken into the lymphatic capillaries and retained within the afferent lymphatics, providing excellent MR imaging of lymphatic flow and the SLN.¹⁰ This dendrimer has also been demonstrated to effectively visualize the thoracic duct of a pig when injected into its hind-foot, illustrating this agent's applicability to larger mammals (Fig. 4). In contrast, the low molecular weight (LMW) MR contrast agent Gd-[DTPA]-dimeglumine demonstrated poor visualization of the lymphatics in mice and furthermore was eliminated quickly from the body, reducing its ability to be used in time-dependent imaging studies.¹¹ Also, because of the many Gd atoms attached to each dendrimer and dendrimers' slower tumbling rate in solution, the relaxivity of such agents is very high and thus, the concentration of den-

FIG. 4. This MRI shows opacification of the thoracic duct of a pig after injection of Gd-chelated G6 PAMAM dendrimer to the hind foot, demonstrating the applicability of this method to larger mammals.

drimers administered can be very low compared to LMW Gd contrast agents.¹¹ Despite the low concentration administered, toxicity concerns still exist due to the potentially long retention time of dendrimers and subsequent leaching of free Gd-ions with the potential for nephrogenic systemic fibrosis necessitating the use of very high affinity chelates.

MR lymphangiography provides a number of distinct advantages over lymphoscintigraphy and the blue dye method. Its resolution is 30–100x greater than lymphoscintigraphy, has excellent temporal resolution, does not require surgery, and does not utilize ionizing radiation. Furthermore, MR images can easily be obtained in 3D providing robust visualization of lymph flow and the SLN. Compared to normal lymph nodes, tumor bearing nodes only take up the G6 dendrimer around their borders. Thus, MR can be used to both locate the SLN and provide information about the metastatic status of the node.¹³

Despite its many benefits, MR is impractical to provide intraoperative guidance. This shortcoming can be solved by combining an MR imaging agent with a near infrared (NIR) probe. Such dual-modality dendrimers have already been developed using Gd-chelated G6 PAMAM dendrimers conjugated to the NIR fluorophore, Cy5.5. After injection into the mammary pads of mice, this agent provided preoperative visualization of multiple lymph nodes using MR and an optical camera, the latter of which could be used for intraoperative guidance (Fig. 5).¹⁴ This concept was further expanded upon by using the Alexa series of NIR fluorophores

FIG. 5. Dual-modality MR and NIR probes preoperatively locate SLNs in mice. Dual-modality Gd-Cy5.5 dendrimer probes demonstrating the SLN are shown on MR (a) and NIR optical imaging (b) in a mouse. Arrows indicate the SLN signal. Lymphatic vessels are visible in the MR image only (a). (Figure originally published in the *Journal of Magnetic Resonance Imaging*, see Ref 14).

conjugated to G6 dendrimers to track the progression of Qdot labeled metastatic melanoma cells along local lymphatic channels into first the SLN, and later into distal nodes.¹⁵ The concepts involved in optical lymphangiography will be expanded upon below.

Optical Lymphangiography

Optical imaging of the lymphatic system allows for the localization of the SLN, mapping of lymphatic flow, and visualization of multiple nodes from different draining regions simultaneously.¹⁶ Aside from being able to visualize systems with multiple colors in real time, optical imaging is relatively inexpensive, easy to use, and does not cause any ionizing radiation. These unique qualities allow for exciting possibilities in the prevention and treatment of lymphedema, as well as the staging and treatment of cancer. Just as the benefits of optical imaging are many, so are the types and characteristics of optical probes.

The principal downside of optical imaging agents remains low depth penetration with the brightest agents only visible through a maximum of 1–2 cm of tissue.¹⁷ Probes that emit in the NIR, approximately 600–800 nm, help to maximize the target to background ratio and have improved depth penetration.¹⁸ Cells, tissues, and biological fluids autofluoresce minimally when stimulated in the NIR, thus, using probes that emit in this spectrum allows for reduced background. Above the NIR, in the true infrared, excitation can induce too much heat damage and is therefore, less desirable than NIR light for clinical purposes.

The target to background ratio can further be enhanced by utilizing cameras with multiple filters and post-processing spectral unmixing algorithms. Unmixing involves taking multiple images filtered at 10 nm increments through the entire visible-NIR spectrum and spectrally resolving these images to separate target fluorescence from autofluorescence that typically has a much broader peak. Unfortunately, doing this in real-time is not possible as each 10 nm increment requires approximately 100 ms to acquire the image and 10–30 such images are obtained. Processing and combining images takes additional time. Further enhancement of target to background ratio can be obtained by reconstructing multiple focal planes into a single image which reduces blurring. This improves the resolution of smaller structures such as the lymphatic vessels.¹⁹

Traditional Fluorophores

Due to their small size, NIR organic fluorophores such as Alexa 700, Cy 7, and Cy 5.5 must be conjugated or incorporated with macromolecules such as immunoglobins, nanoparticles, or other proteins to effectively enter and remain in the lymphatic system without leaking into the surrounding

tissue. It should be noted that the conjugation product of a fluorophore and macromolecule may have a toxicity profile that differs from its constituent parts and thus, the conjugate toxicity must be established before clinical approval. Despite their frequent use, organic fluorophores do have drawbacks. Due to their relatively wide emission spectra, the ability to use them in simultaneous multicolor imaging without detrimental spectral overlap is limited.²⁰ Additionally, because they are less bright and more susceptible to photo-bleaching than quantum dots, their useful imaging depth is reduced and spectral-unmixing algorithms must be used.¹⁶

Despite these limitations, the NIR fluorophores Cy5.5 and Cy7 conjugated to IgG have been used successfully to map the drainage patterns of lymphatic basins and to locate SLNs. IgG was chosen as a carrier due to its low toxicity, favorable uptake and retention by the lymphatics, and low immunogenicity.^{16,21,22} The SLN of mice imaged with Cy5.5 and Cy7-conjugated IgG were visualized within one minute after injection with a depth penetration of approximately 5 mm (Figs. 6 and 7). The mapping demonstrated that axial and lateral thoracic lymph nodes, but not cervical lymph nodes, receive simultaneous input from multiple ipsilateral sites such as the breast and upper extremity. Furthermore, the contri-

FIG. 6. Cy5.5 (red) and Cy7 (green) fluorescently-labeled IgG successfully map the nodes different lymphatic basins. (a) A composite image of spectrally unmixed Cy5.5 and Cy7 before surgical removal of the skin. Yellow arrows indicate a cervical node, which only received drainage from the ipsilateral ear. Blue arrows indicate a lateral thoracic lymph node that received drainage exclusively from the ipsilateral upper extremity. (b) A composite image of unmixed Cy5.5 and Cy7 images after surgical removal of the skin. (c) A color photograph of the same mouse shows that the lymph nodes, without fluorescent enhancement, are indistinguishable from the surrounding tissue. (Figure originally published in the *Journal of Investigative Dermatology*, see Ref. 16).

FIG. 7. Cy5.5 (red) and Cy7 (green) fluorescently-labeled IgG successfully map the drainage territory of axillary and lateral thoracic lymph nodes. The fluorescently-labeled lymph nodes show the regions of the lymph node involved in draining different regions whereas the differential interference contrast images (black and white) do not (a and b). The boundaries (c, white arrow) of the lymph nodes could clearly be discerned, suggesting the possibility of removal of only the diseased portion of a node, sparing the healthy portion of the node and reducing risk of postsurgical lymphedema. (Figure originally published in the *Journal of Investigative Dermatology*, see Ref. 16).

butions from each region to a lymph node could be seen separately, suggesting the possibility of performing a partial lymphadenectomy of only the part of the lymph node that drained the cancer-containing basin.¹⁶ Such precise mapping could greatly improve surgical outcomes and reduce lymphedema.

The FDA-approved NIR dye indocyanine green (ICG) can also be used to image lymphatic flow and the SLN, although with significant limitations. ICG associates readily with serum albumin, once injected in the body, and is readily taken into the lymphatic system *in vivo*, without the need for conjugation to exogenous macromolecules.²³ ICG was used successfully to identify the SLN in breast cancer and melanoma with good detection rates using NIR real time cameras.^{23,24} Recently, ICG was found to visualize the SLN at microdoses between 10–100 μ g.²⁵ Rasmussen et al. expanded this concept by using microdoses of ICG to image lymphatic flow to assess lymphedema in a number of patients.²⁶ Nevertheless, the versatility of ICG is limited. Like many optical imaging agents, its depth penetration restricts its use to very superficial nodes. Furthermore, its lack of functional groups makes conjugation to targeting moieties, radionuclides, MR-contrast agents, or therapeutics difficult.²⁶ Despite these limitations, its clinically approved status and success at low doses makes ICG a promising agent for lymphatic imaging in the near future.

Quantum Dots

Quantum dots (Qdots) are extremely bright fluorescent nanoparticles that overcome some of the depth and spectral limitations of traditional fluorophores. Qdots are excited by a broad spectrum of light, yet emit in a narrow spectrum. Their narrow emission spectra and high quantum yield allows multiple colors in the visible spectrum to be distinguished with the naked eye, allowing for simultaneous multicolor mapping of the lymphatic system without the need for cameras or unmixing algorithms (Fig. 8).²⁷ Furthermore, their extreme brightness provides depth penetration of up to two centimeters.¹⁷ These unique properties could provide real-time guidance during lymphadenectomy, surgery, or endoscopy. Such real-time guidance could allow a surgeon to specifically remove the SLN or the portion of the node receiving drainage from the tumor site, greatly reducing post-surgical lymphedema and scarring. Their hydrodynamic size of 15–19 nm allows for efficient uptake into the lymph vessels without leaking out of the lymphatic endothelial junctions.²⁰ In mice it was shown that they are efficiently retained in the lymph nodes and could be seen for at least 7 days after injection, localized primarily within macrophages and dendritic cells.²⁷ This long retention time could provide the opportunity for minimally invasive Qdot-coupled photodynamic therapy

FIG. 8. Visual spectrum multicolor quantum dots simultaneously map multiple lymph nodes receiving drainage from different basins in real-time. *Arrows* indicate a superficial neck lymph node receiving drainage from two different sites. The *first panel* shows the lymph nodes illuminated through the skin. In the *second panel*, the skin has been removed from the mouse. The *final panel* shows the lymph nodes still illuminated after being surgically removed. (Figure originally published in the *Journal of Investigative Dermatology*, see Ref. 27).

of the SLN. Despite their benefits, toxicity concerns exist. It has been shown that Qdots can cause cell death *in vitro* through the generation of reactive oxygen species.²⁸ Also, heavy metal toxicity due to their Cd/Se or Cd/Te core and potentially long retention time remains a concern.¹¹ To be translated to the clinic, Qdot pharmacokinetics will need to be further elucidated, and strategies to reduce clearance time and mitigate any toxicity of heavy metals will need to be employed.

Upconverting Nanoparticles

Traditional fluorophores are excited at higher frequency (shorter wavelength/higher energy) and emit at a lower frequency (longer wavelength/lower energy), a property known as the Stokes shift. Upconverting nanoparticles offer exciting characteristics that may make them useful agents for lymphatic imaging because they behave in the opposite manner. Unlike traditional fluorophores and quantum dots, upconverting nanoparticles emit photons at a higher frequency (shorter wavelength) than their excitation frequency, known as anti-Stokes fluorescence. Since very few, if any, biological materials are known to display anti-Stokes properties, the light from upconverting nanoparticles can easily be unmixed from the background.¹⁸ Furthermore, like quantum dots but unlike most traditional fluorophores, upconverting nanoparticles are resistant to photobleaching.¹⁸ Though upconverting nanoparticles represent a new field in nanoparticle research, significant progress has already been made. Wu et al. synthesized hexagonal NaYF₄ nanocrystals that are visible with the naked-eye, are nonblinking, are resistant to photobleaching, and are hydrophilic.²⁹ Hilderbrand et al. recently described the synthesis of hydrophilic, biocompatible Y₂O₃ upconverting nanoparticles conjugated to the organic fluorophore carbocyanine dye, both of which emit in the NIR spectrum. They used this novel nanoparticle to image blood vessels of mice with two nanocrystals emitting distinct colors and report that the system was bright enough to be imaged in real-time.¹⁸ Ehlert et al. reported the synthesis of a series of upconverting nanoparticles in five different NIR colors which were approximately 20 nm, an ideal size for lymphatic imaging.³⁰ Promisingly, data suggest that upconverting nano-

crystals and similar nanomaterials are minimally cytotoxic although formal animal toxicity remains to be completed.^{1,18,31} Conversely, Chatterjee et al. describe a 50 nm upconverting nanoparticle which upon excitation generates singlet oxygen which is cytotoxic and could be used as a photodynamic therapy.³¹ Given its size, a similar agent could be ideal for photodynamic therapy of the SLN, offering a minimally invasive alternative to SLN lymphadenectomy. If biocompatibility and high fluorescent yield can be maintained and if their physical properties allow for efficient uptake and retention by the lymphatics, upconverting nanomaterials could be employed to overcome the challenges of traditional fluorophore and Qdot optical lymphangiography.

Future Directions

Mapping the lymphatics and SLN for pre- and intra-operative guidance with a single injection is nearly realized. What remains is the clinical translation of such agents that will require significant financial resources. There are, however, many possibilities for reaching this goal, some of which have already been demonstrated. Fluorescent quantum dots, which are very bright and could easily be seen during surgery with the naked eye, could be conjugated to a radionuclide such as In111 to add preoperative scintigraphic guidance. Alternatively, immunoglobins could be dual-labeled with both an organic fluorophore such as Alexa 700 and a radionuclide. Dual-modality Gd-dendrimers with conjugated fluorophores, which have already been designed, could be further enhanced with a radionuclide providing tri-modality probes. An alternative design is to label an iron ultrasmall particle with a silicate coating in which a NIR fluorophore is embedded and to which a radionuclide could be chelated. Indeed, this has already been designed and tested as a tri-modality lymphatic imaging method. Upconverting nanocrystals could be conjugated to a radionuclide or Gd providing dual-modality imaging. Such multi-modality combinations would allow for SLN localization and subsequent minimally destructive surgery. However, serious challenges remain in documenting safety and efficacy of these agents to enable approval by health regulatory agencies such as the FDA. Moreover, the niche market of lymphatic imaging is relatively small and

therefore, less profitable discouraging pharmaceutical companies from pursuing such compounds.

However, it is easy to imagine combining these imaging options with therapies. The fluorescent properties of the above macromolecules could be used as a trigger for photoactivatable drugs, or the macromolecules could serve as vehicles for photodynamic therapeutics, radiotherapeutics, or cytotoxic drugs. Therapies are more appealing, both from a safety/efficacy viewpoint and also from a profitability standpoint, than are purely diagnostic agents. Additionally, if targeting moieties were added to these macromolecules or if specific antibodies for the lymphatics were used, radiotherapy or otherwise highly-cytotoxic drugs could be delivered directly to the tumor or diseased lymph nodes. However, the advisability of this approach is of some concern and awaits pivotal animal trials before being considered in humans. A number of specific lymphatic markers that could serve as a target have been characterized, including podoplanin, prox1, LYVE-1, and CCL21, to name a few.³² Even without specific targeting, agents such as G6 PAMAM dendrimer have been shown to accumulate in the SLN. These agents could be used for Gd-mediated neutron capture therapy to eradicate cells in the SLN without surgery.¹⁰

Lymphangiogenic markers, such as VEGF-C ligand and the VEGF-R3 receptor, among many others, have recently been elucidated and could provide a means for the diagnosis and treatment of cancer and lymphedema. It has been demonstrated that lymphangiogenesis is induced by primary melanoma tumors both locally and in the SLN and this can precede and increase the likelihood of metastasis.^{2,33} By identifying an increase in these lymphangiogenic markers with fluorescent or otherwise labeled probes, the metastatic or pre-metastatic status of the SLN could be inferred without biopsy. This is particularly interesting as presently no sera-detectable markers for lymphatic metastasis have been identified.² These markers could also serve as therapeutic targets. Antibody and nanoparticle-coupled siRNA against the lymphangiogenic receptor VEGF-R3 and VEGF-C ligand have recently been shown to inhibit metastasis.^{34–36} If these or similar agents were coupled to an imaging agent such as a NIR probe or fluorescent nanoparticle, the effectiveness of the treatment could be monitored in real-time as indicated by a decrease in fluorescent intensity. Furthermore, blocking lymphangiogenesis could inhibit the further spread of metastatic cells to more distal nodes or organs, thus helping to confine the tumor to its primary location. Another therapeutic route could involve the coupling of photodynamic therapeutic, cytotoxic, or radiotherapy agents to probes targeted against lymphangiogenic markers. In this way, the tumor or metastatic cells releasing lymphangiogenic factors, or diseased lymph nodes themselves, could be killed with minimal systemic toxicity. Also, visualizing a decrease in lymphangiogenesis may help predict and thus help prevent lymphedema.

As already mentioned above, simultaneous multicolor imaging of the lymphatic system could provide a presurgical map of the lymphatic system and thus direct the surgeon during lymphadenectomy or biopsy. Such mapping could reveal critical bottle-necks in the lymphatic vasculature that must remain intact to prevent lymphedema, or show lymphedema-resistant collateral connections allowing the surgeon to be more aggressive. Indeed, this mapping could be so precise as to allow the surgeon to remove only the diseased

portions of the lymph node, leaving the healthy portions receiving drainage from other basins intact.

Coupling imaging agents to therapies not only has potential medical benefit but could also help propel new imaging agents through the translational process which is obstructed with significant hurdles. Imaging agents, because they are usually simply diagnostic tools, must demonstrate a high safety margin, but if used in conjunction with a therapeutic, some side effects or toxicities might be more acceptable, given that the patient also stands to benefit from successful treatment. Furthermore, bringing a new medical product to market typically runs in the hundreds of millions of dollars and clear profitability must be shown for large pharmaceutical companies to carry this burden. It is difficult to demonstrate this profitability for a strictly diagnostic instrument, which may only be administered once per patient in a relatively small patient population. However, coupling such an agent to a therapeutic would not only benefit the patient but may make the agent attractive enough to encourage pharmaceutical companies to shoulder additional risk.

Conclusion

Great progress has been made in just the past few years towards designing optimal agents to image the lymphatics. With dynamic and tunable macromolecular lymphatic imaging agents already demonstrated, new insights into the molecular processes of lymphangiogenesis and tumorigenesis can rapidly be translated into functional and molecular imaging probes and coupled therapeutics. Clinical translation of these exciting multi-modality and multi-functional agents will provide material benefit to the treatment of cancer and lymphedema.

Disclosure Statement

No competing financial interests exist for Richard T. Lucarelli, Mikako Ogawa, Nobuyuki Kosaka, Baris Turkbey, Hisataka Kobayashi, and Peter I. Choyke.

References

1. Abdul Jalil R, Zhang Y. Biocompatibility of silica coated NaYF(4) upconversion fluorescent nanocrystals. *Biomaterials* 2008;29:4122–4128.
2. Rinderknecht M, Detmar M. Tumor lymphangiogenesis and melanoma metastasis. *J Cell Physiol* 2008;216:347–354.
3. Cserni G. Metastases in axillary sentinel lymph nodes in breast cancer as detected by intensive histopathological work up. *J Clin Pathol* 1999;52:922–924.
4. Barrett T, Choyke PL, Kobayashi H. Imaging of the lymphatic system: New horizons. *Contrast Media Mol Imaging* 2006;1:230–245.
5. Harisinghani MG, Barentsz J, Hahn PF, Deserno WM, Tabatabaei S, van de Kaa CH, de la Rosette J, Weissleder R. Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. *N Engl J Med* 2003;348:2491–2499.
6. Harisinghani M, Ross RW, Guimaraes AR, Weissleder R. Utility of a new bolus-injectable nanoparticle for clinical cancer staging. *Neoplasia* 2007;9:1160–1165.
7. Allan R. Sentinel node localization: Do or dye alone? *Br J Radiol* 2001;74:475–477.
8. Alazraki N, Glass EC, Castronovo F, Olmos RA, Podoloff D. Procedure guideline for lymphoscintigraphy and the use of

- intraoperative gamma probe for sentinel lymph node localization in melanoma of intermediate thickness 1.0. *J Nucl Med* 2002;43:1414–1418.
9. Gerli R, Solito R, Weber E, Agliano M. Specific adhesion molecules bind anchoring filaments and endothelial cells in human skin initial lymphatics. *Lymphology* 2000;33:148–157.
 10. Kobayashi H, Kawamoto S, Bernardo M, Brechbiel MW, Knopp MV, Choyke PL. Delivery of gadolinium-labeled nanoparticles to the sentinel lymph node: comparison of the sentinel node visualization and estimations of intra-nodal gadolinium concentration by the magnetic resonance imaging. *J Control Release* 2006;111:343–351.
 11. Longmire M, Choyke PL, Kobayashi H. Dendrimer-based contrast agents for molecular imaging. *Curr Top Med Chem* 2008;8:1180–1186.
 12. Longmire M, Choyke PL, Kobayashi H. Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. *Nanomed* 2008;3:703–717.
 13. Kobayashi H, Kawamoto S, Sakai Y, et al. Lymphatic drainage imaging of breast cancer in mice by micro-magnetic resonance lymphangiography using a nano-size paramagnetic contrast agent. *J Natl Cancer Inst* 2004;96:703–708.
 14. Koyama Y, Talanov VS, Bernardo M, Hama Y, Regino CA, Brechbiel MW, Choyke PL, Kobayashi H. A dendrimer-based nanosized contrast agent dual-labeled for magnetic resonance and optical fluorescence imaging to localize the sentinel lymph node in mice. *J Magn Reson Imaging* 2007;25:866–871.
 15. Kobayashi H, Ogawa M, Kosaka N, Choyke PL, Urano Y. Multicolor imaging of lymphatic function with two nanomaterials: Quantum dot-labeled cancer cells and dendrimer-based optical agents. *Nanomed* 2009;4:411–419.
 16. Hama Y, Koyama Y, Urano Y, Choyke PL, Kobayashi H. Two-color lymphatic mapping using Ig-conjugated near infrared optical probes. *J Invest Dermatol* 2007;127:2351–2356.
 17. Kobayashi H, Koyama Y, Barrett T, et al. Multimodal nanoprobe for radionuclide and five-color near-infrared optical lymphatic imaging. *ACS Nano* 2007;1:258–264.
 18. Hilderbrand SA, Shao F, Salthouse C, Mahmood U, Weisleder R. Upconverting luminescent nanomaterials: Application to *in vivo* bioimaging. *Chem Commun (Camb)* 2009;4188–4190.
 19. Hama Y, Koyama Y, Bernardo M, Choyke PL, Kobayashi H. Spectral near-infrared fluorescence imaging of curved surfaces using projection reconstruction algorithms. *Contrast Media Mol Imaging* 2007;2:82–87.
 20. Kobayashi H, Hama Y, Koyama Y, Barrett T, Regino CA, Urano Y, Choyke PL. Simultaneous multicolor imaging of five different lymphatic basins using quantum dots. *Nano Lett* 2007;7:1711–1716.
 21. Pain SJ, Nicholas RS, Barber RW, Ballinger JR, Purushotham AD, Mortimer PS, Peters AM. Quantification of lymphatic function for investigation of lymphedema: Depot clearance and rate of appearance of soluble macromolecules in blood. *J Nucl Med* 2002;43:318–324.
 22. O'Mahony S, Solanki CK, Barber RW, Mortimer PS, Purushotham AD, Peters AM. Imaging of lymphatic vessels in breast cancer-related lymphedema: Intradermal versus subcutaneous injection of ^{99m}Tc-immunoglobulin. *AJR Am J Roentgenol* 2006;186:1349–1355.
 23. Kitai T, Inomoto T, Miwa M, Shikayama T. Fluorescence navigation with indocyanine green for detecting sentinel lymph nodes in breast cancer. *Breast Cancer* 2005;12:211–215.
 24. Fujiwara M, Mizukami T, Suzuki A, Fukamizu H. Sentinel lymph node detection in skin cancer patients using real-time fluorescence navigation with indocyanine green: Preliminary experience. *J Plast Reconstr Aesthet Surg* 2009;62:e373–378.
 25. Sevic-Muraca EM, Sharma R, Rasmussen JC, Marshall MV, Wendt JA, Pham HQ, Bonefas E, Houston JP, Sampath L, Adams KE, Blanchard DK, Fisher RE, Chiang SB, Elledge R, Mawad ME. Imaging of lymph flow in breast cancer patients after microdose administration of a near-infrared fluorophore: Feasibility study. *Radiology* 2008;246:734–741.
 26. Rasmussen JC, Tan IC, Marshall MV, Fife CE, Sevic-Muraca EM. Lymphatic imaging in humans with near-infrared fluorescence. *Curr Opin Biotechnol* 2009;20:74–82.
 27. Kosaka N, Ogawa M, Sato N, Choyke PL, Kobayashi H. *In vivo* real-time, multicolor, quantum dot lymphatic imaging. *J Invest Dermatol* 2009;129:2818–2822.
 28. Lovric J, Cho SJ, Winnik FM, Maysinger D. Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. *Chem Biol* 2005;12:1227–1234.
 29. Wu S, Han G, Milliron DJ, Aloni S, Altoe V, Talapin DV, Cohen BE, Schuck PJ. Non-blinking and photostable up-converted luminescence from single lanthanide-doped nanocrystals. *Proc Natl Acad Sci USA* 2009;106:10917–10921.
 30. Ehlert O, Thomann R, Darbandi M, Nann T. A four-color colloidal multiplexing nanoparticle system. *ACS Nano* 2008;2:120–124.
 31. Chatterjee DK, Yong Z. Upconverting nanoparticles as nanotransducers for photodynamic therapy in cancer cells. *Nanomed* 2008;3:73–82.
 32. Cueni LN, Detmar M. New insights into the molecular control of the lymphatic vascular system and its role in disease. *J Invest Dermatol* 2006;126:2167–2177.
 33. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* 2007;109:1010–1017.
 34. He XW, Liu T, Chen YX, Cheng DJ, Li XR, Xiao Y, Feng YL. Calcium carbonate nanoparticle delivering vascular endothelial growth factor-C siRNA effectively inhibits lymphangiogenesis and growth of gastric cancer *in vivo*. *Cancer Gene Ther* 2008;15:193–202.
 35. Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, Wu Y, Pytowski B, Skobe M. Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res* 2006;66:2650–2657.
 36. Shimizu K, Kubo H, Yamaguchi K, Kawashima K, Ueda Y, Matsuo K, Awane M, Shimahara Y, Takabayashi A, Yamaoka Y, Satoh S. Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer. *Cancer Sci* 2004;95:328–333.

Address correspondence to:

Peter L. Choyke, M.D.
Molecular Imaging Program
National Cancer Institute
Building 10, Room B3B69F
Bethesda, MD 20892

E-mail: pchoyke@nih.gov