Lymphatic Drainage Imaging of Breast Cancer in Mice by Micro-Magnetic Resonance Lymphangiography Using a Nano-Size Paramagnetic Contrast Agent

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Background: The presence of lymph node metastases is an important factor in breast cancer patient prognosis. Therefore, the precise identification of sentinel lymph nodes in these patients is critical. Improving current magnetic resonance (MR) imaging methods using a newly synthesized nano-size paramagnetic molecule, G6, as a contrast agent, provides an attractive means toward attaining this goal.

Methods: A four-dimensional method of micro-MR lymphangiography using G6 (9 nm/240 kd) was developed to visualize the lymphatic ducts and lymph nodes draining mouse mammary tumors over time. The ability of micro-MR lymphangiography with the G6 contrast agent to visualize lymphatic drainage of normal mouse mammary tissue was compared with that of the conventional MR contrast agent, Gd-[DTPA]-dimeglumine (<1 kd). Lymphatic drainage in spontaneous and xenografted breast tumor models was visualized using the G6 contrast agent. Results: Draining lymphatic ducts and lymph nodes were clearly visualized in the mammary tissue of normal mice and in spontaneous and xenografted breast tumor models after a direct mammary gland or peritumoral injection of G6. Gd-[DTPA]-dimeglumine, by contrast, failed to depict lymphatic flow from the mammary tissue in normal mice using the same method. Micro-MR lymphangiography using the G6 contrast agent revealed the absence of filling in the metastatic foci of affected lymph nodes. Conclusions: The superior temporal and spatial resolution of micro-MR lymphangiography using the contrast agent G6 may facilitate the study of tumor lymphatic drainage and lymphatic metastasis in both experimental animals and clinical medicine. In addition, this may be a powerful new method for sentinel lymph node localization in human breast cancer. [J Natl Cancer Inst 2004;96:703–8]

Breast cancer is the most common malignancy among women, resulting in approximately 45 000 deaths annually in the United States (1). The presence of lymph node metastases has major prognostic implications in breast cancer patients (2,3), and it is the major criterion for determining the need for adjuvant chemotherapy (4). For many years, surgical axillary dissection was used to assess lymph node involvement in breast cancer patients. Recently, however, a more limited surgical procedure,
sentinel lymph node (SLN) biopsy, has become increasingly routine because the disease status of the SLN accurately reflects the status of more distant axillary lymph nodes (5–7). Hence, patients who are SLN biopsy–negative for tumor metastases can be spared a more extensive and traumatic lymph node dissection, which is associated with substantial short- and long-term sequelae (8). Although SLN biopsy has advantages over axillary lymph node dissection in regard to postoperative complications, the development of an accurate, reliable, noninvasive method of lymph node assessment would eliminate these complications and be of great clinical benefit to breast cancer patients.

Several methods for localizing SLNs are currently in use; the two most common are peritumoral injections of isosulfan blue dye or a radionuclide-labeled sulfur or albumin colloid. In the first method, the SLN is detected by direct visualization of blue staining of the lymph node; in the second method, the SLN is detected by the local accumulation of the isotope using a handheld gamma ray counter (9). Both methods have limitations. The dye method requires dissection of tissue until the blue-dyed SLN is detected. A number of successful radionuclide imaging studies have been reported using technetium-99m–labeled colloid lymphoscintigraphy or 2-fluoro[18F]-deoxy-n-glucose positron emission tomography (10,11). However, the radionuclide method is limited by low temporal and spatial resolution.

Limitations of the current methodology for localizing SLNs have spurred studies to improve this technology. Experimental studies attempting to detect draining lymph nodes in pigs and dogs with magnetic resonance imaging (MRI) or computed tomography using conventional low-molecular-weight contrast agents have successfully detected some local draining lymph nodes with insufficient signal-to-noise contrast (12). MR lymphography performed in the pig using macromolecular contrast agents, such as ultra-small particles of iron oxide (USPIO) and Gadomer-17, visualized regional draining lymph nodes better than small contrast agents (13,14). Although these techniques could be used to localize SLNs, the dynamics of lymphatic flow could not be imaged. If the main lymphatic channels draining the tumor could be visualized, a better assessment regarding the presence or absence of tumors in the SLN could be made. In an attempt to create a better method for visualizing breast lymphatic flow that could lead to the detection of metastatic tumors in breast cancer patients, we developed a new dynamic three-dimensional (3D) micro-MR mammo-lymphangiography method. We tested this method using a dendrimer-based nano-size paramagnetic contrast agent in mouse models of breast cancer and compared it with MR using a conventional contrast agent.

MATERIALS AND METHODS

Preparation of Paramagnetic Contrast Agent G6

The generation-6 polyamidoamine (PAMAM-G6) dendrimer (Dendritech, Midland, MI) has an ethylenediamine core, 256 terminal reactive amino groups, and a molecular mass of 58 048 d. The PAMAM-G6 dendrimer was then reacted with a 256-fold molar excess of 2-(p-isoctiolcarboxybenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M) at 40 °C for 24 hours. The reaction was maintained at pH 9 by adding 1 M NaOH as necessary. An additional equal amount of 1B4M as a solid was added after 24 hours at room temperature and incubated for another 24 hours at 40 °C. The reaction products were purified by diafiltration using a Centricon 30 filter (Amicon, Beverly, MA). This resulted in more than 98% of the amine groups on the surface of the dendrimer reacting with the 1B4M as determined by 153Gd (NEN; DuPont, Boston, MA) labeling of aliquots as previously described (15).

The purified PAMAM-G6 dendrimer–1B4M conjugate (approximately 3 mg containing 4 μmol 1B4M in 230 μL) was then mixed with 8 μmol of nonradioactive Gd(III) citrate in 0.3 M citrate buffer overnight at 40 °C. The excess Gd(III) in the preparation was removed by diafiltration using a Centricon 30 filter while simultaneously changing the buffer to 0.05 M phosphate-buffered saline. The purified sample (G6) was diluted to 0.2 mL with 0.05 M phosphate-buffered saline, and approximately 5 μL was used in each mouse breast tissue. A replacement assay using 153Gd showed that 84% of the 1B4M on the PAMAM-G6 dendrimer–1B4M conjugate was indeed chelating Gd(III) atoms, as previously described (15).

Mouse Models

All mouse studies were approved by the Animal Care and Use Committee of the National Institutes of Health. Twelve-week-old BALB/c mice (n = 5) or athymic nu/nu mice (n = 9) (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD) were used for the normal mouse studies.

Thirty-one-week-old BALB-neuT mice (n = 3), a generous gift of Dr. G. Forni (University of Turin, Obassano, Italy), were used as a spontaneous breast cancer model because of their spontaneous development of bilateral breast cancers and lymph node metastases. BALB-neuT mice are transgenic for the rat HER-2/ neu (ErbB2) oncogene under the control of the mouse mammary tumor virus promoter, and they exhibit tissue-specific expression of HER-2/neu (16,17). Heterozygous female BALB-neuT mice (BALB/c background) develop mammary gland lobule hyperplasia at 5–6 weeks of age that progresses to atypical hyperplasia by 8–9 weeks, to in situ carcinoma by 14 weeks, and finally to invasive carcinoma, usually by 21 weeks (16–18). Most lymph nodes bearing metastases from the mouse mammary pad are found in the neck, lateral thoracic, or axillary region.

The second mouse breast cancer model was the PT-18 xenograft model. This model was created by injecting 105 PT-18 murine mast cells (19) into the left mammary pad of 10 athymic nu/nu mice. After 3 weeks, six mice developed tumor masses in the left axillary lymph nodes.

Dynamic 3D Micro-MR Mammo-Lymphangiography

Mice were anesthetized with 1.15 mg sodium pentobarbital (Dainabot, Osaka, Japan) by intraperitoneal injection. They were then injected with 0.10–0.16 μmol of Gd in 5–8 μL of the G6 contrast agent. A single G6 contrast agent molecule contained 213 Gd ions. Because the number of Gd ions is responsible for changing the T1-weighted MRI signal, we used Gd ion concentration rather than G6 concentration to describe the dose used for injecting mice. For normal mice, injections were made directly into the mammary gland; for mice used for breast cancer models, injections were made into
mammary tissue surrounding a tumor. Injection of the contrast agents into intra-mammary tissue is generally safer than intravascular injection. Dynamic micro-MR images were obtained using a 1.5-tesla superconductive magnet unit (Signa LX; General Electric Medical Systems, Milwaukee, WI) with a birdcage-type coil 3 cm in diameter held together by a custom-made coil holder. The mice were placed at the center of the coils and were wrapped in gauze to stabilize their body temperature.

To evaluate lymphatic drainage from the normal mammary glands of BALB/c (n = 5) and athymic nu/nu (n = 5) mice, the mice were imaged by 3D fast spoiled gradient echo (3D-fastSPGR [eFGRE3D package; Signa Horizon, General Electric Medical Systems]; repetition time/echo time = 19.2/7.2 ms; inversion time = 47 ms; 31.2 kHz, flip angle 30°, 4 excitations; 36 slice encoding steps; scan time = 4 minutes 49 seconds) with chemical shift fat-suppression (15) at 6, 12, 18, 24, 30, 36, 42, and 48 minutes after injection of the contrast agent. The coronal images were reconstructed with 0.6-mm section thickness at every 0.3 mm. The field of view was 8 cm × 4 cm (512 × 256 pixels). The slice data were processed into 3D images using the maximum intensity projection method (15) with a fixed window and level (window 3500 and level 2100) (Advantage Windows, General Electric Medical Systems). After imaging, the mice were killed by exposure to carbon dioxide gas. Images of the superficial cervical, lateral thoracic, and axillary lymph nodes were independently examined by two U.S. or Japanese board-certified radiologists (S. Kawamoto and H. Kobayashi) who have MRI expertise and have reviewed MR images of mice for more than 5 years. Any discrepancies between the two reviewers were resolved by discussion.

To evaluate the effect of contrast agent molecular mass on the ability to visualize the lymphatic ducts and lymph nodes, 0.15 μmol Gd of Gd-[DTPA]-dimeglumine (Magnevist; Schering AG, Berlin, Germany), an FDA-approved extracellular MRI contrast agent with a molecular mass of 938 d, was injected into the mammary glands of anesthetized athymic nu/nu mice (n = 4). Images were taken at 12, 24, and 36 minutes after injection. The mammary glands of these mice were subsequently injected with 0.15 μmol Gd of G6 contrast agent at the 36-minute time point, and images were taken at 12 and 24 minutes later (i.e., at 48 and 60 minutes after injection of Gd-[DTPA]-dimeglumine).

To evaluate lymphatic drainage from tumor-bearing mammary glands of BALB-neuT transgenic mice (n = 5) or the PT-18 tumor-bearing mammary glands of athymic nu/nu mice (n = 6), tumors were first localized using 2D fast-inversion recovery (2D-fastIR; repetition time/echo time = 8000/96 ms; inversion time = 150 ms; 31.2 kHz, 2 excitations; 16 slices; scan time = 2 minutes 16 seconds). The coronal images were taken before injection of the contrast agent and reconstructed with 1.5-mm-thick sections with no gap between sections. The field of view was 8 cm × 4 cm (512 × 256 pixels). The slice data were processed into 3D images using the maximum intensity projection method with a fixed window and level (window 3500 and level 2100). Then, 0.15 μmol Gd of G6 contrast agent was injected into the mammary tissue around the tumor, and dynamic micro-MR mammo-lymphangiograms were imaged with the 3D-fastSPGR sequence, as described above.

### RESULTS

#### Visualization of Major Draining Lymph Nodes From Normal Breast Tissue by Dynamic Micro-MR Mammo-Lymphangiography

Three major draining lymph nodes—axillary, lateral thoracic, and superficial cervical, with their draining lymphatic vessels—were visualized in normal breast tissue by dynamic micro-MR mammolymphangiography with the G6 contrast agent (Fig. 1 and Supplemental Animations 1 and 2; available at http://jnccancerspectrum.oupjournals.org/jnci/content/vol96/issue9/). (See Supplemental Animations 1 and 2; available at http://jnccancerspectrum.oupjournals.org/jnci/content/vol96/issue9/).

The axillary lymph node and its lymphatic vessels were visualized at the initial (6-minute) time point in all 10 mice (Table 1). However, the superficial cervical and lateral thoracic lymph nodes and their lymphatic vessels were not visualized until later. The lateral thoracic node was visualized in two mice at 18 minutes and in six mice at 42 minutes. The superficial cervical node was visualized in one mouse at the initial time point (6 minutes), in two mice at 24 and 30 minutes, and in three mice at 36, 42, and 48 minutes (Table 1). Initial discrepancies between the findings of the two reviewers occurred only at two time points in two mice (24 minutes for a lateral thoracic node and 30 minutes for a cervical node).

### Table 1. Visualization of three major draining lymph nodes from normal breast tissue

<table>
<thead>
<tr>
<th>Lymph node</th>
<th>No. of visualized lymph nodes/total No. of examined lymph nodes, at time of visualization (in minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial cervical</td>
<td>1/10, 1/10, 1/10, 2/10, 2/10, 3/10, 3/10, 3/10</td>
</tr>
<tr>
<td>Lateral thoracic</td>
<td>0/10, 1/10, 2/10, 4/10, 4/10, 5/10, 6/10, 6/10</td>
</tr>
<tr>
<td>Axillary</td>
<td>10/10, 10/10, 10/10, 10/10, 10/10, 10/10, 10/10, 10/10</td>
</tr>
</tbody>
</table>

*Lymph nodes of 12-week-old BALB/c or athymic nu/nu mice were visualized by three-dimensional micro–magnetic resonance mammolymphangiography using the G6 contrast agent.*
Dynamic micro-MR mammo-lymphangiography images of mice injected with Gd-[DTPA]-dimeglumine were examined and compared with images obtained with the G6 contrast agent in the same mice (Fig. 2). Draining lymph nodes and lymphatic vessels were clearly visualized with the G6 agent but not with Gd-[DTPA]-dimeglumine (Fig. 2).

**Visualization of Lymphatic Drainage From the Breast Tumor Tissue to Its Metastatic Lymph Nodes by Dynamic Micro-MR Mammo-Lymphangiography With Contrast Agent G6**

Because dynamic micro-MR mammo-lymphangiography using G6 could visualize lymphatic drainage in normal mice and was superior to the conventional MRI contrast agent Gd-[DTPA]-dimeglumine, we investigated whether it could be used to visualize lymphatic drainage in mouse breast tumor models. Dynamic micro-MR mammo-lymphangiography was used to image lymphatic drainage in two mouse breast cancer models: a spontaneous breast cancer model using BALB-neuT transgenic mice and a PT-18 mast cell tumor xenograft model. Flow within the draining lymphatic vessels was visualized readily in both models. Fig. 3 and Supplemental Animation 3 (animation available at http://jnccancerspectrum.oupjournals.org/jnci/content/vol96/issue9/) show several dilated lymphatic vessels extending from the breast tumor to two tumors in lymph nodes at the lateral chest wall in the spontaneous breast cancer model. The enhancement of the lymphatic vessels decreased rapidly and was not visible by 30 minutes after injection of the G6 contrast agent. In the PT-18 xenograft model, the metastatic foci in the lymph node tissue did not show enhancement with G6, although normal lymph node tissue located at the rim of the lymph node showed enhancement. In addition, the lymphatic vessel flowing into the lymph node with a metastatic tumor was dilated and showed enhancement (Figs. 4 and 5).

Solid tumor foci located in lymph nodes were not enhanced after administration of the contrast agent G6 because of the absence of normal lymph node tissue in metastatic tumor lesions (Figs. 3, 4, and 5 and Supplemental Animation 3). However, the lymphatic vessel flowing into the lymph node containing a metastatic tumor was dilated, and a small rim of normal lymph node tissue showed enhancement. Histopathologic examination confirmed tumor growth in non-enhanced portions of these nodes (Fig. 5). All six mice with PT-18 tumors showed abnormalities only in the axillary nodes. The axillary node was also the predominant draining node in the tumor-bearing BALB-neuT transgenic mice. Taken together, these results suggest that lymphatic flow from the mouse breast drains primarily to the axillary lymph nodes and readily promotes metastasis of the breast cancer into the axillary lymph node in mice.

**DISCUSSION**

We developed a novel method for dynamic micro-MR mammo-lymphangiography using a G6 dendrimer–based nano-size macromolecular paramagnetic contrast agent. Using this...
four-dimensional method, we were able to visualize both draining lymph nodes and lymphatic vessels from normal breast tissue in mice over time on a 3D display. In addition, the absence of the G6 contrast agent filling into a part of a lymph node suggested the presence of metastatic tumor foci.

Sentinel lymph node localization using lymphoscintigraphy and intraoperative gamma probes is becoming a routine part of the surgical treatment of patients with breast cancer or malignant melanoma (20,21). However, MRI has the following potential advantages over lymphoscintigraphy: 1) the spatial resolution of MRI (0.1–0.3 mm) is 30–100 times greater than that of scintigraphy (1 cm) because breast MRI utilizes surface coils that substantially decrease the field of view (22); 2) the temporal resolution of MRI is greater than 10 times that of lymphoscintigraphy, offering good potential for dynamic studies of lymphatic drainage; 3) 3D images improve anatomic localization; 4) the absence of radiation exposure and contamination, although radiation exposure is small, is beneficial to both surgeons and patients; 5) lymphoscintigraphy with a handheld micro-gamma probe necessitates surgical procedures to detect draining lymph nodes; and 6) a handheld micro-gamma probe is not found as commonly in hospitals as an MRI instrument. Although future studies in humans will be needed to determine the ultimate value of nano-size MRI contrast agents for dynamic micro-MR mammolymphangiography, this method has the potential to circumvent the limitations of standard lymphoscintigraphy and to help distinguish the sentinel lymph node from secondary lymph nodes.

The ideal imaging agent should be small enough to rapidly enter into lymphatic vessels and flow with the lymph fluid yet large enough to stay within the lymphatic system and not leak into capillary vessels. Previous reports (10,23) found that lymphangiographic contrast agents must be at least 4 nm in diameter to be retained efficiently within the lymphatic system. Molecules smaller than 4 nm in diameter penetrate capillary membranes and diffuse into the circulatory system, resulting in poor signal-to-background ratios. Larger molecules, by contrast, diffuse slowly from the interstitial space and likely accumulate more slowly in the sentinel nodes, providing a longer imaging window for visualizing these nodes. The G8 agent (13 nm in diameter) used in our previous report for deep lymphatic imaging studies (24) is too large for rapid uptake by lymphatic vessels (data not shown), whereas the G6 contrast agent (9 nm in diameter) used in this study is large enough to be retained in the lymphatic system but not so large that it cannot be taken up efficiently. The G6 contrast agent is retained by or has an affinity for normal lymph node tissue, resulting in an enhanced signal in normal lymph nodes (24).

FDA-approved MR contrast agents, such as Gd-[DTPA]-dimeglumine, rarely cause serious toxicity after intravenous or...
subcutaneous injection (26). Because adverse events caused by contrast agents are related to dose, the G6 contrast agent was used at less than 1/2500 the molar concentration of the clinical Gd-[DTPA]-dimeglumine dose used in this study to obtain images with sufficient quality and to minimize potential toxicity. Furthermore, the G6 agent was administered directly into the mammary gland tissue because local injection is generally less toxic than intravascular injection. Thus, the dynamic micro-MR mammo-lymphangiography method using nano-size MRI contrast agents may be translatable to clinical practice.

To enhance its use for potential intraoperative localization, the G6 agent can be easily dual-labeled with gadolinium and an optical or fluorescent agent, which will help the surgeon quickly and reliably localize the sentinel lymph node during surgery. We have created dual-labeled dendrimers with gadolinium and fluorescent isothiocyanate dye, and this dual imaging agent will be the subject of a future study.

REFERENCES


NOTE

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