Micro-magnetic Resonance Lymphangiography in Mice Using a Novel Dendrimer-based Magnetic Resonance Imaging Contrast Agent

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Abstract

Major advances in cancer biology and immunology have been gained using mouse models. However, very few methods are currently available to visualize the deep lymphatic system. A new micro-magnetic resonance lymphangiography (MRL) method in mice, which uses dendrimer-based magnetic resonance imaging contrast agents, was developed. Micro-MRL imaging clearly visualized most of the mouse lymphatic system, including both lymphatics and lymph nodes. This method could detect and distinguish among dilation of lymphatic vessels in a lymphangitis model, proliferative or neoplastic lymph node swellings in a lymphoproliferative model, and inflammatory lymph node swellings in an infection/inflammation model. Changes in the lymphoid system of transgenic mice overexpressing interleukin-15 could be visualized. Abnormal enlarged lymph nodes identified by micro-MRL were selectively removed and analyzed to demonstrate their cell type, receptor expression, and clonality in individual mice. We conclude that the enhanced resolution of this noninvasive micro-MRL can detect and classify lymphatic and lymph node abnormalities in mice, which should have wide applicability to the study of immunology and cancer in both experimental animals and clinical medicine.

Introduction

Transgenic and knockout mice are powerful tools to analyze the molecular basis of diseases, in addition to fostering major advances in unraveling the cellular basis of immunity in normal and abnormal states such as cancer, autoimmune diseases, immunodeficiencies, and congenital diseases. Despite the plethora of mouse models of cancer and immune defects, few methods exist to visualize the lymphatic system of mice. Development of a noninvasive and simple screening method for subclinical lymph node metastases would greatly aid translational and clinical researchers in diverse fields such as disease pathogenesis, early detection, biomarkers, therapeutic drug discovery, and high throughput screening of mutant mice. Two methods used to visualize parts of the human lymphatic system have been explored in mice. Direct lymphangiography has been performed using an iodine oil agent injected directly into lower extremity lymph vessels. This method is highly invasive and sometimes causes life-threatening complications, including lung embolization, pulmonary edema, and adult respiratory distress syndrome (1). Lymphoscintigraphy has been attempted with radiolabeled human serum albumin or aggregated albumin that is injected intracutaneously or s.c. (2, 3) but is not suitable for small animals because of poor image resolution. A few groups have recently reported some success in experimental studies of MRL using Gd-DTPA, liposomes, and Gd(III) macromolecular chelates in normal pig, rabbit, and rat models (4–7). Other groups have used i.v. injections of iron oxide particles, including the USPIO, that negatively enhance normal lymph nodes in rabbit and rat models (8–14). However, to the best of our knowledge, none of these methods can visualize the deep lymphatic system of the mouse (Fig. 1).

Apart from conventional imaging modalities, some success in visualizing the lymphatic system in live mice has been reported in studies in which fluorescent dyes were used. These studies analyzed the function of lymphatic networks in vivo, mainly in the mouse tail and surrounding tumors (15–20). Additionally, Leu et al. (21) used fluorescence lymphangiography in patients with systemic sclerosis. Whereas an advantage of fluorescence lymphangiography is the high resolution, its limitation is the depth of the sensitivity as compared with MRI.

Recently, we have synthesized a library of 20 different dendrimer-based macromolecular MRI contrast agents (22–24). PAMAM-G8

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Fig. 1. Schema of the murine lymphatic system.

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2 The abbreviations used are: MRL, magnetic resonance lymphangiography; DTPA, diethylenetriaminepentaacetic acid; USPIO, ultra-small particle of iron oxide; MRI, magnetic resonance imaging; PAMAM, polyamidoamine; G8, generation 8; 1B4M, 2-(p-isothiocyanato benzyl)-6-methyl-diethylenetriaminepentaacetic acid; MR, magnetic resonance; IL-15, interleukin-15; NK-natural killer; IEL, intraepithelial lymphocyte.
was selected because of its long survival in the circulatory system with minimal leakage out of the vessels. We reasoned that the ability of PAMAM-G8 to visualize small vessels might allow it to also visualize fine networks of the lymph vessels as well as lymph nodes (22). We used Gd-(DTPA)-dimeglumine (Schering, Berlin, Germany) as a control. These two MRI contrast agents were tested in mouse inflammation/infection, proliferative lesion/lymphoma, and lymphangitis models.

**Materials and Methods**

**PAMAM Dendrimers.** The PAMAM-G8 dendrimer (Dendritech, Inc., Midland, MI) has an ethylenediamine core, 1024 terminal reactive amino groups, and a molecular weight of 233,383. Gd-(DTPA)-dimeglumine (Magnevist®; molecular weight, 938) was purchased from Schering.

**Conjugation of Chelates to the Dendrimers.** The PAMAM-G8 dendrimer was concentrated to ~5 mg/ml and diafiltered against 0.1 M phosphate buffer at pH 9. The PAMAM-G8 dendrimer was reacted with a 1024-fold molar excess of 1B4M at 40°C and then maintained at pH 9 with 1 M NaOH for 24 h. An additional equal amount of 1B4M was added after 24 h as a solid. The resulting preparations were purified by diafiltration using a Centricon 30 filter (Amicon Co., Beverly, MA). This resulted in >98% of the amine groups on the dendrimer reacting with 1B4M as determined by 153Gd (New England Nuclear DuPont, Boston, MA) labeling of aliquots as described previously (25).
Preparation of MRI Contrast Agents with Nonradioactive Gd(III). PAMAM-G8 dendrimer-1B4M conjugate (−3 mg containing 4 μmol of 1B4M) was mixed with 8 μmol of nonradioactive Gd(III) citrate in 0.3 M citrate buffer overnight at 40°C. The excess Gd(III) in each preparation was removed by diafiltration using a Centricon 30 filter (Amicon Co.) while simultaneously changing the buffer to 0.05 M PBS. The purified samples were diluted to 0.2 ml with 0.05 M PBS, and 5 μl were used in each mouse extremity. A replacement assay using 153 Gd determined that 80% of the 1B4M on the PAMAM-G8 dendrimer-1B4M conjugate was indeed chelating Gd(III) atoms as described previously (25).

Animal Models. All studies were approved by the Animal Care Committee of the NIH. For the concanavalin A-induced lymphangitis model, 7–8-week-old mice were used. To generate the lymphangitis model, 300 μg of concanavalin A (Sigma, St. Louis, MO) were injected i.v. into C57BL6 mice 24 h before micro-MRL. For a chronic lymphoproliferative/neoplastic disease model, 8–10-month-old IL-15 transgenic mice (on a C57BL6 background) were used because they manifested selective expansion of NK, CD8⁺NK-T, γδIELs, and CD8⁺ T cells in the periphery (26). Additionally, nude mice with sporadic bacterial infections were used as an infection model.

Three-dimensional Micro-MRL. Mice were anesthetized with an i.p. injection of 1.15 mg of sodium pentobarbital (Dainabot, Osaka, Japan) and were then given intracutaneous injections of 0.1 μmol of PAMAM-G8 or Gd-DTPA into four mid-fingers in all four extremities. All dynamic micro-MR images were obtained using a 1.5-T superconductive magnet unit (Signa LX; General Electric Medical System, Milwaukee, WI) with a 1-inch round surface coil (Birdcage type) fixed by a custom-constructed coil holder. In each group, six female 6–7-week-old mice of 18–21 g were used, and the PAMAM-G8 contrast agent was prepared two separate times for these imaging studies. The mice, wrapped with gauze to stabilize their body temperature, were placed at the center of the coils. A three-dimensional fast-spoiled gradient echo (efgre3d package, Signa LX; General Electric Medical System; repetition time/echo time = 28.5/7.9; inversion time = 65 ms; 31.2 kHz, flip angle 30°, four numbers of excitation; scan time = 7 min 36 s) with chemical fat suppression was used 45 min after injection of the contrast agents. The coronal images were reconstructed with 0.6-mm section thickness with 0.3-mm overlap (two 512 matrix zips). The field of view was 6 × 3 cm, and the matrix size was 512 × 256. The slice data were processed into three-dimensional images using...
the maximum intensity projection method (Advantage Windows; General Electric Medical System). The image resolution was 117\( \times \)1100\( \times \)600\( \mu \)m.

**Statistical Analysis.** Statistical analysis was performed using either Student’s \( t \) test (SigmaStat; Jandel Scientific, San Rafael, CA).

**Results**

**Three-dimensional Micro-MRL of Normal Mice with PAMAM-G8 Compared with Gd-(DTPA)-dimeglumine.** Most deep lymph nodes throughout the body that were clearly shown on the anatomy schema (Fig. 1) were visualized by three-dimensional micro-MRL with PAMAM-G8 but not by Gd-(DTPA)-dimeglumine (Fig. 2, A and B). In addition, the thoracic duct was visualized in all six mice that were given injections of PAMAM-G8, but not in mice given injections of Gd-(DTPA)-dimeglumine (Fig. 2). Other lymphatic vessels were also better visualized with PAMAM-G8 than with Gd-(DTPA)-dimeglumine.

**Three-dimensional Micro-MRL with PAMAM-G8 in Mouse Disease Models.** Micro-MRL was evaluated in several mouse disease models. Concanavalin A was injected into normal mice to induce lymphangitis. Three-dimensional micro-MRL could detect remarkable dilation of lymphatic vessels throughout the whole body, especially in the liver (Fig. 3a). The enhancement in the liver was seen adjacent to the vascular structures, but the vascular structures themselves did not show enhancement (Fig. 3b). Histology analysis revealed that the lymphocytes mainly infiltrated adjacent to the vascular structures with cavernous dilation (>10\( \mu \)m) of lymphatic ducts filled with massive lymphocytes, corresponding to the enhancement location and consistent with the imaging result (Fig. 3c).

Lymph node changes in a proliferative or neoplastic model were evaluated. IL-15 transgenic mice showed considerable lymph node swelling with nonenhancing central filling defects (Fig. 4a). The abnormal lymph nodes identified by micro-MRL were targeted for
removal, living cell sampling, and subsequent analysis. Immunological and molecular biological analyses to demonstrate the cellular phenotypes, the receptor expressions, and the clonality of the infiltrative cells in individual mice were successfully performed. Pathological examination of these lymph nodes confirmed the observation obtained by the micro-MRL; the germinal center structure no longer seen in these lymph nodes was replaced by a homogenous dense infiltration of lymphoid cells, which restricted access of the contrast agents emulating a filling defect image (Fig. 4b). These observations collectively demonstrated that with age the IL-15 transgenic mouse would develop CD8+ T-cell expansion and proliferation in multiple lymph nodes, which may lead to the onset of lethal pathological conditions such as lymphoma.

Finally, nude mice that develop spontaneous oral ulcers and urinary tract infections were examined. In this infectious model, irregular dilation of the lymphatic vessels in the lymph nodes (Fig. 5) was found. These enlarged infectious lymph nodes could be easily differentiated from the enlarged lymph nodes in the IL-15 transgenic mice that had central filling defects (Fig. 5).

Discussion

PAMAM dendrimers are a class of highly branched spherical polymers that are highly soluble in aqueous solution and have a unique surface of primary amino groups (27, 28). The defined structure and large number of available surface amino groups allow conjugation of antibody molecules (28–31) or chelation of Gd(III) for novel MRI contrast agents (24, 32–35). We have synthesized a library of dendrimer-based MRI contrast agents and reported recently on their pharmacokinetics and imaging properties after i.v. injection (34, 35). The PAMAM dendrimer-core-based macromolecular MRI contrast agents could visualize blood vessels (larger generations) or kidney tubules (smaller generations). In the molecular weight range studied previously, the larger molecules, PANAM-generation-7 and PAMAM-G8 remained in the circulation longer because of less excretion from the kidney, resulting in visualization of finer vessels (22, 34, 36). A large molecule of an appropriate size possessed properties that enabled it to be retained in the vessels longer and thereby also realized the highest signal:noise ratio as compared with a small molecule such as Gd-(DTPA)-dimeglumine.

In our present study, we hypothesized that these special properties of the PAMAM-G8 might make it useful for three-dimensional micro-MRL. We hypothesized that the large size and low transvascular diffusion of PAMAM-G8 (22) might allow it to be retained in fine lymphatic vessels as well as in lymph nodes for an extended period of time, resulting in a high signal:background ratio.

The majority of the MR studies for visualizing the lymphatic system reported previously were performed with i.v. injection of dextran-coated microparticles of iron oxide (USPIO or monocrystalline superparamagnetic iron oxide; Refs. 8–14). However, the USPIO MR contrast agents are not very useful in lymphangiography. Firstly, they preferentially visualize lymph nodes and cannot detect lymphatic vessels. Secondly, the signal obtained with negative MRI contrast agents is much smaller than that obtained with positive contrast agents and can make the lymph node difficult to find. We found that the dendrimer-based MRI contrast agent PAMAM-G8 was able to visualize subtle changes in small lymph nodes and lymphatic vessels of the mouse in part because it is a positive contrast agent, is retained inside lymphatic compartments, and has a high signal:noise ratio. These properties, coupled with the high spatial resolution of three-dimensional micro-MRL, allowed PAMAM-G8 to distinguish between infectious and proliferative or neoplastic lymph node swelling (Fig. 5).

MR contrast agents such as Gd-DTPA rarely cause serious toxicity after i.v. injection (37). Nevertheless, to further minimize potential toxicity, we used 0.0002-fold less PAMAM-G8 (on a molar basis) than typically used for Gd-DTPA for human lymphography. Furthermore, the agent was administered by intracutaneous injection because this route is generally safer than i.v. and intra-arterial injection. Thus, the MRL method might be easier to translate to clinical practice. Micro-MRL of the mouse provides two additional major contributions to immunological studies: (a) we were able to detect abnormalities in the lymphatic system throughout the body in a live animal, allowing evaluation of time-dependent changes in the same mouse (data not shown); and (b) the MRL image was used to target removal and subsequent analysis of involved lymph nodes in IL-15 transgenic mice with lymphoadenopathy. Immunological and molecular biological analyses were then performed to demonstrate the cellular phenotypes, the receptor expressions, and the clonality of the infiltrative cells in

Fig. 5. Three-dimensional micro-MR images of normal (a), infectious (b), and lymphoproliferative or neoplastic (c) external iliac lymph nodes given injections of PAMAM-G8. The images were obtained 45 min postinjection. Maximum intensity projection images are shown. The scale indicates 1 cm.
individual mice. These results will be diagnostically useful in determining the consequences of the expansion of CD8 T lymphocytes in the IL-15 transgenic mouse.

The dilated liver and mesenteric lymphatic systems were enhanced and visualized in the concanavalin A-induced lymphangitis model mice by this method. This type of liver lymphatic enhancement was found just surrounding the vasculature in the disease model mice (lymphoma/lymphangitis). The enhancement tended to locate along the hepatic veins as shown in the Fig. 3. Therefore, a respective amount of contrast agent, which attached on lymphocytes, could migrate from the main trunk of the lymphatic vessels back to liver or mesenteric systems associated with lymphoyyte infiltration. Thus, it might enhance the liver and mesenteric system lymphatic systems, especially under the condition of lymphatic congestion in the disease model mice.

The advantage of this micro-MRL method is its ability to visualize the deep lymph nodes and lymphatic vessels as compared with fluorescence lymphangiography. However, although fluorescence lymphangiography may be limited to visualizing only superficial lymphatic systems, its time and special resolutions are much higher than those of MRI. Therefore, these two techniques are complementary and could be combined to permit imaging of entire lymphatic system.

In conclusion, a method for micro-MRL has been developed that can visualize both lymph nodes and lymphatic vessels with a denliner-based macromolecular MRI contrast agent, PAMAM-G8. This method was able to visualize most of the lymph nodes throughout the bodies of the mice and could distinguish infectious expansion of lymphocytes from that caused by chronic lymphoproliferative conditions. The enhanced resolution of this method should have wide applicability to the study of immunology and cancer in both experimental animals and clinical medicine.

References
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