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Tumor-induced alterations in lymph node lymph drainage identified by contrast - enhanced MRI

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Abstract

Purpose—To use high resolution MRI lymphography to characterize altered tumor-draining lymph node (TDLN) lymph drainage in response to growth of aggressive tumors.

Materials and Methods—Six mice bearing B16-F10 melanomas in one rear footpad were imaged by 3.0-T MRI before and after subcutaneous injection of Gadofosveset trisodium (Gd-FVT) contrast agent into both rear feet. Gd-FVT uptake into the left and right draining popliteal LNs was quantified and compared using Wilcoxon signed-rank test. Fluorescent dextran lymphography compared patterns of LN lymph drainage with the pattern of immunostained lymphatic sinuses by fluorescence microscopy.

Results—TDLNs exhibited greater Gd-FVT uptake than contralateral uninvolved LNs, although this difference did not reach significance ($p < 0.06$). Foci of contrast agent consistently surrounded the medulla and cortex of TDLNs, while Gd-FVT preferentially accumulated in the cortex of contralateral LNs at 5 and 15 min after injection. Fluorescent dextran lymphography confirmed these distinct contrast agent uptake patterns, which correlated with lymphatic sinus growth in TDLNs.

Conclusion—3.0-T MRI lymphography using Gd-FVT identified several distinctive alterations in the uptake of contrast agent into TDLNs, which could be useful to identify the correct TDLN, and to characterize TDLN lymphatic sinus growth that may predict metastatic potential.

Keywords

lymphography; melanoma; lymph node; gadolinium; albumin; mouse

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Introduction

Currently many tumors are overtreated since our toolbox to determine propensity to metastasis is limited (1). Sentinel lymph node (SLN) biopsy has proved useful to test whether the cancer has spread to tumor-draining lymph nodes (TDLNs), as a strong predictor of metastasis to distant organs (2–4). However, the SLN is sometimes misidentified, due to altered or blocked lymphatic drainage (5,6). Additional diagnostic features to not only accurately identify the SLN but also to independently assess metastatic potential of cancers would be useful to improve clinical management and reduce overtreatment.

LN draining malignant tumors develop characteristic alterations that could be useful for diagnosis. First, accumulation of immune cells in the TDLN is associated with hypertrophy, although this parameter on its own is not a strong diagnostic feature (2). Second, growth of lymphatic sinuses is a feature of murine (7–9) as well as human TDLNs (10–12). This LN lymphangiogenesis is not induced by benign tumors, while it is prominent in mice developing carcinomas even before LN metastasis is detected, suggesting that these alterations predict potential for tumor spread (13). Studies of human cancers including, breast, rectal, and squamous cell carcinomas also suggest that TDLN lymphangiogenesis may predict metastatic potential (11,12,14,15). Third, TDLN lymphangiogenesis is associated with accelerated lymph flow through the LN in murine tumor models, as detected by subcutaneous dye injection and optical imaging (9,16). Dynamic contrast-enhanced MRI at 1.5T using gadolinium contrast agent (Gd-DTPA) also demonstrates increased rate and amount of lymph drainage through TDLNs (17). However, the resolution of these prior studies was not high enough for analysis of structural and functional changes in murine LNs, which could relate to LN lymphangiogenesis and increased lymph flow throughout TDLNs. The increased resolution afforded by the development of higher magnetic field strength scanners and optimization of contrast agents for lymphatic imaging could improve visualization of these alterations.

Most MRI lymphography studies thus far have used low molecular weight gadolinium contrast agent formulations (18–20). However these contrast agents rapidly transit through the lymphatics and into the blood circulation, limiting the imaging period (17). Larger size contrast agents have been tested to slow contrast transport through the lymphatics (21,22), as nanoparticles up to 60 nm in diameter can be taken up into blind-ended initial lymphatic vessels in the periphery (23). The nanoparticle Gd-based contrast agents are not yet clinically-approved due to toxicity concerns, limiting their utility for human use (24). However, one contrast agent that has been approved for clinical use is Gadofosveset trisodium (Gd-FVT), which efficiently binds to albumin to form a small nanoparticle (25). In particular, Gd-FVT is appealing for use in lymphography and angiography, due to its increased circulation time and improved resolution relative to low molecular weight gadolinium contrast agents (26). In rabbits (27) and humans (28), Gd-FVT has shown promise for lymphography and LN imaging after subcutaneous injection. In this study, we employed Gd-FVT contrast agent and 3.0-T MRI lymphography to investigate tumor-induced alterations in lymph flow through LNs draining melanomas in mice, with the goal of identifying diagnostic features of SLNs that could be markers of metastatic potential.

MATERIALS AND METHODS

Mouse Tumor Model

C57Bl/6 mice from Jackson Laboratories (Bar Harbor, ME) were maintained in microisolator rooms under specific pathogen-free conditions. Five week-old male or female mice were injected in the left hind leg footpad with 200,000 B16-F10 cells (American Type Culture Collection, Manassas, VA) in 50 microliters of Hanks' Buffered Saline Solution (Gibco Life Technologies, Grand Island, NY), and in the right hindfoot with saline (16,29). Mice were imaged 21 to 23 days later when tumors were 2 to 5 mm in diameter.

Magnetic Resonance Image Acquisition

Mice were imaged *in vivo* on a 3.0-Tesla MR scanner (Philips Achieva, Best, The Netherlands) equipped with high performance gradients (maximum gradient strength of 80 mT/m and maximum slew rate of 200 mT/m/ms) using a dedicated single-channel solenoid mouse RF coil with a built-in heating system to maintain physiological body temperature (Philips Research Laboratories, Hamburg, Germany). Animals were anesthetized with 3% isoflurane through an MR-compatible mobile inhalation system (DRE Inc, Louisville, KY) and sedation was maintained during imaging with 2.5% isoflurane delivered through a nose cone. Animals were positioned supine in the RF coil on a custom platform, with legs loosely taped to a water-filled capped 15 ml test tube to maintain positioning at the same level and to reduce susceptibility-related artifacts. Following MR imaging, mice were euthanized by 5% isoflurane overdose for 5 min, followed by cervical dislocation. LNs were then dissected, examined for melanotic micrometastases (30), and photographed in a stereomicroscope.

For lymphography, the dorsal toes of both rear feet were injected subcutaneously with 25 μ l of gadofosveset trisodium (Gd-FVT; 0.025 mmoles/kg; Ablavar: Lantheus Medical Imaging; N. Billerica, MA). Imaging was performed using a coronal T1-weighted 3D fast gradient echo sequence with fat suppression with TR= 20.5 msec, TE = 9.0 msec, flip angle = 12°, field of view = 44 $\times$ 44 mm, imaging matrix = 316 $\times$ 243, slice thickness = 0.30 mm, number of excitations = 4, with approximately 42 slices for an acquisition time of 10 min, 31 sec. Three timepoints were acquired: a pre-contrast agent acquisition (t = 0 min) followed by two sequential post-contrast agent acquisitions with k-space centered at 5:14 min (t = 5 min) and 15:45 min (t = 15 min) after Gd-FVT injection into the dorsal toe of both feet. Acquired imaging resolution was 0.14 $\times$ 0.18 mm in plane, reconstructed to 0.1 mm in plane with 0.15 mm slice thickness.

Image Analysis

MR images were analyzed on a PC workstation using ImageJ software (National Institutes of Health, Bethesda, MD), incorporating custom in-house plugin software developed using Java (Oracle Corp., Redwood Shores, CA). Signal intensities were measured from sequential pre- and post-contrast agent T1-weighted 3D images by manually delineating regions of interest (ROI) over the entire LN in multiple image slices. The signal intensity values for each voxel in the multiple ROIs were then written out to a text file for subsequent analyses. Integrated density (defined as the sum of the pixel signal intensity values) was calculated for all voxels in the LN, along with other histogram-based statistics (mean, median, standard

deviation, etc). ROIs were drawn separately for each pre- and post-contrast agent time point to account for any motion or misregistration resulting from the injection procedure. LN Gd-FVT uptake was quantified by subtracting pre-contrast from post-contrast agent integrated density measures. Calculations were made for both right and left popliteal LNs. The integrated density metric was used to measure Gd-FVT uptake, as this parameter incorporates both signal enhancement and volume. Change in integrated density was previously observed to provide a more useful measure of LN uptake of contrast agent than change in mean signal intensity (31), as LNs can vary significantly in size and the distribution of contrast uptake tends to be localized with much of the LN remaining unenhanced.

For illustration purposes, contrast enhancement maps representing Gd-FVT uptake were created for individual slices in the LNs, using custom software developed in Matlab (Mathworks, Natick, MA). First, LNs were manually segmented from surrounding tissues and nonlinear image registration was performed to align the pre- and post-contrast agent images. Next, aligned images were subtracted (post- minus pre-contrast agent), and a color map representation was used to indicate the magnitude of the difference.

The Philips Extended MR WorkSpace 2.6.3.4 system (Philips Medical Systems, Best, The Netherlands) was used to create rotated three-dimensional maximum intensity projections (MIPs), which were then saved in movie format.

Fluorescent Dye Lymphography

To facilitate comparisons between MRI and histology, a separate group of 4 mice were imaged using a fluorescent dye lymphography technique (9). Texas Red Dextran of 10,000 MW was used to facilitate comparison with the behavior of the similarly sized 70 kD Gd-FVT-albumin complex. Lysine-fixable Texas Red Dextran (Invitrogen, Grand Island, NY) was injected into both rear dorsal toes, at 8 mg/ml in 25 microliters PBS, while under 2.5% isoflurane anesthesia. Twenty minutes later mice were euthanized by CO₂ overdose, and popliteal LNs were dissected. LNs were oriented for cryosectioning at a cross-section through the cortex and medulla using the white-colored cortical B cell region to orient LN placement into OCT freezing media (Sakura Finetech). Cryosections were dried and fixed in 4% paraformaldehyde for 10 min, immunostained with LYVE-1 (eBioscience, San Diego, CA) and then with Alexa Fluor 488-labelled goat secondary antibody (16). Sections were mounted in Prolong Gold (Invitrogen, Sparks, MD) for photography on a Nikon Eclipse 50i fluorescence microscope, and images were processed using Nikon NIS Elements BR 3.0 software (Nikon, Inc.; Melville, NY).

Statistical Analysis

Integrated density measures were compared between pre- and post-contrast time points by nonparametric Wilcoxon signed-rank test. Analysis was performed using JMP v10.0 (SAS Institute, Cary, NC), with $p < 0.05$ considered significant.

RESULTS

MRI Lymphography of Tumor-draining Popliteal Lymph Nodes

Six mice bearing B16-F10 melanoma tumors in the left rear footpad were imaged for this lymphography study. The left and right rear dorsal toes were injected with Gd-FVT to compare lymph drainage into the tumor-draining left popliteal LN (LPN) with drainage into the uninvolved right popliteal LN (RPN), as illustrated in Fig. 1a. The optimized MRI protocol allowed visualization of the popliteal LNs even before contrast agent injection (Fig. 1b). These pre-contrast agent images demonstrated enlargement of the tumor-draining LPN relative to the control RPN (Fig. 1b).

After Gd-FVT injection, both popliteal LNs showed enhancement (Fig. 1b). Post-injection images show Gd-FVT appearing in both popliteal LNs within 5 min, and continuing through 15 min. Spatially, Gd-FVT enhancement was primarily observed in the margins of the LPN or RPN at 5 or 15 min after injection, which improved delineation of the LN margins relative to pre-contrast images. Interestingly, low signal artifacts appeared in post-contrast agent images of some of the LPNs (e.g. Fig. 1b, arrow), but not in the RPNs. These dark regions were not observed in pre-contrast agent images, suggesting that they represent a contrast agent-induced artifact (32).

Quantitative Analysis of Gd-FVT Uptake into LNs

GD-FVT uptake into the LPN and RPN of each mouse was compared to quantify the effects of tumors on lymph flow in the six imaged mice. Integrated density increased in the left and right popliteal LNs within 5 min after Gd-FVT injection (Fig. 2), and these increases were statistically significant by paired t test ($p < 0.03$). The integrated density increased twice as much in the LPN relative to the RPN, although this difference was not statistically significant ($p < 0.06$). The tumor-draining LPN showed greater uptake of contrast agent in five of six mice imaged at 5 min after contrast injection. Interestingly, Gd-FVT uptake decreased significantly ($p < 0.03$) in the LPN from 5 to 15 min, while the RPN remained stable, suggesting more rapid clearance of the contrast agent from the LPN (Fig. 2).

Tumor-Associated Alterations in Gd-FVT Uptake into LNs

The uptake of contrast agent into the LPN was further examined by reviewing serial image slices through the LNs of a second mouse example. In young mice, the cortical lymphatic sinuses receiving afferent lymph extend over roughly half to two thirds of the medial lymph node surface, while the medullary sinuses draining to efferent lymphatic vessels on the lateral surface variably penetrate into the interior of the LN (33). Thus the popliteal LN can roughly be divided into cortical and medullary halves for comparison of contrast agent uptake patterns. The image slices through the LNs consistently identified medullary and cortical contrast agent uptake in the LPN, while the RPN preferentially acquired contrast agent only in the cortex (Fig. 3). Three-dimensional maximum intensity projections (MIPs) illustrating the flow of contrast agent through LNs of the mouse shown in Fig. 1b also demonstrated these differences in the pattern of lymph drainage through the LPN and RPN at 5 min after injection, with the TDLN accumulating contrast agent in the medulla and

cortex, while the control RPN preferentially acquired Gd-FVT in the cortex (Supplementary Fig. 1 and Supplementary Fig. 2 movies).

Contrast enhancement maps were generated to further illustrate these differences in Gd-FVT uptake. For reference, the actual LNs are shown, illustrating the hypertrophy induced by growth of B16-F10 melanomas (16), so that the LPN is larger and more rounded than the RPN (Fig. 4a), and this enlargement was also detected by MRI before contrast agent injection (Figs. 4b). At 5 or at 15 min after Gd-FVT injection, the tumor-draining LPN acquired contrast agent all around the medulla and cortex of the LN, with a peripheral “nodular” enhancement pattern (Fig. 4c). However, at the same timepoint the control RPN exhibited Gd-FVT concentrated in the cortex. The contrast enhancement maps subtracting pre-contrast pixels confirmed the nodular enhancement of Gd-FVT in the medulla and cortex of the LPN, versus the cortical enhancement in the RPN. (Fig. 4d). This increased Gd-FVT uptake in the medulla of LPNs draining tumors, but not in control RPNs, was consistently identified in all 6 of the mice imaged.

Fluorescent Dye Lymphography Evaluation of Popliteal Lymph Nodes

For comparison and confirmation of MRI findings, lymph transit through the LPN and RPN was examined at higher resolution by using fluorescent Texas Red Dextran lymphography to label the lymph drainage through LNs (9). Microscopic examination showed that Texas Red Dextran was distributed in both the cortex and the medulla, while the Dextran preferentially labeled the cortex of the RPN (Fig. 5a) in all four mice analyzed. These patterns of Texas Red Dextran uptake resemble the patterns of Gd-FVT uptake in the LPN and RPN detected by MRI (Fig. 4d).

The LNs were also immunostained with the LYVE-1 lymphatic marker antibody (34) to compare the pattern of lymphatic sinuses with that of the Texas Red Dextran. The medullary and cortical lymphatic sinuses are expanded in the LPN, while there are few sinuses in the uninvolved RPN (Fig. 5b), as has been previously reported (16). This could account for the expanded region of Texas Red Dextran uptake in the medulla and cortex of the LPN, as demonstrated by merging the green LYVE-1 and Texas Red Dextran images (Fig. 5c). The schematic of Fig. 5d summarizes the different patterns of lymph drainage and lymphatic sinuses in the medulla and cortex of the LPN and RPN.

DISCUSSION

In this study, a novel MR lymphography approach was implemented to investigate tumor-induced alterations in LN lymph drainage. Relative to previous studies using 1.5-T MRI with conventional low MW Gd-DTPA contrast agent (17), the 3.0-T MRI with Gd-FVT greatly improved the assessment of murine LN anatomy. The Gd-FVT contrast agent complexes with albumin to form a small nanoparticle which is better suited for long term vessel imaging than conventional low MW Gd-DTPA contrast agent (26), allowing longer scan times to increase image resolution. This increased resolution allowed visualization of aspects of murine lymph drainage and LN architecture not previously detected by MRI.

A previous study using conventional Gd-DTPA contrast agent and dynamic MRI (with 1 min sampling times) to quantify lymph flow kinetics demonstrated increased lymph flow through TDLNs (17). However, that approach did not provide information on LN anatomy. In this study, the larger Gd-FVT contrast agent was used in an attempt to increase retention of contrast agent within the lymphatic circulation, to allow longer acquisitions to achieve higher imaging resolution. The longer 10 min acquisition times (with k-space centered at 5 min) were able to distinguish tumor-induced lymph flow increases, although these differences were not statistically significant likely due to the long imaging time and small sample size. By 15 min after Gd-FVT injection, differences in enhancement between TDLNs and control LNs were reduced, confirming that even with a larger MW contrast agent, imaging within the first min after contrast agent injection is more useful for identification of tumor-induced lymph flow alterations.

The increased resolution afforded by longer scan times and 3.0-T MRI with Gd-FVT contrast agent allowed identification of several distinct characteristics of lymph flow through TDLNs relative to normal LNs. First, the enlargement of the TDLN was readily visible in pre-contrast agent images. The margins of the LNs were made more distinctly demarcated in post-contrast images, facilitating assessment of TDLN enlargement. Second, as described above, the increased uptake of contrast agent into the LPN distinguished tumor-draining from normal control LNs. Third, the pattern of lymph drainage through the tumor-draining LPN was distinct from that of normal RPNs. The LPN consistently featured multiple foci of GD-FVT uptake in the medulla and cortex, while contrast agent was more restricted to the cortex of the RPN. Taken together, these tumor-induced alterations identify several characteristics of the TDLN detectable by contrast agent-enhanced MRI lymphography. These features of TDLN contrast enhancement were not evident in previous MRI lymphography studies (18,31,35), suggesting that Gd-FVT lymphography at 3.0T allows increased resolution of LN anatomy.

Tumor-enhanced uptake of contrast agent into the medulla and cortex of the LPN was identified by MRI lymphography, and confirmed by Texas Red Dextran lymphography. The LPN consistently featured lymphatic sinus growth in the medulla and cortex, which was accompanied by increased spread of Texas Red Dextran through both regions. In the RPN, Texas Red Dextran was more restricted to the cortical sinuses, which likely explains why the MRI lymphography showed Gd-FVT contrast agent limited to the cortex. These findings suggest that tumor-induced LN lymphangiogenesis can be detected by MRI lymphography. After Gd-FVT injection the LPN often showed darkening of internal regions, a phenotype which has previously been identified in human TDLNs after MRI angiography using Gd-FVT (32,36,37). The basis for this dark artifact remains to be determined, however it could be related to the higher uptake of Gd-FVT into the TDLN lymphatic sinuses.

There were several limitations of our study. Although manual subcutaneous injections were performed with great care not to affect the mouse leg positioning, some level of mis-registration between the LNs on pre and post-contrast images was present in all cases due to removal of the mice from the RF coil and handling to administer the injections. This prevented direct subtraction of pre from post-contrast LN images in order to calculate voxel-wise percent enhancement values. In future studies we plan to implement a catheter-based

remote delivery system, but are still perfecting this approach as it is challenging for these subcutaneous injections of very small amounts of contrast. In addition, our study used a small number of animals, which limited the power of statistical comparisons. Gd-FVT can exhibit lower albumin binding affinity and overall enhancement in mouse serum (38), which may have limited the contrast agent enhancement levels measured in our study. A recent approach pre-mixed the Gd-FVT with human serum prior to injection into the mouse to optimize contrast agent binding to albumin (11,12,14,15,38). This could further improve the measurable differences observed between TDLNs and control LNs using Gd-FVT lymphography. Finally, a more rapid scanning protocol focused in the first min after contrast injection would likely improve detection of tumor-induced lymph uptake into the TDLN.

In conclusion, the tumor-associated alterations in lymph drainage we identified by 3.0-T MRI suggest that lymphography can provide information to correctly identify the TDLNs. MRI lymphography has been used by other groups to identify large TDLN metastases that block lymph drainage (21,27,35). TDLN lymphangiogenesis shows promise to predict metastatic tumors even before TDLN metastases arise in both mice (13) and in humans (11,12,14,15), suggesting that this Gd-FVT lymphography could be used to non-invasively characterize tumor phenotypes. Further investigations are required to establish the utility of contrast-enhanced MRI lymphography as a non-invasive approach to assess tumor phenotype and metastatic potential, which would expand the utility of MRI for cancer diagnosis and management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MR lymphography approach using bilateral subcutaneous Gd-FVT injections

a). Mice bearing B16-F10 melanomas in the left rear footpad were injected subcutaneously in the dorsal toe of each rear foot with Gd-FVT, to trace lymph drainage to the LPN and RPN, which then continue to central iliac (ILN) LNs. b). Images of LPN and RPN from pre-contrast and from 5 and 15 min post-contrast scans. Arrows indicate the popliteal LNs. The arrowhead indicates a dark artifact in the LPN at 5 min which is not found in the pre-contrast images. The orientation of the LN cortex and medulla are indicated.

Figure 2. Quantitation of lymph flow through popliteal lymph nodes

Both the LPN and RPN exhibit significant increases in integrated density within 5 min after Gd-FVT contrast agent injection (*; $p < 0.03$ for both; Wilcoxon signed-rank test).

Increases were greater in the LPN than in the RPN at 5 min, although this difference was not statistically significant ($p < 0.06$). The integrated density of the LPN decreased significantly from 5 to 15 min after injection ($p < 0.03$). Standard error bars are shown.

Figure 3. Slice-by-slice illustration of Gd-FVT uptake distribution through LNs

Serial images sampled through the entire left and right popliteal LNs before and 5 min after subcutaneous agent injection of Gd-FVT. Comparison of pre- and post-contrast images shows Gd-FVT enhancement in the medulla and cortex of the LPN, while enhancement is more restricted to the cortex of the RPN in all slices. Arrows indicate the popliteal LNs, while arrowheads indicate dark artifacts in the LPN that are not present in the pre-contrast image. Note that the RPN images have been flipped to facilitate comparison of the LPN and RPN medulla and cortex.

Figure 4. Altered pattern of lymph drainage through tumor-draining lymph nodes

a). Photographs of dissected LPN and RPN, with orientation of medulla and cortex indicated. b). Pre-contrast image of LPN and RPN from the same mouse. c). Post-contrast MR images (t = 5 min after subcutaneous injection of Gd-FVT). d). Contrast enhancement maps generated by subtracting pre-contrast from post-contrast images.

Figure 5. Texas Red Dextran lymphography identifies lymph node lymphatic sinuses
a). Texas Red Dextran distributes through the medulla and cortex of the LPN, while it preferentially labels the cortex of the RPN. b). Green LYVE-1 immunostaining demonstrates lymphatic sinus growth in the medulla and cortex of the LPN, with very few sinuses in the normal RPN. c). Merged image of Texas Red Dextran and LYVE-1 immunostaining demonstrates that Dextran fills the lymphatic sinuses producing a yellow signal. d). Schematic illustrating the distinct patterns of lymph drainage and lymphatic sinuses (yellow) in the LPN and RPN.