Enhanced sonographic imaging to diagnose lymph node metastasis: importance of blood vessel volume and density

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Précis:

Ultrasonography methods to detect early changes in blood vessel volume and density in lymph nodes containing metastatic cells may offer earlier and more accurate criteria for diagnosis of lymph node metastasis, a critical staging and prognostic marker in many cancers.

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Abstract

Lymph node size is an important variable in ultrasound diagnosis of lymph node metastasis. However, the size criterion often leads to oversight of tumor-positive lymph nodes within the range of "normal" size, such that more accurate diagnostic criteria for lymph node metastasis are required. In this study, we show how diagnosis of lymph node metastasis can be improved by evaluating changes in blood vessel volume and density, using a novel contrast-enhanced high-frequency ultrasound (CE-HFUS) system with Sonazoid. An MRL/MpJ-lpr/lpr (MRL/lpr) mouse model of lymph node metastasis was used in which lymph nodes are similar in size to humans. Metastasis via lymphatic vessels to axillary lymph nodes (proper ALNs) was induced by injection of tumor cells into the subiliac lymph nodes. Within 21 days of injection, significant increases in blood vessel volume and density, but no increases in the size of the proper ALNs, were observed. The increase in blood vessel density was confirmed with immunohistochemical analysis, and was positively related to tumor cell proliferation as measured using bioluminescence imaging. Together, our results demonstrated that alterations in blood vessel volume and density precede alterations in lymph node size in the early stages of lymph node metastasis. Detection of these changes by ultrasonography may offer new criteria for early diagnosis of lymph node metastasis.
INTRODUCTION

Metastasis, a characteristic of many tumor types, is estimated to be responsible for the death of 90% of all cancer patients (1). Early detection of metastasis in tumor-draining lymph nodes, i.e. sentinel lymph nodes, during the management of skin, breast, colon, head and neck, and other cancers should improve assessment of the stage of cancer and facilitate selection of the most appropriate treatment (2). Several non-invasive imaging modalities are currently used for the detection of metastasis in tumor-draining lymph nodes, including computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT) and ultrasound (3-7).

Among these imaging modalities, CT and MRI are used to determine nodal size, which is used to define the status of regional lymph nodes. A nodal size greater than 10 mm in the short-axis diameter is the most widely accepted criterion for the diagnosis of cancer involvement (8). However, this size-based characterization of lymph node metastasis frequently leads to misdiagnosis, as metastatic lymph nodes may be of normal size while non-metastatic lymph nodes may become abnormally enlarged, due to reactive swelling (defined pathologically as reactive lymphadenopathy) (9). Recently, Zhang et al. (10) have reported that both diffusion-weighted and superparamagnetic iron
oxide (SPIO)-enhanced MRI can distinguish tumor metastatic lymph nodes from reactive lymph nodes. However, both methods have limited value in detecting microscopic tumor metastasis to the draining lymph nodes at the early stages.

PET using $^{18}$F-fluorodeoxyglucose (FDG; a glucose analog radiolabeled with fluorine-18) is a functional method for tumor detection, based on the increased glucose metabolism of malignant tumors. Investigators in a previous study (11) have evaluated the role of FDG-PET for the detection of lymph node metastasis in patients with melanoma by comparing its results with those of post-operative histopathology. FDG-PET was reported to be capable of detecting 100% of metastases present in lymph nodes that were 10 mm or greater in their short-axis diameter, 83% of metastases that were 6 to 10 mm and 23% of metastases that were 5 mm or less. However, FDG-PET was found to be highly sensitive (93%) only for the detection of metastases with more than 50% lymph node involvement or with capsular infiltration.

Current clinical ultrasound systems use a frequency of 3 to 15 MHz (12). The criteria used for diagnosing lymph node metastasis with gray-scale sonography are lymph node size (short-axis diameter/long-axis diameter), cortical thickness, shape, nodal borders, central necrosis, absence of echo-rich hilar structures and extracapsular spread (13-15). Among these criteria, nodal size and depiction of necrosis are the most
important sonographic criteria (16). As technology has developed, and with the introduction of power Doppler sonography, it has become possible to evaluate the pattern of intra-nodal vessels and measure blood flow velocity and vascular resistance with spectral Doppler gates (15, 17). Kagawa et al. (18) have reported that blood flow signals become scattered and that the scattering index increases as the metastatic lymph node size increases.

However, among the current ultrasound techniques available, even high-resolution ultrasound (6 to 11 MHz) combined with power Doppler sonography is unable to detect metastatic deposits smaller than 4.5 mm in diameter in positive sentinel lymph nodes (19, 20); in fact, it can detect only 22.2% of metastatic deposits larger than 4.5 mm (20). This is due to limitations in spatial resolution, operator dependence, the short time window available for imaging and the limited field of view. Another limiting factor in the development of diagnostic ultrasound imaging has been a lack of appropriate animal models for studying lymph node metastasis (21). Since the lymph node size of conventional mice is 1 to 2 mm, changes in the internal structure of murine lymph nodes from the onset of metastasis cannot be detected using current ultrasound systems. To overcome the limitations imposed by the use of traditional mouse models and sonography, the present study used three-dimensional (3D) contrast-enhanced
high-frequency ultrasound (CE-HFUS) in combination with the contrast medium, Sonazoid, to examine a mouse model of lymph node metastasis established by our previous research (22). Specifically, a CE-HFUS system, set at a frequency of 35 MHz, was used to examine tumor development from the onset of metastasis in MRL/lpr mice, which have lymph nodes that are enlarged to approximately 8 mm in diameter (similar in size to human lymph nodes). The injection of tumor cells into the subiliac lymph nodes (SiLNs) of these mice was used to induce metastasis through a single lymphatic vessel to the proper axillary lymph nodes (proper ALNs), the anatomical name given to these lymph nodes in a previous paper (23).
MATERIALS AND METHODS

All in vivo studies were carried out in strict accordance with the recommendations in the Guide for the Proper Conduct of Animal Experiments and Related Activities in Academic Research and Technology, 2006. The protocol was approved by the Institutional Animal Care and Use Committee of Tohoku University (Permit Number: 2010BeLMO-76-20-255, 2009BeA-6, 2010BeA-7).

Cell culture

KM-Luc/GFP cells (22), which stably express a fusion of the luciferase (Luc) and enhanced-green fluorescent protein (EGFP) genes, were prepared by transfection of MRL/MpTn-gld/gld malignant fibrous histiocytoma-like (MRL/N-1) cells (24, 25) (obtained from M. Ono, Tohoku University, Japan, on January 24, 2007), using pEGFPLuc (BD Biosciences, Franklin Lakes, NJ, USA) with Lipofectin Transfection Reagent (Invitrogen, Carlsbad, CA, USA). MRL/N-1 cells were established from the spleen of an MRL/gld mouse in 1997 (24). Expression of mutant Fas antigen on the cell surface had been confirmed using flow cytometric analyses (24). KM-Luc/GFP cells had characteristics of malignant fibrous histiocytoma-like cells, which were confirmed using histopathological assessment (22). KM-Luc/GFP cells were cultured in
Dulbecco's Modified Eagle Medium (DME M) supplemented with 10% fetal bovine
serum (FBS) containing 1% L-glutamine-penicillin-streptomycin (Sigma-Aldrich, Tokyo, Japan) and 1% Geneticin G418 (Wako Pure Chemical Industries, Ltd, Tokyo, Japan). Before conducting the metastasis experiments, a Mycoplasma detection kit (R&D Systems Inc., Minneapolis, MN, USA) was used to ensure the absence of Mycoplasma contamination of the cell cultures.

Induction of metastasis in the proper ALN

MRL/lpr mice, which develop systemic lymphadenopathy (26), were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred and maintained at the Institute for Animal Experimentation, Graduate School of Medicine, Tohoku University. Twelve mice were used (weights 35 - 45 g; 14 - 18 weeks old). The mean longitudinal diameters of the SiLN and proper ALN of the 12 mice, measured using a digital caliper, were 7.7 ± 1.7 mm (mean ± SD; n = 12) and 7.4 ± 1.5 mm (n = 12) respectively. Lymph node metastasis was induced in the proper ALN of mice in the metastasis group (n = 6) by injection of \(1 \times 10^5\) KM-Luc/GFP cells, suspended in 30 μl of phosphate-buffered saline (PBS), into the SiLN of mice anesthetized with 2% isoflurane (Abbott Japan Co., Ltd, Tokyo, Japan) in oxygen. PBS (30 μl) was injected
into the SiLN of mice in the negative control group \((n = 5)\). The day of inoculation was defined as day 0.

To visualize the lymphatic vessel between the proper ALN and the SiLN, indocyanine green (ICG; excitation wavelength, 805 nm; emission wavelength, 840 nm; Daiichi Sankyo, Tokyo, Japan) was injected into the SiLN of one mouse, and its flow to the proper ALN was visualized with a Photo Dynamic Eye (PDE) infrared photodynamic camera (Hamamatsu Photonics, Hamamatsu, Japan), which emitted light at 760 nm and detected light at wavelengths greater than 820 nm.

Detection of metastasis in the proper ALN by \textit{in vivo} luminescence imaging

Metastasis to the proper ALN was assessed using an \textit{in vivo} luminescence imaging system (IVIS; Xenogen, Alameda, CA, USA) (27). Each mouse was anesthetized with 2.0\% isoflurane in oxygen before intraperitoneal injection with 150 mg/kg of luciferin (Promega Co., Madison, WI, USA). After 10 min, luciferase bioluminescence activity was measured for 30 s, using the IVIS. This procedure was carried out on days 0, 4, 9, 14 and 21 post-inoculation.

Evaluation of time-dependent changes in signal intensity in the proper ALN
Sonazoid (Daiichi Sankyo, Tokyo, Japan), a lipid-stabilized suspension of perfluorocarbon microbubbles, was used as an ultrasound contrast agent (28). A zeta potential and particle size analyzer (ELSZ-2; Otsuka Electronics, Osaka, Japan) revealed that Sonazoid had a median diameter of 2.46 ± 0.12 μm (n = 3, mean ± SD) and a mean zeta potential of -37.0 ± 1.1 mV (n = 3). To investigate the in vivo stability of Sonazoid, two-dimensional (2D) dynamic contrast-enhanced imaging was performed using a CE-HFUS system (VEVO770; VisualSonics Inc., Toronto, Canada) with a 35 MHz transducer (RMV-703; axial resolution 50 μm, focal length 10 mm; VisualSonics Inc.) set at 50% transmission power. For these experiments, an additional group of mice was used that had received no tumor cells (n = 5). Mice were anesthetized with 2% isoflurane in oxygen and placed onto a stage maintained at 38°C (TM150, VisualSonics Inc.). While the transducer remained in a stable position, 2D contrast-enhanced images of the center of the proper ALN were taken serially, until 12 min after slow injection of 100 μl Sonazoid into the tail vein; respiration gating was used to suppress imaging artifacts due to respiration. A region of interest was subsequently drawn around the proper ALN and a time-intensity curve was created for determination of the optimal diagnostic window.
Three-dimensional reconstruction of the microvasculature in the proper ALN using CE-HFUS with Sonazoid

A 3D image of the microvasculature within the proper ALN was reconstructed and time-dependent blood vessel development in the proper ALN was evaluated on days 0, 4, 9, 14 and 21 post-inoculation, using a CE-HFUS system with a 35 MHz transducer fixed to a 3D stage control system (Mark-204-MS, Sigma Koki Co., Ltd., Tokyo, Japan). Before, and 180 s after intravenous bolus injection of 100 μL Sonazoid into the tail vein, consecutive B-mode images, with a slice thickness of 100 μm, were captured throughout the entire proper ALN for 3D imaging during the diagnostic window. The difference in the video intensity between the pre-injection and post-injection image frames was highlighted as a green overlay in the B-mode anatomical images, using the accompanying software (VisualSonics). The highlighted green areas were considered to be the extracted blood vessel images. Blood vessel densities and volumes in the proper ALN (whose boundaries were manually traced using sequential, parallel ultrasound scans, according to the anatomical and acoustic characteristics) were calculated using analysis software (VisualSonics Inc.). Values on day 0 were set at 100%. Changes in each individual animal, measured on days 4, 9, 14 and 21, were calculated and expressed as percentage changes. Throughout the imaging session, mice remained on July 21, 2017. © 2013 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on July 21, 2017. © 2013 American Association for Cancer Research.
anesthetized, while resting on a heated stage, by administration of 1 l/min of 2%
isoflurane in oxygen, in accordance with the manufacturer’s protocol. Respiratory
gating was used to synchronize data acquisition with the mouse respiratory cycle to
reduce motion artifacts during image analysis. A 2D image of the maximum
cross-section of the proper ALN was extracted from the 3D image.

Immunohistochemical analysis

After IVIS and ultrasound imaging had been performed on day 21 post-inoculation,
metastatic lymph nodes were excised in a manner that allowed the researcher to remain
blind as to whether they had been excised from mice in the control (4 of 5 mice) or
metastasis (4 of 6 mice) group. Lymph nodes were fixed overnight in 18.5%
formaldehyde in PBS at 4°C (Rapid Fixative, Kojima Chemical Industry, Inc., Saitama,
Japan), dehydrated and then embedded in paraffin. The embedded specimens were cut
into 2 μm serial sections, and either stained with hematoxylin and eosin (H&E) or
immunostained for detection of LYVE-1-positive and CD31-positive cells using a
Discovery XT automated staining processor (Ventana Medical Systems, Inc., Tucson,
AZ, USA). Immunostaining of lymphatic endothelial cells was performed using a
polyclonal rabbit anti-mouse LYVE-1 antibody (4 μg/ml; 103-PA50AG, Cosmo Bio Co.
Ltd., Tokyo, Japan) for 2 h at room temperature, an anti-rabbit IgG Histofine MAX-PO (R) kit (Nichirei Biosciences Inc., Tokyo, Japan) for 16 min at room temperature and diaminobenzidine (DAB). Immunostaining of vascular endothelial cells was carried out using a pre-diluted polyclonal rabbit anti-CD31 antibody (1/100 dilution; sc-1506-R, Santa Cruz Biotechnology, Inc., California, USA) for 2 h at room temperature, in combination with an anti-rabbit IgG Histofine MAX-PO (R) kit for 16 min at room temperature.

To obtain the mean blood vessel density of the macrovessels, defined as vessels with a diameter greater than or equal to 30 μm, the total macrovessel area was measured and the value obtained was divided by the total area of the specimen. The specimen boundary was measured under low magnification (×40 or ×100) using a microscope (BX51; Olympus Co., Tokyo, Japan) and digital camera (DP72; Olympus). The hot-spot method was used to calculate the mean blood vessel density of the microvessels, defined as vessels with a diameter less than 30 μm (29). Specifically, 5 hot-spot fields with the highest microvessel density were selected under low magnification (×40 or ×100) and the blood vessel density was calculated by dividing the total microvessel area, consisting of vasculature with a minor axis of 5 to 30 μm (× 200), by the CD31-positive hotspot area (523 μm × 695 μm). Measurements of blood-vessel density were made by
two researchers to reduce the measurement error.

3 **Statistical analysis**

4 All measurements are presented as either mean ± SD (standard deviation) or mean ± SEM (standard error of the mean) values. Differences between groups were determined by two-way analysis of variance (ANOVA) followed by the Tukey-Kramer test (Fig. 1D, Fig. 4B, and Fig. 5A) or Student’s *t*-test (Fig. 6B). Measurement of the correlation between luciferase activity (bioluminescence imaging) and blood vessel density (ultrasound imaging) in metastatic lymph nodes was determined using the Spearman’s rank correlation coefficient test (Fig. 5B). A *P* value of < 0.05 was considered to represent a statistically significant result. Statistical analyses were performed using Excel 2007 (Microsoft, WA, USA) with Statcel2 software.
RESULTS

Metastasis in the proper ALN

Fig. 1A shows an anatomical drawing of a 24-wk-old MRL/lpr mouse that developed systemic lymphadenopathy to a remarkable extent. A single lymphatic vessel (which cannot be seen by the naked eye) and a single superficial epigastric vein extend in parallel from the SiLN to the proper ALN (Fig. 1A). When ICG solution was injected in the vicinity of the efferent lymphatic vessel in the SiLN, it reached the proper ALN through the lymphatic vessel (Fig. 1B).

Fig. 1C and D illustrate measurement of the luciferase activity of cells growing in both the SiLN and the proper ALN, on days 0, 4, 9, 14 and 21 post-injection of either KM-Luc/GFP cells into the SiLN of the metastasis group (n = 6) or PBS into the SiLN of the control group (n = 5). The results show that the luciferase activities in both the SiLN and the proper ALN increased over time in the metastasis group. In the SiLN, there was a steep increase in luciferase activity on day 4, which was followed by a further slight increase up to day 21 (P < 0.01 on day 21, control-SiLN vs. treated-SiLN); in the proper ALN, a gradual increase up to day 21 was observed (P < 0.05 on day 21, control-proper-ALN vs. treated-proper-ALN). In the control group, the luciferase activities of both the SiLN and the proper ALN remained at baseline levels over the
Determination of the diagnostic window

To determine the optimal diagnostic window in which to perform blood vessel imaging in the presence of Sonazoid, we investigated the time-intensity relation for B-mode imaging of the proper ALN without metastasis (Fig. 2). As an injected solution is generally not evenly distributed throughout the circulation immediately after injection, a concentration difference is produced in the bloodstream, which varies depending on the duration of the administration. In the present study, dense Sonazoid acoustic signals were detected near the hilum of the proper ALN, 1 s after injection, and throughout the proper ALN, 10 s after injection, while scattered acoustic signals were detected throughout the proper ALN after 60 s. We found that the concentration of Sonazoid in the blood became uniform over time. Fig. 2B shows the contrast intensity throughout the proper ALN over time. Immediately after tail-vein injection of Sonazoid, the mean gray-scale value initially increased dramatically but then decreased gradually until 168.0 ± 13.7 s post-inoculation (mean ± SD; n = 5); subsequently, it remained at this decreased level. Based on this determination of the optimal diagnostic window, all ultrasound imaging was performed between 180 and 420 s.
Evaluation of the changes in blood vessel volume and lymph node size in the proper ALN

As changes in vascularization and tumor size are important parameters that determine tumor progression (30, 31), changes in blood vessel volume and nodal size of the proper ALN, with \((n = 6)\) and without \((n = 5)\) metastasis, were measured using 3D CE-HFUS.

Fig. 3 shows 2D images of the maximum cross-section of the proper ALNs, extracted from the 3D images. It may be seen that the acoustic signals in the lymph nodes did not change over time in the control group (Fig. 3A), but increased over time throughout the lymph nodes in 5 of the 6 mice in the metastasis group (Fig. 3B), with localized dense acoustic signals found in 1 of the 6 mice in the metastasis group (Fig. 3C).

In general, 2D images provide data regarding only one cross-section, unless there is 3D symmetry and uniformity that allows for extrapolation. Thus, a 2D image cannot be used to evaluate internal structural changes when vessels are localized to a particular area, as shown in Fig. 3C. Therefore, the changes in blood vessel volume and nodal size in the proper ALN were measured using 3D CE-HFUS. Fig. 4 illustrates the 3D vessel structures of the proper ALN with metastasis, on days 0, 4, 9, 14 and 21; the increase in blood vessel volume over time is shown in Fig. 4A and the changes in blood
vessel volume and nodal size are presented in Fig. 4B. The day of cell injection into the SiLN was set at day 0 and blood vessel volume and lymph node size on day 0 were each set at 100%. The lymph node size of the control group ($n = 5$) was observed to remain constant over the experimental period ($96 \pm 2\%$ at day 21; $P > 0.05$, day 0 vs. day 21), while that of the metastasis group ($n = 6$) appeared to increase slightly, although statistical significance was not reached ($116 \pm 10\%$ at day 21; $P > 0.05$, day 0 vs. day 21). The blood vessel volume of the control group ($n = 5$) also remained constant up to day 21 ($87 \pm 2\%$ at day 21; $P > 0.05$, day 0 vs. day 21) whereas that of the metastasis group ($n = 6$) increased over time. Blood vessel volume in the metastasis group at day 21 was significantly different from that at day 0 ($190 \pm 28\%$ at day 21; $P < 0.01$, day 0 vs. day 21). Importantly, the magnitude of the change in blood vessel volume in the metastasis group at day 21 was found to be significantly different from the magnitude of the change in lymph node size ($P < 0.05$). This finding suggests that angiogenesis is a more important parameter than nodal size for evaluating the progression of lymph node metastasis in the early stages.

Quantitative analysis of blood vessel density in the proper ALN with metastasis

Blood vessel density in the proper ALN was investigated using 3D CE-HFUS, with
vessel density normalized to the density measured on day 0. Fig. 5A shows that there was no significant change in normalized blood vessel density over time in the control group \((n = 5)\), but a 1.62-fold increase on day 21 compared with day 0 in the metastasis group \((n = 6; P < 0.05, \text{day 0 vs. day 21})\). In addition, the normalized blood vessel density of the metastasis group was found to be 1.72-fold greater than that of the control group on day 21 \((P < 0.05, \text{control vs. metastasis})\). Fig. 5B shows the correlation between the normalized blood vessel density, measured using 3D CE-HFUS, and the normalized luciferase activity, reflecting the proliferation of tumor cells as measured using IVIS. There was a positive correlation between the two factors (Spearman's rank correlation coefficient \([rs] = 0.763, n = 28\)).

**Evaluation of blood vessel density by immunohistochemical analysis**

Analysis of structural changes in the proper ALNs, with and without metastasis, revealed that the basic structures of the lymph nodes, such as the medulla, paracortex, and cortex, and the lymphatic channels, had been preserved in the control group. Fig. 6 shows that blood vessels could be discretely observed in the control group. In contrast, continuous invasion of tumor cells from the marginal sinus was detected in the cortex of the metastasis group, as well as a reduction in the size of LYVE-1-positive regions by
the expansion of metastasis and an increase in the number and size of the blood vessels of the lymph node, even in non-metastatic regions. Quantification of blood vessel density (measured as blood vessels per unit area) in the proper ALNs, with and without metastasis, on day 21 revealed that the densities of both the microvessels (vessels with a width < 30 μm) and macrovessels (vessels with a width ≥ 30 μm) of the metastasis group were greater than those of the control group. Specifically, the total vessel density of both microvessels and macrovessels in the metastatic proper ALNs was found to be 1.5-fold greater than that of the control proper ALNs (P < 0.01). These changes correspond well with the 1.72-fold increase in the metastasis group compared with the control group, shown in Fig. 5A.
DISCUSSION

The present study is the first to demonstrate, using a 3D CE-HFUS system in combination with Sonazoid, that in lymph nodes undergoing metastasis, increases in blood vessel volume and density precede changes in nodal size. Despite the fact that microscopic lymph node metastases may be present that cannot be observed macroscopically, and that there may be small lesions that cannot be detected even by current ultrasonography techniques, it is nonetheless very important to use ultrasonography before treatment to assess lymph nodes less than 10 mm in diameter that cannot be detected by CT or MRI, as a diagnostic procedure for lymph node metastasis (32). In this study, using a 3D CE-HFUS system with Sonazoid, the increases in blood vessel volume and density on day 21 were found to be 1.9-fold and 1.6-fold greater, respectively, than values on day 0, and the increase in blood vessel density was positively related to tumor cell proliferation. In contrast, no significant difference was found between the nodal size of the proper ALN on day 0 and that on day 21, most likely because the normal lymph node tissue had been partially replaced by infiltrating tumor cells, causing only a slight increase in nodal size. Additionally, neither the emergence of an echogenic region due to central necrosis (33) nor the absence of hilum (16) was detected in metastatic lymph nodes until the last day of the experiment (day 21, July 21, 2017). © 2013 American Association for Cancer Research.
These results indicate that blood vessel volume and density may be used as ultrasound parameters for diagnosis of early lymph node metastasis.

In general, normal tissue is invaded and replaced by tumor cells at an early stage of metastasis, resulting in changes in the microenvironment. These changes include release of metalloproteinases that degrade the extracellular matrix, secretion of growth factors to promote neovascularization (34), and development of sinus hyperplasia (35). In the diagnosis of lymph node metastasis by B-scan sonography, the clinical criteria currently used are increases in nodal size and cortical thickness, changes in nodal shape, infiltration of surrounding structures, presence of inhomogeneous internal echo patterns (including necrosis), absence of echo-rich hilar structures, and extracapsular spread (14, 36). However, these changes only become pronounced once the tumor has already grown to some extent. In fact, other studies have found that the size and shape of lymph nodes with small metastatic tumors are within the normal range for their anatomical positions (37), or only slightly larger than those of tumor-free lymph nodes (38). Anticipatory changes in blood vessel volume and density at earlier stages may be overlooked when using conventional ultrasonography techniques for diagnosis.

Our research group has previously reported that use of 2D CE-HFUS with
acoustic liposomes was capable of evaluating antitumor angiogenic effects in a
subcutaneous tumor model (39). Combined with Sonazoid, the 3D CE-HFUS system
utilized in the present study allows for in vivo quantitative monitoring of tumor
vascularity during the progression of lymph node metastasis. This approach provides
several advantages. Sonazoid is a microbubble ultrasound agent with a stable shell,
resulting in a relatively long time window being available for imaging. The use of a 3D
stage control system avoids the operator-dependence of selection that occurs for a 2D
scan. In addition, compared with conventional vascular visualization methods (40), the
combination of this approach with a respiratory gating technique allows for
visualization of vascular regions with reduced effects of noise and tissue movement
during ultrasound measurements. Finally, whereas conventional functional ultrasound
(power Doppler imaging) is more sensitive for large, mature vessels (41), the imaging
technique presented in this study enables the assessment of both large and small vessels,
through which microbubbles normally pass freely.

We used the 3D CE-HFUS system, set at a frequency of 35 MHz, to examine
the proper ALNs of MRL/lpr mice with lymph nodes enlarged to approximately the size
of human lymph nodes (7.6 ± 1.3 mm, mean ± SD; n = 15). This technique allows for
evaluation of tumor development from the onset of metastasis. Furthermore, this
method was able to overcome the limitations imposed by the use of conventional clinical ultrasound systems, set at frequencies of 3 to 15 MHz, and by mouse models with normal-sized (1 to 2 mm) lymph nodes (42). MRL/lpr mice show lymphadenopathy due to an accumulation of lpr T cells (B220+/Thy1.2+/CD4−/CD8+) in the paracortical regions of the lymph nodes (43, 44). However, as these lpr T cells do not infiltrate the lymphoid follicle and the follicular dendritic cell network (45), the basic lymph node structures, including the medulla, paracortex and cortex, and the lymphatic channels are maintained, allowing MRL/lpr mice to serve as an animal model of lymph node metastasis.

In general, multiple steps are required for tumor cells to metastasize from their primary site to regional lymph nodes, including detachment from the primary tumor mass, invasion into lymphatic vessels, transport through draining lymphatic vessels, arrest in lymph nodes, and survival and growth in lymph nodes (46). In an experiment using BALB/c mice and athymic BALB/c nu/nu mice (47), organization of the lymphatic channels and the vasculature was observed before the establishment of metastasis in the sentinel lymph node, with enlargement of lymph node sinuses, and emergence of functional blood vessels that developed from high endothelial venules. This may be the reason why the number and size of the blood vessels increased not only
in the metastatic foci but also in non-metastatic regions. The total number of lymphatic
evessels in metastatic lymph nodes decreases, because these vessels may collapse or find
it difficult to penetrate into expanding primary tumor (Fig. 6A) (48, 49). In the mouse
model used in the present study, direct injection of cells into the SiLN via a lymphatic
vessel resulted in the delivery of tumor cells to the proper ALN. This was followed by
continuous invasion of cells that had metastasized into the marginal sinus, into the
cortex and paracortex, and then into the medulla, possibly through the lymphatic and
medullary sinuses; this invasion pattern in the proper ALN was similar to that of
spontaneous lymph node metastasis. However, our previous experiments found that the
incidence of metastasis to the proper ALN from the SiLN depended on the number of
cells injected into the SiLN, the duration of the injection and the SiLN volume (22). The
spread of metastasis and subsequent blood vessel development may depend on the
uniformity of the cells in the injection solution (Fig. 3B and C). The molecular
biological aspects of metastasis could not be investigated directly in the present study,
whose methodology and findings were limited to the use of imaging techniques. In
addition, potential differences between tumor metastatic lymph nodes and inflammatory
lymph nodes were not investigated.

In conclusion, the results of the present study indicate that blood vessel volume
and density are more important parameters than nodal size for sonographic evaluation of the progression of lymph node metastasis in the early stages. As a correlation was identified between vessel density and luciferase activity, a molecular marker of tumors, the use of 3D CE-HFUS in combination with PET (50) or MRI (10), together with specific molecular probes, should be investigated as a means of further improving diagnostic accuracy. In future, our findings may allow for the early diagnosis of metastasis of some malignant tumors to superficial lymph nodes (such as melanoma, breast cancer, and head and neck cancers), or intraoperative diagnosis of metastatic lymph nodes.

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FIGURE LEGENDS

Fig. 1. Progression of metastasis from the subiliac lymph node (SiLN) to the proper axillary lymph node (proper ALN).

A. Anatomical drawing of a male MRL/lpr mouse at 24 weeks.

B. Indocyanine green (ICG) flow to the proper ALN from the SiLN via the efferent lymphatic vessel, after injection into the area near the SiLN helix. The photograph was taken 45 min after inoculation of ICG.

C. Representative images of metastasis in the proper ALN, observed using an in vivo luminescence imaging system. KM-Luc/GFP cells (1 \times 10^5 cells/30 μl) were injected into the SiLN of the metastasis group (n = 6), and PBS (30 μl) was injected into the SiLN of the control group (n = 5).

D. Quantitative analysis of luciferase activity in a metastasized proper ALN and an inoculated SiLN, on days 0, 4, 9, 14 and 21 post-inoculation. Mean ± SEM values are shown. Significance was determined using two-way ANOVA followed by Tukey-Kramer testing. *P < 0.05 and **P < 0.01 vs. day 0 in the metastasis group. #P < 0.05 and ##P < 0.01 vs. the control group on day 21.

Fig. 2. Time-dependent changes in signal intensity in the proper ALN without
metastasis.

A. 2D CE-HFUS ultrasound images of proper ALNs without metastasis, obtained 1 s, 2 s, 3 s, 10 s, 60 s, 3 min, 5 min and 10 min after administration of Sonazoid. Vessels in the lymph node are shown in green. Scale bar = 3 mm.

B. Contrast-enhanced images, taken using the B-mode, showing maintenance of a stable plateau from approximately 180 s after injection of Sonazoid. The black bar represents the scan time during which 3D measurements were performed.

**Fig. 3.** Temporal changes in the vessel structures of control \((n = 5)\) and metastatic \((n = 6)\) lymph nodes assessed using 2D CE-HFUS. Single-slice reconstructed vessel images with a thickness of 100 \(\mu\)m are presented. Vessels in the proper ALN are highlighted in green, and red circles indicate the boundaries of the lymph node. In the metastasis group (B and C), no obvious changes in vessel structure were observed until day 9. On day 21, the vessel structure showed different patterns, with panel B showing diffusely scattered blood vessels \((n = 5)\) and panel C showing localized dense blood vessels \((n = 1; \text{blue circle})\).
**Fig. 4.** Changes in blood vessel volume and nodal size of proper ALNs, determined using 3D CE-HFUS.

A. 3D images of the blood vessel structure of the proper ALN with metastasis, obtained on days 0, 14 and 21. The vessels are highlighted in green.

B. Changes in blood vessel volume and lymph node size. ‘Metastasis’ refers to a proper ALN with metastasis (1 × 10^5 cells/min; n = 6), and ‘Control’ to a proper ALN without metastasis (n = 5). Open and closed circles represent the blood vessel volumes within the proper ALNs of the control and metastasis groups, respectively. Open and closed triangles represent the sizes of the proper ALNs of the control and metastasis groups, respectively. Values at different time points were normalized by comparison to those on day 0. On day 21, the magnitude of the change in blood vessel volume was larger than the magnitude of the change in lymph node size. * Indicates a temporal change within each group; # indicates a comparison between groups. * or #, P < 0.05; ** or ##, P < 0.01; calculated using two-way ANOVA followed by Tukey-Kramer testing. Error bars indicate the SEM.
Fig. 5. Quantitative assessment of metastasis in the proper ALN using 3D CE-HFUS and an *in vivo* bioluminescence imaging system (IVIS). Values were normalized by comparison with those obtained on day 0.

A. Quantitative assessment of the change in blood vessel density of control (*n* = 5) and metastatic (*n* = 6) proper ALNs using 3D CE-HFUS. The blood vessel density of the metastatic proper ALNs increased with time and the difference between groups became significant on day 21. *P* < 0.05 vs. day 0 in the metastasis group; *P* < 0.05 vs. control on day 21; determined by two-way ANOVA followed by Tukey-Kramer testing. Mean ± SEM values are shown.

B. Relationship between normalized blood vessel density, determined using 3D CE-HFUS, and normalized luciferase activity, determined with IVIS. Using analysis of Spearman’s rank correlation coefficient, a linear correlation (rs = 0.763) was found between normalized luciferase activity and normalized blood vessel density during tumor progression (*P* < 0.01). This relationship indicates that both these non-invasive imaging methods may be used to follow the progression of metastasis.

Fig. 6. Evaluation of blood vessel density by immunohistochemical analysis.
A. Results of staining control (n = 4) and metastatic (n = 4) proper ALNs with hematoxylin and eosin (H&E), anti-LYVE-1 antibody and anti-CD31 antibody. In the control group, basic structures such as the cortex (cor), paracortex (paracor) and medulla (med), and the lymphatic channels were preserved; the blood vessels remained discretely distributed. In the metastasis group, the area of the lymphatic channels decreased with expansion of metastasis, while the number and size of the blood vessels of the lymph nodes increased even in the non-metastatic region. ‘Meta’ refers to tumor cells detected in the cortex that continuously invaded from the marginal sinus.

B. Quantitative analysis of the CD31-positive area in the proper ALN, with and without metastasis on day 21 after inoculation. Vessels greater than or equal to 30 μm in size were defined as macrovessels and vessels less than 30 μm in size as microvessels.

(a) Microvessel and (b) macrovessel density was significantly greater in the metastasis group compared with the control group. **P < 0.01 and *P < 0.05, respectively, determined by Student’s t-test; mean ± SEM values are shown. (c) Total vessel density was significantly (1.5-fold) greater in the metastasis group compared with the control group. **P < 0.01, Student’s t-test; mean ± SEM values are shown.
Fig. 1

(A) Diagram showing the proper axillary lymph node, subiliac lymph node, superficial epigastric vein, and lymphatic vessel.

(B) Image showing the subiliac lymph node.

(C) Images showing luciferase activity over time:
- Day 0
- Day 4
- Day 9
- Day 14
- Day 21

(D) Graph showing luciferase activity (photons/s) over days after inoculation:
- SiLN (Control)
- SiLN (Metastasis)
- Proper ALN (Control)
- Proper ALN (Metastasis)

Color Bar:
- Min = 1e+06
- Max = 5e+08

Photons/s/cm²: 5, 4, 3, 2, 1, 0

Days after inoculation:
0, 4, 8, 12, 16, 20, 24

Luciferase activity (photons/s):
- 10^14
- 10^12
- 10^10
- 10^8
- 10^6
- 10^4

Significance:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
- #: p < 0.1
Fig. 2

A

B
A. Almost stable intensity $(n = 5)$

Day 0  | Day 9  | Day 21
--- | --- | ---
Control

B. Diffusely-scattered intensity increase $(n = 5)$

Day 0  | Day 9  | Day 21
--- | --- | ---
Metastasis

C. Localized intensity increase $(n = 1)$

Day 0  | Day 9  | Day 21
--- | --- | ---
Fig. 4

A

Day 0  Day 14  Day 21

B

Change in blood vessel volume and lymph node size in the proper ALN (%)

-○-  Vessel (Control)
-●-  Vessel (Metastasis)
-△-  Size (Control)
-◆-  Size (Metastasis)

Days after inoculation

0  4  8  12  16  20  24  28

Day 0  Day 14  Day 21

18  220  260

2.2  2.6  s

Vessel (Control)  Vessel (Metastasis)

1.0  1.4  1.8

Chang

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Fig. 5

A

Normalized blood vessel density by 3D CE-HFUS (day 0)

Days after inoculation

Control
Metastasis

* #

B

Normalized blood vessel density by 3D CE-HFUS (day 0)

Normalized luciferase activity by IVIS (day 0)

Spearman’s rs = 0.763
P < 0.01
R² = 0.552
Fig. 6

A

Control

HE (× 4)

LYVE-1 (× 4)

CD31 (× 20)

1 mm

1 mm

250 µm

Med

Paracor

Cor

Lymphatic endothelia

Vascular endothelia

Metastasis

HE (× 10)

LYVE 1 (× 10)

CD31 (× 20)

500 µm

500 µm

250 µm

Med

Paracor

Cor

Meta

Lymphatic endothelia

Vascular endothelia

B

(a) (b) (c)

Microvessel density (% area)

P = 0.0004

Macrovascular density (% area)

P = 0.023

Total blood vessel density (% area)

P = 0.0008

** 1.5-fold

Control

Metastasis

Control

Metastasis

Control

Metastasis

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Enhanced sonographic imaging to diagnose lymph node metastasis: importance of blood vessel volume and density

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