Preparing the “Soil”: The Primary Tumor Induces Vasculature Reorganization in the Sentinel Lymph Node before the Arrival of Metastatic Cancer Cells

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Abstract

Sentinel lymph node (SLN) metastasis is the first step in the spreading of cancer in many malignancies. Tumor-reactive lymphadenopathy in SLNs has been observed for decades, but alterations of the lymphatic channels and vasculature in these nodes before the arrival of metastatic tumor cells remain unexplored. Using animal models, we show here that, before the establishment of metastasis in the SLN, there are reorganizations of the lymphatic channels and the vasculature. The node becomes a functional blood vessel–enriched and lymph vessel/sinus-enriched organ before metastasis. The enlargement of the lymph sinuses is correlated with the primary tumor weight. The newly emerged functional blood vessels develop from high endothelial venules (HEV), in which the proliferation rate of the endothelial cells is also significantly increased. Similar alterations of the HEVs are also characterized in the axillary lymph nodes from human breast cancer patients without the evidence of metastasis. These findings support the hypothesis that modification of the microenvironment for a secondary tumor (i.e., vasculature reorganization in the SLN) can be initiated by a primary tumor before and independent of the physical presence of metastatic cancer cells. (Cancer Res 2006; 66(21): 10365-76)

Introduction

The most important survival predictor in patients with head and neck cancers is cervical lymph node metastasis, regardless of the extension of the primary tumor (1). Detection of lymph node metastasis is therefore important for tumor staging and therapy planning. Current imaging approaches to evaluate lymph node metastasis mainly rely on the size and the shape of the involved lymph node, and controversial interpretations of these imaging results remain due to the limited knowledge of the angiogenesis and lymphangiogenesis within the lymph node.

It has been known for decades that regional lymph nodes draining tumor areas may be enlarged without evidence of metastasis. The term for this condition is tumor-reactive lymphadenopathy (2). However, the processes of lymphangiogenesis and angiogenesis in tumor-reactive lymphadenopathy are not well understood. Angiogenesis is essential to the growth and metastasis of solid tumors (3). Recently, intumoral lymphangiogenesis has been characterized in head and neck carcinomas, related to regional cervical lymph node metastasis (4, 5). Increased expression of vascular endothelial growth factor (VEGF)-C, which can induce lymphangiogenesis, is also correlated with regional lymph node metastasis of cancer cells in both animal model and human tumors (6, 7). Expression of platelet-derived growth factor in murine fibrosarcoma cells can induce tumor lymphangiogenesis, leading to enhanced metastasis in lymph nodes (8). Lymphangiogenesis in the lymph nodes induced by VEGF-A contributes to cancer metastasis (9).

Nasopharyngeal carcinoma (NPC) has the highest incidence rate of lymph node metastasis among head and neck cancers (10, 11). In breast cancer, axillary lymph node involvement is a significant prognostic factor (12, 13), although 20% to 30% of node-negative patients will develop distant metastases within 10 years (14). To characterize the process of lymph node metastasis, we established a spontaneous lymph node metastasis animal model by using a human, poorly differentiated NPC cell line, CNE-2 (15), and the murine breast cancer cell line DA3. The premetastatic alterations of the sentinel lymph node (SLN), which receives direct lymphatic drainage from the tumor lesion (16), were analyzed. Human axillary lymph nodes from breast cancer patients were used to confirm the findings from the animal models.

Materials and Methods

Cell culture and clone selection. The human NPC cell line CNE-2 and its clones were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The individual clones from the CNE-2 parental cell line, each grown from a single cell, were isolated by limited dilution methods.

In vitro migration and invasion assays. For migration assays, 5.0 × 104 NPC cells in 500 μL serum-free DMEM was added to the cell culture inserts with an 8-μm microporous filter without extracellular matrix coating (Becton Dickinson Labware, Bedford, MA). DMEM containing 10% FBS was added to the bottom chamber. After 18 hours of incubation, the cells on the lower surface of the filter were fixed and stained followed by microscopic examination. The number of cells in five random optical fields (×200 magnification) from triplicate filters was averaged. For in vitro invasion assays, the inserts of the chambers to which the cells were seeded were coated with Matrigel (Becton Dickinson Labware).
Animals and spontaneous lymph node metastasis assay. Female BALB/c mice and athymic BALB/c nu/nu mice (5-6 weeks old) were maintained in the vivarium of the Van Andel Research Institute (VARI; Grand Rapids, MI). All of the animal studies were conducted under protocols approved by the Institutional Animal Care and Use Committee of VARI.

Tumor cells (8 × 10^6 in 20 μL Hank's solution) were injected s.c. into the footpad of left hind limb of each nude mouse. On the terminal day, the primary tumor weight was calculated by subtracting the weight of the foot without tumor from the weight of foot carrying the primary tumor. The popliteal lymph nodes from both hind limbs were isolated using a dissecting microscope. The lymph node volumes were calculated as follows:

\[ \text{volume} = \text{width} \times \text{length} \times \text{height} / 2 \]

To trace the lymph drainage, 10 μL Evans blue dye (0.4%) in PBS was injected s.c. at the end of the limb 15 minutes before sacrifice.

Real-time quantitative PCR. Single CNE-2 cells were identified by limited dilution method in 96-well plates. Total RNA from cells and lymph nodes was extracted using the High Pure RNA Tissue kit (Roche Applied Science, Penzberg, Germany). To prove the validity of this method in identifying a few cancer cells in the lymph node, varying numbers of cancer cells were combined with a certain number of normal popliteal lymph nodes for homogenization. Briefly, tumor cell suspension in culture medium was plated into 96-well plates. Four hours later, the cell number was counted, and 100 μL lysis/binding buffer was added into each well. The lysate was then transferred to a tube containing a certain number of fresh-frozen popliteal lymph nodes from normal mice. Another 300 μL lysis/binding buffer was then added followed by homogenization. Specific primers for human hypoxanthine phosphoribosyltransferase (HPRT) were used (17). The PCR products were continuously measured with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Forster City, CA) during 50 cycles. The experiments were done in duplicate, and the average of the two samples was calculated.

Ultrasonography and image analyses. Blood flow in the popliteal lymph node was detected in vivo using the Acuson Sequoia 512 (Mountain View, CA) instrument with a 15L8 (15 MHz) linear transducer (18). Mice were anesthetized using continuous inhalation of isoflurane. Ultrasonography was done on the popliteal lymph node 6 to 26 days after tumor cell inoculation into the footpad. No detectable popliteal lymph node was found in the hind limbs of normal healthy mice or in the right hind limbs of the mice carrying left hind footpad tumors. Any sentinel popliteal lymph nodes with histologically verified metastases were excluded from the analyses. The blood flow in large blood vessels of the popliteal lymph node was evaluated by color Doppler ultrasound. The application of contrast medium, which are air bubbles 2 μm in diameter, enables the detection of blood flow in smaller vessels (40-70 μm in diameter). First-pass, low-intensity, nondestructive contrast medium-enhanced ultrasound imaging was done using Definity Perflutren Liquid Microspheres (Bristol-Myers Squibb Medical Imaging, Inc.) by tail-vein injection at 15 μg/mouse. Contrast medium ultrasound imaging analysis was done using ULA dedicated functional molecular imaging analysis software (iLAB's Israel).

Histologic evaluation and immunohistochemical and immunofluorescent staining. Paraffin-embedded human lymph node tissue samples were obtained from the Shared Pathology Informatics Network at VARI (19).

Mouse lymph nodes were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 μm throughout the lymph node. To exclude lymph nodes containing micrometastases, H&E staining and histologic examination were done on all of the rest of the continuous tissue sections from a whole lymph node after several sections were selected for immunohistochemical staining. To quantify the lymph vessel/sinus area, the largest H&E-stained cross-section of each nonmetastatic lymph node was used for image analyses. Rat anti-mouse CD31 monoclonal antibody (mAb; Research Diagnostics Inc., Concord, MA), the rabbit anti-mouse LYVE-1 antibody (rsAb; Abcam, Inc., Cambridge, MA) and the rabbit anti-mouse MECA-79 (9A) antibody (BD PharMingen), and the mouse anti-human CD31 (JC70A, DAKO, Carpinteria, CA) mAbs were used at a dilution of 1:50. The mouse anti-human CD34 (QBend10, DAKO) mAb was used at a dilution of 1:100. The anti-mouse MECA-79 antibody reacts with both mouse and human PNAd carbohydrate antigen. The proteinase K (DAKO) antigen retrieval method was applied to the staining of CD31 in mouse lymph nodes. The avidin-biotin complex method Elite kit (Vector Laboratories, Burlingame, CA) was used to detect the primary antibodies followed by rabbit polyclonal antibodies to human PNAd carbohydrate antigen. The proteinase K (DAKO) antigen retrieval method was applied to the staining of CD31 in mouse lymph nodes.

Endotoxin-induced lymphadenopathy. Lipopolysaccharides from Escherichia coli O127:B8 (Sigma, Saint Louis, MO) were dissolved in PBS (1 mg/mL) and injected into the left hind footpads of 10 BALB/c nude mice at the dosage of 20 μg daily for 20 days. An equal volume of PBS was injected into another group of 10 mice as a vehicle control. After 20 days of injection, the mice were euthanized and the sentinel popliteal lymph nodes were isolated for evaluation.

Proliferation rates of high endothelial venule endothelial cells. Rabbit anti-proliferating cell nuclear antigen (PCNA) polyclonal antibody (Abcam, Inc., Cambridge, MA) and rat anti-mouse MECA79 were applied at 1:100 dilution simultaneously onto the lymph node sections followed by TRITC-conjugated donkey anti-rabbit and FITC-conjugated goat anti-rat secondary antibodies. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). At least 200 high endothelial venule (HEV) endothelial cells were randomly counted for each lymph node to calculate the percentage of proliferating endothelial cells. For routine immunohistochemical staining, the rabbit anti-PCNA polyclonal antibody was used at 1:300 dilution.

Image analysis. Image analysis was done using interactive image analysis software developed by our laboratory. Regions positively stained with the HEV marker MECA-79 within the images were identified by combining two binary masks using a logical AND operator. The first mask was generated by identifying pixels that were bright in the yellow image channel (after converting the red, green, and blue (RGB) color image to cyan, magenta, yellow, and black color space). The second mask was generated by identifying pixels that were dark in the green image channel (from the RGB color space). Pixels that were "bright" or "dark" were automatically identified using the thresholding algorithm of Ridler and Calvard (1978; ref. 21). Small staining artifacts (mainly resulting from nonstaining endothelial nuclei) were removed by applying a two-pixel opening function. Vessels within the binary mask were identified using connected component analysis (22). Several variables for each connected component within the binary mask were then calculated:

1. The total number of pixels within each component, representing the cross-sectional area of the HEV wall.
2. The length of edge of each component, representing the sum of the inner and outer circumferences of the vessel wall.
3. The number of pixels enclosed by the vessel wall, representing the luminal area (if any).
4. The "longest chord" of the luminal area (if any). This variable was used to correct for nonperpendicular sectioning of the vessels. Once the longest chord is known, the minor axis (i.e., the longest chord perpendicular to the major axis) may be calculated. The area of an ellipse is defined in Eq. 1, where \( a_1 \) and \( a_2 \) are half the major and minor axes, respectively [half for the same reason that half the diameter (i.e., the radius) is used for calculating the area of a circle].

\[
A = \pi a_1 a_2 
\]

\[
a_2 = \sqrt{\frac{A \pi}{a_1}}
\]

\[
A = \pi a_2^2
\]

Because we measure the area directly (by enumerating pixels within the lumen), we may rearrange this equation to solve for half the minor axis (\( a_2 \)).
of gates. All tests were two-tailed. A number that follows the sign is a SD.

Results

Spontaneous lymph node metastasis in a mouse model of NPC. There is considerable evidence of heterogeneity in the metastatic population of tumor cells (23–25). We isolated 29 clones from the parental cell line CNE-2 and screened for their migratory and invasive abilities in vitro. Clone-18 statistically produced the greatest migration and invasion when compared with the parental cell line and other clones (Fig. 1A).

To study the process of spontaneous lymph node metastasis, an animal model was established by injecting CNE-2 tumor cells into the left hind footpad of immunodeficient nude mice, resulting in metastasis to the popliteal lymph node, which is the SLN for this model. Relative to the parental cell line and other clones, Clone-18 had a significantly greater potential of metastasis (Fig. 1B and C), although the primary tumor growth rates were not different among these cell populations (Fig. 1D). Metastases of Clone-18 were observed by day 20 and reached 100% metastatic incidence on day 50 after inoculation of $8 \times 10^7$ cells into the hind footpad of the mouse (Fig. 1E). The footpad model was used because (a) there is only a single popliteal lymph node in the anatomic structure of the murine rear leg and (b) the anatomic location of the popliteal lymph node is constant, superficial, and easy to identify by palpation, favoring noninvasive imaging by ultrasonography. To study the histologic alteration before metastasis, we restricted our analyses to the lymph node that contained no CNE-2 micrometastases functioning before metastasis.

Lymphangiogenesis in SLNs before metastasis. An increase in the number of lymph vessels/sinuses, as well as their level of dilatation, was evident in the popliteal SLNs by staining with Evan’s blue dye (Fig. 2B). To histologically evaluate the process of lymphangiogenesis, the anti-mouse LYVE-1 antibody was used to identify the lymphatic endothelium (20, 28). The absence of colocalization of LVYE-1 and CD31 staining was evident in the lymph node tissue (Fig. 3A-D), confirming that LYVE-1 did not stain blood vessels in the lymph node. Normally, the lymph vessels/sinuses with an open lumen in the popliteal lymph node of young adult mice are not common. The collapsed lymphatic endothelium branching from the hilum to the medulla and paracortex of the lymph node is usually observed (Fig. 3E). In the popliteal SLNs for CNE-2 tumor-bearing mice, these lymph vessels/sinuses were clearly dilated (Figs. 3F and 4) before metastasis.

To assess the correlation between lymph sinus dilatation and primary tumor weight, 12 nonmetastatic sentinel popliteal lymph nodes from the nude mice carrying Clone-2 tumors were examined. Thirty days after inoculation were studied. The largest cross-section of each lymph node stained with H&E was completely captured in several images (Supplementary Fig. S1A). The total lymph sinus area was then objectively calculated by the computer program (Supplementary Fig. S1B). The total area of lymph vessels/sinuses was significantly correlated with the primary tumor weight (Supplementary Fig. S1C), implying that the dilatation of the lymph vessels/sinuses in SLN was induced by the primary tumor.

The HEV is responsible for tumor-induced vascularization in the lymph node. Histologic examination confirmed that the enlarged blood vessels in popliteal SLNs contained numerous RBC...
besides the typically thin, attenuated capillary vessels (<15 μm in diameter), HEVs (>20 μm in their largest diameter) were conspicuous in the normal mouse popliteal lymph node (Figs. 4 and 5A), with their characteristically cuboidal endothelial cells expressing peripheral node addressin, which could be recognized by the MECA-79 antibody (Fig. 5B; refs. 29, 30) as well as by traditional blood vessel markers. Unlike lymph vessels/sinuses, which concentrate in and branch from the hilum to the medulla/paracortex, HEVs were evenly distributed in both medullary and paracortical regions in the mouse popliteal lymph node (Fig. 4). In the popliteal SLNs of mice bearing CNE-2 tumors, however, the HEVs were dilated, with increased lumenal diameter (Figs. 4 and 5D) and the branching out of thin-walled blood vessels (Fig. 5C). Few HEV with flattened lumens (like those in normal lymph nodes) were observed in the SLN 20 days after tumor cell inoculation.

The remodeling of HEV in SLN could be induced in nude mice carrying low-metastatic clones as well as in immunocompetent mice carrying syngeneic mouse breast cancer cells. To determine if the premetastatic lymphangiogenesis and vascularization observed in the murine SLNs are universal, we inoculated low-metastatic CNE-2 Clone-22 and Clone-26 cells into the immunodeficient mice and mouse breast adenocarcinoma DA3 cells into the immunocompetent BALB/c mice. The popliteal lymph nodes were collected 15 to 30 days after inoculation. The dilation of lymph sinuses/vessels was consistent with the findings from Clone-18 experiments (Fig. 3G). Computerized quantitative analyses were done to measure the HEV number, the wall thickness, and the adjusted cross-sectional lumen area of HEV. The mean HEV sections per field (0.26 mm²) of the popliteal SLNs versus the contralateral popliteal lymph node in the animal models of Clone-22, Clone-26, and DA3 tumors were 8.97 ± 2.3 versus 20.95 ± 5.75 (P < 0.001), 8 ± 2.1 versus 14.57 ± 3.7 (P < 0.001), and 9.65 ± 3.18
versus 14.9 ± 5.68 (P < 0.001), respectively. In these mouse models, the decrease of the HEV density in SLNs can be explained by the dilation of the HEV lumen, the dilation of the lymph sinuses, and the proliferation of the lymphocytes during lymphadenopathy. In all of these groups, the wall thickness of HEVs was significantly decreased in the SLNs, whereas the adjusted cross-sectional lumen area of HEVs was significantly increased (Fig. 6A and B). These results suggest that the premetastasis remodeling of HEVs in SLNs is a common phenomenon in animal models and is independent to the metastatic ability of cancer cells.

Remodeling of HEVs in the axillary lymph nodes of human breast cancer patients. To determine if the remodeling of HEVs also occurred in human neoplasia, we examined a series of archived axillary lymph node tissues. The normal thick-walled HEVs devoid of RBC were easily identified in human axillary lymph nodes (Fig. 5E). However, in the nonmetastatic axillary lymph nodes of breast cancer patients, the HEVs had increased lumen diameters, contained RBC, and transitioned into thin-walled enlarged blood vessels (Fig. 5F-H). To quantitatively evaluate these changes, 10 axillary lymph nodes from 6 patients with noncancerous chronic inflammation were used to generate 28 images of the regions of greatest HEV density (“hotspots”) within the lymph node sections for a noncancerous control (Fig. 6C). Fourteen metastatic and 11 nonmetastatic axillary lymph nodes from 7 breast cancer patients were selected to generate 32 and 31 images of HEV hotspots, respectively (Fig. 6D-F).

The mean HEV sections per field (0.26 mm²) of metastatic, nonmetastatic, and noncancerous control lymph nodes were 20.21 ± 7.22, 24.44 ± 10.25, and 36.69 ± 15.94, respectively. The difference between the mean HEV numbers per field in metastatic versus nonmetastatic lymph node sections was statistically significant (P = 0.00004). The difference in the mean HEV numbers per field between metastatic versus control lymph nodes, as well as between nonmetastatic versus control lymph nodes, was likewise statistically significant (P = 0.0014 and 0.0315, respectively).

The mean adjusted cross-sectional lumen areas for images of metastatic, nonmetastatic, and noncancerous control lymph nodes were 215.44 ± 491.20, 156.84 ± 301.41, and 34.70 ± 30.52 mm², respectively. The difference between the mean adjusted lumen area in metastatic versus nonmetastatic lymph node sections was not statistically significant (P = 0.26). The difference in the mean adjusted lumen cross-sectional area between images of metastatic versus noncancerous control lymph node, as well as between nonmetastatic versus noncancerous control lymph nodes, was statistically significant (P < 0.00001 for each).

Of particular interest was the measurement of the emergence of functional venules from the population of nascent HEVs. We selected 80 μm² as a minimum luminal cross-sectional area for functional vessels, corresponding to the minimum caliber of physiologic venules (31). Figure 6G compares the proportion of HEVs having any measurable open lumen at all, as well as those
with open lumens of at least 80 μm², among metastatic, nonmetastatic, and noncancerous control nodes. Significantly more vessels had open lumens among the HEVs imaged in metastatic lymph nodes (38%) relative to both nonmetastatic lymph node (27%) and noncancerous control lymph nodes (11%; \( P < 0.001 \) for each), as well as among nonmetastatic (27%) relative to noncancerous control lymph node (11%; \( P < 0.001 \)). Similarly, the proportion of HEVs with adjusted luminal areas of at least 80 μm² was significantly larger in metastatic lymph nodes (18%) compared with both nonmetastatic (13%) and noncancerous control lymph nodes (0.8%; \( P < 0.001 \) for each), as well as among nonmetastatic (13%) relative to noncancerous control lymph nodes (0.8%; \( P < 0.001 \)).

Comparing the proportion of vessels with any open lumen at all which exceeded the theoretical minimum caliber for function (80 μm²), there was no significant difference between the metastatic lymph node (47%) and nonmetastatic lymph node (45%; \( P = 0.8 \)). However, the difference between each of these groups and the noncancerous control lymph node (8%) was highly significant (\( P < 0.00001 \) for both).

Among HEVs with open lumens, the mean estimated vessel wall thickness for HEVs imaged in noncancerous control, nonmetastatic, and metastatic lymph nodes was 11.96 ± 3.93, 8.57 ± 3.52, and 9.12 ± 4.80 μm, respectively. Whereas there was no significant difference between mean HEV wall thickness in metastatic and nonmetastatic nodes (\( P = 0.14 \)), the mean wall thickness for each group was significantly thinner than in noncancerous control nodes (\( P < 0.00001 \) for both).

Figure 6H compares the wall thicknesses of those HEVs that had open lumens among noncancerous control, nonmetastatic, and

Figure 3. Identification of lymphatic channels in popliteal lymph nodes by using anti-LYVE-1 antibody. A to C, immunofluorescent images of the same lymph node section. A, staining with anti-LYVE-1 primary antibody followed by FITC-conjugated secondary antibody (green fluorescence). B, staining with anti-CD31 primary antibody followed by rhodamine-conjugated secondary antibody (red fluorescence). C, staining with DAPI blue fluorescence to indicate the nucleus. D, merged image of (A-C), showing no colocalization of LYVE-1 and blood vessels. E to G, images of routine immunohistochemical staining using anti-LYVE-1 antibody. E, a section of a normal popliteal lymph node. Inset, the higher magnification of the framed area. F, a section of a sentinel popliteal lymph node from a nude mouse 10 days after CNE-2 Clone-18 cell inoculation; enlargement of the lymph sinuses is evident. G, a section of a sentinel popliteal lymph node without metastasis from an immunocompetent BALB/c mouse 15 days after DA3 cell inoculation.
metastatic lymph nodes. There was no difference in the proportion of HEVs with walls <5-μm thick between metastatic (9.8%) and nonmetastatic nodes (7.4%) or between nonmetastatic and control lymph nodes (2.5%), but there was a significantly larger proportion of HEVs with walls <5-μm thick when comparing metastatic to control lymph nodes (P = 0.035). Furthermore, there was no difference in the proportion of HEVs with walls 5- to 10-μm thick or 10- to 15-μm thick when comparing metastatic (59% and 22%, respectively) and nonmetastatic lymph nodes (63% and 23%, respectively). However, each of these groups had a significantly greater proportion of HEVs with walls 5- to 10-μm thick and a significantly smaller proportion with walls 10- to 15-μm thick relative to noncancerous controls (29% and 49%, respectively; P < 0.0001 for all).

Finally, there was a significantly larger proportion of HEVs with walls >15-μm thick in the control lymph node (18%) relative to either the metastatic (8%; P = 0.006) or nonmetastatic lymph node (6%; P = 0.0018). There was an inverse correlation between lumen size and wall thickness for those vessels that have lumens (Spearman’s ρ = −0.318; P < 0.0001), implying that the larger the HEV lumen, the thinner the vessel wall. All these results suggested that, in human axillary lymph nodes of breast cancer patients, the remodeling of HEV started before metastasis and continued after the arrival of metastatic cancer cells. The decrease of HEV density in the cancerous condition can be explained by the dramatic dilation of the HEV lumen space.

Proliferation of endothelial cells during the remodeling of HEV. For the proliferation study in mouse lymph node, 10 normal mouse popliteal lymph nodes were collected at the age of 2 months old. Ten nonmetastatic and 10 metastatic mouse sentinel popliteal lymph nodes were also collected from nude mice 30 days after inoculation of CNE-2 Clone-18 cells into the left hind footpad. The combined fluorescent images of triple stained HEVs were used to calculate the proliferation rates of HEV endothelial cells (Fig. 7A). The proliferation rate of HEV endothelial cells was significantly increased before metastasis (Fig. 7E), which is consistent with the process of HEV remodeling. The endothelial cell proliferation rates were not different between nonmetastatic and metastatic lymph

![Figure 4](https://www.aacrjournals.org/10371) The enlargement of lymph sinuses and blood vessel lumens in the SLN before metastasis. The normal popliteal lymph nodes was collected from a 8-week-old female nude mouse without tumor cell inoculation. The contralateral and sentinel popliteal lymph nodes were collected from another mouse with the same age 20 days after tumor cell inoculation. Continuous sections from each lymph node were used for the lymphatic, blood vessel, and H&E staining. The lymphatic and blood vessel endothelia were stained using anti-LYVE-1 and anti-CD31 antibodies, respectively, H&E staining shows the RBC in the enlarged and functioning blood vessels in sentinel popliteal lymph node, in which enlargement of lymphatic sinuses is also evident. Bars, 0.1 mm.
nodes, implying that the proliferation of HEV endothelial cells was mainly influenced by the duration of the carcinogenic condition.

To confirm that proliferation of HEV endothelial cells is also involved in the remodeling process of human HEV, the above-mentioned 10 noncancerous, 11 nonmetastatic, and 14 metastatic axillary lymph nodes from patients were immunofluorescently stained (Fig. 7B). The typical proliferating HEV endothelial cells in a metastatic lymph node detected by routine immunohistochemical staining are also shown in Fig. 7C and D. The proliferation rate of the HEV endothelial cells was significantly increased in the nonmetastatic lymph nodes (Fig. 7F), which was consistent with our results from animal models. Interestingly, the proliferation rate of HEV endothelial cells was significantly higher in the metastatic human lymph nodes than in nonmetastatic lymph nodes, implying that the lymph nodes with more robust HEV proliferation may be associated with a higher metastasis possibility in human lymph node.

Characteristics of endotoxin-induced lymphadenopathy and tumor-reactive lymphadenopathy. The use of athymic nude mice, which lack T cells, clearly excludes the possibility that node proliferation is due to the generation of a primary immune response to micrometastases or to tumor antigens from the primary lesion being presented by antigen-presenting cells arriving via the afferent lymphatics. The use of DA3 cells in the syngeneic BALB/c mice further excludes the involvement of immune response. However, the injection of dead CNE-2 Clone-18 cells fixed with 70% alcohol (8 × 10⁵ cells twice with a 5-day interval), saline (30 μL daily for 20 days), or the serum from nude mice

Figure 5. Remodeling of HEVs in the SLN of animal models and in the regional lymph nodes of breast cancer patients is evident. Normal popliteal lymph nodes in BALB/c nude mice, stained with anti-CD31 antibody (A) and stained with anti-MECA79 antibody (B). Popliteal SLN before metastasis 10 days after Clone-18 inoculation, stained with anti-CD31 antibody (C) and stained with anti-MECA79 antibody (D). Remodeled HEVs are seen to be dilated and are branching out into thin-walled, functional blood vessels containing RBC (asterisks in C), which are typical of an angiogenic phenotype. E, human noncancerous axillary lymph node with a normal HEV (arrow). F to H, nonmetastatic axillary lymph nodes from breast cancer patients. F, a dilated HEV. G, a dilated and remodeled HEV with a part of the vessel wall transforming into thin wall (arrow). H, another transforming HEV with the changing of the high endothelial cells into flat endothelial cells (arrow). Staining is with anti-MECA79 antibody (E and F) and H&E (G and H).
carrying CNE-2 tumors (30 µL daily for 20 days) all failed to induce any morphologic and functional alteration of the lymph channel or HEV vasculature in the SLNs (data not shown). Moreover, in any of our footpad models, we found no morphologic alteration of the lymph channel or HEV vasculature in the second lymph node station (e.g., the inguinal lymph nodes; data not shown).

It is unclear whether morphologic alterations of the lymph channel and HEV vasculature share the same patterns in inflammatory lymphadenopathy and tumor-reactive lymphadenopathy. In nude mice, the absence of T cells accounts for the resistance to endotoxin-induced inflammation (32, 33). Therefore, prolonged administration of endotoxin for 20 days was done to induce significant lymphadenopathy in the sentinel popliteal lymph node (Supplementary Fig. S1D). Staining for LYVE-1 showed that the dilated lymph vessels/sinuses in endotoxin-induced lymphadenopathy were full of lymphocytes (Supplementary Fig. S1E). This was in contrast to the dilated lymph vessels/sinuses in tumor-reactive lymphadenopathy, which contained few cellular structures, implying that it was carrying a large amount of fluid rather than a large amount of lymphocytes. Interestingly, the morphology of HEVs was not altered at all in the endotoxin-induced lymphadenopathy (Supplementary Fig. S1E), suggesting that the inflammatory reaction and cancerous reaction in the SLN are induced by different mechanisms.

Premetastatic remodeled HEVs could integrate into the postmetastatic tumor vasculature with further differentiation. The standard marker for HEVs is MECA-79. The MECA-79-reactive ligand is known as peripheral node addressin (PNAd). Anti-MECA-79 antibody specifically stains HEVs in lymph nodes and other secondary lymphoid organs in many species, including humans and mice (34). Within metastatic tumor nests in the axillary lymph nodes from human breast cancer patients, some

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**Figure 6.** Quantitative analyses of the remodeling of HEVs in both animal SLNs and human regional lymph nodes. A, the increase of the adjusted HEV lumen area in the sentinel popliteal lymph node before metastasis is statistically significant in nude mice carrying human cancers (CNE-2 Clone-22 and Clone-26) as well as in immunocompetent BALB/c mice carrying mouse DA3 breast adenocarcinoma (*P* < 0.001). B, the decrease of the HEV wall thickness is statistically significant in Clone-22 (*P* < 0.001), Clone-26 (*P* < 0.001), and DA3 (*P* = 0.035). C to F, immunohistochemical staining of HEVs using anti-MECA79 antibody in a human noncancerous lymph node from a cancer-free patient (C), a nonmetastatic lymph node from a breast cancer patient (D), and a metastatic lymph node (E and F) showing the margin of a metastatic tumor lesion (T in E) and a lymphoid tissue area distant to the tumor lesion (F). G, proportion of HEVs with lumens (any or >80 µm²) for control, nonmetastatic, and metastatic lymph nodes. *P* = 0.001 relative to control; **P* < 0.001 relative to nonmetastatic. H, proportion of open lumen HEVs with various wall thicknesses for control, nonmetastatic, and metastatic lymph nodes. *P* < 0.05 relative to control.
identified HEVs were full of RBC, implying fully functioning vessels (Supplementary Fig. S2A). Interestingly, the remodeled HEVs gradually lost their specific marker MECA-79 from the tumor margin to the central portion of the tumor nests (Supplementary Fig. S2B), implying further differentiation of the HEVs when they integrated into the metastatic tumor vasculature. Actually, most of the HEVs lost their specific marker MECA-79 after integrating into the tumor vasculature, leaving the surrounding remnant lymphoid tissue, indicating that they were pre-existing vessels (Supplementary Fig. S2C). These findings implied that the remodeled HEVs in the premetastatic phase continued to nurture the metastatic lesions after the arrival of cancer cells and therefore played an important role in the secondary tumor growth inside the involved lymph node.

**Discussion**

The lumens of the lymph sinuses/vessels were dilated in SLN before metastasis. The SLN is rebuilt by the primary tumor to become a functional blood vessel–enriched organ before and independent of metastasis, with the morphologic and functional alterations of the HEVs to become main blood flow carrier in the lymph node. The processes of vascularization in the SLN were consistent in both animal model and human tissues. This study presents functional and structural data that the primary tumor is manipulating the “soil” to improve the “seeding” of subsequent metastases.

The extent of lymph sinus dilation in the SLN was significantly correlated with the primary tumor weight, suggesting that the lymph from the primary tumor induced the persistent alteration of the lymph channel in SLN. These results are consistent with a recent finding that, in contrast to angiogenesis, in which blood flow proceeds only after the vessel develops, lymphangiogenesis can be induced by interstitial fluid channeling (35). The alteration of lymph channels in SLNs can also occur during an inflammatory reaction, which facilitates the migration of inflammatory cells (36). Interestingly, the dilated lymph vessels/sinuses in endotoxin-induced lymphadenopathy were full of lymphocytes, but they contained very few cellular structures in tumor-reactive lymphadenopathy, suggesting different roles of the SLN lymphatic channels in different pathologic processes.

It has been well characterized that the HEVs of lymph nodes play an important role in recruiting lymphocytes for the generation of immune responses. By expressing homing receptors on their surface, which blood lymphocytes can recognize as they pass in
circulation, HEVs provide a unique location where naïve lymphocytes can enter the lymph node (30, 37, 38). In this study, we found that the role of HEV was shifted to become the main blood flow carrier in the SLN before metastasis. Not only could the HEV morphology change dramatically to carry more blood flow, but the proliferation rate of HEV endothelial cells was also increased before metastasis. However, the HEV morphology did not alter at all in endotoxin-induced lymphadenopathy, implying a selective reaction of HEV in the cancerous condition.

The adaptive response of the vascular wall has been noted when a vein segment is transposed as a bypass graft into the arterial circulation, with an increase in wall thickness and the appearance of multiple cellular layers in the venous endothelium (39, 40). Distinct, differentiated gene expression has also been reported when the endothelial cells respond to the changing of their microenvironment (41). In our own study, we found that the cellular morphology of the tall endothelial cells forming HEVs changed dramatically to become flat endothelial cells in cancerous condition. As a consequence, the HEV was remodeled from a thick-walled, endothelial vessel with a small lumen to a thin-walled, large-lumen vessel, shifting its function from recruiting lymphocytes to becoming a blood flow carrier. These facts suggest again that the blood vessel endothelium has tremendous potential to adapt its biomechanical environment.

The confined lymph and blood channel alterations within the SLN but not in the next station lymph node imply that an inducer from the primary tumor is functioning locally with the existence of a liable primary tumor. VEGF-A has been found to be an inducer of lymphangiogenesis in SLNs (9). We also found that the serum level of VEGF-A was elevated in patients with late-stage NPC (42). Because VEGF-A is a secreted protein that can travel via the circulation, HEVs provide a unique location where naïve lymphocytes can enter the lymph node (30, 37, 38). In this study, we found that the role of HEV was shifted to become the main blood flow carrier in the SLN before metastasis. Not only could the HEV morphology change dramatically to carry more blood flow, but the proliferation rate of HEV endothelial cells was also increased before metastasis. However, the HEV morphology did not alter at all in endotoxin-induced lymphadenopathy, implying a selective reaction of HEV in the cancerous condition.

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Acknowledgments
Received 8/15/2006; accepted 8/18/2006.

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We thank Dawna Dylewski, Elissa Boguslawski, Bryn Eagleson, and the rest of the VARI Vivarium staff for assistance in animal husbandry; JC Goolshy and the rest of the VARI Histology Core Facility for technical support; and David Nadziejka for critically reading this article.

References
Preparing the "Soil": The Primary TumorInduces Vasculature Reorganization in the SentinelLymph Node before the Arrival ofMetastatic Cancer Cells

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