Real-time Imaging of Lymphogenic Metastasis in Orthotopic Human Breast Cancer

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Abstract
Metastatic spread to regional lymph nodes is one of the earliest events of tumor cell dissemination and presents a most significant prognostic factor for predicting survival of cancer patients. Real-time in vivo imaging of the spread of tumor cells through the lymphatic system can enhance our understanding of the metastatic process. Herein, we describe the use of in vivo fluorescence microscopy imaging to monitor the progression of lymph node metastasis as well as the course of spontaneous metastasis through the lymphatic system of orthotopic MDA-MB-231 human breast cancer tumors in severe combined immunodeficient mice. High-resolution noninvasive visualization of metastasizing cancer cells in the inguinal lymph nodes was achieved using cells expressing high levels of red fluorescent protein. Sequential imaging of these lymph nodes revealed the initial invasion of the tumor cells through the lymphatic system into the subcapsular sinuses followed by intrusion into the parenchyma of the nodes. FITC-dextran injected i.d. in the tumor area enabled simultaneous tracking of lymphatic vessels, labeled in green, and disseminated red cancer cells within these vessels. Fast snapshots of spontaneously metastasizing cells in the lymphatic vessels monitored the movement of a few tumor cells and the development of clumps clustered at lymphatic vessel junctions. Quantification of high interstitial fluid pressure (IFP) in the tumors and fast drainage rates of the FITC-dextran into the peritumoral lymphatic vessels suggested an IFP-induced intravasation into the lymphatic system. This work presents unprecedented live fluorescence images that may help to clarify the steps occurring in the course of spontaneous lymphogenic metastasis.

Introduction
Metastasis of malignant tumors to regional lymph nodes is one of the earliest signs of cancer spread and critically influences the prognosis and choice of therapy, particularly in patients with invasive breast cancer (1). In general, the dissemination of tumor cells to form distant metastases can occur through the hematogenous system, the lymphatic system, or both. Thus far, much greater insight has been gained into the molecular, cellular, and mechanistic aspects of the hematogenic metastasis rather than lymphogenic metastasis (2). However, over the past few years, advances have been made in understanding the cellular and molecular aspects of tumor-induced lymphangiogenesis (3). Clarifying the nature and mechanisms of lymphogenic metastasis requires an experimental animal model, in which tumor cells originating from the primary tumor spontaneously metastasize into the lymphatic system. In such a model, the metastatic process is influenced by critical forces, such as lymphatic flow and interstitial fluid pressure (IFP), which drive the extravasation of tumor cells (4).

Recent developments in optical imaging techniques are providing a clearer understanding of the functional-mechanistic aspects of metastatic processes (5). In animal models, intravital imaging or whole-body imaging using fluorescent or bioluminescent probes has mainly been used to monitor experimental metastases formed by injecting tumor cells into the bloodstream or spontaneous metastasis through the blood circulation (6, 7). However, real-time noninvasive optical imaging has not yet been applied to discern the various steps of spontaneous lymphatic metastasis (7). In the present study, we applied high-resolution in vivo fluorescence imaging to investigate, noninvasively, spontaneous lymphogenic metastasis of orthotopic human breast cancer.

Materials and Methods
Cells. MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and were routinely cultured as described previously (8). Plasmid-encoding red fluorescent protein (RFP) pDsRed2-N1 (Clontech Laboratories, Palo Alto, CA) was transfected into MDA-MB-231 cells. Highly efficient transfection was obtained by using polyethylenimine (Sigma-Aldrich, Rehovot, Israel). Cells were serially diluted 48 hours after transfection and seeded to provide a wide range for screening. Following 2 weeks of growth in culture medium containing the selective antibiotic G418 (0.8 mg/mL), hundreds of isolated fluorescent clones were observed. Forty-eight clones with the highest levels of red fluorescent emission were picked up and cultivated. Clone 7, which consistently expressed RFP with ~5-fold higher fluorescence emission than the rest of the clones, was used in this study.

Mouse model. All animal procedures were carried out in accordance with the Guidelines for the Care and Use of Research Animals at the Weizmann Institute of Science (Rehovot, Israel). MDA-MB-231 clone 7 cells (5 × 10⁵ in 0.1 mL PBS) were inoculated into the mammary fat pads of 7- to 8-week-old female CB-17 severe combined immunodeficient (SCID) mice (n = 24).

Fluorescence imaging. Mice were anesthetized by i.p. injection with a mixture of 5 mg/kg ketamine and 1 mg/kg of 2% xylazine (85:15%, v/v). The hair was removed using depilatory cream to avoid light scattering and hair autofluorescence. For visualization of the lymphatic vessels, ~5 to 10 μL of 10 mg/mL FITC-dextran conjugate (molecular weight, 5 × 10⁵; Sigma-Aldrich) were injected i.d. into the periphery of the tumor. Mice were placed under a Zoom Stereo Microscope SZX-RFL-2 (Olympus, Tokyo, Japan) equipped with a fluorescence illuminator and a Pixelfly QE CCD camera.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Results

Efficient transfection of MDA-MB-231 human breast cancer cells with RFP and a broad-based screen for highly red emitting cells yielded a stable, intensely fluorescent clone, amenable to noninvasive in vivo imaging. The tumorigenic and invasive properties of MDA-MB-231 tumors, described previously (8, 10), were retained in the transfected clone. The cells inoculated into the mammary fat pad of SCID mice formed tumors that reached a size of 10 \( \pm \) 5 mm\(^3\) within 2 weeks and 314 \( \pm \) 81 mm\(^3\) within 5 weeks. From 3 weeks onward, we detected metastases in these mice that were confirmed by histology, mostly to the lymph nodes and lungs (Table 1) and occasionally to the bones and other inner organs, such as the spleen, pancreas, and colon. The incidence of metastasis to the ipsilateral inguinal lymph node (same side as the tumor) was 75% by weeks 3 to 5 and 95% by weeks 6 to 7, whereas to the contralateral inguinal lymph node the incidence was only 50% by weeks 6 to 7. By weeks 8 to 11, all lymph nodes were involved. The incidence of invasion to the lungs was 75% by weeks 6 to 7 and 100% by weeks 8 to 11.

![Figure 1](image)

Figure 1. In vivo fluorescent images of spontaneous metastasis into lymph nodes of the RFP-expressing MDA-MB-231 human breast cancer tumors. Spontaneous metastases to the inguinal lymph nodes (LN), to the ipsilateral node within 4 to 6 weeks after inoculation (A), and to both inguinal lymph nodes within 6 to 8 weeks after inoculation (B). Green background, body autofluorescence. It is important to note that the tumor size in this figure appears larger than its actual size by \( \approx \) 40% due to light scattering and saturation. C to E, higher magnification of metastatic progression (6, 8, and 9 weeks after inoculation of the tumor cells) in a contralateral inguinal lymph node.

Tumor growth and metastatic progress of the cells within the lymph nodes were tracked weekly by in vivo monitoring of the fluorescent signal in the inoculated regions and in the inguinal lymph nodes. Invasion of cancerous cells into the ipsilateral inguinal lymph node preceded their invasion into the contralateral node (Fig. 1A and B). Increased fluorescence intensity in the area of the lymph nodes was detected during progression of the metastases (Fig. 1C-E).

Further confirmation of the metastatic progression within the lymph nodes was obtained by subsequent histopathologic examination and cytokeratin staining for tumor cells in the lymph nodes (Fig. 2). Specifically, 6 weeks after cell inoculation, early metastasis into the contralateral inguinal lymph node appeared as a fluorescent ring 100 to 200 μm thick. The spatial ring-like distribution of tumor cells in this lymph node was confirmed by cytokeratin staining for epithelial cells, which showed an invasion of cancer cells into the subcapsular sinus of the lymph node (Fig. 2A and B). At \( \approx \) 8 weeks after cell inoculation, a massive invasion of cancerous cells into lymph nodes was visualized as an increase in fluorescence intensity and area. This was further confirmed by cytokeratin staining (Fig. 2C and D).

The strong fluorescent signal emitted by the metastasizing cells made it possible to monitor in vivo their dissemination through the lymphatic vessels and to trace their movement within these vessels. The overall direction of the flow of cells within the lymphatic vessels was from the tumor periphery toward the inguinal lymph nodes. This flow pattern exhibited regional oscillations in both directions typical of lymphatic fluid movement (3) as shown in Supplementary Movie 1 online. In this experiment, the average rate of tumor cells flow within the lymphatic vessels was 23 \( \pm \) 11 μm/s, which is within the reference range of lymphatic flow in mice (11).

### Table 1. Metastatic incidence of RFP-transfected MDA-MB-231 (clone 7) in SCID mice

<table>
<thead>
<tr>
<th>End point (wk)</th>
<th>Ipsilateral lymph node</th>
<th>Contralateral lymph node</th>
<th>Lungs</th>
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<tbody>
<tr>
<td>3-5</td>
<td>3/4*</td>
<td>NF</td>
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<tr>
<td>6-7</td>
<td>15/16</td>
<td>8/16</td>
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<tr>
<td>8-11</td>
<td>6/7</td>
<td>7/7</td>
<td>3/3</td>
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NOTE: Organs were inspected by histology at the end of the experiment in 24 mice. Eleven animals were inspected for metastases in the lungs.

Abbreviation: NF, not found at this time point.

*Three animals were inspected by fluorescence microscopy. The earliest metastases were found at week 3 after inoculation.
To verify that the tumor cells were located within the lymphatic vessels, we used green fluorescent high molecular weight FITC-dextran that has been shown to specifically mark lymph vessels (12). This compound was injected i.d. into the outer surface of the tumor; thus, the detected green fluorescent lymph vessels outlined the pathway through which tumor cells could travel toward the lymph nodes (Fig. 3A). Simultaneous observation of tumor cells (red) and lymphatic vessels (green) confirmed the distribution of metastatic cells within the lymphatic network (Fig. 3B). Sequential observations of tumor cell movement within lymphatic vessels at the same location in the same mouse showed the clumping of cells at junctions of the lymphatic vessels (Fig. 3C and D). This clumping was also accompanied by a slowdown in the rate of the lymphatic flow. (Fig. 3; see also Supplementary Movie 2 online).

After ∼ 8 to 10 weeks of implantation, the sequential monitoring also revealed local metastases in the vicinity of lymphatic vessels (Fig. 4). Small metastatic loci were observed underneath the skin at truncated edges of dermal lymphatic vessels (Fig. 4A and B). Histologic examination of the involved tissues revealed cancerous invasion from a lymph vessel into subdermal tissue (Fig. 4B). Another fluorescent locus, observed near lymphatic vessels (Fig. 4C), was revealed by the cytokeratin staining as a large metastasis into a perivascular tissue (Fig. 4D).

Injection of FITC-dextran also provided a means by which to measure the rate of entry of this macromolecule into the lymphatic system. We found that this rate was much faster (90-300 μm/s) when the FITC-dextran was injected i.d. into the tumor rim compared with that injected i.d. into abdominal areas far from the tumor (see Supplementary Movie 3 online). The rapid outflow of lymphatic fluid from the tumor rim suggested the involvement of high IFP, which is typical of invasive tumors (13). To validate the presence of high IFP in these tumors, we measured the fluid pressure using the wick-in-needle technique. We found that IFP within the tumor rim varied from 15 to 23 mm Hg, with a mean value of 19.4 ± 3 mm Hg (n = 5); these values were significantly higher than the IFP in the normal muscle tissue of these mice, which was close to 0.

**Discussion**

Efforts to improve the understanding of the various stages involved in tumor progression and metastasis have led to the development of new tumor models and optical imaging techniques, which enabled the intravital imaging of both primary and metastatic lesions (6, 14). Although these novel methods provided real-time quantitative tracking of the progression of the primary tumors, clear delineation of the metastases required additional invasive stages, such as skin removal or skin flap surgery, to expose the tissue, or the prior preparation of skinfold chambers or s.c. windows (7). In our study, which focused on lymphogenic metastasis, it was critical to use noninvasive imaging procedures to avoid any interruption of lymph flow and disturbance of interstitial fluid gradients. Imaging in the visible range is limited in depth penetration due to light absorption by the tissue and light scattering. When using fluorescent proteins, the depth can reach a few millimeters depending on the fluorescent yield. Another drawback is the overlap of fluorescent proteins with the tissue autofluorescence. However, the use of RFP, which emits fluorescence at a longer wavelength than green fluorescent protein, provides less overlap with tissue autofluorescence, thereby increasing the detection capacity (5). Despite these limitations, by obtaining high expression levels of RFP in the breast cancer cells, we were able to visualize noninvasively at high resolution and in real-time the migration of cancer cells within the skin lymph vessels as well as the invasion of these cells into the lymph nodes. Inner metastases, both under the skin and under the abdominal wall (to a depth of ≥3 mm), were also clearly seen using those highly fluorescent cells.

Thus far, visualization of circulating cancer cells by means of fluorescence microscopy was primarily achieved by direct injection of cancerous cells into the bloodstream and the subsequent monitoring of hematogenic metastasis (7, 15). Nevertheless, the lymphatic system is the primary route of invasion for the majority of human carcinomas (1). The high preference of invasion into the lymphatics can be attributed to the loose structure of lymphatic capillaries or to the function of the lymphatic system as a drainage network, by which tumor cells can reach distant sites with the tide of lymph flow (2, 16). To achieve a spontaneous metastatic model, in which tumor cells originate from the primary tumor and in which the critical forces driving the lymphatic flow and interstitial pressure are maintained, we used orthotopic inoculation of the tumor cells. It should be noted that the SCID mouse used as our animal model has only small rudimentary lymph nodes consisting of reticuloendothelial (phagocytic) cells. However, the overall architecture of the lymphatic nodes in these mice is conserved, with a subcapsular sinus, efferent and afferent lymphatic flow, and blood circulation parallel to the lymph system. Hence, the sequential events that we have monitored are most likely similar to the physiology of lymphogenic metastasis of invasive breast cancer in humans.
Microscopic imaging of the regional inguinal lymph nodes revealed various stages of tumor cell dissemination within the nodes. This pattern of dissemination, which are frequently seen in human carcinomas (1), follows the normal pathways of lymphatic fluid drainage, first entering the subcapsular sinus through capsular pores and then diffusing through efferent lymphatic vessels. It is interesting that the observation of metastases in the lymph nodes preceded the observation of cell clumps within the lymph vessels. It was previously established that mechanical entrapment of tumor cells in the subcapsular sinus of draining lymph nodes increases local tumor cell concentration, leading to colonization of tumor cells within the lymph nodes (1). We suggest that once the free flow within the lymphatic system is disturbed by the tumor cells blockage of the lymph nodes, tumor burden within the lymphatic system increases. This leads to the accumulation of tumor cells at lymph vessel junctions, forming cell clusters, which eventually break through the lymph vessel walls and penetrate outer tissues, forming local metastases.

It has been suggested that the lack of a functional lymphatic network within tumors leads to elevated IFP in tumors (17). High IFP can also be the driving force behind the directional motility of metastasizing cells toward peritumoral lymphatic vessels (4, 16). Indeed, we showed previously that, in MDA-MB-231 tumors, there is a disparity between the influx and outflux transcapillary transfer constants, with the outflux exceeding the influx rate, which increases with tumor growth (8). This disparity most likely reflects increased IFP during tumor growth. In this study, we confirmed the presence of high IFP in these tumors by directly measuring the fluid pressure 6 weeks after tumor cells inoculation; by that time, most tumors exhibited early metastasis to the inguinal lymph nodes. Moreover, we found that the rate of lymphatic flow from the tumor rim outwards at that time was much faster than the rate of lymphatic flow observed in normal tissues in the same mice and the normal lymphatic flow previously measured in mice, ranging from 0 to 29 μm/s (11). Thus, it is reasonable to suggest that elevated levels of IFP in these tumors result in an increased outflow of lymphatic fluid to peritumoral regions and in enforced drainage of tumor fluids into the lymphatic network. This elevated flow may increase the probability that tumor cells will enter the peritumoral lymphatic network, thus facilitating lymphogenic metastasis. Previous studies also showed that lymph node metastasis was associated with high IFP (18), suggesting that interstitial hypertension increases the probability of metastatic dissemination.

In summary, a real-time monitoring of spontaneous breast cancer lymphogenic metastasis was achieved using in vivo fluorescence microscopy. Substantiation of the stages associated with lymph node metastasis and with the intravasation and flow of tumor cells from lymphatic vessels to abdominal sites is shown in Figure 4.

Figure 3. Real-time fluorescent images of metastases within lymphatic vessels. A, lymph node metastasis (red fluorescence) and lymphatic vessels (green fluorescence). The lymphatic vessels were marked by i.d. injection of FITC-dextran. B, high magnification of rectangle in (A), revealing a few tumor cells (arrows) and tumor cell clusters (arrowheads) within lymphatic vessels. C, image of the same area as in (A) obtained 3 days later. D, high magnification of rectangle in (C), revealing increased tumor cells clustering at a lymphatic vessel junction (arrowhead).

Figure 4. Metastatic infiltration from lymphatic vessels to abdominal sites. A, fluorescent image of metastatic cells (red) within a lymphatic vessel (green) underneath the skin (arrow) observed 11 weeks after inoculation of tumor cells. B, cytokeratin staining (brown) of the region imaged in (A). Note the tumor cells extravasating from a lymph vessel (LV) and invading the surrounding tissue. C, fluorescent image revealing a large metastatic focus observed in the vicinity of lymphatic vessels. D, cytokeratin staining (brown) of the lesion seen in (C) showing penetration into peritoneal tissues.
cells within the lymphatic vessels enabled us to offer clues on the mechanism of lymphatic metastasis. In addition, this technique can provide a means for testing new drugs that could intervene in the progression of lymphatic metastasis.

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References

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