Microenvironment and Immunology

Vascular Endothelial Growth Factor-C Induces Lymphangitic Carcinomatosis, an Extremely Aggressive Form of Lung Metastases

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Abstract
The lymphatic system is an important pathway for tumor dissemination to the lymph nodes, but to which extent it contributes to the formation of distant metastases remains unknown. We report that induction of lymphangiogenesis by vascular endothelial growth factor-C (VEGF-C) at the secondary site, in the lung, facilitates expansion of already disseminated cancer cells throughout the lung tissue. By using orthotopic spontaneous metastasis models in nude mice, we show that VEGF-C expression by tumor cells altered the pattern of pulmonary metastases from nodular to diffuse and facilitated disease progression. Metastases expressing VEGF-C were tightly associated with the airways, in contrast to the control cells that were scattered in the lung parenchyma, throughout the alveolar region. VEGF-C induced lung lymphangiogenesis and promoted intralymphatic spread of metastases in the lung and formation of tumor emboli in the pulmonary arteries. This pattern of metastasis corresponds to lymphangitic carcinomatosis metastatic phenotype in human cancer patients, an extremely aggressive pattern of pulmonary metastases. In accordance, pulmonary breast cancer metastases from patients which were classified as lymphangitic carcinomatosis showed high levels of VEGF-C expression in cancer cells. These data show that VEGF-C promotes late steps of the metastatic process and identify the VEGF-C/VEGF receptor-3 pathway as the target not only for prevention of metastases, but also for treatment of established metastatic disease. Cancer Res; 70(5); 1814–24. ©2010 AACR.

Introduction
The lung is an extremely common site for metastases. Most cancers spread to the lungs, including common cancers, such as breast, colon, gastric, pancreatic, and kidney cancer. The lung is particularly susceptible for metastasis, because it receives the entire cardiac output from the right side of the heart. Regardless of their preference for the hematogenous or lymphatic pathway of spread, disseminated tumor cells ultimately enter into the lung through the pulmonary arteries, following the venous circulation (1). Whereas some cancer cells enter into the blood stream directly, tumor cells disseminated to the lymph nodes may spread along the route of lymphatic drainage: through the efferent lymphatic vessels, thoracic duct, subclavian vein, and into the pulmonary artery. Although the importance of lymph node metastases for tumor staging and patient prognosis has been well established (2, 3), the extent to which the lymphatic system contributes to the formation of distant metastases is a subject of debate. The lymphatic system is no doubt recognized as an important pathway for spread of tumor cells from the primary tumor to the lymph nodes, but whether lymphatic vessels in a distant organ, such as the lung, play a role in the seeding of distant metastases and colonization of the secondary site remains unknown.

Recent studies have shown that vascular endothelial growth factor-C (VEGF-C) plays a critical role in facilitating tumor metastases. VEGF-C induces lymphangiogenesis by activating the VEGF receptor-3 (VEGFR-3) receptor tyrosine kinase on lymphatic endothelial cells (2, 4–6), and it has been shown to promote tumor dissemination to the regional lymph nodes in several mouse models (7–13). Many studies also showed a correlation between the expression of VEGF-C and lymph node metastases in human tumors (3). In addition, VEGF-C has been shown to facilitate lung metastases in experimental models. Our work showed that VEGF-C increased lung metastases of MDA-MB-435 tumor cells and that this could be suppressed by blocking VEGFR-3 signaling with function-blocking antibodies (7, 14). VEGF-C also promoted lung metastases in other mouse models, including breast cancer, prostate cancer, and melanoma (9, 11, 15). Conversely, inhibition of VEGF-C activity by shRNA silencing,
sVEGFR-3-Ig, or function-blocking antibodies to VEGFR-3 reduced lung metastases in mouse models of breast cancer (9, 14, 16), prostate cancer (17), and melanoma (15) without affecting primary tumor growth. VEGF-C gene silencing was also shown to enhance survival of mice bearing mammary tumors (16). In human tumors, high levels of VEGF-C have been correlated with poor patient prognosis, particularly in breast, gastric, and colorectal cancer (2, 18–22).

The mechanism by which VEGF-C facilitates distant metastasis remains elusive. The VEGF-C/VEGFR-3 pathway is thought to exert its effects primarily by acting at the primary tumor site, inducing tumor lymphangiogenesis, and promoting dissemination to the lymph nodes. Hence, blocking VEGF-C/VEGFR-3 is thought to prevent distant metastases by targeting the very early steps of the metastatic process. In this study we show that, in addition to its effects at the primary tumor site and in the lymph nodes, VEGF-C has profound effects on metastatic progression by acting directly in the lung tissue. We show that VEGF-C alters the pattern of lung metastases and induces an extremely aggressive metastatic phenotype, which corresponds to the lymphangitic carcinomatosis metastatic disease in human cancer patients.

Materials and Methods

Cell culture. MDA-MB-435/green fluorescent protein (GFP) cells transfected with the human VEGF-C cDNA (cl.13) or pcDNA/control vector (cl.2) have been established and cultured as previously described (7) and are called MDA/VEGF-C and MDA/pcDNA, respectively. Cells were transduced with a retroviral vector encoding RFP/firefly luciferase fusion reporter gene as described (23). Cells were sorted for double-positive GFP/RFP expression using fluorescence-activated cell sorting (FACSvantage, Becton Dickinson).

Metastasis assay and tissue collection. Tumor cells were injected bilaterally into the second mammary fat pads of athymic, female, 8-wk-old NCR nu/nu mice (2 × 10⁶/100 μL serum-free culture medium) as described (7, 14). Three independent experiments were performed, with 10 mice in each group. Mice were sacrificed after 12 to 16 wk, and primary tumors and organs were collected. Thoracic lymph nodes and lungs were fixed in formalin, and tissues were embedded in OCT compound and frozen, or processed and embedded in paraffin as described (7, 14). Mouse experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee.

Antibodies. Mouse tissue was stained with rabbit anti-mouse/human α-smooth muscle actin (α-SMA; Abcam, 1:200), rabbit anti-mouse LYVE-1 (Upstate Cell Signaling Solutions, 1:800), rat anti-mouse CD34 (BD Pharmingen, 1:100), rabbit anti-VWF (DAKO, 1:600), goat anti-mouse VEGFR-3 (R&D Systems, 1:40), hamster anti-mouse podoplanin (Angio-Bio, 1:200), and FITC-conjugated goat anti-GFP (Abcam, 1:1,000). Human paraffin sections were stained with rabbit anti-human D2-40 (DAKO, 1:1,000) and rabbit anti-human VEGF-C (Santa Cruz, 1:100). Corresponding secondary antibodies used were labeled with AlexaFluor-488, AlexaFluor-555, AlexaFluor-594, AlexaFluor-647 (Molecular Probes, 1:300), Cy5 (Jackson Labs, 1:300), or biotin (Vector Laboratories, 1:100). Cell nuclei were counterstained with Hoechst bisbenzimide (Sigma-Aldrich, 20 μg/mL).

Immunofluorescent staining and microscopy. Cryosections (12–14 μm) of lungs and thoracic lymph nodes were stained as previously described (24), and stained tissues were examined with a Nikon Eclipse E600 fluorescence microscope equipped with a Nikon DS-Q1MC camera. Images were captured using NIS Elements software (Nikon). Tissue sections were also analyzed with a Leica SP5-DMI confocal microscope, and images were captured using LAS AF v1.8.2 software (Leica Microsystems). Thick lung sections (30–50 μm) were stained as described (25, 26). Images were captured with the confocal microscope at 0.2-μm intervals, and three-dimensional reconstruction was performed using Velocity v4.5 software (Improvision).

Evaluation and quantification of metastases. Lungs and lymph nodes were harvested, fixed in formalin, and immediately examined for metastases by evaluating GFP and RFP fluorescence with the Leica MZ16FA stereomicroscope. Presence of metastases was further confirmed by staining frozen sections with an anti-GFP antibody or by examining RFP fluorescence, and by H&E staining. For quantification, computer-assisted morphometric analysis of digital images was performed using the NIS Elements software (Nikon). Size of the individual metastatic foci was measured and expressed in square micrometers (μm²). For MDA/pcDNA group, 45 metastatic foci were measured in 12-μm sections from three lung samples from individual mice. For MDA/VEGF-C group, 64 metastatic foci were examined from lungs of seven mice. Statistical significance was determined using the unpaired Student’s t test.

Quantification of lymphangiogenesis. Lung sections were stained with antibodies to LYVE-1 and VEGF-3 to visualize lymphatics. Analysis of digital images was performed using the NIS Elements software (Nikon). For quantification, five mice were examined in each experimental group. For each lung sample, five sections were evaluated at 10× in each section, five areas containing lymphatic vessels were evaluated. Total lymphatic vessel area was calculated per lung, and data were expressed as the percentage of the lung area occupied with the vasculature. To evaluate lymphatic vessels associated with the individual metastatic foci, lymphatic area was measured within 200 μm of the foci (n = 13 in each group). Statistical significance was determined using the unpaired Student’s t test.

Immunohistochemistry. VEGF-C staining was performed by using the Avidin-Biotin Peroxidase System (Vector Laboratories), and LYVE-1 or α-SMA stainings were performed by using the catalyzed signal amplification system (CSA Ancillary System, DAKO) according to the manufacturer’s protocol.

Human tissues. Archived samples of paraffin-embedded lung tissue containing metastatic breast adenocarcinoma were obtained from the files of the Department of Pathology, Mount Sinai School of Medicine. All specimens were from patients who have undergone wedge excision biopsies. All specimens were diagnosed as lymphangitic carcinomatosis...
spread of breast adenocarcinoma by board-certified pathologists based on H&E staining and immunohistochemistry. Six anonymized samples were retrieved and analyzed. Use of human tissue samples was approved by the Institutional Review Board of Mount Sinai School of Medicine.

Results

Lung metastases expressing VEGF-C are associated with the airways. We have shown previously that VEGF-C expression by MDA-MB-435 tumor cells potently increased metastatic burden in the lungs and that the systemic treatment with anti-VEGFR-3 blocking antibodies reduced the amount of lung metastases (7, 14). MDA/VEGF-C cells express high levels of the 33-kDa form of VEGF-C, but they do not express VEGFR-3 (Supplementary Fig. S1). To gain insight into the mechanism by which VEGF-C and VEGFR-3 activation facilitates formation of distant metastases, we examined phenotypes of spontaneous lung metastases formed by MDA/pcDNA and MDA/VEGF-C cells after orthotopic injection into nude mice. Histopathologic analysis revealed a remarkably different pattern of pulmonary metastases formed by tumor cells expressing high levels of VEGF-C (Fig. 1). The vast majority of MDA/VEGF-C metastases were found in tight association with the airways, localizing along the air conducting portion of the respiratory tract (Fig. 1). Metastases were observed along the entire bronchial tree: around the main bronchi, smaller bronchi, and the terminal bronchioles (Fig. 2). Early metastases in the peribronchial area were presented as evenly contoured small foci. In the advanced stage, metastases had spread along the bronchial wall and ultimately enclosed large parts of the bronchial plexus (Fig. 2).

In contrast, metastases formed by MDA-MB-435 control cells were scattered in the lung parenchyma and were rarely associated with the bronchial tree (Figs. 1 and 2). MDA/pcDNA metastases colonized mainly the respiratory portion of the lung, i.e., the alveolar region. Metastatic lesions were presented as small nodules with irregular margins and were found dispersed throughout the alveolar region, in more peripheral lung parenchyma. Whereas MDA/VEGF-C metastases could also be observed in the lung parenchyma, the majority of the metastatic burden localized in the peribronchial area. Remarkably, MDA/VEGF-C metastatic lesions, which were seen in the lung parenchyma, were comparable in size with MDA/pcDNA lesions, whereas the MDA/VEGF-C lesions associated with the airways were 21-fold larger (Fig. 1C). Collectively, these results show that increased VEGF-C production by metastatic cells promotes peribronchial spread of metastases.

VEGF-C promotes formation of pulmonary tumor emboli. In contrast to the MDA/pcDNA metastatic nodules, which had mostly irregular margins, MDA/VEGF-C metastases frequently displayed a defined boundary (Fig. 2A, A5–A7). Combined α-SMA and CD34 immunostaining revealed that the majority of VEGF-C–expressing metastases were intravascular (Fig. 3), localizing in large pulmonary blood vessels (51 of 55 metastatic foci showed blood vessel involvement, 92%). Histopathologic examination showed that tumor emboli were present in the pulmonary arterial system, which runs in parallel with the bronchial plexus and carries deoxygenated blood. Large and small pulmonary arteries, as well as arterioles, frequently contained aggregates of tumor cells (Fig. 3A and C).

Metastases from MDA/pcDNA cells showed no association with the pulmonary arterial system (Fig. 3D, D1–D3), in agreement with our data showing that MDA/pcDNA cells were rarely found adjacent to the bronchial tree. In rare cases in which MDA/pcDNA cells were detected adjacent to the large pulmonary vessels, intravascular embolism was not observed. However, whereas MDA/pcDNA cells were not associated with pulmonary arteries, they were sometimes found in pulmonary capillaries surrounding the alveoli (Fig. 3D, D4–D5). In summary, these results show that the occlusion of pulmonary arteries with tumor cells is a common manifestation of the metastatic phenotype induced by VEGF-C.

VEGF-C promotes intralymphatic spread of metastases in the lung. In normal lung tissue, lymphatic vessel distribution is restricted to the peribronchial and perivascular interstitium, i.e., to the proximity of bronchial plexus and large blood vessels, respectively, and to the pleura. Because the majority of the VEGF-C–overexpressing metastases were found adjacent to bronchi, we investigated the relationship between MDA/VEGF-C metastases and the deep lymphatic plexus associated with the bronchial tree. Lymphatic vessels were detected by using a combination of anti–LYVE-1, anti-podoplanin, and anti-VEGFR-3 antibodies. Strikingly, the bulk of MDA/VEGF-C metastases observed in the peribronchial area was seen inside of greatly distended lymphatic vessels (31 of 55 metastatic foci showed lymphatic vessel involvement, 56%; Fig. 4). Lymphatic vessels in the proximity of pulmonary veins were also massively infiltrated with tumor cells (Fig. 4C, C3–C4). Furthermore, MDA/VEGF-C metastases were frequently detected in the pleura (MDA/VEGF-C in five of seven mice; MDA/pcDNA in one of six mice), which is another area of lung tissue rich in lymphatics. In sharp contrast, MDA/pcDNA metastases were rarely seen as intravascular or even adjacent to the lymphatics (Supplementary Fig. S2). These data indicate that VEGF-C facilitates intralymphatic spread of metastases in the lung.

VEGF-C induces dilation of pulmonary lymphatics and metastatic lymphangiogenesis. Lymphatic vessels in lungs infiltrated with MDA/pcDNA tumor cells were detected in their normal anatomic location, i.e., surrounding bronchi, large pulmonary vessels, and in the pleura. There was no lymphangiogenesis associated with MDA/pcDNA nodules (Supplementary Figs. S2 and S3). In fact, only seldom were lymphatics seen in the vicinity of metastases (number of metastatic foci with lymphatics present within 200 μm: MDA/pcDNA 6 of 48, 13% versus MDA/VEGF-C 37 of 39, 95%), and these were not altered in their appearance compared with normal lungs (Supplementary Fig. S2). In contrast, VEGF-C–overexpressing metastatic lesions were characterized by pronounced lymphangiogenesis (Fig. 5; Supplementary Fig. S3). Lymphatic vessel area associated with MDA/VEGF-C metastatic foci was on average 75-fold greater than...
the lymphatic vessel area associated with MDA/pcDNA foci, which had lymphatics in proximity (Fig. 5B). An expansion of the lymphatic network paralleled an increase in the size of metastases. Notably, the original anatomic location of lymphatic vessels was preserved and lymphangiogenesis was not induced de novo in lung areas that are naturally devoid of lymphatics, such as in lung parenchyma. In addition, lymphatic vessels were greatly dilated throughout the lungs and not only at the metastatic site. In summary, these data show that VEGF-C can drastically alter the architecture of pulmonary lymphatic vasculature by inducing lymphangiogenesis and lymphatic vessel enlargement.

Intravascular localization is a characteristic feature of metastases expressing VEGF-C. MDA/VEGF-C metastases in the peribronchial region were without exception intravascular, and 100% of the metastatic foci showed either lymphatic or blood vessel involvement (Supplementary Table S1). In fact, the majority of metastases were confined to the intravascular space. Invasion of the adjacent peribronchial interstitium was associated primarily with large metastases, and metastases invading

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**Figure 1.** Metastases expressing VEGF-C are associated with the airways. Fluorescence stereomicroscopy of thick lung sections (150 μm) showing distribution of MDA/pcDNA (A1, A2) and MDA/VEGF-C (B1, B2) metastases (RFP, red) in the lung. Confocal analysis of MDA/pcDNA (A3) and MDA/VEGF-C (B3) metastases. Note the tight association of MDA/VEGF-C metastases with the airways (green, autofluorescence). C, comparison of the size of metastatic foci. b, bronchi; arrows, metastases. Scale bars, 500 μm (A1, A2, B1, B2) and 150 μm (A3, B3). ***, P < 0.001.
the peribronchial interstitium without involvement of the vasculature were not observed. In contrast, MDA/pcDNA metastases were rarely found in the peribronchial region and, when detected, were neither seen in the form of intralymphatic nor arterial emboli. Data on the distribution of MDA/VEGF-C metastases in the peribronchial space in relationship to the vasculature are summarized in Supplementary Table S1.

**VEGF-C increases thoracic lymph node metastases.** To investigate whether VEGF-C promotes secondary metastatic dissemination to the thoracic nodes, we analyzed lung-draining lymph nodes for the presence of metastases. Mediastinal and hilar lymph nodes from tumor-bearing mice were evaluated by fluorescence stereomicroscopy to detect GFP-labeled tumor cells. The incidence of lymph nodes positive for metastases from mice bearing VEGF-C–expressing tumors was significantly higher (4-fold) than the incidence in mice bearing control tumors (Supplementary Fig. S4). Histopathologic analyses showed that metastases localized mostly in the subcapsular sinus and, at later stages, infiltrated deeper into the lymph node. These data show that VEGF-C promotes secondary spread of lung metastases to the thoracic lymph nodes.

**Metastatic phenotype induced by VEGF-C resembles lymphangitic carcinomatosis, an aggressive form of lung metastasis.** The data shown above have shown that VEGF-C induced a distinct pattern of pulmonary metastasis characterized by peribronchial localization, intralymphatic spread, lymphangiogenesis, and arterial tumor emboli (summarized in Supplementary Table S2). Remarkably, this pattern of malignant disease is strikingly similar to the lymphangitic carcinomatosis metastatic phenotype described in human cancer patients. Pulmonary lymphangitic carcinomatosis is characterized by lymphatic spread throughout the lung, resulting in a diffuse presentation of metastases, and conveys an extremely poor prognosis (1, 27, 28). The similarities between the clinical picture of lymphangitic carcinomatosis and pulmonary metastatic disease induced by VEGF-C are shown in Supplementary Table S2. Based on these findings, we asked...
Figure 3. Pulmonary tumor emboli are characteristic of MDA/VEGF-C metastases. A, MDA/VEGF-C tumor cells present in pulmonary arteries (arrows) associated with the airways, stained with α-SMA. B, MDA/pcDNA metastases are not found in pulmonary arteries (arrows; B1–B2) nor in veins (v; B3). C, confocal images of MDA/VEGF-C tumor emboli in the pulmonary arteries. C1, metastases labeled with RFP (red). C2, immunofluorescent staining for α-SMA (red) showing tumor embolus (GFP, green) in a small artery (arrow) adjacent to the bronchus (b). C3, staining for blood-endothelial marker CD34 (purple) showing small artery (arrow) with tumor embolus and pulmonary capillaries. C4, large metastatic lesion (GFP, green) involving pulmonary arteries (α-SMA, red, arrows) and the surrounding peribronchial interstitium. D, MDA/pcDNA metastases (GFP, green) are generally distant from the large vasculature (D1). Large pulmonary vessels (arrow) do not contain MDA/pcDNA tumor emboli (D2–D3). Red, α-SMA; purple, CD34. D4–D5, tumor cells (green, arrowheads) localized within pulmonary capillaries (vWF, red). Blue, Hoechst nuclear stain; b, bronchi; t, tumor cells. Data are representative of at least three experiments. Scale bars, 200 μm (A–B), 150 μm (C1), 50 μm (C2–C4, D1–D3), and 25 μm (D4–D5).
whether pulmonary breast cancer metastases classified as lymphangitic carcinomatosis express VEGF-C. Immunostaining of human lung tissue from patients with metastatic disease showed high levels of VEGF-C expression in metastases of breast adenocarcinoma (Fig. 6). Notably, intralymphatic metastases were strongly positive for VEGF-C in all samples examined (n = 6), regardless of the VEGF-C expression levels in the rest of the metastatic lesion, which localized in the lung parenchyma (Fig. 6). Together, these data show that VEGF-C induces an aggressive pattern of pulmonary metastasis designated as lymphangitic carcinomatosis.

**Discussion**

We report here that VEGF-C induced an aggressive pattern of lung metastases in a mouse model of breast cancer, which closely resembles lymphangitic carcinomatosis metastatic phenotype in human cancer patients. A hallmark of this disease is a diffuse pattern of metastases, reflecting involvement of lung lymphatics with cancer. Tumor emboli are also frequently detected in pulmonary arteries. Prognosis for the patient with this clinical picture is extremely poor, and 50% of the patients die within 3 months of diagnosis (1, 27–29). Malignant cancers most commonly presenting as pulmonary lymphangitic carcinomatosis are breast, lung, gastric, pancreatic, and prostate cancer.

The finding that VEGF-C facilitates progression of metastatic disease by promoting expansion of metastases within the lung and secondary spread to the thoracic nodes indicates that VEGF-C exerts its prometastatic effects directly in the target organ to which the tumor has spread. We and others have shown previously that VEGF-C enhanced tumor spread to the regional lymph nodes (7–9, 12, 13) and expression of VEGF-C in human tumors has been associated with...
Figure 5. Evolution of lymphangiogenesis associated with expansion of metastases. A, immunofluorescent staining for VEGFR-3 (red) showing lymphatic vessels in relation to the MDA/VEGF-C metastases. A1-A3, small metastatic nodule (t, green) next to the lymphatic vessel. Note that during early stages of metastases lymphatics are not changed in number or appearance. A4-A6, dilated lymphatics (arrows) surround larger metastatic lesions and new lymphatics line the edge of metastases. A7-A9, large metastatic lesion infiltrated with lymphatics; many lymphatics contain tumor cells. A10-A12, drastic expansion of the lymphatic network and extreme dilation of lymphatics are associated with very large metastases. Note that metastases and the associated lymphatics always localize in the proximity of the airways. Green, GFP-labeled MDA/VEGF-C metastases; blue, Hoechst nuclear stain; red, VEGFR-3; t, tumor cells; b, bronchi; arrows, dilated lymphatic vessels. Scale bar, 100 μm. B, quantification of the total lymphatic vessel area in the lungs bearing metastases (left). Quantification of the lymphatic vessel area per metastatic foci (right). **, P < 0.01.
increased incidence of lymph node metastases and poor prognosis (3). Studies in mouse models of breast cancer, prostate cancer, and melanoma (7, 9, 11, 14, 15) showed that VEGF-C also increased distant metastasis, and this has been attributed to the increased rate of dissemination from the primary tumor to the regional lymph nodes and beyond. Our data show that, in addition to increasing a risk of distant metastases by promoting tumor lymphangiogenesis and spread to the lymph nodes, VEGF-C accelerates growth and secondary spread of cancer cells already disseminated to the lung. To date, targeting of lymphatic vessels has been viewed as an approach to inhibit lymphatic spread from the primary tumor to the regional lymph nodes and consequently prevent distant metastasis. In a clinical setting, however, a major
chance is treatment of metastatic disease after the primary tumor has been surgically removed. The data presented herein show that VEGF-C promotes late steps of the metastatic process and identify lymphatic vessels and the VEGF-C/VEGFR-3 pathway as the targets for treatment of established metastatic disease.

VEGF-C-mediated increase of metastatic burden in the lung was due to the selective increase in the size of metastases surrounding bronchi. Specifically, MDA/VEGF-C metastases were typically seen inside of the peribronchial lymphatic vessels, suggesting that it is the intralymphatic niche that facilitates tumor growth and/or survival. Factors produced by lymphatic endothelium and lymph fluid itself may have growth-promoting activity, but the exact mechanisms of preferential intralymphatic spread and growth of VEGF-C-expressing lung metastases remain to be elucidated. Importantly, whereas VEGF-C expression resulted in drastic expansion of peribronchial metastases, the size of the metastatic foci in the lung parenchyma was not influenced by VEGF-C. This could be explained by the fact that alveolar spaces are normally devoid of lymphatics, and as such, this environment is not receptive for signals inducing lymphangiogenesis. A recent study suggested that in fibrotic lung disease, idiopathic pulmonary fibrosis, lymphatic vessels could be formed de novo in the lung parenchyma from CD11b+ alveolar macrophages (30). Our data show that, in the lungs with metastases, new lymphatic vessels were formed only in their original anatomic locations, i.e., in the peribronchial and perivascular interstitium, indicating that the proximity of metastatic foci to the preexisting lymphatic network is a prerequisite for the induction of lymphangiogenesis. These findings underscore the critical importance of the lung microenvironment (i.e., "soil") for metastatic growth.

How does VEGF-C facilitate metastatic expansion in the lungs? By inducing lymphangiogenesis tumor cells can create pathways of least resistance to spread, which allow for rapid lung colonization without a necessity to interface directly with the lung environment. This model is supported by our data, which showed that the main bulk of the VEGF-C-expressing lung metastases in a mouse model was intravascular and that the invasion of the adjacent peribronchial interstitium was observed only when massive metastatic lesions were present in the lymphatics. Intralymphatic localization, in turn, may protect the organ from immediate destruction. Because the bulk of the tumor does not invade into the alveolar region, lung function remains preserved until the disease is in a very advanced stage. Accordingly, lung architecture remained normal in mice bearing MDA/VEGF-C metastases, as it does in patients with pulmonary lymphangitic carcinomatosis, who typically do not experience symptoms until very late in the disease progression. Dry cough and shortness of breath are characteristic symptoms caused by the tumor, which exerts pressure on the airways and narrows air passages, whereas the alveolar region of the lung may be largely unaffected.

Occlusion of pulmonary arteries and arterioles by aggregates of tumor cells was another common manifestation of the VEGF-C metastatic phenotype. In human metastatic disease, coinvolvement of lymphatic vessels and pulmonary arteries with metastatic cancer has been frequently observed. Autopsy studies revealed the arterial involvement in 87% of the cases with lymphatic spread in the lung (31) and the lymphatic involvement in 86% of the cases when pulmonary arteries contained tumor emboli (32). This clinical picture is important as pulmonary artery tumor embolism could lead to the complications ultimately contributing to a patient’s death (33). The mechanism by which VEGF-C promotes the formation of pulmonary tumor emboli remains to be elucidated. Tumor cells could induce coagulation that may facilitate binding of tumor cells to the endothelium or exploit other specific adhesion mechanisms. The affinity of VEGF-C-expressing tumor cells for pulmonary arteries could be a reason for preferential seeding of tumor cells in the peribronchial region. Conventional wisdom implies that tumor cells arrest in pulmonary capillaries, from which they extravasate and subsequently form metastatic nodules (34). Based on our data showing that VEGF-C–expressing metastases disseminated predominantly through the peribronchial lymphatic vessels and pulmonary arterial system, we propose an alternative model for arrival and spread of metastases in the lung. Metastatic tumor cells arriving to the lung from the lymph nodes through the pulmonary arteries could arrest in the pulmonary arterial system before reaching the alveolar capillaries. Because VEGF-C has been shown to significantly increase lymph node metastases, it is conceivable that the occlusion of pulmonary arterial system with tumor cells reflects in part massive arrival of tumor cells into the lung. From the pulmonary arteries, tumor cells could spread directly into the adjacent lymphatics and eventually infiltrate the peribronchial interstitium. Tumor cell dissemination is further facilitated by lymphangiogenesis induced by VEGF-C. This model of preferential seeding of VEGF-C–expressing tumor cells in the pulmonary arterial system and enhanced growth in pulmonary lymphatics may explain the affinity of metastases for the peribronchial area.

These data also imply that tumor cell extravasation from the blood vasculature may not be a prerequisite for metastasis. The prevailing view seems to favor the notion that tumor cells survive and colonize more efficiently once they have extravasated the pulmonary capillaries (34, 35). Some studies, however, challenged this concept and suggested that metastasis may grow intravascularly (36, 37). Our data show that lung colonization can occur by massive dissemination of tumor cells through the pulmonary arteries and lymphatics, in support of the concept that extravasation into the lung parenchyma may not be obligatory step for the initiation of metastatic growth and organ colonization.

In conclusion, our data show that VEGF-C directly facilitates metastatic expansion in the lung, indicating that the VEGF-C/VEGFR-3 pathway and lymphatic vessels could be promising therapeutic targets for treatment of established metastatic disease.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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