Inhibition of VEGFR-3 Activation with the Antagonistic Antibody More Potently Suppresses Lymph Node and Distant Metastases than Inactivation of VEGFR-2

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Abstract

Lymph nodes are the first site of metastases for most types of cancer, and lymph node status is a key indicator of patient prognosis. Induction of tumor lymphangiogenesis by vascular endothelial growth factor-C (VEGF-C) has been shown to play an important role in promoting tumor metastases to lymph nodes. Here, we employed receptor-specific antagonist antibodies in an orthotopic spontaneous breast cancer metastasis model to provide direct evidence for the key role of VEGFR-3 activation in metastasis. Inhibition of VEGFR-3 activation more potently suppressed regional and distant metastases than inactivation of VEGFR-2, although VEGFR-2 blockade was more effective in inhibiting angiogenesis and tumor growth. Despite prominent proliferation, metastases were not vascularized in any of the control and treatment groups, indicating that the growth of metastases was not dependent on angiogenesis at the secondary site for the duration of the experiment. Systemic treatment with either VEGFR-2 or VEGFR-3 antagonistic antibodies suppressed tumor lymphangiogenesis, indicating that VEGFR-3 signaling affects the rate of tumor cell entry into lymphatic vessels through both lymphangiogenesis-dependent and independent mechanisms. Combination treatment with the anti-VEGFR-2 and anti-VEGFR-3 antibodies more potently decreased lymph node and lung metastases than each antibody alone. These results validate the concept of targeting the lymphatic dissemination and thereby very early steps of the metastatic process for metastasis control and suggest that a combination therapy with antiangiogenic agents may be a particularly promising approach for controlling metastases. (Cancer Res 2006; 66(5): 2650-7)

Introduction

Metastasis is the main cause of treatment failure and death for cancer patients. For most types of cancer, the first site of metastasis are lymph nodes, and the extent of lymph node involvement is a major criterion for evaluating patient prognosis and the choice of therapy (1, 2). The number of positive axillary lymph nodes, their location, and the size of lymph node metastases are evaluated to assess the stage of the disease progression. To form metastatic lesions at distant sites, cancer cells need to escape immune surveillance, survive in the blood circulation, arrest in the vasculature of the target organ, and grow at the secondary site (3). Although each step of the process represents a potential target for the antimetastatic therapy, metastatic growth is currently considered the most promising stage of the metastatic process for therapeutic targeting (4).

Angiogenesis is essential for the growth of most primary tumors and their metastases, and antiangiogenic therapy has been effective in suppressing tumorigenicity and metastases in experimental models of cancer. Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 play a central role in tumor angiogenesis, and agents that block VEGF signaling pathways have shown promising results in clinical trials (5). Recent studies showing the ability of tumors to induce lymphangiogenesis have identified also lymphatic vessels as a potential target for the antimetastatic therapy (6). Lymphatic vessel invasion by tumor cells, increased numbers of tumor-associated lymphatics and enlarged lymphatic vessels have been frequently correlated with lymph node metastases and poor patient prognosis (2, 7). Notably, a large number of studies showed a correlation between the expression of the lymphangiogenic factor VEGF-C in human tumors and lymph node metastases (2). VEGF-C plays a key role in lymphangiogenesis by activating the VEGFR-3 receptor tyrosine kinase on lymphatic endothelial cells (1, 8–10). Mature form of VEGF-C also binds and activates VEGFR-2 (11), but whether VEGFR-2 plays a direct role in lymphangiogenesis is less clear.

Several studies in animal tumor models have provided direct evidence for the causal role of VEGF-C in tumor lymphangiogenesis and metastasis. VEGF-C has been shown to induce tumor lymphangiogenesis and facilitate tumor spread to the regional lymph nodes in mouse models of breast (12–14), pancreatic (15), gastric (16), and colorectal cancer (17). Our previous studies in a mouse xenograft model of breast cancer showed increase of not only lymph node but also distant metastases upon VEGF-C overexpression (12). Here, we evaluated the VEGF-C/VEGFR-3 signaling pathway as a target for the antimetastatic therapy and have compared the effectiveness of antiangiogenesis versus anti-lymphangiogenesis approach for control of tumor metastases. Our results show that inhibition of VEGFR-3 signaling with the function-blocking antibody mF4-31C1 was more effective in suppressing metastases than inactivation of VEGFR-2, and that the combination therapy was more potent in suppressing metastases than single-antibody treatments. These results show a key role for VEGFR-3 activation in metastasis and validate the concept of targeting the lymphatic endothelium and lymphatic spread for metastasis control.
Materials and Methods

**Spontaneous metastasis assay.** MDA-MB-435/GFP cells (18) transfected with the human VEGF-C cDNA (c13) or pcDNA/control vector (c12) have been established and cultured as previously described (12) and will be referred to as MDA/VEGF-C and MDA/pcDNA, respectively. Cells were injected bilaterally into the second mammary fat pads of athymic, female, 8-week-old NCR nu/nu mice (1 × 10^6/100 μL serum-free culture medium). Tumor growth was monitored weekly. Upon sacrifice, mice were perfused through the heart with PBS, and lungs, liver, left and right axillary, and brachial lymph nodes were harvested and processed for evaluation of metastases by flow cytometry as described below. For analysis of green fluorescent protein (GFP)--labeled metastases in tissue sections, tissues were processed as described previously (12), embedded in OCT and frozen. Tumors were embedded in OCT and frozen for immunostaining or snap-frozen in liquid nitrogen for protein extractions.

**Tail vein metastasis assay.** MDA/pcDNA or MDA/VEGF-C cells were injected i.v. into the lateral tail vein of 8-week-old NCR female, athymic nu/nu mice (1 × 10^6/100 μL HBSS). Mice were sacrificed after 10 weeks, and lymph nodes, lungs, and liver were collected and processed for evaluation of metastases.

**Blocking antibodies.** Neutralizing rat monoclonal antibodies specific for mouse VEGFR-3 (mF4-31C1; ref. 19) and mouse VEGF-2 (DC101; refs. 20, 21) were generated by ImClone Systems Inc. (New York, NY). The mF4-31C1 antibody has been shown to block VEGF-C--induced phosphorylation of mouse VEGFR-3 and VEGF-C--induced cell proliferation *in vitro* and to inhibit physiologic regeneration of lymphatic vessels *in vivo* (19).

**Treatment modalities.** Animals were randomly assigned to one of the four treatment groups (mF4-31C1, DC101, mF4-31C1+DC101, and control), 10 mice per group. In the prevention regimen, treatment was initiated at the time of orthotopic tumor cell injection. In the intervention regimen, treatment commenced when tumors and metastases were established, 4 weeks after the orthotopic tumor cell inoculation. Blocking antibodies were administered i.p. at 800 μg/mouse every second day, over the 6-week period. For the combination treatment, both antibodies were administered concurrently at 800 μg/mouse.

**Quantification of metastases by flow cytometry.** Tissue dissociation, sample preparation, and fluorescence-activated cell sorting (FACS) analysis were done essentially as described (22), with some modifications. Upon sacrifice, organs were collected and dissociated individually by mechanical disruption and incubation with 400 units/ml collagenase D (Roche, Indianapolis, IN) for 30 minutes at 37°C. Collagenase activity was stopped with 10 mmol/L EDTA, and samples were passed through a 70-μm cell strainer (BD Biosciences, Bedford, MA). Cell suspensions were centrifuged and incubated in ice-cold 0.17 mol/L NH₄Cl for 10 minutes to lyse RBC. Finally, cells were washed with HBSS, stained with 3 μg/ml propidium iodide, and re suspended in FACS buffer (1% fetal bovine serum/PBS).

Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Entire samples of each lymph node were analyzed. Total number of cells in lymph nodes typically ranged from 5 × 10⁴ to 2 × 10⁵. At least 2 × 10⁸ cells were analyzed in each lung or liver sample. Background fluorescence was determined by using tissue from mice that were not implanted with tumor cells. Nonviable cells were excluded based on propidium iodide staining. Data acquisition and analysis were done with Cell Quest software (BD Biosciences). Tumor burden in organs was calculated as the percentage of fluorescent tumor cells detected in the total population of viable cells in the sample. As few as 1 fluorescent tumor cell in 1 × 10⁸ host cells (0.001% total cells) could be accurately detected. Taking into consideration the minimal number of cells in lymph nodes and the sensitivity of detection, <1 fluorescent cell in 1 × 10⁸ host cells (<0.001%) was considered negative.

**Immunofluorescent staining.** Cryosections (~7 μm) of tumors, lymph nodes, and lungs were stained as previously described (23), using antibodies against mouse CD31 and CD34 (1:50; BD Biosciences Pharmingen, San Diego, CA), LYVE-1 (1:800; United Biomedical, Inc., Hauppauge, NY), VEGFR-3 (1:40; R&D Systems, Minneapolis, MN), VEGFR-2 (1:100; R&D Systems), Ki67 (1:1,000; Novocastra, Newcastle, United Kingdom), and corresponding secondary antibodies labeled with Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR). Cell nuclei were counterstained with 10 μg/ml Hoechst bisbenzimide (Sigma-Aldrich, St. Louis, MO). Specimens were examined with a Nikon E-600 microscope (Nikon, Melville, NY), and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

**Quantification of tumor vasculature.** Tumor sections were double-stained with antibodies to LYVE-1 and CD31, to visualize lymphatic (LYVE-1) and blood vessels (LYVE-1-/CD31+). Computer-assisted morphometric analysis of digital images was done using the IPLab software (Scanalytics, Fairfax, VA). Five tumors were examined in each experimental group, and five areas of each tumor were evaluated at ×20 magnification. Lymphatic and blood vasculatures were quantified in the tumor areas with the highest density of respective vessels ("hotspots"). Total vessel area was calculated per tumor, and data were expressed as the average vessel area of five tumors, or as the percentage of the tumor area occupied with the vasculature. The average vessel size was also determined for each tumor.

**Statistical analyses.** The data were analyzed using a mixed model ANOVA, to take into account correlation structure in the data (four lymph nodes in each mouse). Because the outcome variables of percent GFP+ cells and the area of the vasculature both exhibited right-skewed distributions, these variables were log-transformed before analysis. The least-squares means for each treatment group were back-transformed to the original scale to provide the geometric means. The Ps for the differences between the means of treatment group versus control were calculated using Dunnett's adjustment for multiple comparisons of treatment means versus a single control. The Tukey-Kramer--adjusted Ps were used for the comparisons between the different treatments. All statistical analyses were done using SAS statistics software (SAS Institute, Inc., Cary, NC).

**Results**

**Tumor metastasis model.** We have previously shown that VEGF-C overexpressed in MDA-MB-435/GFP human breast cancer cells promotes tumor metastasis to lymph nodes and to lungs 12 weeks after the orthotopic injection of tumor cells (Table 1; Fig. 1; ref. 12). To determine the onset of metastasis in this tumor model, we have analyzed the kinetics of tumor dissemination by a highly sensitive flow cytometry--based assay for detection of GFP-labeled cells in tissues. Flow cytometric analysis showed that the metastasis of MDA/VEGF-C cells to regional lymph nodes is an early event, with tumor cells detectable in the lymph nodes of most animals after 1 week (8 of 10), and in all animals within the 3 weeks (10 of 10) after the orthotopic injection. In contrast, MDA/pcDNA cells had disseminated to lymph nodes in only 50% of the mice (5 of 10) by 12 weeks, indicating that VEGF-C significantly accelerates the metastatic process. Earlier onset of metastasis was independent of tumor size, as both tumor cell lines showed comparable growth rates *in vivo*, reaching an average tumor volume of ~35 mm³ within 3 weeks after tumor cell injection.

Lung metastases were also formed early, with tumor cells detectable in the lungs of 80% of the mice 1 week after the orthotopic injection. By 3 weeks, lung metastases were established in 100% of the mice. To determine at which step in the metastatic process is VEGF-C/VEGFR-3 signaling important for promoting dissemination to distant sites, we compared the ability of MDA/pcDNA and MDA/VEGF-C cells to form colonies in the target organs after i.v. injection. Both cell lines metastasized to the lungs and liver at the same frequency 10 weeks after the inoculation (Table 2). Interestingly, tumor cells also colonized lymph nodes after the i.v. injection; however, no difference was observed between the control and VEGF-C--overexpressing cells (Table 2). These results show that, whereas VEGF-C clearly promotes dissemination from the primary tumor, once tumor cells are
present in the bloodstream, VEGF-C does not provide an advantage for the formation of metastases in distant organs.

**Systemic treatment with antagonist antibody to VEGFR-3 potently inhibits lymph node metastases.** To evaluate the role of VEGFR-3 in tumor dissemination, we examined the effects of systemic inhibition of VEGFR-3 activation on metastasis of MDA/VEGF-C cells in two different treatment regimens. In the prevention regimen, blocking antibody mF4-31C1 that is specific for mouse VEGFR-3 was administered concurrently with the tumor cell injection. In the intervention regimen, the antibody treatment commenced 6 weeks after the tumor cell injection, when metastases were established. Systemic treatment with the mF4-31C1 antibody significantly reduced the incidence of lymph node metastases in the prevention regimen (Table 1). In fact, the effect of VEGF-C on promoting tumor metastases was completely abolished with the anti-VEGFR-3 antibody treatment, as the incidence of lymph node metastases in mF4-31C1–treated mice bearing MDA/VEGF-C tumors was comparable with that of control mice bearing tumors devoid of VEGF-C expression.

**Table 1.** Effects of treatments with mF4-31C1 and DC101 antibodies on the incidence of lymph node metastases

<table>
<thead>
<tr>
<th>Prevention</th>
<th>Intervention</th>
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<tbody>
<tr>
<td>pcDNA, PBS</td>
<td>VEGF-C, 31C1</td>
</tr>
<tr>
<td>No. mice with positive LN</td>
<td>5/10</td>
</tr>
<tr>
<td>No. positive LN</td>
<td>5/39</td>
</tr>
<tr>
<td>% Positive LN</td>
<td>13%</td>
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</table>

NOTE: Tumor cells were injected orthotopically, and blocking antibodies were given systemically at 800 µg/mL, every 2 days. Antibodies were administered over the 6-week period commencing at the time of tumor cell inoculation (prevention regimen), or 4 weeks after the tumor cell injection (intervention regimen). Abbreviation: LN, lymph node.

We next examined the effect of VEGF-C on promoting tumor metastases. To evaluate the role of VEGFR-2 in tumor dissemination, we examined the effects of systemic inhibition of VEGFR-2 activation on metastasis of MDA/VEGF-C cells in two different treatment regimens. In the prevention regimen, blocking antibody DC101 decreased overall tumor burden in the lymph nodes by 74% as compared with the control-treated mice (% GFP+ tumor cells: 0.0092 ± 0.0344 versus 0.035 ± 0.0836, respectively; P < 0.05; Fig. 1A). The low average number of tumor cells reflects mainly the lesser number of lymph nodes containing tumor cells in anti-VEGFR-3–treated mice. The number of tumor cells in the lymph nodes that contained the metastatic cells, however, was comparable between the control and anti-VEGFR-3–treated mice. The number of tumor cells in the lymph nodes that contained the metastatic cells, however, was comparable between the control and anti-VEGFR-3–treated mice. The number of lymph nodes with metastases was comparable between the mF4-31C1–treated and control group, but the number of lymph nodes with metastases in each mouse was lower in the anti-VEGFR-3–treated group (Table 1).

In the intervention treatment regimen, mF4-31C1 antibody lowered tumor burden in the lymph nodes by 47% (Fig. 1B). The number of mice with lymph node metastases was comparable between the mF4-31C1–treated and control group, but the number of lymph nodes with metastases in each mouse was lower in the anti-VEGFR-3–treated group (Table 1).

**Systemic treatment with the anti-VEGFR-3 blocking antibody inhibits lung metastases.** Blocking antibody to VEGFR-3 drastically reduced tumor burden in the lungs in the prevention and less prominently in the intervention treatment regimen (Fig. 1C and D). In the prevention regimen, blocking VEGFR-3 decreased tumor burden in the lungs by 87% compared with the control (% GFP+ tumor cells: 0.016 ± 0.01 versus 0.119 ± 0.12, respectively; P < 0.05). The effect of VEGF-C on promoting lung metastases was abolished with the anti-VEGFR-3 antibody treatment. In the intervention regimen, the inhibition was much less pronounced (% GFP+ tumor cells mF4-31C1 treated 0.05 ± 0.07 versus control 0.07 ± 0.1; P > 0.05).

**Blocking VEGF-2 is less effective in halting metastases than blocking VEGFR-3.** Inhibition of VEGF-2 signaling had only minor effect on the incidence of metastases to lymph nodes in the prevention regimen and no effect in the intervention treatment regimen (Table 1). However, anti-VEGFR-2 blocking antibody DC101 decreased overall tumor burden in the lymph nodes by 64% in the prevention regimen (control, 0.035 ± 0.0836 versus DC101, 0.0128 ± 0.019; P > 0.05), although the effect was not statistically significant. In the intervention regimen, blocking VEGF-2 also did not show a significant effect (control, 0.833 ± 1.51 versus DC101, 0.633 ± 0.88; P > 0.05; Fig. 1A and B). Because the number of lymph nodes with metastases was comparable between the control and anti-VEGFR-2–treated mice, reduced

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**Table 1.** Effects of treatments with mF4-31C1 and DC101 antibodies on the incidence of lymph node metastases

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PC</th>
<th>VEGF-C</th>
<th>VEGF-C</th>
<th>VEGF-C</th>
<th>VEGF-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA, PBS</td>
<td>5/10</td>
<td>10/10</td>
<td>3/10</td>
<td>8/10</td>
<td>8/10</td>
</tr>
<tr>
<td>Positive LN</td>
<td>5/39</td>
<td>24/40</td>
<td>6/39</td>
<td>21/40</td>
<td>32/38</td>
</tr>
<tr>
<td>% Positive LN</td>
<td>13%</td>
<td>60%</td>
<td>15%</td>
<td>53%</td>
<td>83%</td>
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</table>

*NOTE:* Tumor cells were injected orthotopically, and blocking antibodies were given systemically at 800 µg/mL, every 2 days. Antibodies were administered over the 6-week period commencing at the time of tumor cell inoculation (prevention regimen), or 4 weeks after the tumor cell injection (intervention regimen).

**Abbreviation:** LN, lymph node.
Relative potency of blocking VEGF-R2 or VEGF-R3 on tumor angiogenesis and growth. Anti-VEGFR-3 antibody reduced blood vessel densities and decreased the size of tumor blood vessels. Quantitative analysis showed a 69% reduction of the tumor area occupied with the blood vessels (%tumor area: control, 4.7 ± 1.37% versus mF4-3IC1, 1.45 ± 0.48%; P < 0.0001). Likewise, DC101 antibody decreased the total blood vessel area in tumors by 71% (control, 4.7 ± 1.37% versus DC101, 1.36 ± 0.39%; P < 0.0001). However, whereas in the DC101-treated group large tumor areas were completely avascular and the remaining vessels seemed large and mature, in the mF4-3IC1–treated group, small blood vessels were scattered throughout the tumor (Fig. 5A-D). The average blood vessel size in the mF4-3IC1–treated group was significantly smaller than in the DC101-treated group (DC101, 8.631 ± 2.170 μm² versus mF4-3IC1, 2.846 ± 930 μm²; P < 0.001). Results were comparable in both treatment regimens. As determined by

tumor burden in lymph nodes of DC101-treated mice reflects primarily the lower number of tumor cells in lymph nodes with metastases. Indeed, the average number of metastatic cells in lymph nodes was decreased by 59% with the DC101 treatment (control, 0.058 ± 0.1 versus DC101, 0.024 ± 0.02; P < 0.05).

Blocking VEGFR-2 also proved less effective in reducing lung metastases. In the prevention regimen, DC101 antibody decreased lung tumor burden, by 42% (%GFP+ tumor cells: control, 0.119 ± 0.12 versus DC101, 0.069 ± 0.082; P > 0.05) compared with 87% decrease of lung tumor burden with the mF4-3IC1 antibody (Fig. 1C). In the intervention regimen, the extent of lung metastases was comparable between control and DC101-treated groups (%GFP+ tumor cells: 0.07 ± 0.102 versus 0.1 ± 0.084, respectively; P > 0.05; Fig. 1D).

Blocking of either VEGFR-3 or VEGFR-2 inhibits tumor lymphangiogenesis. Our previous studies showed that overexpression of VEGF-C in MDA-MB-435/GFP cells resulted in increased intratumoral lymphangiogenesis and enlargement of peritumoral lymphatic vessels (12). Thus, we investigated whether the different effects of VEGFR-3 and VEGFR-2 blocking on tumor metastasis can be attributed to distinct roles of these two receptors in tumor lymphangiogenesis. Lymphatic vessels were visualized by immunostaining with the antibody for LYVE-1, a specific marker of lymphatic endothelium (24, 25). Blocking VEGFR-3 activation resulted in the complete normalization of peritumoral lymphatic vasculature (Fig. 2). In contrast to the hyperplastic lymphatic vessels observed around the tumors of control animals, lymphatic vessels surrounding tumors of mice treated with mF4-3IC1 were small, compressed, and indistinguishable from the lymphatic vessels in normal skin. Moreover, anti-VEGFR-3–treated mice displayed complete inhibition of intratumoral lymphangiogenesis compared with control-treated mice (Fig. 3). Intratumoral lymphatic vessel densities were decreased by 95% in the prevention and in the intervention treatment regimen (P < 0.001). Notably, blocking VEGFR-2 also resulted in the normalization of peritumoral lymphatic vasculature as well as in the significant reduction of intratumoral lymphangiogenesis (Fig. 2). DC101 antibody decreased intratumoral lymphatic vessel densities by 81% (P < 0.001) in both treatment regimens (Fig. 3).

As determined by immunostaining, VEGFR-2 was strongly expressed by peritumoral lymphatic vessels (Fig. 4). However, in contrast to VEGFR-3 that was present on all peritumoral and intratumoral lymphatics, VEGFR-2 was weakly expressed or absent from the intratumoral lymphatic vessels (Fig. 4).

Table 2. VEGF-C has no effect on organ metastases in experimental metastasis model

<table>
<thead>
<tr>
<th></th>
<th>Experimental metastases (i.v.)</th>
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<tbody>
<tr>
<td></td>
<td>Lymph node</td>
<td>Lung</td>
<td>Liver</td>
</tr>
<tr>
<td>MDA/pcDNA</td>
<td>0.9 ± 0.5</td>
<td>6.7 ± 2.3</td>
<td>6.7 ± 3.1</td>
</tr>
<tr>
<td>MDA/VEGF-C</td>
<td>0.8 ± 0.6</td>
<td>5.2 ± 1.6</td>
<td>6.4 ± 2.3</td>
</tr>
</tbody>
</table>

NOTE: MDA/pcDNA or MDA/VEGF-C cells were injected i.v., and metastases were quantified after 10 weeks. Tumor burden is expressed as the percentage of total cells (mean ± SD) in lymph nodes (n = 28), lungs (n = 7), or liver (n = 7).

Figure 2. Blocking VEGFR-3 or VEGFR-2 inhibits tumor lymphangiogenesis. Immunofluorescent staining for LYVE-1 (green, arrows) revealed small, mostly collapsed lymphatic vessels in the skin overlaying control-transfected tumors (A) and a few intratumoral lymphatic vessels (B). The skin adjacent to the MDA/VEGF-C tumors was characterized by highly enlarged lymphatics (C), and the tumors were extensively infiltrated throughout with the lymphatic vessels (D). In contrast, lymphatic vessels at the periphery of MDA/VEGF-C tumors treated with the anti-VEGFR-3 antibody (mF4-3IC1) had returned to their normal size (E), and very few intratumoral lymphatics were detected (F). Likewise, tumors treated with the anti-VEGFR-2 antibody (DC101) were surrounded with small lymphatics (G), and the intratumoral lymphatic vessel density was dramatically decreased (H). Note that the appearance of lymphatic vessels in the mF4-3IC1 (E and F) and DC101-treated tumors (G and H) is comparable with the control (A and B). Cell nuclei are counterstained with Hoechst (blue). ep, epithelium; d, dermis; t, tumor. Bar, 100 μm.
immunostaining, VEGF-3 and VEGF-2 were expressed by the subpopulation of tumor blood vessels (Fig. 4).

Tumor growth rate was moderately attenuated with the anti-VEGFR-3 treatment (Fig. 5E). In the prevention regimen, blocking VEGFR-3 resulted in the 45% decrease of tumor volume 6 weeks after the tumor cell injection. Blocking VEGFR-2 reduced tumor volume by 84%. In the intervention regimen, anti-VEGFR-3 antibody diminished tumor size by 35%, but the difference was not statistically significant. Because differences in tumor size may result from changed expression of VEGFs, we examined the effects of blocking VEGFR-3 or VEGFR-2 on expression of VEGFs in tumors. As determined by Western analysis, expression levels of VEGF-A, VEGF-C, or VEGF-D were not altered with the antibody treatments (data not shown).

**Phenotypic characteristics of metastases.** To gain insight into the mechanism by which inactivation of VEGFR-2 and VEGFR-3 signaling suppresses metastases, we evaluated lymph node and lung metastases by histology and examined metastatic nodules for proliferation and angiogenesis. At the time points examined (i.e., 6 weeks for the prevention and 10 weeks for the intervention experiments), we have not observed any macrometastases on the surface of the organs examined. Micrometastases, which were detected in the lymph node sections by GFP fluorescence, were examined the effects of blocking VEGFR-3 or VEGFR-2 on expression of VEGFs in tumors. As determined by Western analysis, expression levels of VEGF-A, VEGF-C, or VEGF-D were not altered with the antibody treatments (data not shown).

**Combination treatment is more effective in suppressing metastases than single antibody treatments.** The effects of the combined treatment with the anti-VEGFR-2 and anti-VEGFR-3 large lymph node metastasis is shown in Fig. 6A. Unexpectedly, we found that all lymph node metastases, including the largest nodules in the nontreated group, were avascular, as shown by the CD31 and CD34 immunostaining (Fig. 6C and D). Tumor cell proliferation in the lymph node was apparently high, as shown by Ki67 labeling (Fig. 6B). Similarly, even the largest lung metastases were not vascularized yet clearly showed proliferative activity (Fig. 6E-G). Upon different antibody treatments, both the size and the number of the metastatic lesions were decreased (Supplementary Fig. S2A-D). Although the appearance and the size of the metastatic lesions were rather heterogeneous within the same group of specimens (Supplementary Fig. S2E and F), the phenotype of metastases was not notably different between the control and the treated groups.

**Figure 3.** Quantification of tumor lymphangiogenesis. Computer-assisted image analysis showed that mF4-31C1-treated mice displayed nearly complete inhibition of tumor lymphangiogenesis compared with control-treated mice; intratumoral lymphatic vessel area was also markedly decreased with the DC101 treatment yet to a somewhat lesser extent than with the mF4-31C1 treatment. The results were comparable between the prevention (open columns) and the intervention (filled columns) treatment regimen. Because mice were sacrificed later in the intervention then in the prevention experiment (10 versus 6 weeks, respectively), the overall lymphatic vessel densities are always higher in the intervention group. Columns, mean lymphatic vessel area (n = 5 for each treatment group); bars, SD. **, P < 0.01.

**Figure 4.** Expression of VEGFR-2 and VEGFR-3 on tumor lymphatic and blood vasculature. A-D, double immunofluorescent staining for LYVE-1 (green) and VEGFR-2 (red) showed that VEGFR-2 is strongly expressed on all peritumoral lymphatic vessels (arrowheads; A and B) but is partially down-regulated or absent from the intratumoral lymphatics (C and D). E-H, in contrast, VEGFR-3 is strongly expressed by all peritumoral (E and F) and intratumoral (G and H) lymphatic vessels (arrowheads). A fraction of tumor-associated blood vessels (arrows) expressed VEGFR-2 (A-D) or VEGFR-3 (E and H), MDA/VEGF-C control tumors. Cell nuclei are counterstained with Hoechst (blue). ap, epidermis; d, dermis; t, tumor. Bar, 100 μm.
antibodies on metastases were evaluated in the intervention treatment regimen. Combining the DC101 and mF4-31C1 antibodies very prominently reduced the incidence of lymph node metastases (Table 1). The number of mice with metastases and the number of lymph nodes with metastases in each mouse were both significantly lower in the combined treatment group compared with the control. Moreover, combined treatment was more effective than single treatment with the anti-VEGFR-2 or anti-VEGFR-3 antibodies. Combination treatment also decreased the overall tumor burden in the lymph nodes by 70% (control, 0.833 ± 0.151 versus DC101+mF4-31C1, 0.25 ± 0.47; P = 0.05; Fig. 1B). Lung tumor burden was decreased by 54% (%GFP+ tumor cells: control, 0.07 ± 0.01 versus DC101+mF4-31C1, 0.033 ± 0.03; P > 0.05), the combination being more effective than mF4-31C1 single treatment (Fig. 1D). In addition, only 6 of 10 mice developed lung metastases in the double-treated group compared with 9 in 10 mice in the control. Combination of DC101 and mF4-31C1 antibodies decreased tumor angiogenesis by 79% (compared with control; P < 0.001), which was slightly more potent than with the two antibody alone (69% and 71% inhibition with mF4-31C1 and DC101 antibody, respectively; P < 0.01). Lymphangiogenesis was not inhibited more than with the single-antibody treatments. Finally, the combined treatment was not more effective in inhibiting tumor growth than blocking VEGFR-2 alone (Fig. 5E).

**Discussion**

In this study, we evaluated the specific roles of VEGFR-3 versus VEGFR-2 signaling in metastasis and show that the selective inhibition of VEGFR-3 signaling is more effective in suppressing regional and distant tumor metastases than inhibition of VEGFR-2.

Our previous studies have shown that MDA-MB-435 breast cancer cells producing high levels of VEGF-C induce tumor lymphangiogenesis, and that the VEGF-C–mediated activation of tumor-associated lymphatic vessels facilitates metastases in a mouse xenograft model (12). By using this tumor model, we show here that the systemic treatment with the function-blocking antibody mF4-31C1, which selectively inhibits murine VEGFR-3 signaling (19), potently reduced tumor cell dissemination to the lymph nodes, indicating that the activation of VEGFR-3 signaling is critical for lymphogenous spread. Notably, the blockade of VEGFR-3 signaling also reduced lymph node metastases when the antibody was administered after the metastases were established, indicating for the first time a potential use of lymphatic vessel targeting for halting metastases after the tumor dissemination has occurred and not only as a prevention strategy.

The soluble form of VEGFR-3 has been reported to prevent formation of lymph node metastases in several experimental tumor

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**Figure 5.** Effects of treatments with mF4-31C1 and DC101 antibodies on tumor angiogenesis and growth. The density of intratumoral blood vessels (CD31+/LYVE-1−) was comparable between the MDA/pcDNA (A) and MDA/VEGF-C tumors (B). Blocking VEGFR-3 (C) or VEGFR-2 (D) decreased tumor angiogenesis. DC101-treated tumors were characterized by large tumor areas devoid of the vasculature, and the remaining blood vessels seemed large and mature (D). In contrast, blood vessels in mF4-31C1–treated tumors were small and scattered throughout the tumor (C). Bar, 100 μm. E, tumor growth rates were not significantly different between the MDA/pcDNA and MDA/VEGF-C cells. Tumor growth was delayed significantly with the mF4-31C1 treatment (P < 0.001), albeit less dramatically than with the DC101 antibody treatment (P < 0.001). Combination treatment was comparable with the DC101 alone. Points, average size of 20 tumors (10 mice, 2 tumors each; prevention regimen).
models. Adenoviral administration of soluble VEGFR-3-Ig fusion protein reduced incidence of lymph node metastases from lung, melanoma, and prostate tumors implanted s.c. in immunodeficient mice (26, 27). In another study, VEGFR-3-Ig overexpressed in breast cancer cells suppressed formation of lymph node metastases in immunocompetent rats (14). Most recently, small interfering RNA (siRNA)–mediated VEGF-C gene silencing has been shown to inhibit tumor lymphangiogenesis and metastasis (28). Because soluble VEGFR-3 and the VEGF-C siRNA approach interfere with the activation of both VEGFR-2 and VEGFR-3, these studies could not assess the specific role of each receptor in metastasis. It has been reported that the monoclonal antibody AFI4 specifically blocks murine VEGFR-3 signaling and inhibits corneal lymphangiogenesis (29). In experimental tumor models, AFI4 has been found to inhibit angiogenesis and tumor growth (30) and to suppress lymph node metastases (31). In contrast, Pytowski et al. reported that AFI4 binds to VEGFR-3 but does not block VEGFR-3 signaling and have suggested that the observed effects of AFI4 may be mediated by nonantagonist mechanisms, such as the steric hindrance of VEGFR-3 dimerization or antibody-induced reduction in surface receptor expression (19). In our own studies, we have not observed any effects of the AFI4 antibody on tumor lymphangiogenesis or metastasis. In view of the above, the results presented in this report provide the first direct evidence for the pivotal role of VEGF-3 signaling in lymphogenous metastasis.

Importance of VEGFR-3 signaling in metastases to lymph nodes is further underscored by our findings that blocking VEGFR-2 was less effective in halting metastases then blocking VEGFR-3, although VEGFR-2 blockade more potently inhibited angiogenesis and primary tumor growth. Because blocking of VEGFR-2 mainly affected angiogenesis and tumor growth, we hypothesized that inactivation of VEGFR-2 suppresses metastases by restraining metastatic growth, whereas VEGFR-3 inactivation mainly inhibits tumor spread. To test this hypothesis, we analyzed lymph node and lung metastases for angiogenesis and proliferation. We found, to our surprise, that despite prominent proliferation, metastases were not vascularized in any of the control and treatment groups, indicating that the growth of metastases was not dependent on angiogenesis at the secondary site within the duration of the experiment. This could explain the limited efficacy of the antiangiogenic therapy with the DC101 antibody for metastasis control in this experimental model. It is conceivable that the metastatic cells were not highly sensitive to the antiangiogenesis treatment because the metastatic lesions were too small to depend on angiogenesis for growth. It has also been suggested in the literature that the vascular dependence of malignant cells may be heterogeneous, particularly, that in the late stages of tumor progression vascular demand may be lowered (32–34). Furthermore, in well-vascularized organs, such as lungs, the dependence on angiogenesis may be diminished because of the extensive preexisting vascular network which can be co-opted by the metastatic cells (35, 36). Hence, the observed suppression of metastases with the DC101 antibody could be due to the significant decrease in the size of a primary tumor and decreased lymphangiogenesis, which may limit the rate of tumor cell escape from the tumor.

Effects of VEGFR-3 inhibition on metastases could be explained, at least in part, by inhibition of lymphangiogenesis. We and others have shown that increased lymphangiogenesis and recruitment of lymphatic vessels into the tumor increase the propensity of tumors to metastasize (12–15, 17, 37). Accordingly, decreasing the number of lymphatic vessels in the vicinity of tumor cells may restrain tumor spread by restricting tumor cell access to the lymphatic vasculature. Because VEGFR-2 blockade also inhibited tumor lymphangiogenesis but without decreasing the incidence of lymph node metastases, the reduction of lymphatic vessel densities alone can not explain the suppression of metastases by VEGFR-3 inactivation, raising a question about the mechanism by which VEGF-C and VEGFR-3 activation promote tumor dissemination. We have proposed previously that activation of lymphatic endothelium by VEGF-C may facilitate tumor cell entry into the lymphatics by promoting molecular interactions between tumor cells and lymphatic endothelium (12, 38, 39). Activation of lymphatic endothelial cells by VEGF-C and other VEGF-C ligands may facilitate the escape of cancer cells from the primary tumor by promoting release of chemo-kines that may attract tumor cells into the lymphatics or by altering adhesive properties of lymphatic endothelium to support tumor invasion.

In support of this concept, high levels of VEGF-C expression have been associated with lymphatic vessel invasion and lymph node metastases in the multitude of human tumors, whereas tumor metastases have not always been associated with increased lymphangiogenesis (2). In the present study, we have observed fewer lymphatic vessels infiltrated with tumor cells in tumors treated with the anti-VEGFR-3 antibodies compared with anti-VEGFR-2–treated tumors. Furthermore, our in vitro studies showed that lymphatic endothelial cells potently promote tumor cell chemotaxis. Taken together, these results suggest that in addition to lymphangiogenesis, activation of lymphatic vessels via the VEGFR-3 pathway may facilitate tumor metastases by promoting entry of tumor cells into the peripheral lymphatics.

To gain insight into the mechanism by which VEGF-C/VEGFR-3 signaling promotes distant metastases, the ability of control and VEGF-C–overexpressing tumor cells to form colonies in target organs was assessed in the experimental metastasis assay. In this assay, which models later stages of the metastatic process, there was no difference in the colonization of lungs or liver between control and VEGF-C–expressing cells. These findings show that VEGF-C does not provide an advantage for tumor cell survival in the bloodstream, arrest, extravasation, or growth at the secondary site and further support the concept that VEGF-C increases formation of distant metastases via the lymphatic pathway. Nevertheless, a possibility remains that VEGF-C may also facilitate tumor cell invasion of the blood vasculature at the primary tumor site. Interestingly, when injected directly into the blood circulation, tumor cells also entered lymph nodes. In this case, tumor burden in the lymph nodes was not increased by VEGF-C, reinforcing the conclusion that VEGF-C does not increase growth of metastases in lymph nodes but facilitates tumor cell arrival from the primary tumor.

In summary, we show that selective inhibition of the VEGF-C/VEGFR-3 signaling pathway is an effective strategy for control of regional and distant metastases. It has been suggested that targeting the late steps of the metastatic process, such as growth of metastases at the secondary site, may be the most effective

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3 N. Roberts and M. Skobe, unpublished data.

4 S.K. Das and M. Skobe, unpublished data.
antimetastatic strategy (4, 40). The present study validates the concept of targeting the lymphatic endothelium, and thereby very early stages of the metastatic process, for metastasis control. Our results, showing the combination treatment with mF4-31C1 and DC101 antibodies to be more potent in suppressing metastases than either treatment alone are encouraging and suggest a potential promise for strategies which combine targeting both tumor dissemination and growth for the treatment of metastatic disease.

References

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Inhibition of VEGFR-3 Activation with the Antagonistic Antibody More Potently Suppresses Lymph Node and Distant Metastases than Inactivation of VEGFR-2

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