Down-Regulation of Vascular Endothelial Cell Growth Factor-C Expression Using Small Interfering RNA Vectors in Mammary Tumors Inhibits Tumor Lymphangiogenesis and Spontaneous Metastasis and Enhances Survival

Zhengtang Chen,1,3 Michelle L. Varney,1 Matthew W. Backora,1 Kenneth Cowan,2 Joyce C. Solheim,2 James E. Talmadge,1 and Rakesh K. Singh1

1Department of Pathology and Microbiology and 2Epley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska; and 3Cancer Center of People's Liberation Army, Xin Qiao Hospital, Third Military Medical University, Chongqing, PR China

Abstract

Tumor production of vascular endothelial cell growth factor (VEGF)-C is associated with tumor lymphangiogenesis and lymph node metastasis. In this study, we examined the effects of small interfering RNA (siRNA)-mediated inhibition of VEGF-C on murine mammary tumor growth, metastasis, and survival. The mRNA and protein expression of VEGF-C in murine mammary tumor cells stably transfected with a VEGF-C siRNA vector were significantly lower compared with VEGF-C-control vector-transfected cells. Cl66-siVEGFC tumors had lower levels of lymphangiogenesis and lymph node and spontaneous lung metastasis than Cl66-control tumors. However, we did not observe significant differences in primary tumor growth and experimental lung metastasis between mice injected with Cl66-siVEGFC and Cl66-control cells. In addition, mice bearing Cl66-siVEGFC tumors lived significantly longer than mice bearing Cl66-control tumors. Furthermore, our data suggest that inhibition of VEGF-C modulates immune cell infiltration and their function, which might be critical in tumor immunity. In summary, our data show that inhibition of VEGF-C expression using siRNA-mediated gene silencing vectors reduces lymphangiogenesis and lymph node and spontaneous lung metastasis, and enhances survival. (Cancer Res 2005; 65(19): 9004-11)

Introduction

At the time of diagnosis, the majority of breast cancer patients have developed lymph node metastases, which function as an important prognostic indicator. Recent studies in animal models suggest that lymphangiogenesis can promote metastasis (1–4). Series of lymphangiogenic factors, such as vascular endothelial growth factor (VEGF)-C and VEGF-D (5–7), and lymphatic markers, such as lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1; refs. 8–13), VEGF receptor 3 (VEGFR-3; ref. 14), podoplanin (15–17), and prox-1 (3, 18, 19), have been identified. The VEGF-C protein belongs to the platelet-derived growth factor family (20) and is a known ligand for VEGFR-3 (Flt4; refs. 21, 22).

VEGF-C participates in the development of lymphatic vessels (21, 22). As such, VEGF-C is thought to be involved in tumor lymphangiogenesis and lymph node and spontaneous lung metastasis (23–33). Overexpression of VEGF-C in breast cancer cells can potentially increase intratumor lymphangiogenesis, resulting in significantly enhanced metastasis to regional lymph nodes (4, 7, 34, 35). Despite the continuing accumulation of correlative data, a functional role of VEGF-C in tumor lymphangiogenesis and regional and distant metastasis remain unclear. In the present study, we examined whether inhibition of VEGF-C by gene silencing using mammalian expression vectors expressing small interfering RNA (siRNA) might lead to inhibition of lymphangiogenesis and eventual metastases. We examined the effects of these siRNA-vectors on the inhibition of VEGF-C expression, lymphangiogenesis, primary tumor growth, experimental and spontaneous metastasis, and host survival using a murine mammary tumor model. Our data show that the inhibition of constitutive VEGF-C expression in murine mammary tumors reduces lymphangiogenesis and spontaneous metastasis and increases survival.

Materials and Methods

Mice and mammary tumor cells. Female BALB/c mice (6-8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH. The murine mammary tumor cell line Cl66 was propagated and maintained in culture as an adherent monolayer in MEM supplemented with 5% fetal bovine serum (FBS; Life Technologies, Inc., Gaithersburg, MD), 2 mmol/L L-glutamine, sodium pyruvate, nonessential amino acids, vitamin solution, and gentamicin (Mediatech, Herndon, VA). The cells were maintained at 37°C with 5% CO2.

Construction of small interfering RNA expression vectors. We used the pSuppressorNeo siRNA plasmid vector-based GeneSilencer system (Imgenex Corp., San Diego, CA), in which siRNA expression is driven by the U6 RNA promoter, to produce siRNA following stable transfection and selection. We generated two different siRNA vectors. The oligonucleotides 5′-TGAATACAGTGGCTTCTTACACATGGAGGTAGTCAGTGAATTACCAGCAAAAGTTCTCTTTGCTGGTAA-3′ and 3′-CTGGCTTTACTCGCATCTTGAATT-5′ were used to generate anti–VEGF-C siRNA vector, and oligonucleotides 5′-TGAATACAGTGGCTTCTTACACATGGAGGTAGTCAGTGAATTACCAGCAAAAGTTCTCTTTGCTGGTAA-3′ and 3′-CTGGCTTTACTCGCATCTTGAATT-5′ were used to generate a control siRNA vector. Each oligonucleotide pair (100 pmol) was annealed by incubation at 95°C for 5 minutes and slow cooling. One microliter of this mixture was then...
ligated into XhoI and XbaI sites on the pSuppressorNeo vector. As a result, two vectors, psi–VEGF-C and psi-control, were generated.

Transfection of murine mammary tumor cells. Cl66 murine mammary tumor cells were transfected with 2 μg DNA using Lipofect-AMINE Plus reagent (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. Transfected Cl66 cells were selected with 400 μg/mL G418-sulfate. Stably transfected Cl66-siVEGFC and Cl66-control cells were obtained and routinely maintained in selection media containing 250 μg/mL of G418-sulfate to avoid overgrowth of nontransfected cells.

Messenger RNA analysis. Total cellular RNA was isolated from Cl66-control or Cl66-siVEGFC cells and murine mammary tumors using Trizol reagent (Invitrogen) and cDNA was synthesized using total RNA (5 μg), oligo(dT)18 primer, and Superscript RT (Invitrogen). Quantitative real-time PCR (qRT-PCR) was done using 2 μL of a 1:100 dilution of first-strand cDNA in FastStart DNA Master SYBR Green I with specific primers and iCycler (Bio-Rad, Hercules, CA). Primers used were as follows: VEGF-C, 5'-CTACAGATGTGGGGTGCT-G and 5'-GCTGCCTGACACTGTGGTAA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH); 5'-AGCCTCGTCCCGTAGACAAA-3' and 5'-GATGACAAGCTCCATTTCTCG-3'; LYVE1, 5'-AGACACTTGAAGTCGCCGGA-3' and 5'-GCCCACACTCCGTATACAT-3'; VEGFR-3, 5'-CCCCAGCCATGTACAGAAGGT-3' and 5'-GGCTGAGTCAGAGGAGTTG-3'; VEGF-A, 5'-TTACTGCTGTACCTCCACC-3' and 5'-TCAGGCGTCCTGAAGATG-3'. The fluorescence intensity of the double-stranded specific SYBR Green I, reflecting the amount of PCR product, was monitored at the end of each cycle. The PCR efficiency was examined by serially diluting the template cDNA and the melting curve data were collected to check the PCR specificity. Each cDNA sample was run triplicate and the corresponding no cDNA sample was included as a negative control. The mRNA levels of each gene were normalized to that of the GAPDH mRNA (36).

In vitro proliferation assay. Cl66-control or Cl66-siVEGFC cells (5 × 10^3) were seeded into 38 mm^2 wells of 96-well flat-bottomed plates.

Figure 1. Inhibition of VEGF-C production in Cl66 cells using a VEGF-C siRNA-expressing mammalian expression vector. The levels of VEGF-C in Cl66 cells stably transfected with control or VEGF-C-siRNA vector were examined for mRNA and protein expression using qRT-PCR, Western blot, and immunohistochemistry. A, expression of VEGF-C–specific mRNA transcript analyzed by qRT-PCR. Columns, relative mRNA levels for VEGF-C; bars, SE. B, expression of VEGF-A–specific mRNA transcript analyzed by qRT-PCR. Columns, relative mRNA levels for VEGF-C; bars, SE. C, expression of VEGF-C protein levels examined by Western blotting analysis. Columns, arbitrary units (A.U.) representing levels of VEGF-C protein quantitated by densitometry; bars, SE. D, immunohistochemical analysis of VEGF-C expression in Cl66-control cells and Cl66-siVEGFC cells using anti–VEGF-C antibody. *Significantly different from control.
in triplicate and allowed to adhere overnight. The cultures were then washed and refed with medium containing different concentrations of FBS (0%, 1.25%, 2.5%, and 5.0%). Following 24, 48, and 72 hours of culture, proliferative activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using a microtiter plate reader (Bio-Tek Instruments, Inc., Winooski, VT) at 570 nm. Growth stimulation or inhibition was calculated as \( \% = \left[ \frac{A - B}{A} \right] \times 100 \), where \( A \) is the absorbance of treated cells and \( B \) is the absorbance of untreated control cells.

**Tumorigrenicity, metastasis, and survival assays.** C66-siVEGFC and C66-control cells were harvested with 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS and cells were washed once and resuspended in HBSS for injection. Only single-cell suspensions with >90% viability (tested by trypan blue exclusion) were used for injections. C66-control or C66-si-VEGFC cells (1 \( \times \) 10\(^5\) cells) in 0.1 mL HBSS were injected into mammary fat pads of female BALB/c mice (20 mice per group). Tumors were measured with calipers twice a week. Tumor volume was calculated using the following formula: volume = \( W^2 \times L / 2 \), where \( W = \) short diameter and \( L = \) long diameter.

Ten mice per group were sacrificed 6 weeks following tumor cell injection and mammary tumors were harvested. Mice were necropsied for examination of regional lymph node and distant metastases. Mammary tumors, lymph nodes, livers, lungs, and femurs were processed for immunohistochemical analysis (of micrometastasis and lymphangiogenesis) and for Western blot and mRNA analysis (of VEGF-C expression). The remaining 10 mice from each group were monitored for survival studies.

To examine experimental lung metastasis, C66-control or C66-siVEGFC cells (1 \( \times \) 10\(^5\) cells/0.1 mL HBSS/animal) were injected i.v. into female BALB/c mice (20 mice per group). Ten mice from each group were killed 3 weeks following tumor cell injection and their lungs were harvested and fixed in Bouin's solution and scored under a dissecting microscope for the number of metastatic nodules on the lung surface. The remaining 10 mice from each group were monitored for survival studies.

**Immunohistochemical analysis.** C66-control or C66-siVEGFC cells cultured in chamber slides were fixed with a cold solution of methanol/acetone (50:50) for 10 minutes. The tumors from C66-control or C66-siVEGFC–injected mice were put into optimal cutting temperature compound (Sakura, Tokyo, Japan), and 6 to 8 \( \mu \)m thick sections were cut. VEGF-C, VEGFR-3 (for lymphangiogenesis), and CD31 (for angiogenesis) positivity was analyzed by immunohistochemical techniques. Anti–pan-cytokeratin antibody (Serotec, Inc., Raleigh, NC) was used to determine lymph node metastasis. Briefly, tissue sections were processed for immunohistochemistry using rabbit anti–VEGF-C or goat anti-mouse VEGFR-3 (PharMingen) or anti-CD31 or anti-pan-cytokeratin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and Serotec). Immunoreactivity was detected using the ABC Elite kit and 3,3′-diaminobenzidine substrate kits (Vector Laboratories, Burlingame, CA) per instructions of the manufacturer. A reddish brown precipitate in the cytoplasm indicated a positive reaction. Negative controls had all reagents included except the primary antibody. The number of positive cells was counted at \( \times 400 \) magnification in area of highest intensity. Each section was examined by two independent observers.

**Analysis of tumor-infiltrating leukocytes.** T cells and dendritic cell accumulation in C66-control and C66-siVEGFC tumors was examined by flow cytometry using anti-CD4 (clone H129.19), anti-CD8a (clone 53-6.7), and anti-CD11c (clone HL3) antibodies purchased from BD PharMingen. Tumors were measured, excised, and nonneoplastic tumor tissue was minced. The tumor samples were transferred to 15 mL tubes, pelleted on a 400-gspin, and washed twice in sterile HBSS. The samples were then resuspended in freshly prepared HBSS containing collagenase (200 units/mL) and DNase I (270 KU/mL) and incubated at 37°C with constant agitation. Following collagenase dissociation, the cells were passed through 70 \( \mu \)m nylon cell strainer, washed once in HBSS, and layered over Lympholyte-M (Cedarlane, Hornby, Ontario, Canada). After centrifugation, mononuclear cells were collected from interface, washed, and adjusted to a concentration of 1 \( \times \) 10\(^7\) cells/mL in 2% FBS/0.1% sodium azide/PBS. Cells (0.1 mL/ aliquot) were distributed into 96-well U-bottomed plate and nonspecific binding blocked with 1.5% normal murine serum in 0.1% sodium azide/PBS. Next, samples were stained with primary antibodies labeled with FITC, biotin, or phycoerythrin. Biotinylated antibodies were revealed with allopheocyanin-streptavidin (Molecular Probes, Eugene, OR). Data on the stained samples were acquired with a FACScan Libar flow cytometer (BD ImmunoCytometry System, San Jose, CA), Cell populations were gated on forward and side scatter before analysis of antibody staining. Between 30,000 and 100,000 events were collected per sample and the data were analyzed with Attractors or Cell Quest software (BD ImmunoCytometry System).

**Lymphocyte proliferation.** Splenocytes from C66-control and C66-siVEGFC tumor-bearing mice were plated at 0.2 \( \times \) 10\(^6\) cell/well and cultured with different concentrations of concanavalin A for 5 days. \(^{[3H]}\)Thymidine incorporation was quantified by scintillation counting as cpm. Splenocytes from normal nontumor-bearing mice was used as control.

**Western blot analysis.** C66-control or C66-siVEGFC cells were seeded in 100 \( \times \) 20 mm culture dishes. After 48 hours of incubation, cells were washed four times with cold PBS and lysed in M-PER reagent (Pierce Chemicals, Rockford, IL) supplemented with protease inhibitors (15 \( \mu \)g/mL phenylmethylsulfonyl fluoride, 0.5 \( \mu \)g/mL leupeptin, and 0.7 \( \mu \)g/mL pepstatin). The mammary tumors from C66-control or C66-siVEGFC cells were lysed in T-PER reagent (Pierce Chemicals) supplemented with protease inhibitors. Cellular debris was removed by centrifugation and the proteins (20 \( \mu \)g/lane) were separated on SDS-PAGE and electrotransferred to 0.45 \( \mu \)m nitrocellulose membranes blocked with 5% nonfat dry milk in Trition X-100 containing TBS. Western blot analysis was done with anti–VEGF-C (1:500; #9006 www.aacrjournals.org Downloaded from cancerres.aacrjournals.org on August 30, 2017. © 2005 American Association for Cancer Research.)
Santa Cruz Biotechnology) as the primary antibody. After extensive washing, the membranes were incubated with anti-rabbit antibody conjugated with horseradish peroxidase (Amersham, Piscataway, NJ) at a 1:2,000 dilution for 1 hour and then incubated with avidin-AP (Molecular Dynamics, Sunnyvale, CA). After the scan, the membranes were stripped and reprobed with rabbit anti-α-actin (1:1,000 Sigma Chemical Co. St. Louis, MO) for determining the relative amounts of protein loaded.

**Statistical analysis.** The significance of the data was determined by the Student’s t test (two-tailed) for all in vitro studies and the Mann-Whitney U-test for in vivo studies. Survival rates were graphed using the Kaplan-Meier method and compared among different groups by log-rank statistical analysis. \( P < 0.05 \) was deemed significant. All statistical analyses were done using SPSS software (SPSS, Inc, Chicago IL).

**Results**

**Inhibition of the VEGF-C expression in vitro and in vivo by stable expression of small interfering RNA in murine mammary tumor cells.** We constructed a mammalian expression vector to express VEGF-C siRNA constitutively to inhibit endogenous VEGF-C expression. We stably transfected Cl66 murine mammary tumor cells, which express high levels of VEGF-C, with psi–VEGF-C (to generate Cl66-siVEGFC cells) and psi-control (to generate Cl66-control cells). We examined mRNA expression of VEGF-C and VEGF-A, a closely related member of the VEGF family, in these transfected cells to show the specificity of VEGF-C gene silencing. We observed significant inhibition of VEGF-C mRNA expression in Cl66-siVEGFC cells compared with Cl66-control cells (Fig. 1A). We did not observe any difference in the mRNA expression of VEGF-A in Cl66-siVEGFC and Cl66-control cells (Fig. 1B). In addition, Western blot and immunohistochemical analyses showed that Cl66-siVEGFC cells had a significant decrease in VEGF-C protein levels compared with Cl66-control cells (Fig. 1C and D). Further, there was no significant difference in the rate of cell proliferation between Cl66-siVEGFC and Cl66-control cells (data not shown).

**Effect of the VEGF-C gene silencing primary tumor growth.** We examined the impact of siRNA-mediated VEGF-C gene silencing on murine mammary tumor growth and metastasis. Stably transfected Cl66-siVEGFC and Cl66-control cells (1 \( \times \) \( 10^5 \) cells) were injected orthotopically into BALB/c female mice and tumor growth was monitored twice a week. We did not observe a statistically significant difference in tumor growth between mice injected with Cl66-siVEGFC or Cl66-control cells (Fig. 2A).

We confirmed VEGF-C gene silencing in tumors produced by Cl66-siVEGFC or Cl66-control cells. Specifically, we observed significantly lower expression of VEGF-C mRNA expression in Cl66-siVEGFC tumors compared with Cl66-control tumors (Fig. 2B).

**Effect of the VEGF-C gene silencing on tumor lymphangiogenesis.** We next examined the effect of VEGF-C gene silencing on lymphangiogenesis in Cl66-siVEGFC and Cl66-control tumors produced by immunohistochemical analysis using an anti–VEGFR-3 antibody. Our data show fewer lymphatics in Cl66-siVEGFC tumors compared with Cl66-control tumors (Fig. 3A). We further confirmed this observation by qRT-PCR for

---

**Figure 3.** A, lymphangiogenesis in mammary tumors expressing different levels of VEGF-C. Cl66-siVEGFC tumor (a) and Cl-66 tumor (b) were immunostained using anti-VEGFR-3 antibody for analysis of lymphangiogenesis. In addition, Cl66-siVEGFC (c) and CI-66 (d) tumors were immunostained with anti-CD31 antibody for neovascularization analysis. B and C, expression levels of lymphatic markers VEGFR-3 and LYVE-1 in tumors produced by Cl66-siVEGFC and Cl66-control cells as examined by qRT-PCR. Columns, relative mRNA levels for VEGFR-3 or LYVE-1; bars, SE. *Significantly different from control.
VEGFR-3 and LYVE-1 using mRNA from tumors produced by Cl66-siVEGFC and Cl66-control cells (Fig. 3B and C). As expected, we observed no difference in neovascularization (by immunohistochemistry using anti-CD31 antibody) between tumors produced by Cl66-siVEGFC and Cl66-control cells (Fig. 3A, C and D). These data suggest that inhibition of VEGF-C in primary tumors by siRNA-mediated gene silencing leads to decreased lymphangiogenesis but not angiogenesis.

**Effect of the VEGF-C gene silencing on spontaneous metastasis and survival.** Overexpression of VEGF-C has been shown to enhance lymph node metastasis in breast cancer. We examined the spontaneous metastatic potential of Cl66-siVEGFC and Cl66-control cells. Mice injected with Cl66-siVEGFC and Cl66-control cells were sacrificed on day 21 and lymph node, lung, liver, and bone marrow were examined for metastasis by immunohistochemistry. We observed a significant inhibition in the incidence of lymph node metastasis in mice injected with Cl66-siVEGFC cells (30%) compared with mice injected with Cl66-control cells (70%; Fig. 4A). In addition, the frequency of pan-cytokeratin–positive metastatic cells in lymph node was significantly lower in mice injected with Cl66-siVEGFC cells compared with mice injected with Cl66-control cells (Fig. 4A).

Furthermore, the incidence and number of spontaneous lung metastases in mice injected with Cl66-siVEGFC cells were lower compared with mice injected with Cl66-control cells (Fig. 4B). These data suggest that silencing of VEGF-C gene inhibits regional and distant metastasis in murine mammary tumor cells.

We also examined the difference in the survival of mice injected in the mammary fat pad with Cl66-siVEGFC and Cl66-control cells and observed significantly enhanced survival of mice injected with Cl66-siVEGFC cells compared with mice injected with Cl66-control cells (Fig. 4C). Taken together, these data show that siRNA-mediated VEGF-C gene silencing in murine mammary tumor cells inhibits lymphangiogenesis and spontaneous lymph node and lung metastasis, leading to increase in survival.

**Effect of the VEGF-C gene silencing on experimental lung metastasis and survival.** We examined the experimental metastatic potential of Cl66-siVEGFC and Cl66-control cells following i.v. injection into the female BALB/c mice. We sacrificed mice in both groups on day 21 and examined the lungs for micrometastasis. Using this approach, we did not observe significant differences in the incidence and number of lung metastatic nodules in mice injected with Cl66-siVEGFC or Cl66-control tumor cells (Fig. 5A).

Mice injected i.v. with Cl66-siVEGF-C cells had significantly enhanced survival compared with mice injected with Cl66-control cells (Fig. 5B). Further, necropsies of the dead mice showed similar

![Figure 4](image_url)

**Figure 4.** Spontaneous lymph node and lung metastasis and survival following mammary fat pad injection of Cl66-siVEGFC and Cl66-control cells. A, lymph node metastasis was examined by pan-cytokeratin staining of tumor cells. Columns, incidence and frequency of pan-cytokeratin–positive cells in the lymph nodes. B, spontaneous lung micrometastasis was determined by H&E staining. The values are incidence, median numbers of micrometastasis, and range of metastatic lung nodules. C, survival rate of mice bearing Cl66-control or Cl66-siVEGFC tumors at different time points. *Significantly different from control.

<table>
<thead>
<tr>
<th></th>
<th>Incidence</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl66-control</td>
<td>100%</td>
<td>18 (4-22)</td>
</tr>
<tr>
<td>Cl66-siVEGFC</td>
<td>30%</td>
<td>4 (0-7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Incidence</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl66-control</td>
<td>100%</td>
<td>43 (4-52)</td>
</tr>
<tr>
<td>Cl66-siVEGFC</td>
<td>100%</td>
<td>49 (7-57)</td>
</tr>
</tbody>
</table>
numbers of metastatic lung nodules in mice injected with Cl66-siVEGFC cells and Cl66-control cells (data not shown). These data show that siRNA-mediated VEGF-C gene silencing enhanced survival of mice with similar lung tumor burdens.

Inhibition of VEGF-C modulates the tumor-infiltrating immune cell phenotype. BALB/c mice were injected with Cl66-control and Cl66-siVEGFC tumor cells orthotopically. When tumors reached ~1 cm in diameter, mice were sacrificed and tumors were harvested. The frequency of T cell and dendritic cell subsets in tumor-associated leukocytes was determined using cell-specific markers and multicolor flow cytometry. The infiltration of CD4+ T cells in Cl66-control tumors was significantly higher compared with Cl66-siVEGFC tumors (Fig. 6A). In contrast, the infiltration of CD8+ T cells in Cl66-siVEGFC tumors was significantly higher compared with Cl66-control tumors (Fig. 6A and B). In addition, the infiltration of CD11b+CD11c+ cells in Cl66-siVEGFC tumors was significantly higher compared with Cl66-control tumors (Fig. 6C). Next, we examined the proliferation of tumor-associated leukocytes in response to concanavalin A. We observed higher levels of concanavalin A–induced proliferation in tumor-associated leukocytes from Cl66-siVEGFC tumor-bearing animals compared with tumor-associated leukocytes from Cl66-control tumor-bearing animals (Fig. 6D). These data suggest that inhibition of VEGF-C modulates immune cell infiltration and function, which might be critical in tumor immunity.

Discussion

In the present study, we showed that siRNA-mediated VEGF-C gene silencing in murine mammary tumors cells inhibited lymphangiogenesis and spontaneous lymph node and lung metastasis, and enhanced survival. For many carcinomas, transport of tumor cells via lymphatics is the pathway of initial dissemination (37), and VEGF-C–mediated enhancement of lymphangiogenesis plays an important role in tumor metastasis (38, 39). To our knowledge, the data described here represent the first evidence that siRNA-mediated VEGF-C gene silencing leads to inhibition of lymphangiogenesis and spontaneous metastasis to lymph nodes and lungs in a metastatic mammary tumor model. Recent reports show that blockade of VEGF-C receptor VEGFR-3 inhibits lymphangiogenesis and metastasis in highly metastatic human lung cancer cell line (40). In this case, He et al. (40) used a receptor antibody fusion protein (VEGFR-3-Ig fusion protein) that traps

![Figure 6](https://example.com/figure6.png)

**Figure 6.** T cell and dendritic cell phenotype in Cl66-control and Cl66-siVEGFC tumors. The number of CD3+CD4+ (A) and CD3+CD8+ (B) T cells and CD11b+CD11c+ (C) dendritic cells in Cl66-control and Cl66-siVEGFC tumors was determined by flow cytometry. The values are number of immune T cells and dendritic cells in per mm² of tumor. D, tumor-associated leukocytes proliferative response to concanavalin A. Tumor-associated leukocytes from Cl66-siVEGFC and Cl66-control tumors were examined for their proliferative response. Points, cpm from triplicate cultures; bars, SD. This is a representative experiment of three.
both VEGF-C and VEGF-D (40). In the present study, we show that specific silencing of VEGF-C gene alone can inhibit tumor lymphangiogenesis in murine mammary tumors.

Clinical and pathologic observations have suggested that initial dissemination of breast cancer is via the lymphatics. Compared to the blood vasculature, little is known about the biology of tumor lymphatics, the regulation of tumor lymphangiogenesis, or mechanisms that determine the interaction of tumor cells with lymphatic vessels (35). Although peritumoral lymphatic vessels contribute to tumor metastasis, opposite views exist as to whether intratumoral lymphatics have any role in tumor metastasis (39). Recent reports using various rodent models provide evidence that tumor VEGF-C–mediated tumor lymphangiogenesis facilitates lymphatic metastasis (41). In addition, using a genetic model, it has been shown that VEGF-C mediates lymphangiogenesis and promotes tumor metastasis (35).

In the present study, we provide evidence that VEGF-C gene silencing inhibited lymphangiogenesis and spontaneous metastasis, and enhanced survival. When overall survival was compared, mice bearing Cl66-siVEGFC tumors lived approximately twice as long as Cl66-control tumor-bearing mice, although the primary mammary tumor size and metastatic burden (in the experimental metastasis studies) were the same for both groups. Our studies suggest that inhibition of tumor VEGF-C production had no direct effect on the regulation of the tumor cell phenotype but might have led to modulation of the tumor microenvironment (such as lymphangiogenesis or recruitment of inflammatory and immune cells). Because VEGF-C might also modulate the immune response, we hypothesize that a decrease in VEGF-C levels might enhance an antitumor immune response, resulting in enhanced survival even if tumor burden is not decreased.

The relationship between immune cell phenotype and VEGF-C expression in cancer remains unclear. A recent report suggests a correlation between tumor expression of VEGF-C with tumor-infiltrating dendritic cell in gastric cancer (42). In this study, there was a direct correlation between VEGF-C expression and poor survival in gastric cancer patients (42). We observed significantly higher number of CD8+ T cells and CD11b+CD11c+ dendritic cells in Cl66-siVEGFC tumors compared with Cl66-control tumors. In addition, we observed significantly enhanced canceravalin A–induced proliferation in tumor-associated leukocytes from Cl66-siVEGFC tumors compared with Cl66-control tumors. The functional role of VEGF-C in the modulation of tumor-specific immune response is not yet clear. Our data suggest that inhibition of VEGF-C expression modulates T cell and dendritic cell phenotype and function, which might affect antitumor immunity. We are currently investigating this possibility by examining the role of VEGF-C in immune cell recruitment and activation and tumor-induced immunosuppression.

In summary, the present study shows that (a) siRNA-mediated VEGF-C gene silencing is an effective tool to inhibit VEGF-C expression in murine mammary tumors cells in vitro and in vivo; (b) inhibition of VEGF-C had no effect on primary tumor growth and experimental lung metastasis; (c) inhibition of VEGF-C inhibited lymphangiogenesis and spontaneous lymph node and lung metastasis, and enhanced survival; and (d) inhibition of VEGF-C modulates dendritic cell and T cell infiltration in tumors. Based on these studies, we suggest that down-regulation of lymphangiogenesis might provide a therapeutic strategy for inhibiting tumor metastasis and for enhancing the survival of patients with breast cancer.

References

Acknowledgments
Received 3/17/2005; revised 7/1/2005; accepted 7/25/2005.
Grant support: CA72781 (R.K. Singh) from National Cancer Institute, NIH, and Nebraska Research Initiative Molecular Therapeutics Program (R.K. Singh).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Down-Regulation of Vascular Endothelial Cell Growth Factor-C Expression Using Small Interfering RNA Vectors in Mammary Tumors Inhibits Tumor Lymphangiogenesis and Spontaneous Metastasis and Enhances Survival

Zhengtang Chen, Michelle L. Varney, Matthew W. Backora, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/19/9004

Cited articles
This article cites 41 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/19/9004.full#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/19/9004.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.