

## Suppression of Tumor Lymphangiogenesis and Lymph Node Metastasis by Blocking Vascular Endothelial Growth Factor Receptor 3 Signaling

Yulong He, Ken-ichi Kozaki, Terhi Karpanen, Katsumi Koshikawa, Seppo Yla-Herttuala, Takashi Takahashi, Kari Alitalo

**Background:** Vascular endothelial growth factor C (VEGF-C) stimulates tumor lymphangiogenesis (i.e., formation of lymphatic vessels) and metastasis to regional lymph nodes by interacting with VEGF receptor 3 (VEGFR-3). We sought to determine whether inhibiting VEGFR-3 signaling, and thus tumor lymphangiogenesis, would inhibit tumor metastasis. **Methods:** We used the highly metastatic human lung cancer cell line NCI-H460-LNM35 (LNM35) and its parental line NCI-H460-N15 (N15) with low metastatic capacity. We inserted genes by transfection and established a stable N15 cell line secreting VEGF-C and a LNM35 cell line secreting the soluble fusion protein VEGF receptor 3-immunoglobulin (VEGFR-3-Ig, which binds VEGF-C and inhibits VEGFR-3 signaling). Control lines were transfected with mock vectors. Tumor cells were implanted subcutaneously into severe combined immunodeficient mice (n = 6 in each group), and tumors and metastases were examined 6 weeks later. In another approach, recombinant adenoviruses expressing VEGFR-3-Ig (AdR3-Ig) or  $\beta$ -galactosidase (AdLacZ) were injected intravenously into LNM35 tumor-bearing mice (n = 14 and 7, respectively). **Results:** LNM35 cells expressed higher levels of VEGF-C RNA and protein than did N15 cells. Xenograft mock vector-transfected LNM35 tumors showed more intratumoral lymphatic vessels (15.3 vessels per grid; 95% confidence interval [CI] = 13.3 to 17.4) and more metastases in draining lymph nodes (12 of 12) than VEGFR-3-Ig-transfected LNM35 tumors (4.1 vessels per grid; 95% CI = 3.4 to 4.7;  $P < .001$ , two-sided  $t$  test; and four lymph nodes with metastases of 12 lymph nodes examined). Lymph node metastasis was also inhibited in AdR3-Ig-treated mice (AdR3-Ig = 0 of 28 lymph nodes; AdLacZ = 11 of 14 lymph nodes). However, metastasis to the lungs occurred in all mice, suggesting that LNM35 cells can also spread via other mechanisms. N15 tumors overexpressing VEGF-C contained more lymphatic vessels than vector-transfected tumors but did not have increased metastatic ability. **Conclusions:** Lymph node metastasis appears to be regulated by additional factors besides VEGF-C. Inhibition of VEGFR-3 signaling can suppress tumor lymphangiogenesis and metastasis to regional lymph nodes but not to lungs. [J Natl Cancer Inst 2002;94:819–25]

Tumor metastasis involves a series of complex processes that include the detachment of tumor cells from the primary tumor mass, microinvasion of tumor cells into stromal tissues, intravasation of tumor cells into the lymphatic or blood vessels, and extravasation and growth of tumor cells in secondary sites (1,2).

The capacity of tumor cells to induce angiogenesis and lymphangiogenesis may regulate the probability of blood vascular or lymphatic metastasis.

The five mammalian vascular endothelial growth factor (VEGF) family members identified to date, VEGF, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF), play crucial roles in the physiologic and pathologic regulation of vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis, and vascular permeability (3–5). Almost all types of cancer cells express VEGF, which uses VEGF receptor 1 (VEGFR-1) and VEGFR-2 for signaling. Associations have been observed for VEGF expression, the vascular density in human tumors, and patient prognosis (3,6). VEGF-C and VEGF-D have been identified as specific lymphangiogenic factors, which bind to and induce tyrosine phosphorylation of VEGFR-3 (7–9). These factors stimulate lymphangiogenesis *in vivo*, for example, in the avian chorioallantoic membrane and in the skin of transgenic mice (10–12). Both factors are produced as prepropeptides that are proteolytically processed to mature forms. The mature factors have higher affinities for VEGFR-3 and can bind to and activate VEGFR-2. Both factors can also exert angiogenic activity through VEGFR-2 (13–15).

VEGFR-3 signaling regulates the formation of the primary cardiovascular network in embryos (16) and the development and growth of the lymphatic system (4,17). Expression of VEGFR-3 becomes restricted mainly to lymphatic vessels after midgestation (18,19). However, VEGFR-3 expression in tumors has been detected in both blood and lymphatic vessels (20–22). An association of VEGF-C expression with tumor lymphangiogenesis and with lymph node metastasis has been observed in many cancers including thyroid, prostate, gastric, colorectal, and lung cancers (2). Several studies have demonstrated that overexpression of VEGF-C or VEGF-D induces lymphangiogenesis and promotes tumor metastasis in mouse tumor models (23–26). These results provide one mechanism for the observed involve-

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ment of lymphatic vessels in tumor metastasis. Furthermore, a soluble VEGFR-3-Ig fusion protein has been shown to inhibit VEGF-C-induced tumor lymphangiogenesis (26). To investigate whether the inhibition of lymphangiogenesis is sufficient to block tumor metastasis, we studied the human lung cancer cell line NCI-H460-LNM35, because it has a high frequency of lymphatic metastasis (27), and its parental cell line NCI-H460-N15, which has a low metastatic capacity.

## MATERIALS AND METHODS

### Cell Lines and Transfections

The human lung cancer cell line NCI-H460-LNM35 (hereafter LNM35) is a subline of NCI-H460-N15 (hereafter N15), a human large-cell carcinoma of the lung, established as previously described (27,28). LNM35 cells and N15 cells were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% fetal bovine serum (Autogen Bioclear, Calne, U.K.). LNM35 cells were transfected with the pEBS7/VEGFR-3-Ig vector (26) or with the pEBS7 vector, and N15 cells were transfected with the pEBS7/VEGF-C (26) vector or with pEBS7 by the use of liposomes (FuGENE 6; Roche, Indianapolis, IN). Transfected cells were then selected in hygromycin (400 µg/mL; Calbiochem, La Jolla, CA) until resistant cell pools were obtained.

### Xenotransplantation and Analysis of Tumors

All experiments performed on animals were in accord with institutional guidelines. Approximately  $1.0 \times 10^7$  LNM35/VEGFR-3-Ig, LNM35/pEBS7, N15/VEGF-C, or N15/pEBS7 cells in 100 µL serum-free medium were implanted in the subcutaneous tissue of the right abdominal wall of female severe combined immunodeficient (SCID) mice (7–8 wk old, one tumor per mouse,  $n = 6$  mice per group). Tumors were measured with digital calipers, and the tumor volumes (as cubic millimeters) were calculated as follows: volume = length  $\times$  width<sup>2</sup>  $\times$  0.52. Mice were killed after 6 weeks, and tumors, some internal organs including the lungs, and axillary lymph nodes were collected. The length and width of lymph nodes were also measured, and the lymph node volumes (as cubic millimeters) were calculated as volume =  $(\pi/6) \times (\text{length} \times \text{width})^3/2$ , as described (29). Samples of each tumor were snap-frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  for protein analysis or fixed immediately in 4% paraformaldehyde overnight at  $4^\circ\text{C}$  and then processed for further histologic analysis.

In separate experiments, approximately  $1.0 \times 10^7$  LNM35 cells were also subcutaneously implanted into SCID mice (one tumor per mouse). Recombinant adenoviruses expressing the VEGFR-3-Ig fusion protein (AdR3-Ig) (26) or  $\beta$ -galactosidase (AdLacZ) (30) ( $10^9$  plaque-forming units) were administered via the tail vein on the day of tumor implantation ( $n = 14$  and 7 mice, respectively). Blood from the treated and control mice was collected, and the serum concentration of VEGFR-3-Ig was determined by enzyme-linked immunosorbent assay (ELISA), as previously described (31). Mice were killed 5 weeks later, and their tissues were collected and processed as above.

### Histology

Paraffin sections (6 µm) of tumors were immunostained with monoclonal antibodies against platelet endothelial cell adhesion molecule 1 (PECAM-1; PharMingen, San Diego, CA), poly-

clonal antibodies against lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (32) (a gift from Dr. David G. Jackson, University of Oxford, U.K.; and from Dr. Erkki Ruoslahti, The Burnham Institute, La Jolla, CA), or biotin-conjugated mouse anti-human IgG1 antibodies (Zymed, San Francisco, CA) as previously described (21). Sections of the lungs and axillary lymph nodes were stained with hematoxylin and eosin.

### Immunoprecipitation and Western Blot Analysis

The cells were metabolically labeled in methionine-free and cysteine-free modified Eagle medium supplemented with [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine (Promix, Amersham Pharmacia Biotech, Uppsala, Sweden) at 100 µCi/mL for about 8 hours. Conditioned medium was then harvested and cleared of particulate material by centrifugation. VEGF-C was immunoprecipitated from the medium with polyclonal antibodies against VEGF-C (33), and then the antigen-antibody complexes were incubated with protein A-Sepharose (Amersham Pharmacia Biotech). Complexes were then washed twice with 0.5% bovine serum albumin/0.02% Tween 20 in phosphate-buffered saline (PBS), washed once with PBS, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

Expression of the soluble VEGFR-3-Ig fusion protein in conditioned medium from cell cultures and cell lysates from tumor samples was examined. Briefly, frozen tumor samples were homogenized on ice in the lysis buffer (20 mM Tris-HCl [pH 7.6], 1 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton-X100, supplemented with 1 mM phenylmethylsulfonyl fluoride, aprotinin [1 mU/mL], 1 mM Na<sub>3</sub>VO<sub>4</sub>, and leupeptin [10 µg/mL]). The fusion protein in LNM35/VEGFR-3-Ig cell-conditioned medium or tumor lysates (equal amounts of total protein [1.5 mg]) was bound to protein A-Sepharose, as determined by the BCA protein assay (Pierce, Rockford, IL). Bound proteins were dissolved in reducing SDS-PAGE sample buffer, separated by SDS-PAGE in 8% gels, and transferred to nitrocellulose. Blots were probed with horseradish peroxidase-conjugated goat anti-human IgG (Fc) antibody (KPL, Gaithersburg, MD) and developed with the enhanced chemiluminescence detection system.

### Analysis of RNA

RNA was extracted from LNM35 and N15 cells, and northern blot and hybridization analyses were performed. Complementary DNA probes were generated by a polymerase chain reaction using the following primers: VEGF sense primer = AGCTC-TAGACATCACGAAGTGGTGAAGTT and antisense primer = AGCGAATTCTTGTCACATCTGCAAGTACG; VEGF-B sense primer = AGCTCTAGAAGATGTGTATACTCGCGCTA and antisense primer = AGCGAATTCCTTGGAACGGAGGAAGCT; VEGF-C sense primer = AGCTCTAGACCAGTGTAGAT-GAACTCATG and antisense primer = AGCGAATTCAGCCAG-GCATCTGCAGATGT; VEGF-D sense primer = AGCTCTA-GATGTAAGTGCTTGCCAACAGC and antisense primer = AGCGAATTCAGCGCAATGCTTTGCACAT.

### Statistical Analysis

Statistical analysis was performed with the unpaired *t* test. All statistical tests were two-sided.

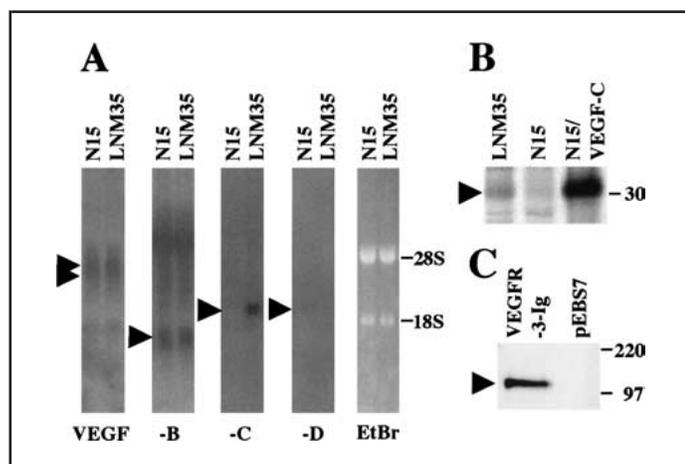
## RESULTS

### Analysis of VEGF-C and VEGF-D Expression

We examined the expression of VEGFs in LNM35 cells, which, in mice, consistently show lymphogenous metastasis, and its parental cell line, N15, which is derived from a human large-cell carcinoma of the lung and does not show lymphogenous metastasis (27). By use of northern blot and hybridization analyses, we detected equal levels of VEGF and VEGF-B mRNA in both cell lines, but the level of VEGF-C mRNA was considerably higher in LNM35 cells than in N15 cells (Fig. 1, A). VEGF-D mRNA was not detected in either line. Enhanced secretion of VEGF-C protein by LNM35 cells was confirmed by immunoprecipitation with polyclonal antibodies against VEGF-C (Fig. 1, B). We also confirmed that N15 cells transfected with a VEGF-C expression vector secreted high levels of VEGF-C into the culture medium (Fig. 1, B).

### Establishment of LNM35 Cells Overexpressing VEGFR-3-Ig

To study the effect of soluble VEGFR-3-Ig expression on tumor lymphangiogenesis, LNM35 cells were transfected with the pEBS7/VEGFR-3-Ig expression vector, and pools of hygromycin-selected cell clones were isolated. These pools were characterized for protein secretion by metabolic labeling and immunoprecipitation (data not shown) and by western blot analysis. VEGFR-3-Ig fusion protein was detected in serum-free medium conditioned by VEGFR-3-Ig-transfected cells (LNM35/VEGFR-3-Ig cells) but not in that of LNM35 cells transfected with pEBS7 (LNM35/pEBS7) (Fig. 1, C). Cells from both pools were microscopically indistinguishable and had similar growth rates *in vitro* (data not shown).



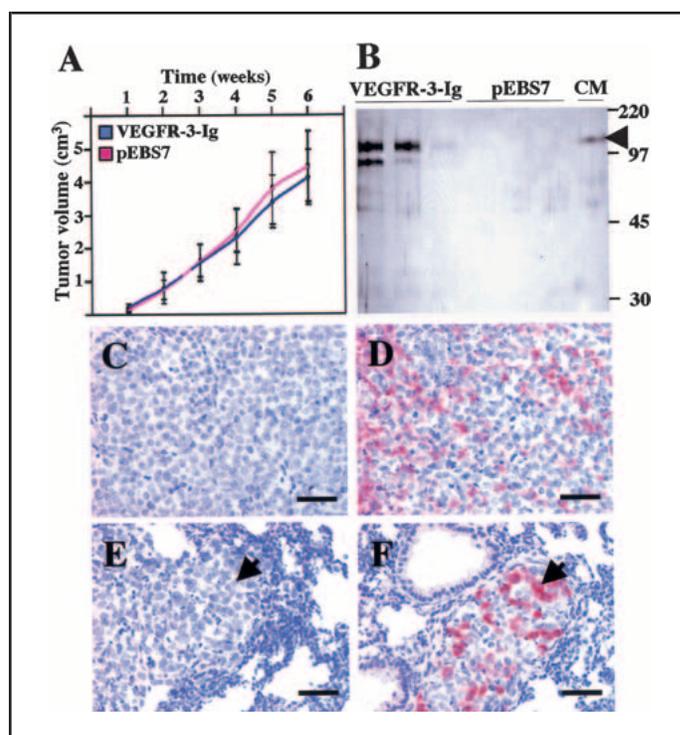
**Fig. 1.** Analysis of vascular endothelial growth factors (VEGFs) produced by the tumor cells and secretion of recombinant proteins by the transfected cells. (A) Northern blot and hybridization analyses of VEGF mRNAs (arrowheads), as indicated, in LNM35 and N15 cells. Lane EtBr = total RNA stained with ethidium bromide. (B) Immunoprecipitation analysis of secreted VEGF-C protein in the conditioned medium from the LNM35, N15, and N15/VEGF-C cells. Bands were visualized by exposure to Kodak Biomax MR film. (C) Analysis of the vascular endothelial growth factor receptor-3-Ig (VEGFR-3-Ig) fusion protein (arrowhead) in the conditioned medium of LNM35 cells transfected with the pEBS7/VEGFR-3-Ig or pEBS7 vector plasmids. Bands were visualized by exposure to Fuji x-ray film.

### Persistent Expression of VEGFR-3-Ig in Tumors

LNM35/pEBS7 and LNM35/VEGFR-3-Ig cells were subcutaneously injected into the right abdominal wall of SCID mice. As shown in Fig. 2, A, the tumors formed by the two cell lines *in vivo* had similar growth rates. Tumors were excised 6 weeks after implantation, and tumor lysates were analyzed by western blotting with antibodies against the Fc portion of human IgG. Expression of VEGFR-3-Ig protein was detected in LNM35/VEGFR-3-Ig tumors (Fig. 2, B) and then confirmed by immunohistochemical analysis of tumors (Fig. 2, D) and lung metastases (Fig. 2, F). No staining was obtained in tumors or metastases generated by vector-transfected cells (Fig. 2, C and E).

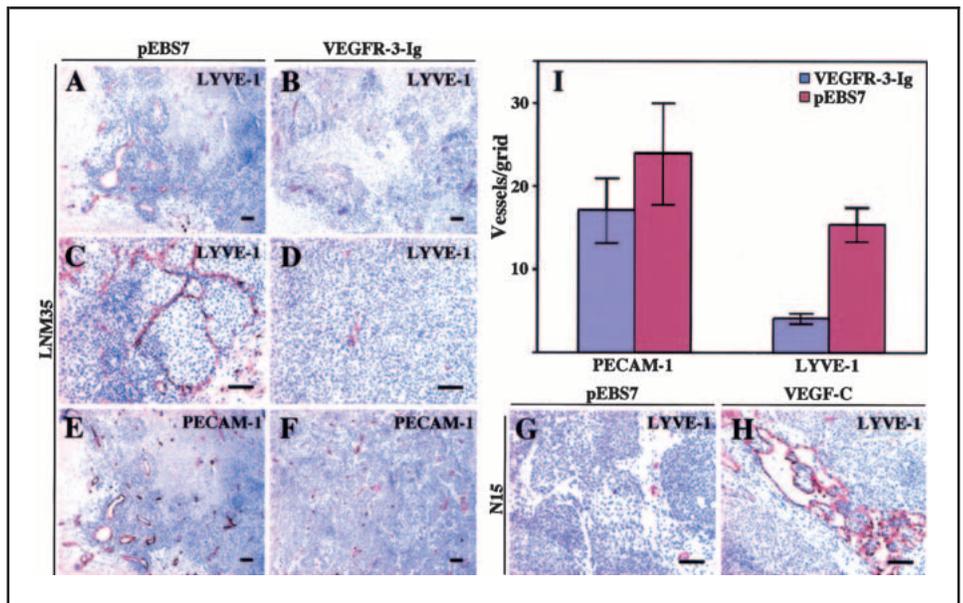
### Inhibition of Tumor Lymphangiogenesis by VEGFR-3-Ig

Lymphatic vessels in the tumors were analyzed by immunostaining with antibodies against the lymphatic endothelial marker LYVE-1 (32,34). LYVE-1-positive vessel-like structures were found in control LNM35 tumors (Fig. 3, A), often in clusters, and hyperplastic lymphatic vessels filled with tumor cells were observed in the tumor periphery (Fig. 3, C). In contrast, few LYVE-1-positive vessel-like structures were observed in VEGFR-3-Ig tumors (Fig. 3, B and D). The mean number of LYVE-1-positive vessels was determined from three microscopic fields with the highest vessel density, as shown in Fig. 3, I, as follows: VEGFR-3-Ig = 4.1 vessels per grid (95% confi-



**Fig. 2.** Analysis of tumor growth and expression of soluble vascular endothelial growth factor receptor 3 Ig (VEGFR-3-Ig). (A) Mean volumes and 95% confidence intervals (error bars) for tumors generated with the LNM35/VEGFR-3-Ig and LNM35/pEBS7 cells presented as a function of time ( $n = 6$  tumors). (B) Western blot analysis of lysates from three VEGFR-3-Ig tumors and three control tumors using horseradish peroxidase-conjugated goat anti-human IgG(Fc) antibodies. Lane CM = LNM35/VEGFR-3-Ig cell conditioned medium used as a positive control. (C-F) Immunohistochemical staining for human IgG1 in LNM35 control (C) and VEGFR-3-Ig tumor (D) sections and in lung sections (E, F) from the respective mice. Arrows = metastatic tumor cells in the lungs. Bars in (C-F) = 50  $\mu$ m.

**Fig. 3.** Blood and lymphatic vessels in the LNM35 and N15 tumors. Immunohistochemical staining is shown for LYVE-1 (A–D, G, H) to identify lymphatic vessels and for PECAM-1 (E, F) to identify both blood and lymphatic vessels. (A, C, E) Sections of the control LNM35/pEBS7 tumors. (B, D, F) Sections from the LNM35/vascular endothelial growth factor receptor-3-Ig (VEGFR-3-Ig) tumors. (G, H) Sections of the N15/pEBS7 and N15/VEGF-C tumors, respectively. (I) LYVE-1-stained or PECAM-1-stained vessels in three microscopic fields of the highest vessel density were counted, and the results were compared by use of the unpaired *t* test. **Error bars** = 95% confidence intervals. There is a statistically significant decrease of LYVE-1-stained vessels ( $P < .001$ ) and PECAM-1-stained vessels ( $P = .022$ ) in VEGFR-3-Ig-expressing tumors. **Bars** in (A, B, E, F) = 100  $\mu\text{m}$ ; in (C, D, G, H) = 80  $\mu\text{m}$ .



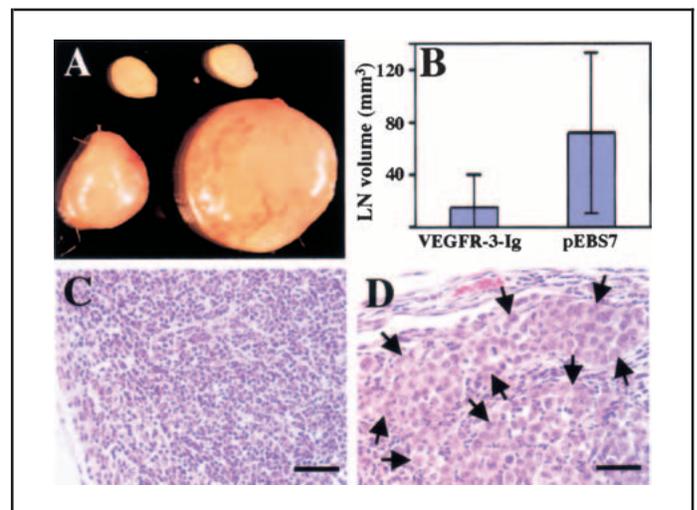
dence interval [CI] = 3.4 to 4.7) and control = 15.3 (95% CI = 13.3 to 17.4;  $n = 6$  tumors per group;  $P < .001$ , two-sided *t* test). PECAM-1 (Fig. 3, E and F), a panendothelial marker, detected a statistically significant difference in vessel density between the control LNM35 tumors and transfected LNM35 tumors secreting soluble VEGFR-3-Ig, as shown in Fig. 3, I, as follows: VEGFR-3-Ig = 17.1 vessels per grid (95% CI = 13.2 to 20.9) and control = 23.9 vessels per grid (95% CI = 17.7 to 30.0;  $n = 6$ ;  $P = .022$ ). A slight trend toward higher PECAM-1 vessel density and the occurrence of larger vessels in the vector-transfected tumors may depend on the fact that lymphatic vessels were also stained by the anti-PECAM-1 antibodies. Inhibition of intratumoral and peritumoral lymphangiogenesis was also observed in mice treated with AdR3-Ig (data not shown).

Much lower levels of lymphangiogenesis were detected in N15 tumors than in LNM35 tumors (Fig. 3, G). However, hyperplastic lymphatic vessels were evident between the expanding tumor foci in the N15/VEGF-C tumors (Fig. 3, H).

### Suppression of Axillary Lymph Node Metastasis

Fig. 4, A, shows representative axillary lymph nodes found in tumor-bearing mice. The mean lymph node volume was 14.6  $\text{mm}^3$  (95% CI = 0 to 39.9) in the VEGFR-3-Ig group ( $n = 12$  lymph nodes total), and the mean volume was 71.6  $\text{mm}^3$  (95% CI = 10.7 to 132.6) in the control group ( $n = 12$  lymph nodes total; Fig. 4, B). One large metastatic lymph node was detected in the VEGFR-3-Ig group, but only low levels of soluble receptor were detected in the corresponding tumor. Thus, a statistically significant difference was not found in lymph node sizes between the two groups ( $P = .070$ ). Histologic analysis revealed that all 12 lymph nodes from the control group but only four of 12 lymph nodes from the VEGFR-3-Ig group contained metastases. Typical lymph node sections stained with hematoxylin and eosin are shown in Fig. 4, C and D.

LNM35 tumor-bearing mice treated with AdR3-Ig had high serum concentrations of the VEGFR3-Ig fusion protein (Fig. 5, A). Six of seven mice receiving AdLacZ in the control group showed macroscopic lymph node metastasis, and 11 of 14 axillary lymph nodes examined were histologically confirmed to contain metastasized cells. However, no macroscopic or mi-



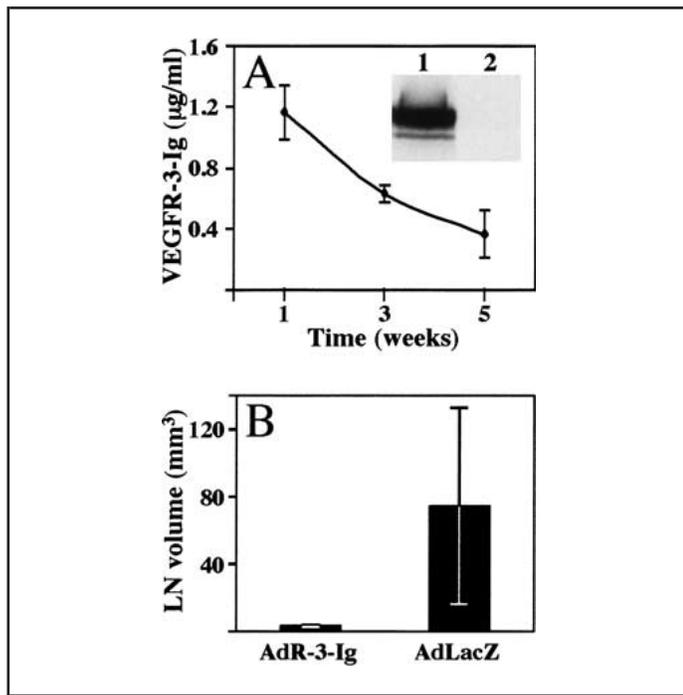
**Fig. 4.** Macroscopic and microscopic analysis of axillary lymph nodes. (A) Typical lymph nodes in mice bearing the vascular endothelial growth factor receptor 3 Ig (VEGFR-3-Ig) tumors (upper pair) and control LNM35 tumors (lower pair). (B) Lymph node (LN) volume and 95% confidence intervals (error bars) ( $n = 12$ ) ( $P = .070$ ). (C, D) Histologic staining of lymph node sections from mice with the VEGFR-3-Ig-overexpressing and control tumors, respectively. **Arrows** = tumor cells in the lymph node. **Bars** in C and D = 50  $\mu\text{m}$ .

croscopic metastases were observed in 14 mice treated with AdR3-Ig. In the AdR3-Ig-treated group, the mean lymph node volume was 3.2  $\text{mm}^3$  (95% CI = 2.8 to 3.7,  $n = 14$ ), and in the control group the mean lymph node volume was 74.26  $\text{mm}^3$  (95% CI = 16.0 to 132.5;  $n = 7$  mice total; Fig. 5, B). A statistically significant difference in the lymph node sizes was found between the two groups ( $P < .001$ ).

No difference in lymph node size was observed between the N15/VEGF-C and N15/pEBS7 groups (data not shown), and metastasis to the lymph nodes was not increased in mice bearing N15/VEGF-C tumors.

### Lung Metastasis

Numerous metastatic tumor nodules in the lungs were observed in the VEGFR-3-Ig-expressing group (Fig. 6, A and B)



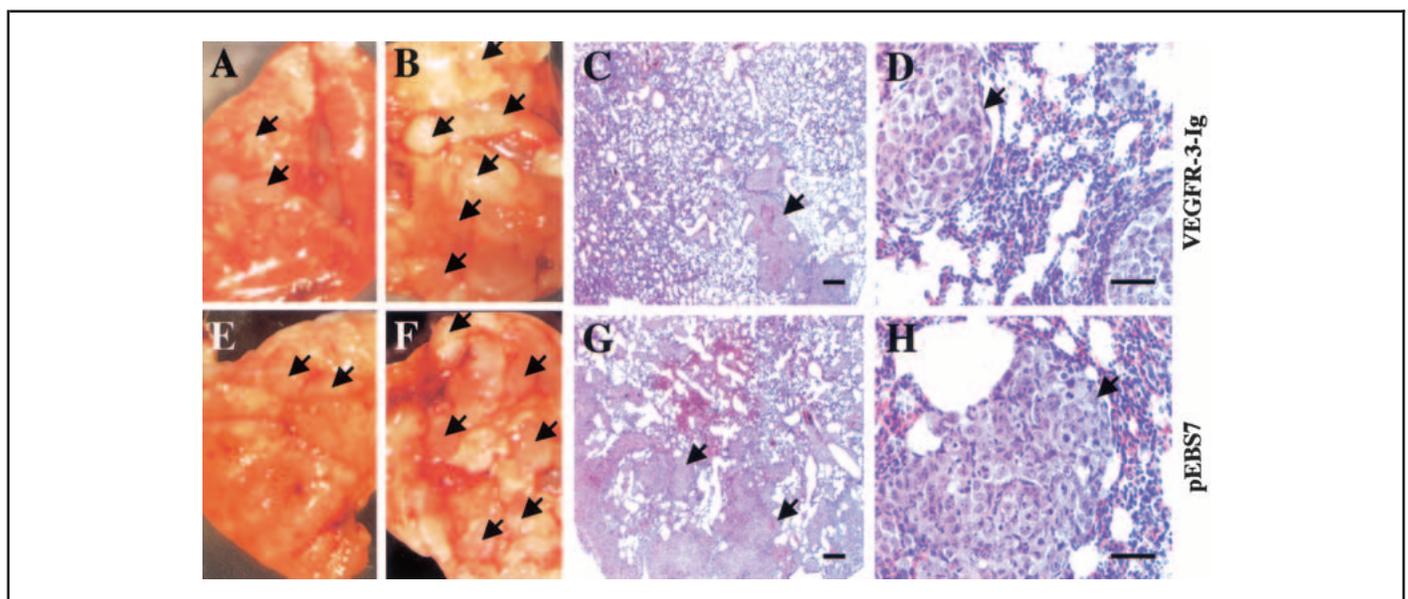
**Fig. 5.** Inhibition of lymph node metastasis by adenoviral expression of the vascular endothelial growth factor receptor 3 Ig (VEGFR-3-Ig) fusion protein. (A, inset) Immunoprecipitation of the VEGFR-3-Ig fusion protein from the supernatant of metabolically labeled LNM35 cells infected with AdR3-Ig (recombinant adenoviruses expressing the VEGFR-3-Ig fusion protein) (lane 1) and AdLacZ (recombinant adenoviruses expressing the  $\beta$ -galactosidase) (lane 2). The immunoprecipitated complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (A) Serum concentration of VEGFR-3-Ig at 1 week, 3 weeks, and 5 weeks after the administration of AdR3-Ig. AdLacZ-infected mice had no measurable level of immunoreactive protein. (Error bars = 95% confidence intervals [CIs].) (B) Lymph node (LN) volume and 95% CIs (error bars) (n = 14 for AdR3-Ig; n = 7 for control;  $P < .001$ ).

and the control group (Fig. 6, E and F). Lung metastases were also observed in AdR3-Ig-treated mice and control mice. No statistically significant difference was observed in the number of tumor nodules between the two groups (data not shown). More visible tumor nodules were always found on the anterior side of the lungs (Fig. 6, B and F) than on the posterior side (Fig. 6 A and E). Histologic analysis revealed that metastatic cells invaded lung tissue and did not grow just by expansion (Fig. 6, C, D, G, and H).

## DISCUSSION

This study demonstrates, to our knowledge for the first time, that inhibition of VEGFR-3 signaling with a soluble fusion protein, VEGFR-3-Ig, can suppress tumor lymphangiogenesis and lymphatic metastasis but not lung metastasis. Thus, the mechanisms of lymphatic and lung metastasis may differ, at least in this model. According to our results, the overexpression of VEGF-C and the associated *de novo* formation of lymphatic vessels are necessary but not sufficient for the metastatic dissemination of tumor cells to the lymph nodes. Factors in addition to VEGF-C are thus apparently required for metastatic spread.

LNM35 cells can spontaneously metastasize to regional lymph nodes and lungs with an incidence of 100%, even when subcutaneously implanted (27). Because VEGF family members play important roles in tumor angiogenesis, growth, lymphangiogenesis, and metastasis, we compared expression of the VEGF family members in the highly metastatic LNM35 cells with that in the less metastatic parental N15 cells. Northern blot and immunoprecipitation analyses demonstrated that VEGF-C expression was higher in LNM35 cells than in N15 cells, but VEGF-D mRNA was not detected in either cell line. In addition, essentially no difference was detected in the expression of VEGF and VEGF-B in the two cell lines. Consistent with the VEGF-C expression results, LYVE-1-positive vessel-like structures were found at a much higher density in LNM35 tumors than in N15 tumors. These findings are in agreement with the



**Fig. 6.** Macroscopic (A, B, E, F) and microscopic (C, D, G, H) analysis of lung metastasis from mice bearing the vascular endothelial growth factor receptor 3 Ig (VEGFR-3-Ig) (A–D) or control LNM35 tumors (E–H). Arrows = metastatic tumor nodules. Bars in (C, G) = 100  $\mu$ m; in (D, H) = 50  $\mu$ m.

recently published direct evidence for the role of VEGF-C in tumor lymphangiogenesis and tumor cell dissemination (23,24,26). Thus, enhanced VEGF-C expression may at least partially account for the invasion of lymphatic vessels by LNM35 cells.

Overexpression of VEGF-C in the low metastatic N15 cells was not sufficient to convert these cells to a high metastatic phenotype, and thus, additional factors may also regulate the metastatic phenotype. Our previous work showed differences in gene expression profiles between LNM35 cells and parental N15 cells (28). Enhanced expression of cyclooxygenase-2, an enzyme associated with tumor metastasis, was detected in LNM35 cells compared with N15 cells. Furthermore, N15 cells grow as clusters but LNM35 cells are only loosely attached to each other (27), and no obvious change of cell morphology was observed in N15 cells after transfection with the VEGF-C expression vector (data not shown). Such observations indicate that decreased expression of adhesion molecules may contribute to the metastatic phenotype. Finally, it is also possible that microenvironmental factors in subcutaneous tissues do not favor metastasis of the VEGF-C-overexpressing N15 lung carcinoma cells.

Hyperplastic lymphatic vessels filled with tumor cells were observed in the periphery of the LNM35 tumors. These vessels resembled the lymphatic vessels previously observed in VEGF-C-overexpressing tumors (26). In addition, lymphatic vessel clusters with open lumens were present but not evenly distributed in the tumor. This observation may reflect variations in the local tumor microenvironment, such as differences in growth factor concentrations. In contrast, in clinical cancer, intratumoral lymphatic vessels are rare (35) although regional lymph node metastases are frequently observed. Thus, peritumoral lymphatics may be responsible for lymphatic tumor spread. It has been proposed that lymphatics collapse within the tumor because of mechanical stress generated by proliferating cancer cells and the high interstitial pressure caused by plasma leakage (2,20). Thus, at least some of the intratumoral lymphatic vessels in the control LNM35 tumors may result from the trapping of peritumoral lymphatic vessels in between the growing tumor foci.

Expression of the VEGFR-3-Ig fusion protein under control of the K14 promoter in transgenic models induces apoptosis in lymphatic endothelial cells and results in the regression of lymphatic vessels formed in embryonic skin (31). However, the blood vasculature was not affected. Soluble VEGFR-3-Ig also blocked the lymphatic hyperplasia in the skin of doubly transgenic mice overexpressing VEGF-D or a mutant form of VEGF-C (VEGF-C156S) (12). These results suggested that inhibition of VEGFR-3 signaling could inhibit developmental lymphangiogenesis. To investigate whether blocking VEGFR-3 signaling affected the metastatic phenotype, we used VEGFR-3-Ig vector-transfected LNM35 cells, which persistently expressed the soluble receptor in the tumors during the study. Greater inhibition of tumor-associated lymphatic vessels was obtained in tumors produced by these cells than in tumors from the LNM35 vector-transfected cells. Intratumoral and peritumoral lymphangiogenesis was also inhibited by adenoviral expression of VEGFR-3-Ig in the liver (data not shown). This result is consistent with the inhibition of peritumoral lymphatic vessels in VEGF-C-transfected MCF-7 breast carcinoma xenografts of SCID mice infected with the recombinant adenovirus (26).

Only lymphatic vessels were stained by anti-VEGFR-3 antibodies in the skin and peritumoral areas, whereas both lymphatic

and blood vessels were VEGFR-3 positive in the tumors (data not shown). VEGFR-3 induction in endothelial cells of tumor blood vessels may play a role in tumor angiogenesis (20–22,36). However, in this study, the soluble VEGFR-3 fusion protein overexpressed in the tumor cells did not affect the tumor growth rate, and there was also essentially no difference in the density of intratumoral blood vessels. This result agrees with the previous result, that a soluble form of VEGFR-3 (VEGFR-3-Ig) did not inhibit tumor angiogenesis or growth (37). Thus, VEGFR-3 expressed in the endothelial cells of tumor blood vessels may have a redundant function in tumor angiogenesis, at least in some tumors.

Tumor metastasis to regional lymph nodes is common in many types of human cancers, and an association between lymphangiogenesis and tumor metastasis recently has been shown (23–26). Our study extends these findings by showing that inhibition of lymphangiogenesis suppresses lymph node metastasis of a tumor that secretes high levels of endogenous VEGF-C. Histologic analysis demonstrated that most axillary lymph nodes of mice bearing the control LNM35 tumors were almost completely occupied by the tumor cells, but only one lymph node had macroscopic evidence of metastasis in mice bearing VEGFR-3-Ig-expressing tumors. A similar observation was also made in mice with a recombinant adenovirus expressing the VEGFR-3-Ig fusion protein that was injected via the tail vein at the same time that LNM35 cells were implanted subcutaneously. Strikingly, no macroscopic or microscopic metastases were observed in the axillary lymph nodes of the AdR3-Ig-treated mice by 5 weeks after tumor implantation. However, macroscopically detectable lymph node metastases were observed in control mice injected with AdLacZ. Yet lung metastases occurred in all these mice. Thus, LNM35 tumor cells can also use other mechanisms or routes, such as the bloodstream, for metastasis. Infiltration of LNM35 tumor cells into blood vessels has been described previously (27) and was also observed in this study (data not shown).

The spread of tumor cells to local lymph nodes is an early event in tumor metastasis. Results of our study show that VEGF-C expression is increased in a tumor cell line selected for lymphatic metastasis. Our results also provide, to our knowledge for the first time, direct evidence for the hypothesis that inhibition of VEGFR-3 signaling can block tumor lymphangiogenesis and suppress lymph node metastasis. However, in our cells, VEGF-C was not the only metastasis rate-limiting factor. Furthermore, evidence was obtained that the inhibition of visceral metastasis requires the blocking of other routes, such as tumor spreading via blood vessels. Further studies on the mechanisms underlying the initiation of tumor cell dissemination and the interactions of tumor cells with blood and lymphatic endothelial cells should advance our current understanding of cancer invasion.

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## NOTES

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