Suppression of Tumor Growth and Metastasis by Simultaneously Blocking Vascular Endothelial Growth Factor (VEGF)--A and VEGF-C with a Receptor-Immunoglobulin Fusion Protein

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Abstract

The major cause of cancer mortality is the metastatic spread of tumor cells that can occur via multiple routes, including the vascular system and the lymphatic system. In this study, we developed an IgG-like fusion protein molecule [vascular endothelial growth factor (VEGF) receptor 31–immunoglobulin (VEGFR31-Ig)] which could simultaneously bind the angiogenic growth factor VEGF-A and the lymphangiogenic growth factor VEGF-C. Importantly, VEGFR31-Ig exhibited VEGF-A-binding affinity similar to that of VEGFTrap, the most potent VEGF-A binder, and VEGF-C-binding affinity comparable with that of the soluble fusion protein VEGFR3-Ig (sVEGFR3). Pharmacokinetic analysis in mice showed that VEGFR31-Ig had improved pharmacokinetic properties compared with either VEGFTrap or sVEGFR3. In a highly metastatic human hepatocellular carcinoma (HCCLM3) model in severe combined immunodeficient mice, VEGFR31-Ig potently blocked both tumor angiogenesis and lymphangiogenesis, effectively inhibiting primary tumor growth and metastasis to lungs and lymph nodes. In contrast, VEGFTrap only suppressed primary tumor growth and metastasis to lungs by inhibiting tumor angiogenesis, whereas VEGFR3 was only effective in suppressing tumor metastasis to lymph nodes by blocking tumor lymphangiogenesis. Although a combination of VEGF-Trap (25 mg/kg twice weekly) and sVEGFR3 (25 mg/kg twice weekly) can achieve the same therapeutic effect as VEGFR31-Ig (25 mg/kg twice weekly) in the HCCLM3 xenograft mouse model, developing two separate receptor-Ig fusion proteins for clinical use as combination therapy is impractical, mainly owing to regulatory hurdles and cost. Taken together, the VEGFR31-Ig fusion protein presented here has been suggested to have great potential for the treatment of metastatic cancer. Cancer Res; 70(6); 2495–503. ©2010 AACR.

Introduction

The development of a vascular supply is a key factor in the growth and metastatic spread of malignant tumor (1–3). Vascular endothelial growth factor-A (VEGF-A) is a potent angiogenic cytokine with critical roles in tumor angiogenesis. VEGF-A mediates its biological effects by interacting with two high-affinity transmembrane tyrosine kinase receptors [VEGF receptor-1 (VEGFR-1) and VEGFR-2] that are selectively, although not exclusively, expressed by vascular endothelium (4, 5). Blockade of the VEGF-A pathway has been achieved by many different means, such as blocking antibodies targeted against VEGF-A (6) and soluble decoy receptors that prevent VEGF-A from binding to its normal receptors (7). A humanized monoclonal anti-VEGF-A antibody (8), bevacizumab (Avastin), has been approved for use in treating colorectal cancer, breast cancer, and lung cancer. Aflibercept (VEGFTrap) is a derivative of the most potent VEGF-A binder, VEGFR1. Soluble forms of FVGR1 suffer from poor pharmacokinetic properties, which seem to correlate with their non-specific interactions with extracellular matrix. VEGFTrap was engineered to have dramatically enhanced pharmacokinetic profile (9). It has much higher VEGF-A-binding affinity than bevacizumab and has shown ability to potently suppress tumor growth and vascularization in vivo (9). Currently, VEGFTrap is being tested in clinical trials for a wide range of solid tumors.
The metastatic spread of tumor cells is known to occur via both blood and lymphatic vessels (10). In various carcinomas, the presence of tumor foci in lymph nodes is the most important adverse prognostic factor in apparently localized disease (11). However, lymphangiogenesis has traditionally been overshadowed by the greater emphasis placed on angiogenesis. This is in part due to the lack of identification of lymphangiogenic factors, as well as suitable markers that distinguish blood from lymphatic vascular endothelium (12). In the past decade, there has been a dramatic increase in studies of mechanisms of tumor-associated lymphangiogenesis and lymphatic metastasis. VEGF-C, a member of the VEGF family, is the first lymphangiogenic growth factor to be identified. Mice lacking both VEGF-C alleles showed failure in lymphatic vessel development and died of edema (13). VEGF-C overexpression in cancer cells significantly increases tumor-associated lymphangiogenesis, resulting in enhanced metastasis to regional lymph nodes (14–16).

VEGF-C stimulates lymphangiogenesis and lymphatic endothelial cell growth and migration upon binding to its receptor, VEGFR3, a receptor tyrosine kinase that is similar to VEGFR1 and VEGFR2 in structure but does not bind VEGF-A (11, 17). VEGFR3, which has been proposed as a marker for lymphatic endothelial cells, is expressed throughout the embryonic vasculature, but during development and in the adult, its expression is limited to lymphatic endothelial cells (5, 18). The induction of lymphangiogenesis by the VEGF-C/VEGFR3 axis increases tumor metastasis via the lymphatic system (14, 19). Blockade of VEGF-C/VEGFR3 signaling has been shown to potently inhibit tumor lymphangiogenesis and metastases in many metastatic tumor models (20–22).

In the present study, we first developed a receptor-immunoglobulin (Ig) fusion protein, which could bind VEGF-A and VEGF-C simultaneously. This fusion protein, denoted as VEGFR31-Ig, was shown to potently block both tumor angiogenesis and lymphangiogenesis in a highly metastatic hepatocellular carcinoma severe combined immunodeficient (SCID) mouse model. The ability of VEGFR31-Ig to inhibit tumor growth and metastasis was further evaluated in this model. Our findings suggest a potential promise for the strategies that combine targeting angiogenic and lymphangiogenic growth factors for the treatment of metastatic cancer.

Materials and Methods

Materials. Human hepatocellular carcinoma cell lines HepG2, Huh7, and Hep3B and Chinese hamster ovary (CHO-K1) cell line were purchased from American Type Culture Collection. HCCLM3, a human hepatocellular carcinoma cell line with highly metastatic potential (23, 24), was obtained from the Liver Cancer Research Institute and Zhong Shan Hospital of Fudan University. These cell lines were obtained within 1 y before being used in this study. All the cell lines were authenticated twice by morphologic and isoenzyme analyses during the study period. Cell lines were routinely checked for contamination by Mycoplasma using Hoescht staining and were consistently found to be negative. Human umbilical vein endothelial cells (HUVEC) and human dermal lymphatic endothelial cells (HDLEC) were purchased from Cascade Biologics and ScienCell Research Laboratories, respectively. Recombinant human VEGF165 and VEGF-C were purchased from R&D System. VEGF-C was lyophilized from a 0.2-μm filtered solution in PBS. It is the proteolytically processed form (amino acid residues Thr103 to Arg227 and containing a Cys156Ser substitution) with a 10× histidine tag at the COOH terminus. Six-week-old female C57BL/6 mice and male BALB/c SCID mice were obtained from the Planned Parenthood Research Institute. All animals in this study were housed in pathogen-free conditions and were treated in accordance with the guideline of the Committee on Animals of the Second Military Medical University.

Construction, expression, and purification of Trap proteins. The VEGFR1-R2 fusion gene was generated by genetically fusing the second Ig domain of VEGFR1 to the third Ig domain of VEGFR2. The VEGFR1-R2 gene was fused in frame to the 5′ terminus of the human IgG1 Fc gene, and the resulting fusion gene was cloned into the pcDNA3.1 vector (Invitrogen), yielding the expression vector for VEGF-Trap (Fig. 1A). Similarly, the expression vector for sVEGFR3...
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(Fig. 1A) was obtained using the same method as described above, except that the VEGFR1R2 gene was replaced with the gene encoding the first three Ig domains of VEGFR3. VEGFR31g (the first two domains of VEGFR3), VEGFR1R2s, and IgG1 Fc domain were genetically linked in tandem to generate the VEGFR31-Fc gene, and then this fusion gene was inserted into the pcDNA3.1 vector, yielding the expression vector for VEGFR31-Fc (Fig. 1A). To construct the expression vector for VEGFR31-Ig, VEGFR31g2, and VEGFR1R2s were genetically fused to the heavy chain constant region of human IgG1 and the human κ light chain constant region, respectively. The resultant heavy and light chain fusion genes were cloned into the pcDNA3.1 vector, yielding the heavy and light chain expression vectors for VEGFR31-Ig (Fig. 1A). For the expression of sVEGF3, VEGF-Trap, and VEGFR31-Ig, their expression vectors were transfected into CHO-K1 cells using Lipofectamine 2000 reagent (Invitrogen). To express VEGFR31-Ig, its heavy and light chain expression vectors were cotransfected into CHO-K1 cells. After transfection, the stable transfecteds were isolated by limiting dilution in the presence of 600 μg/mL G418. The cell clones producing the highest amount of recombinant proteins were selected and grown in serum-free medium. Finally, recombinant proteins were purified by affinity chromatography on protein A-Sepharose (Amersham Biosciences) from the serum-free culture supernatants. The purity of the recombinant proteins was analyzed on 8% SDS-PAGE under nonreducing conditions and on 12% SDS-PAGE under reducing conditions, followed by Coomassie Brilliant Blue staining.

**Binding activity assays.** Different concentrations of VEGF-Trap, sVEGF3, VEGF-Trap, and VEGFR31-Ig were added to 96-well plates coated with VEGF-A (0.2 μg/mL) or VEGF-C (2 μg/mL), followed by incubation at room temperature for 1 h. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (KPL) was added and the plates were further incubated for 1 h. Finally, 3,3′,5,5′-tetramethylbenzidine was added as a substrate for HRP, and the absorbance was measured at 450 nm.

**Binding affinity measurement.** Binding affinities of VEGF-Trap, sVEGF3, VEGF-Trap, and VEGFR31-Ig were determined by the method described by Holash and colleagues (9). Briefly, we measured binding affinities of recombinant fusion proteins for VEGF165 by using a commercially available VEGF-A ELISA kit (R&D Systems) for detecting free human VEGF165 in mixtures of the recombinant proteins (ranging in concentration from 0.1 to 2,000 pmol/L) with human VEGF165 (at 10 pmol/L). Likewise, binding affinities of recombinant fusion proteins for VEGF-C were determined by a VEGF-C ELISA kit (R&D Systems) for detecting free VEGF-C in mixtures of the recombinant proteins (ranging in concentration from 0.06 to 132 nmol/L) with human VEGF-C (at 1 nmol/L).

**Animal study.** HCCLM3 cells (1 × 10⁶ per mouse) were inoculated s.c. into the right flank of male BALB/c SCID mice. One day after inoculation, the mice were randomized into groups of 12. Then the mice were injected s.c. with 25 mg/kg of different fusion proteins twice weekly. Tumors were measured with a caliper every other day, and tumor volume was calculated using the following formula: tumor volume (mm³) = length × (width)² / 2. After 6 wk, animals were euthanized and tumors, lungs, and axillary lymph nodes were collected. The length and width of lymph nodes were measured, and the lymph node volumes were calculated as volume = (a / 6) × (length × width)⁴ / 2, as described previously (20). Samples were fixed in 4% paraformaldehyde overnight at 4°C and then processed for further histologic analysis.

Sections of the lungs and axillary lymph nodes were stained with H&E. Lung metastatic foci were counted as described previously (24). Briefly, a total of 100 serial sections were made for every half of the lung tissue blocks, and the first of every decade of sections were analyzed under the microscope for the presence of the tumor cell clusters.

**Quantification of the vessel density.** Paraffin sections (6 μm) of tumors were immunostained with a rat anti-mouse CD31 antibody (BioLegend) or a rat anti-mouse lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) monoclonal antibody (R&D System). The number of vessels was counted in five fields of the highest vascular density (vascular hotspots) in a section. Microvessel density (MVD) and lymphatic vessel density (LVD) were determined from the mean of CD31-positive vessel counts and LYVE-1-positive vessel counts in three sections of the tumor, respectively.

**Statistical analysis.** Statistical analysis was performed by Student's unpaired t test to identify significant differences unless otherwise indicated. Differences were considered significant at a P value of <0.05.

Details about cell proliferation assays, pharmacokinetic analysis, and quantitative real-time PCR are presented as Supplementary Data.

**Results**

**Construction and characterization of VEGFR-Ig fusion proteins.** The structure of the various Trap proteins is shown in Fig. 1A. The purity and the molecular weight of the purified fusion proteins were determined by SDS-PAGE (Fig. 1B). Under reducing conditions, VEGFR31-Ig yielded two protein bands with molecular mass of 70 kDa (heavy chain) and 34 kDa (light chain), whereas the sVEGFR3, VEGF-Trap, and VEGFR31-Fc migrated as a single band of 75, 60, and 85 kDa, respectively, corresponding approximately to the calculated molecular mass of the monomeric polypeptides. Under nonreducing conditions, a single band of ~210 kDa for VEGFR31-Ig, 150 kDa for sVEGF3, 120 kDa for VEGF-Trap, and 180 kDa for VEGFR31-Fc was observed (Fig. 1B). These results indicated that sVEGF3, VEGF-Trap, or VEGFR31-Fc formed the disulfide-linked dimer and that VEGFR31-Ig was composed of two heavy and light chains held together with disulfide bonds.

Firstly, the binding of VEGFR31-Fc to VEGF-A and VEGF-C was assessed by ELISA. As shown in Fig. 2A and B, VEGFR31-Fc exhibited a markedly reduced VEGF-A–binding activity in comparison with VEGF-Trap and almost totally lost the binding activity to VEGF-C. Because VEGFR31g2 (VEGF-A–binding domain) was directly linked to VEGFR1g2.
(VEGF-C–binding domain) in the structure of VEGFR31-Fc (Fig. 1A), we speculated that these two binding domains might pose substantial steric hindrance to each other, thus affecting the binding activity of VEGFR31-Fc. In an attempt to develop a receptor-Ig fusion protein, which can bind both VEGF-A and VEGF-C with high affinity, next we designed and constructed an IgG-like receptor-Ig molecule, VEGFR31-Ig. In VEGFR31-Ig, VEGFR312 and VEGFR12R23 were, respectively, fused to the constant region of human IgG1 and the human κ light chain constant region (Fig. 1A). Therefore, these two binding domains in VEGFR31-Ig molecule may have the potential to function independently without posing significant steric hindrance to one another. We investigated and compared the binding of VEGFR31-Ig and VEGFR31-Fc to VEGF-A and VEGF-C. The results shown in Fig. 2A and B clearly indicated that VEGFR31-Ig had much better VEGF-A– and VEGF-C–binding activity than that of VEGFR31-Fc. The VEGF-A–binding affinities of VEGFR31-Ig and VEGFR31-Fc were also compared. The data (Fig. 2C) indicated that the VEGF-C–binding affinity of VEGFR31-Ig to VEGF-A was significantly lower than that of VEGFR31-Ig. Further study showed that VEGFR31-Ig not only bound VEGF-A with a comparable affinity to VEGF-Trap but also had the similar VEGF-C–binding affinity as sVEGFR3 (Fig. 2C and D). These results indicated that VEGFR31-Ig could bind both VEGF-A and VEGF-C with high affinity. Therefore, we chose VEGFR31-Ig for further investigation.

**VEGFR31-Ig inhibits cell proliferation induced by VEGF-A and VEGF-C.** We determined the activity of VEGFR31-Ig in inhibiting the growth of VEGF-A– and VEGF-C–stimulated HDLECs. Both VEGF-A– and VEGF-C–induced mitogenesis was blocked in a concentration-dependent manner by VEGFR31-Ig. VEGFR31-Ig suppressed the growth of VEGF-A–stimulated HUVECs with an IC50 value (mean ± SD) similar to that of VEGF-Trap (Fig. 3). It also exhibited similar potency as sVEGFR3 in inhibiting VEGF-C–induced mitogenesis of HDLECs (Fig. 3). The potent ability of VEGFR31-Ig to block the mitogenic activity of VEGF-A and VEGF-C was in consistent with its high affinity for both of the two cytokines.

**Pharmacokinetic properties.** The pharmacokinetics of VEGFR31-Ig were measured in mice and compared with VEGF-Trap and sVEGFR3. As shown in Supplementary Fig. S1, the maximal concentration (Cmax) of VEGFR31-Ig was 10.5 μg/mL, ∼0.25-fold and 1.44-fold higher than those for VEGF-Trap (8.4 μg/mL) and sVEGFR3 (4.3 μg/mL), respectively. Correspondingly, VEGFR31-Ig had an area under the curve (AUC) of 37.91 μg d/mL, which was ∼1.3 times that of VEGF-Trap (28.56 μg d/mL) and 3.7 times that of sVEGFR3 (10.12 μg d/mL).

**VEGFR31-Ig potently suppresses primary tumor growth.** Here we determined VEGF-A and VEGF-C mRNA expression levels by quantitative real-time PCR in several human hepatocellular carcinoma cell lines (HepG2, Huh7, Hep3B, and HCCLM3). Our results indicated that HCCLM3 cells expressed the highest levels of VEGF-A and VEGF-C mRNAs in these cell lines (Supplementary Fig. S2). Next we evaluated the antitumor activity of VEGFR31-Ig in SCID mice bearing HCCLM3 tumors. After s.c. implantation of HCCLM3 cells, mice were allowed a brief recovery period and then
received s.c. injections of 25 mg/kg of VEGF-Trap, sVEGFR3, VEGFR31-Ig, or VEGF-Trap in combination with sVEGFR3 twice weekly for the duration of the experiment (6 weeks). The tumors became palpable ∼7 days after injection, and the tumor formation was 100%. However, the mice treated with VEGFR31-Ig, VEGF-Trap, and VEGF-Trap plus sVEGFR3 showed a longer latency period, taking ∼5 weeks to reach a mean tumor volume of ∼300 mm³, whereas sVEGFR3-, PBS-, and control human IgG-treated groups only took ∼2 weeks (Fig. 4). The tumors in sVEGFR3 treatment group grew at a very fast rate similar to that of those in PBS- or control human IgG-treated mice, showing that sVEGFR3 was ineffective in suppressing primary tumor growth. VEGFR31-Ig, VEGF-Trap, and VEGF-Trap plus sVEGFR3 seemed to be equally effective in inhibiting primary tumor growth (Fig. 4). Compared with control human IgG, these three treatments were shown to significantly arrest primary tumor growth from day 15 onwards ($P < 0.01$, Mann-Whitney test; Fig. 4). Six weeks after tumor inoculation, animals were killed and tumors, lungs, and axillary lymph nodes were harvested and processed for histologic or immunohistochemical examination.

**Inhibition of lung metastases by VEGFR31-Ig.** Sections of the lungs were stained with H&E, and representative lung sections were shown in Fig. 5A. Lung metastases were observed in 10 of 12 (83%) control human IgG-treated mice. Only 1 of 12 (8%) mice injected with VEGFR31-Ig or VEGF-Trap plus sVEGFR3 and 2 of 12 (16%) VEGF-Trap-treated mice had metastatic lesions in the lungs, whereas a high incidence of lung metastasis (11 of 12) was seen in sVEGFR3 group. The number of lung metastatic foci in mice from different treatment groups was also compared. The results shown in Fig. 5B clearly indicated that the number of metastatic foci was significantly reduced by treatment with VEGFR31-Ig, VEGF-Trap, and VEGF-Trap plus sVEGFR3 ($P < 0.01$ for each compared with control human IgG, Mann-Whitney test). VEGFR31-Ig and VEGF-Trap were shown to be equally effective in reducing lung metastasis ($P > 0.05$, Mann-Whitney test). There was no statistically significant difference in the number of metastatic foci between control human IgG and sVEGFR3 treatment groups ($P > 0.05$, Mann-Whitney test), suggesting that sVEGFR3 failed to inhibit tumor metastasis to lungs.

**Suppression of axillary lymph node metastasis by VEGFR31-Ig.** Sections of axillary lymph nodes were stained with H&E to identify metastases. Lymph node metastases were observed in 11 of 12 (92%) control human IgG-treated mice and in 9 of 12 (75%) mice injected with VEGF-Trap. In contrast, only 17% (2 of 12), 8% (1 of 12), and 17% (2 of 12) of mice developed lymph node metastases after receiving VEGFR31-Ig, sVEGFR3, and VEGF-Trap plus sVEGFR3, respectively. The mean lymph node volumes of different treatment groups were also calculated and compared. The results shown in Supplementary Table S1 indicated that the control human IgG-treated mice had a mean lymph node volume (mean ± SD) of 63.23 ± 12.31 mm³, which showed a statistically significant difference ($P < 0.001$, Mann-Whitney test) when compared with that of each of groups treated with VEGFR31-Ig (3.95 ± 1.09 mm³), sVEGFR3 (4.98 ± 0.78 mm³), and VEGF-Trap plus sVEGFR3 (4.27 ± 1.21 mm³). The mean lymph node volume was 55.77 ± 18.98 mm³ in the VEGF-Trap

Figure 4. VEGFR31-Ig effectively suppresses HCCLM3 tumor growth in SCID mice. HCCLM3 cells (1 × 10⁶ per mouse) were inoculated s.c. into the right flank of male SCID mice. One day after inoculation, the mice were injected s.c. with 25 mg/kg of VEGF-Trap, sVEGFR3, VEGFR31-Ig, or VEGF-Trap plus sVEGFR3 twice weekly. Points, mean tumor volume ($n = 12$); bars, SD.
group, and no significant difference in lymph node size was observed between the control human IgG group and VEGFTrap treatment group. These data suggest that VEGFR31-Ig and sVEGFR3 are effective in blocking lymph node metastasis, whereas VEGFTrap is not.

**Inhibition of tumor angiogenesis and lymphangiogenesis by VEGFR31-Ig.** Blood vessels in the tumors were visualized by staining with antibodies against the endothelial marker CD31. As shown in Fig. 6, MVD was significantly decreased in the VEGFR31-Ig, VEGFTrap, and VEGFTrap plus sVEGFR3 groups in comparison with the control human IgG group (P < 0.001, Mann-Whitney test). In contrast, there was no significant difference in MVD between the human IgG group and sVEGFR3 group (P > 0.05, Mann-Whitney test).

Lymphatic vessels in the tumors were analyzed by immunostaining with antibodies specific for the lymphatic endothelial marker LYVE-1. The results illustrated in Fig. 6 showed that VEGFR31-Ig and sVEGFR3 had similar ability to inhibit tumor lymphangiogenesis and LVD in either of the two groups was much lower than in the control human IgG group. However, inhibition of lymphangiogenesis was not observed in mice treated with VEGFTrap. These results suggest that VEGFR31-Ig is able to block both tumor angiogenesis and lymphangiogenesis, whereas VEGFTrap alone only suppresses angiogenesis and sVEGFR3 alone is only effective in inhibiting lymphangiogenesis.

**Discussion**

Tumor metastasis to regional lymph nodes is common in many types of human cancers, and an association between lymphangiogenesis and tumor metastasis has been shown (14, 16, 25). In contrast to VEGF-C, there is comparatively little evidence to support that VEGF-A may be involved in lymphangiogenesis. But recent studies reveal that lymphatic endothelial cells can express VEGFR2 and that VEGF-A can support their survival and promote tube formation in vitro (26–29). Further studies reported by Hirakawa and colleagues (30) indicated that targeted overexpression of VEGF-A potently induced tumor lymphangiogenesis in cutaneous squamous cell carcinoma and promoted tumor metastasis to sentinel lymph nodes. However, our current study showed that blocking VEGF-A by VEGFTrap failed to suppress lymphangiogenesis and lymph node metastasis in the HCCLM3 hepatocellular carcinoma model, suggesting that VEGF-A might be ineffective at inducing tumor lymphangiogenesis. The contradictory results in Hirakawa’s and our studies might be explained by the use of different mouse strains and tumor models. In Hirakawa’s study, they generated transgenic mice that overexpress VEGF-A and green fluorescent protein specifically in the skin and subjected them to a standard chemically induced skin carcinogenesis regime. Moreover, because VEGFR31-Ig, but not VEGFTrap, was shown to effectively inhibit lymphangiogenesis and lymph node metastasis in this study, we speculated that VEGF-C, which can be blocked by VEGF31-Ig but not by VEGFTrap, might play a major role in tumor lymphangiogenesis in the HCCLM3 hepatocellular carcinoma model.

The VEGF-C/VEGFR3 signaling system is currently the most attractive target for antilymphangiogenic therapeutics designed to restrict cancer metastasis. Blockade of VEGF-C/VEGFR3 signaling may be expected to effectively control systemic metastases in tumors that metastasize exclusively via the lymphatic route but may be ineffective or less effective in tumors that metastasize via the vasculature or via the lymphatics and vasculature. Lin and colleagues (22) reported that stable systemic expression of sVEGFR3 following a recombinant adeno-associated virus-mediated gene transfer potently inhibited lung metastases in a prostate tumor (PC-3-mlg2) model but was only partially effective in blocking lung metastases in a melanoma (A375-mln1) model, suggesting that the PC-3-mlg2 tumors may metastasize to systemic sites using the lymphatic route and that A375-mln1 tumors may metastasize to systemic sites using the vasculature or both the vasculature and the lymphatics (22). In the studies presented here, we show that sVEGFR3 potently inhibits tumor lymphangiogenesis and metastasis to the lymph nodes.
in the HCCLM3 hepatocellular carcinoma model but fails to suppress tumor angiogenesis and lung metastasis, suggesting that HCCLM3 tumors might metastasize to systemic sites using the blood vasculature.

For complex diseases in which multiple mediators contribute to overall disease pathogenesis by distinct mechanisms, simultaneous blockade of multiple targets may yield better therapeutic efficacy than inhibition of a single target. The major cause of cancer mortality is the metastatic spread of tumor cells that can occur via multiple routes, including the blood and lymphatic vasculature. Previous studies indicated that VEGFTrap, which could bind VEGF-A with significantly higher affinity than previously reported VEGF-A antagonists (31), potently suppressed tumor growth in a variety of tumor xenograft models (9, 32, 33). In the present study, VEGFTrap effectively inhibited tumor angiogenesis and primary tumor growth and lung metastasis in the HCCLM3 hepatocellular carcinoma model but failed to suppress lymphangiogenesis and lymph node metastasis. Because sVEGFR3 has proved to be effective in inhibiting tumor lymphangiogenesis and lymph node metastasis in this model, a combination of VEGFTrap and sVEGFR3 is predicted to have the potential to block both tumor angiogenesis and lymphangiogenesis, thus effectively inhibiting primary tumor growth and tumor metastasis to lungs and lymph nodes. Our data have shown the benefit of this combination treatment in the HCCLM3 hepatocellular carcinoma model. However, developing two separate receptor-Ig fusion proteins for clinical use as

![Figure 6. VEGFR31-Ig inhibits tumor angiogenesis and lymphangiogenesis in the HCCLM3 hepatocellular carcinoma tumor model. At 6 wk following tumor inoculation, HCCLM3 primary tumors from mice treated with PBS, control human IgG, VEGFTrap, sVEGFR3, VEGFR31-Ig, or VEGFTrap plus sVEGFR3 were harvested and analyzed for blood vessels using a rat anti-mouse CD31 antibody and for lymphatic vessels using a rat anti-mouse LYVE-1 antibody by immunohistochemistry. A, representative sections for immunohistochemical staining using CD31 antibody (magnification, ×200). Scale bars, 50 μm. B, representative sections for immunohistochemical staining using LYVE-1 antibody (magnification, ×200). Scale bars, 50 μm. C and D, quantification of CD31-positive vessels (C) and LYVE-1-positive vessels (D) in HCCLM3 primary tumors from mice treated with PBS, control human IgG, VEGFTrap, sVEGFR3, VEGFR31-Ig, or VEGFTrap plus sVEGFR3. Columns, mean; bars, SD.](image)
combination therapy is impractical, owing to regulatory hurdles and cost. In addition, the preclinical safety and efficacy of the combination of two different receptor-Ig fusion proteins have not been adequately addressed. In this study, we first show that a soluble fusion protein, VEGFR31-Ig, can potentially suppress both tumor angiogenesis and lymphangiogenesis. The VEGFR31-Ig fusion protein exhibits VEGF–A and VEGF–C-binding affinity similar to that of VEGFTrap and sVEGFR3, respectively. Importantly, it is capable of binding VEGF-A and VEGF-C simultaneously, showing that each binding domain can function independently without posing substantial steric hindrance to one another. The complex molecules frequently encounter the problems of poor expression, which has hampered clinical use of these molecules as therapeutics. In this study, the expression level of VEGFR31-Ig was ∼750 mg/L in a 14-day shake-flask fed-batch culture comparable with that of the conventional IgG molecule, suggesting that this fusion protein could be efficiently produced by mammalian expression systems. The in vitro stability was analyzed by incubation of VEGFR31-Ig in PBS at 37°C and subsequent measurement of VEGF–A–binding activity in ELISA. The results showed that VEGFR31-Ig was highly stable under this condition, with only 6% loss of binding activity for a 14-day incubation (data not shown). Pharmacokinetic analysis in mice further showed that VEGFR31-Ig had markedly improved pharmacokinetic properties in comparison with sVEGFR3. Even when compared with VEGFTrap, which was engineered to have a favorable pharmacokinetic profile (9), a slight increase in Cmax and AUC was observed for VEGFR31-Ig. In HCCM3 hepatocellular carcinoma mouse model, VEGFR31-Ig and VEGFTrap were equally effective in inhibiting primary tumor growth and lung metastasis. VEGFR31-Ig also showed similar potency as sVEGFR3 in blocking the development of lymph node metastasis in this model. It is particularly noteworthy that the VEGFR31-Ig protein at 25 mg/kg dose can achieve the same therapeutic effect as the combination of VEGFTrap (25 mg/kg) and sVEGFR3 (25 mg/kg).

In summary, the data shown here suggest a potential promise for the strategies that combine targeting angiogenic and lymphangiogenic growth factors for the treatment of metastatic cancer. The VEGFR31-Ig fusion protein specifically binding to both VEGF-A and VEGF-C simultaneously may provide a novel agent for prevention and treatment of metastatic diseases through inhibition of both tumor angiogenesis and lymphangiogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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