The Propeptides of VEGF-D Determine Heparin Binding, Receptor Heterodimerization, and Effects on Tumor Biology

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Background: VEGF-D is a secreted protein that promotes cancer metastasis; it comprises a receptor-binding domain flanked by cleavable propeptides but the functions of the propeptides were unclear.

Results: Propeptides determine heparin binding, VEGF receptor heterodimerization, and rates of metastasis.

Conclusion: Propeptides influence molecular interactions of VEGF-D and its bioactivity in cancer.

Significance: This study defines the biological significance of VEGF-D propeptides.

VEGF-D is an angiogenic and lymphangiogenic glycoprotein that can be proteolytically processed generating various forms differing in subunit composition due to the presence or absence of N- and C-terminal propeptides. These propeptides flank the central VEGF homology domain, that contains the binding sites for VEGF receptors (VEGFRs), but their biological functions were unclear. Characterization of propeptide function will be important to clarify which forms of VEGF-D are biologically active and therefore clinically relevant. Here we use VEGF-D mutants deficient in either propeptide, and in the capacity to process the remaining propeptide, to monitor the functions of these domains. We report for the first time that VEGF-D binds heparin, and that the C-terminal propeptide significantly enhances this interaction (removal of this propeptide from full-length VEGF-D completely prevents heparin binding). We also show that removal of either the N- or C-terminal propeptide is required for VEGF-D to drive formation of VEGFR-2/VEGFR-3 heterodimers which have recently been shown to positively regulate angiogenic sprouting. The mature form of VEGF-D, lacking both propeptides, can also promote formation of these receptor heterodimers. In a mouse tumor model, removal of only the C-terminal propeptide from full-length VEGF-D was sufficient to enhance angiogenesis and tumor growth. In contrast, removal of both propeptides is required for high rates of lymph node metastasis. The findings reported here show that the propeptides profoundly influence molecular interactions of VEGF-D with VEGF receptors, co-receptors, and heparin, and its effects on tumor biology.

Angiogenesis and lymphangiogenesis are key processes in embryogenesis, wound healing, and immune function, and in diseases including metastatic cancer, inflammatory disorders and lymphangioleiomyomatosis (LAM) 1–4. Members of the VEGF family of secreted glycoproteins promote lymphangiogenesis and/or angiogenesis by activating cell surface receptor tyrosine kinases on endothelial cells, such as VEGF receptor (VEGFR)-2 and VEGFR-3. For example, human VEGF-D activates both VEGFR-2 and VEGFR-3 (5), and drives growth of blood vessels and lymphatics in various settings (6–12). In animal models of cancer, expression of VEGF-D promotes growth of blood vessels and small lymphatics in and around tumors, facilitating tumor growth and enhancing lymph node and distant organ metastasis (13–15). VEGF-D also promotes dilatation of tumor-draining collecting lymphatics, which facilitates transport of tumor cells and further enhances metastasis (16).

VEGF-D is expressed in a range of human tumors, and can correlate with lymph node metastasis and poor patient outcome (Refs. 17–21); for review see Refs. 2, 22). Clinicopathological as well as experimental data indicate that the VEGF-D signaling pathway may be a therapeutic target for restricting the spread of cancer (23, 24). Further, it has been proposed that VEGF-D is an alternative mediator of tumor angiogenesis to VEGF-A (25, 26), that might contribute to mechanisms of resistance to bevacizumab, a widely used anti-cancer drug targeting VEGF-A (27). The analysis of VEGF-D in clinical samples, such as tumor tissues, is complicated by the fact that this is a proteolytically processed protein (28), which can be detected in various forms of different sizes and subunit compositions. It will be essential to understand the functions of the various domains of VEGF-D to design optimal therapeutic and diagnostic approaches targeting the clinically important form(s) of the protein.

The full-length form of VEGF-D is an antiparallel homodimer consisting of subunits comprising a central VEGF

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homology domain (VHD), containing binding sites for VEGFR-2 and VEGFR-3, and N- and C-terminal propeptides flanking the VHD (5). The full-length form can be proteolytically processed outside the cell, by enzymes such as proprotein convertases (29) and plasmin (30), to generate a partially processed form, in which the C-terminal propeptide is cleaved from the VHD, but remains attached to the molecule via disulfide bonding between the N- and C-terminal propeptides on different subunits (28, 31). This partially processed form can be converted to mature VEGF-D, consisting of VHD dimers, via cleavage of the N-terminal propeptide. The mature form has enhanced binding affinities for VEGFR-2 and VEGFR-3 (28).

The capacity of cells to promote proteolytic processing varies (29, 32), indicating this is a regulated process, and the relative abundance of the various forms of VEGF-D in the conditioned cell culture media of primary cells or cell lines can consequently be very different. For example, transfected LoVo colon carcinoma cells, which cannot promote VEGF-D processing, accumulate only full-length VEGF-D in the media (29); mouse primary embryonic fibroblasts, Balb/c/3T3 cells, and transfected 293EBNA-1 cells, which have an intermediate capacity for VEGF-D processing, accumulate almost all forms of VEGF-D in media including full-length and partially processed material (28, 29, 33); transfected Capan-1 pancreatic cancer cells, which process VEGF-D very efficiently, accumulate predominantly mature VEGF-D (32).

It has been shown that proteolytic processing is absolutely required for VEGF-D-mediated tumor growth and spread (34); however, little is known about the functions of the propeptides in these events. Analysis of propeptide function is complicated by difficulty in purifying, or specifically expressing, partially processed VEGF-D (in which either propeptide has been cleaved from the VHD) in the absence of full-length or mature material (28, 34). Here we circumvent this problem using forms of VEGF-D, in which either propeptide has been deleted, and processing of the other propeptide is blocked by mutation. This allowed us to test the hypothesis that propeptides modulate a variety of interactions of VEGF-D, and its effects in cancer. We show that propeptides determine binding of VEGF-D to VEGF receptor heterodimers, co-receptors and heparin, and influence key aspects of tumor biology such as rates of primary tumor growth and metastasis.

EXPERIMENTAL PROCEDURES

Cell Culture—293EBNA-1 and human microvascular endothelial cells (HMVECs) were cultured as previously (28, 34), and Suspension Freestyle™ 293-F cells according to the supplier (Invitrogen, Carlsbad, CA).

VEGF-D Protein Constructs—The VEGF-D derivatives used in this study are as follows. (i) VEGF-D_{SSTS,ISS}: an N-terminally FLAG-tagged full-length form of human VEGF-D containing mutations in the propeptidic cleavage sites that prevent cleavage of both propeptides (29); (ii) VEGF-D_{ΔNAC}: a mature form of human VEGF-D consisting of the VHD tagged with FLAG at the N terminus (5, 28); (iii) VEGF-D-CPRO: the C-terminal propeptide of human VEGF-D (amino acid residues 206–354) tagged at the N terminus with FLAG (35); (iv) VEGF-D_{ΔISS}: an N-terminally FLAG-tagged form of VEGF-D (encompassing amino acids 89–354) lacking the N-terminal propeptide, and containing mutations (R204S and R205S) that prevent cleavage of the C-terminal propeptide; (v) VEGF-D_{ΔC_{SSTS}}: an N-terminally FLAG-tagged form of VEGF-D (encompassing amino acids 24–205) lacking the C-terminal propeptide, and containing mutations (R85S and R88S) that prevent cleavage of the N-terminal propeptide. VEGF-D_{ΔISS} and VEGF-D_{ΔC_{ISS}} contain the N-terminal region of the VHD thought to be important for interaction with VEGFR-3 (36). Expression vectors for these proteins were generated from pApex-3 (Alexion Pharmaceuticals, Cheshire, CT).

Transfection and Protein Purification—293EBNA-1 cells were transfected, and stably expressing clones selected, as described (34). 293F cells were transfected according to the supplier (Invitrogen). VEGF-D proteins were purified from conditioned media by affinity chromatography on M2 (anti-FLAG) gel as described previously (28), and quantitated spectrophotometrically.

Immunoprecipitation and Western Blotting—VEGF-D derivatives were immunoprecipitated and detected in Western blots as described (33, 34). For analysis of VEGF-2/VEGF-3 heterodimers, HMVECs were treated with growth factors for 10 min, after which cells were lysed for 15 min in ice-cold buffer (1% Nonidet P-40, 20 mM Tris-Cl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). VEGF-2 was immunoprecipitated and targeted in Western blots with a mab to the C terminus of human VEGF-2 (55B11, Cell Signaling Technologies, Beverly, MA), and VEGF-3 with a polyclonal antibody to the C terminus of human VEGF-3 (sc-321, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Phosphotyrosine residues were targeted with a mab against Phosphotyramine (4G10, Millipore, Billerica, MA), and VEGF-A with a mab against VEGF_{A_{165}} (Santa Cruz Biotechnology Inc.). For detection, secondary 800 IRDye®-conjugated IgG antibodies (LI-COR Biosciences, Lincoln, NB) were used. Proteins were visualized, and relative band intensities were measured, on an Odyssey Infrared Imaging System (LI-COR Biosciences).

Biosensor Analysis—Protein interactions were monitored in real-time on an instrumental optical biosensor using surface plasmon resonance detection (Biacore 3000, GE Healthcare, Giles, UK). Fusion proteins, consisting of the extracellular domains of human VEGF-2 or VEGF-3 and the Fc portion of human IgG (R&D Systems, Minneapolis, MN), were immobilized onto a CM5 sensor surface using amine coupling chemistry [N-hydroxysuccinimide and N-ethyl-N-dimethylaminopropyl-carbodiimide] at a flow rate of 5 ml/min as reported previously (29, 37, 38). Immobilization levels were 5,500 and 5,000 RU for VEGF-2 and VEGF-3, respectively. VEGF-D derivatives, purified by anti-FLAG affinity chromatography, were subjected to BSA-depletion, and samples were analyzed by SDS-PAGE (under reducing and non-reducing conditions) to confirm the presence of dimers, although this approach did not exclude the possibility that aggregated protein was present in the samples. VEGF-D derivatives were diluted in Biacore buffer (10 mM Hepes, pH 7.4, containing 3.4 mM EDTA, 0.15 mM NaCl and 0.005% (v/v) Tween 20) to the appropriate concentrations (125–2,000 nM) for kinetic analysis. Samples (30 μl)
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were injected sequentially over the sensor surfaces at a flow rate of 5 µl/min. Following completion of the injection phase, dissociation was monitored in Biacore buffer alone at the same flow rate with running temperature of 25 °C (see supplemental Fig. S1 for representative sensorgrams). The sensor surface was regenerated between injections using 10 µl of 1 M diethanolamine, pH 10, at a flow rate of 20 µl/min. This treatment did not denature the sensor surface as shown by equivalent signals on repeat injection of the same sample. Kinetic rate constants were determined using the BIAevaluation software version 4.1 (GE Healthcare). $K_D$ values were determined from kinetic analyses.

ELISAs for Receptor Activation—HMVECs were stimulated with growth factors for 10 min, and samples used in Total and Phospho VEGFR-2 or VEGFR-3 ELISAs (R&D Systems, Minneapolis, MN) as per the manufacturer’s instructions.

Heparin Affinity Chromatography—Proteins were loaded onto a 1-ml column of heparin-Sepharose (GE Healthcare). The column was washed with PBS to remove unbound protein, and bound protein eluted with a 0.1–2.0 M gradient of NaCl in PBS in 2-ml fractions which were subsequently analyzed by Western blotting.

Binding to Neuropilins—Binding of VEGF-D derivatives to neuropilins was assessed using fusion proteins consisting of the extracellular domains of rat neuropilin-1 or human neuropilin-2, and the Fc portion of human IgG (R&D Systems, Minneapolis, MN), as described previously (34).

Mouse Tumor Xenograft Model—Female SCID/NOD mice (Animal Resources Centre, Perth, Australia), 11–12 weeks old, were used. Tumors were generated by injecting 293EBNA-1 cell lines subcutaneously into the mouse flank, and tumor volume determined as described (13). Each study group contained 10 mice and the experiment was conducted twice with similar results in each case. Detection of PECAM-1 or LYVE-1 in tissue sections, immunoprecipitation and Western blotting of tumor tissue to monitor levels of VEGF-D proteins and detection of tumor cells in lymph nodes were carried out as described previously (34), when primary tumors reached ~2,500 mm³. Statistical analyses were as described previously (34).

RESULTS

Expression and Receptor Binding of VEGF-D Propeptide Mutants—To study the role of propeptides in the molecular interactions and biological functions of VEGF-D, mutants were created in which either propeptide had been deleted, and the processing site for the remaining propeptide mutated to block processing. These proteins, tagged with the FLAG octapeptide, were designated VEGF-ΔNHISS and VEGF-ΔCSTTS, and are represented in Fig. 1A. Expression of these proteins in 293F or 293EBNA-1 cells, and reducing SDS-PAGE analysis, revealed that the subunits of VEGF-ΔNHISS and VEGF-ΔCSTTS were ~43 kDa and ~31 kDa, respectively (Fig. 1B), as expected based on the known sizes of the VHD and propeptides (28). Neither protein was processed to generate mature VEGF-D, the subunit of which is ~21 kDa (5).

We used surface plasmon resonance to assess binding affinities of VEGF-ΔNHISS and VEGF-ΔCSTTS for the extracellular domains of VEGFR-2 and VEGFR-3 (Fig. 1C; see supplemental Fig. S1 for representative sensorgrams). VEGF-ΔNHISS and VEGF-ΔCSTTS had ~50- and ~100-fold greater VEGFR-2 affinity, respectively, than VEGF-ΔCSTTS (a form of full-length VEGF-D, which cannot be processed (29)). In contrast, VEGF-ΔNAC (a form of mature VEGF-D (5)) had much stronger VEGFR-2 affinity than both VEGF-ΔNHISS and VEGF-ΔCSTTS. The VEGFR-3 affinities of VEGF-ΔCSTTS and VEGF-ΔCSTTS were similar, 17–20-fold lower than that of VEGF-ΔNAC. The VEGFR-3 affinity of VEGF-ΔNHISS was 3–4-fold lower than those of VEGF-ΔCSTTS and VEGF-ΔCSTTS, and 68-fold lower than that of VEGF-ΔNAC. These data show that removal of either propeptide significantly enhances VEGF-2 affinity, which is further enhanced when both propeptides are removed. However, removal of either propeptide does not greatly affect VEGFR-3 affinity (full-length VEGF-D exhibits reasonable VEGFR-3 affinity (29)), but removal of both propeptides significantly enhances this affinity. The ability of VEGF-D variants to activate VEGFR-2 and VEGF-3 on HMVECs was analyzed (Fig. 1D). Both VEGF-ΔNHISS and VEGF-ΔCSTTS activated VEGFR-2 (in contrast, VEGF-ΔCSTTS cannot activate VEGFR-2 (34)) indicating that removal of either propeptide allows VEGFR-2 activation. VEGF-ΔNHISS and VEGF-ΔCSTTS activated VEGFR-3 (Fig. 1D), as can VEGF-ΔCSTTS (34).

VEGF-D Propeptides Determine Receptor Heterodimerization and Interactions with Neuropilins—VEGF-D induces formation of VEGFR-2/VEGFR-3 heterodimers on endothelial cells, which are thought to positively regulate angiogenic sprouting (39), although it was unclear which forms of VEGF-D promote this heterodimerization. To monitor the influence of propeptides on the formation of VEGFR-2/VEGFR-3 heterodimers, HMVECs were stimulated with VEGF-D variants, and receptor heterodimers assessed by immunoprecipitation and Western blotting (Fig. 2A). VEGF-2/VEGFR-3 heterodimers were induced by either VEGF-ΔNHISS or VEGF-ΔCSTTS at 500 ng/ml, but not at 200 ng/ml (data not shown). In contrast, receptor heterodimers were not observed following stimulation with VEGF-ΔCSTTS at 500 ng/ml. As expected from previous studies (39), VEGF-ΔNAC at 200 ng/ml induced receptor heterodimers. Tyrosine phosphorylation of both VEGFR-2 and VEGFR-3 was observed in response to all forms of VEGF-D tested (Fig. 2A), with the exception that VEGF-ΔCSTTS did not induce VEGFR-2 phosphorylation. These results indicate that VEGF-ΔNHISS and VEGF-ΔCSTTS activate VEGF-2 and VEGF-3, and drive heterodimer formation, but suggest that processing to remove both propeptides enhances these effects.

It has been reported that partially processed, but not full-length, VEGF-D binds neuropilin (NP)1 and NP2 (34, 40) which act as co-receptors with VEGFR-2 and VEGFR-3 (41, 42) and are key molecules in the development of blood vessels and lymphatics (43, 44). To further characterize the binding of VEGF-D to NPs, we tested the capacity of VEGF-D variants to bind fusion proteins comprised of the extracellular domains of NP1 or NP2 and the Fc portion of human IgG1 (Fig. 2B). These experiments were conducted in the presence or absence of heparin, a molecule which can be required for binding of VEGF-C to NP1 (40). VEGF-ΔNHISS did not bind either the NP1 or NP2
construct irrespective of the presence or absence of heparin, however, VEGF-D\(\Delta C\)\(_{\text{SSTS}}\) bound both constructs. Heparin slightly enhanced the binding of VEGF-D\(\Delta C\)\(_{\text{SSTS}}\) to NP2, but not to NP1. To further assess the domain(s) of VEGF-D that binds NPs, NP constructs were used to precipitate VEGF-D\(\Delta N\)\(_{\text{NIISS}}\) and VEGF-D\(\Delta N\)\(_{\text{NIISS}}\) bound both NPs, interactions that were slightly enhanced by heparin. However, binding of VEGF-D\(\Delta C\)\(_{\text{SSTS}}\) to NP1 and NP2 generated more intense bands indicating that the N-terminal propeptide enhances NP interactions.
The C-terminal Propeptide Mediates Heparin Binding—The ability of VEGF family members, including VEGF-A, VEGF-B, and PlGF, to bind heparan sulfate proteoglycans (HSPGs) in the extracellular matrix or at the cell surface can influence the concentration gradient and bioavailability of growth factor, which can in turn modulate bioactivity (45). As the ability of VEGF-D to bind HSPGs or heparin has not been documented, we subjected VEGF-D variants to heparin Sepharose affinity chromatography using VEGF-A165, which binds HSPGs (46, 47), as positive control. VEGF-A165 was retained on the column and subsequently eluted from the heparin Sepharose at NaCl concentrations from 0.9 to 1.4 M, reflecting its interaction with heparin (Fig. 3). Likewise, significant proportions of VEGF-DSSTS,IIISS and VEGF-DΔNΔC were retained on the column, and subsequently eluted at 0.6 to 1.1 M and 0.6 to 1.2 M NaCl, respectively, indicating that these derivatives bind heparin. In
VEGF-D propeptides in a mouse tumor xenograft model, whereas most of the VEGF-D-CPRO did bind and was eluted at 0.6 to 1.2 M NaCl. These data indicate that binding of VEGF-D to heparin is modest, and that the interaction of VEGF-D to heparin is mediated predominantly by the C-terminal propeptide.

Propeptides Influence Primary Tumor Growth and Angiogenesis—The data presented above indicate that the two VEGF-D propeptides modulate interactions with distinct signaling molecules known to influence angiogenesis or lymphangiogenesis, which are important processes in the growth and spread of cancer. We therefore monitored the effects of the VEGF-D propeptides in a mouse tumor xenograft model, involving 293EBNA-1 cell lines stably expressing VEGF-D\textsubscript{NIISS} or VEGF-D\textsubscript{CSSTS}. The 293EBNA-1 model was chosen because it does not express significant quantities of VEGF family members and it produces slow growing and poorly vascularized epithelioid-like tumors in mice (13). Hence it provides an appropriate “background” in which to monitor the effects of VEGF-D on tumor biology. Further, expression of mature VEGF-D in this model (via stable transfection) promotes angiogenesis, lymphangiogenesis, primary tumor growth, and lymph node metastasis, whereas a full-length form of VEGF-D that cannot be processed (i.e. VEGF-D\textsubscript{CSSTS}) did not promote any of these key features of tumor biology (34). For our study, a 293EBNA-1 cell line stably transfected with expression vector lacking VEGF-D sequence (designated “Vector Control”) was used as negative control, and a cell line stably expressing VEGF-D\textsubscript{NIISS} was the positive control. The cell lines expressing forms of VEGF-D produced comparable levels of these N-terminally FLAG-tagged proteins as assessed by Western blotting using an anti-FLAG antibody (data not shown). These cell lines were injected subcutaneously into the flanks of SCID/NOD mice to generate tumors. Tumors did not process either VEGF-D\textsubscript{NIISS} or VEGF-D\textsubscript{CSSTS} to mature VEGF-D (Fig. 4A).

The growth rates of tumors expressing VEGF-D\textsubscript{NIISS} or VEGF-D\textsubscript{CSSTS} were similar, and they grew significantly faster than Vector Control tumors or tumors expressing VEGF-D\textsubscript{CSSTS} (Fig. 4B; morphology of the tumors is shown in Fig. 4C). The growth of tumors expressing VEGF-D\textsubscript{NIISS} was not statistically different from Vector Control tumors. Given that VEGF-D\textsubscript{CSSTS} a full-length form that cannot be processed, does not promote tumor growth in this model at all (34), these findings indicate that removal of the C-terminal propeptide, but not the N-terminal propeptide, from full-length VEGF-D enhances tumor growth.

Blood vessel endothelium in the tumors was quantitated by immunostaining for PECAM-1 (Fig. 4D). There was no statistically significant difference in staining between VEGF-D\textsubscript{NIISS} and VEGF-D\textsubscript{NIISS} tumors, and both tumor groups contained significantly more blood vessel endothelium than VEGF-D\textsubscript{CSSTS} or Vector Control tumors. Given that VEGF-D\textsubscript{CSSTS} does not promote angiogenesis in this model (34), these data indicate that removal of the C-terminal propeptide, but not the N-terminal propeptide, from full-length VEGF-D promotes angiogenesis which likely led to the fast growth of VEGF-D\textsubscript{CSSTS} tumors.

Propeptides Influence Lymph Node Metastasis and Tumor Lymphangiogenesis—As VEGF-D can promote lymph node metastasis (13), axillary lymph nodes were assessed for the presence of tumor cells which were easily identified due to their dark staining, enlarged nuclei and irregular shape (Fig. 5A). The expression of VEGF-D\textsubscript{NIISS} in tumors significantly enhanced lymph node metastasis compared with Vector Control tumors, which did not exhibit any metastatic spread to lymph nodes (Fig. 5B). When tumors expressed VEGF-D\textsubscript{NIISS} or VEGF-D\textsubscript{CSSTS}, metastatic spread to lymph nodes did occur but was significantly reduced compared with VEGF-D\textsubscript{NIISS} tumors. These findings indicate that removal of both propeptides is required for VEGF-D to efficiently promote lymph node metastasis.
The abundance of lymphatic vessel endothelium in tumors was monitored by immunohistochemistry for LYVE-1 (Fig. 5C). VEGF-DΔNAC tumors contained regions with abundant lymphatics, particularly at the tumor periphery. Vector Control and VEGF-DΔCSTS tumors both had a low abundance of lymphatic endothelium, *i.e.* significantly less than for VEGF-DΔNAC tumors. VEGF-DΔNISS tumors contained regions at the tumor periphery with abundant lymphatics and exhibited significantly greater overall LYVE-1 staining than Vector Control or VEGF-DΔCSTS tumors. Given that VEGF-DΔSTSISS does not promote lymphangiogenesis in this model (34), these data indicate that removal of the N-terminal propeptide from full-length VEGF-D (as indicated by VEGF-DΔNISS) leads to an increase in tumor lymphangiogenesis.

**DISCUSSION**

The findings reported here show that the propeptides of VEGF-D are critical determinants of a range of molecular inter-
actions with receptors, co-receptors and heparin. For example, the presence of both propeptides in the full-length protein prevents VEGF-D-mediated formation of VEGFR-2/VEGFR-3 heterodimers, whereas removal of either, or both, propeptides allows receptor heterodimerization to occur. The inability of full-length VEGF-D to drive receptor heterodimerization likely reflects its poor affinity for VEGFR-2 (29). However, full-length VEGF-D can drive activation of VEGFR-3 (34). Given that VEGF-2/VEGFR-3 heterodimers have altered trans-phosphorylation of tyrosine residues in the VEGFR-3 subunit compared with those of VEGFR-3 homodimers (48), the removal of propeptides from full-length VEGF-D would result in mechanistically distinct modes of signaling via VEGFR-3. Importantly, VEGF-2/VEGFR-3 heterodimers are thought to positively regulate angiogenic sprouting (39). It is therefore noteworthy that VEGF-D is localized in vascular smooth muscle surrounding large arteries in adult human tissues (35, 49, 50). This indicates that VEGF-D could access blood vessels and promote angiogenesis, possibly via VEGF-2/VEGFR-3 heterodimers on angiogenic sprouts, in response to tissue damage. It was recently shown that mature VEGF-C binds heparan sulfate from lymphatic endothelial cells as well as heparin-Sephose (51), however, the capacity of other forms of VEGF-C to bind heparin is unknown. Here we show that mature VEGF-D binds heparin, although this interaction did not appear to be of high affinity. Further, the C-terminal propeptide of VEGF-D binds heparin strongly, and confers this property on full-length VEGF-D indicating that proteolytic processing to remove the C-terminal propeptide would be a determinant of this interaction. Although there is no obvious heparin-binding domain in the C-terminal propeptide of VEGF-D (as assessed by comparison of the primary structure with VEGF-A (46, 52) or other heparin-binding proteins), it is possible that basic residues are clustered together in the 3-dimensional structure of this pro-

FIGURE 5. Impact of N- and C-terminal propeptides on lymph node metastasis and lymphangiogenesis. A, effects on lymph node metastasis. Representative images of lymph node tissue sections, stained with hematoxylin and eosin, that were taken from mice with primary tumors 2,500 mm3 in size. Top panel shows lymph node from a mouse with a metastatic VEGF-DΔNΔC tumor; bottom panel shows a lymph node from a mouse with a non-metastatic Vector Control tumor. Arrowheads indicate clusters of tumor cells. B, occurrence of lymph node metastasis in tumor xenografts. Mice were scored positive for metastasis if tumor cells were observed in the axillary lymph nodes. Numbers indicate the proportion of mice with primary tumors that scored positive for lymph node metastasis. Asterisks indicate those groups with significantly lower rates of metastasis than the VEGF-DΔNΔC study group, as determined by the Kruskal-Wallis test (p < 0.05). Data generated from VEGF-DΔNΔC tumors has been reported previously (34) and is shown for comparison. The data from two independent experiments is combined in the table. C, analysis of tumor lymphatic vessels. Tissue sections were analyzed by immunohistochemistry with antibodies to LYVE-1 (brown staining) for lymphatic vessels (left panels). Graph shows quantitation of lymphatic endothelium in tumor sections as assessed by the number of pixels positively stained for LYVE-1. ΔN-ISS denotes VEGF-DΔNISS and ΔC-SSTS denotes VEGF-DΔCSSTS. Asterisks indicate statistically significant differences in abundance of lymphatic endothelium (p < 0.01: Student’s t test). Error bars represent S.E. The experiments described in this figure were conducted twice with similar results in each case, the results of one experiment are shown here in panels A and C.
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peptide to allow heparin binding. However, the crystal structure of the C-terminal propeptide remains to be elucidated. The interactions of VEGF-A isoforms with HSPGs can influence VEGF-A stability and bioavailability, and the amplitude and duration of signaling interactions with receptors (45), whether there is similar functional relevance to the interaction of VEGF-D with HSPGs remains to be determined. A variety of cells (i.e. cell lines or primary cell cultures) have been reported to produce significant quantities of full-length VEGF-D and the most abundant partially processed form, in which the C-terminal propeptide has been cleaved but the N-terminal propeptide has not (28, 29). The latter is thought to retain the C-terminal propeptide due to disulfide bonding between N- and C-terminal propeptides on different subunits in the antiparallel dimer. The presence of the C-terminal propeptide in these forms of VEGF-D should allow them to bind HSPGs. If so, cleavage of both propeptides would be required to maximally enhance the solubility of VEGF-D in a biological setting.

NP1 and NP2 act as co-receptors with VEGF-R2 and VEGF-R3 (41, 42), and are important during embryogenesis for development of blood vessels and lymphatics (43, 44). It was already known that full-length VEGF-D cannot bind NPs but processing of the C-terminal propeptide allows binding (34, 40). Here we show that mature VEGF-D binds both NP1 and NP2, and that binding is enhanced for both receptors by the presence of the N-terminal propeptide (i.e. VEGF-DΔCSTSS binds better than VEGF-DΔNΔC). However, the C-terminal propeptide inhibits binding to both NPs, i.e. VEGF-DΔNΔISS cannot bind NPs despite the presence of the VHD. The inhibitory effect of the C-terminal propeptide also explains why full-length VEGF-D does not bind NPs.

It has been reported that mature VEGF-D promotes tumor angiogenesis and lymphangiogenesis, as well as tumor growth and lymph node metastasis, but a form of full-length VEGF-D which cannot be processed (i.e. VEGF-DΔCSTSS,IS) does not promote any of these aspects of tumor biology (34). However, the contribution of each propeptide to these observations was unknown. We observed that tumors expressing VEGF-DΔCSTSS contained significantly more blood vessel endothelium and grew faster than VEGF-DΔCSTSS,ISS and VEGF-DΔNΔC tumors. This may reflect the enhanced affinity of VEGF-DΔCSTSS for VEGF-R2 compared with VEGF-DΔCSTSS,ISS. While VEGF-DΔNΔISS has a similar affinity for VEGF-R2 as VEGF-DΔCSTSS, it is not able to bind NPs. Interactions with both NP1 and VEGF-R2 may be central to the enhanced angiogenesis induced by VEGF-DΔCSTSS compared with VEGF-DΔNΔISS. This finding is consistent with previous studies which have demonstrated that the formation of a complex of NP1 and VEGF-R2 can enhance VEGF-A binding and promote VEGF-A activation, thereby enhancing angiogenesis (41, 53, 54). The extensive angiogenesis in tumors expressing VEGF-DΔCSTSS likely accounts for their fast growth rate, and shows that a species of VEGF-D containing the N-terminal propeptide and VHD is angiogenic. These findings are the first to indicate that removal of the C-terminal propeptide is rate-limiting for VEGF-D to promote angiogenesis and tumor growth.

It was previously shown that a partially processed form of VEGF-D, containing the N-terminal propeptide and VHD, bound NP2 (40) and VEGF-R3 (28), and that interaction between these receptors mediates lymphatic vessel sprouting in response to VEGF-C (55). Hence it was unexpected that VEGF-DΔCSTSS did not promote significant lymphangiogenesis in the tumor model. In contrast, VEGF-DΔNΔISS induced growth of lymphatics, indicating that a species of VEGF-D containing the VHD and C-terminal propeptide is lymphangiogenic. As the affinities of VEGF-DΔNΔISS and VEGF-DΔCSTSS for VEGF-R3 as assessed by biosensor are comparable, the explanation for why VEGF-DΔCSTSS lacks lymphangiogenic activity is unclear. However, it is possible that the capacity of VEGF-DΔNΔISS to bind HSPGs, which is not shared by VEGF-DΔCSTSS, may explain these observations. Importantly, a recent study indicated that heparan sulfate, present on lymphatic endothelial cells (LEC), enhances binding of VEGF-C to VEGF-R3, phosphorylation of VEGF-3, and migration, proliferation and sprouting of LEC in response to VEGF-C (51). If VEGF-D is similar to VEGF-C in these respects, the capacity of VEGF-DΔNΔISS to bind HSPGs should enhance its potency, relative to VEGF-DΔCSTSS, for driving signaling via VEGF-3 in LEC, and for promoting lymphangiogenesis. Our observation that VEGF-DΔNΔISS can induce lymphangiogenesis at comparable levels to VEGF-DΔCSTSS, despite having a 68-fold lower VEGF-3 affinity, may also be due to the stronger binding of VEGF-DΔNΔISS to HSPGs.

Our data show that the propeptides of VEGF-D are critical determinants of VEGF receptor heterodimerization, and interactions with neuropilin co-receptors and heparin. The propeptides exert profound effects on the capacity of VEGF-D to drive tumor angiogenesis and lymphangiogenesis, and thereby promote the growth and spread of cancer.

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