VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development

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SUMMARY

The vascular endothelial growth factor family has recently been expanded by the isolation of two new VEGF-related factors, VEGF-B and VEGF-C. The physiological functions of these factors are largely unknown. Here we report the cloning and characterization of mouse VEGF-C, which is produced as a disulfide-linked dimer of 415 amino acid residue polypeptides, sharing an 85% identity with the human VEGF-C amino acid sequence. The recombinant mouse VEGF-C protein was secreted from transfected cells as VEGF-R3 (Flt4) binding polypeptides of 30-32×10^3 M_r and 22-23×10^3 M_r which preferentially stimulated the autophosphorylation of VEGF-R3 in comparison with VEGF-R2 (KDR). In in situ hybridization, mouse VEGF-C mRNA expression was detected in mesenchymal cells of postimplantation mouse embryos, particularly in the regions where the lymphatic vessels undergo sprouting from embryonic veins, such as the perimetanephric, axillary and jugular regions. In addition, the developing mesenterium, which is rich in lymphatic vessels, showed strong VEGF-C expression. VEGF-C was also highly expressed in adult mouse lung, heart and kidney, where VEGF-R3 was also prominent. The pattern of expression of VEGF-C in relation to its major receptor VEGF-R3 during the sprouting of the lymphatic endothelium in embryos suggests a paracrine mode of action and that one of the functions of VEGF-C may be in the regulation of angiogenesis of the lymphatic vasculature.

Key words: VEGF-C receptor, VEGFR-3, vascular system, endothelial cell, mouse, lymphatic system, angiogenesis

INTRODUCTION

The cardiovascular system is the first organ system to begin functioning in the developing embryo. The inner layer of blood and lymphatic vessels as well as the endocardium are formed by endothelial cells that play a critical role in physiological and pathological processes of the vasculature. The process known as vasculogenesis is restricted to the embryonic period of development. Vasculogenesis involves the formation of the earliest blood vessels by in situ differentiation of endothelial cells from mesodermal precursor cells known as angioblasts (Risau et al., 1988). Angiogenesis is the subsequent formation of blood vessels via sprouting and intussusception from pre-existing ones. This mechanism also occurs where neovascularization is required in adults, and is of particular significance in wound healing, maturation of ovarian follicles and tumor development. The mechanisms regulating the latter processes are of particular interest as they may generate targets for the therapeutic control of pathological processes dependent on angiogenesis (Folkman and Shing, 1992; Hanahan and Folkman, 1996).

Several inhibitors and stimulators of angiogenesis have been described, but only a few of them appear to be endothelial cell-specific. Many factors affect the proliferation and differentiation of the endothelium indirectly. Vascular endothelial growth factor (VEGF) is currently held to be the major endothelial-cell-specific angiogenesis and permeability factor, whereas the related placenta growth factor is expressed only in a restricted set of tissues (for a review, see (Dvorak et al., 1995; Ferrara et al., 1992; Neufeld et al., 1994). Recently two new endothelial-cell-specific growth factors VEGF-B and VEGF-C have been cloned (Joukov et al., 1996; Lee et al., 1996; Olofsson et al., 1996). Both share a striking structural similarity with VEGF, thus expanding the family of known VEGF-like growth factors.

VEGF-C protein was purified and its cDNA cloned from human prostatic carcinoma cells (Joukov et al., 1996). While being homologous with other members of the VEGF/platelet derived growth factor (PDGF) family, the C-terminal half of VEGF-C contains extra cysteine-rich motifs characteristic of the protein component of silk produced by the larval salivary glands of the midge, Chironomus tentans. Human VEGF-C is proteolytically processed, binds the Flt4 receptor tyrosine kinase, which we have renamed the VEGF receptor-3 (VEGFR-3), and induces tyrosine autophosphorylation of VEGF-R3 and VEGF-R2 (Joukov et al., 1996). In addition, VEGF-C stimulates the migration of bovine capillary endothelial cells in collagen gels. VEGF-C is thus a novel regulator of endothelia.

Despite their homology, the VEGFs probably have different
functional roles that may overlap, as regulatory factors of the endothelium. In order to clarify of the function of VEGF-C in vivo (Joukov et al., 1996), we isolated the mouse VEGF-C cDNA and analysed its protein product and mRNA expression pattern in developing mouse embryos.

MATERIALS AND METHODS

Isolation of mouse cDNA clones for VEGF-C

To isolate mouse VEGF-C cDNAs, approximately 1×10^6 bacteriophage lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda lambda 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product showed that the open reading frame encodes a polypeptide of 415 amino acid residues (Fig. 1B). A hydrophobic sequence of 31 amino acid residues, fulfilling the criteria for a secretory signal sequence, is located in the N-terminus and its cleavage site is predicted to occur between residues A31 and F32 (von Heijne, 1986). By analogy with the human sequence, the mouse VEGF-C mRNA 3' of the segment encoding the signal sequence should encode an N-terminal propeptide, which is predicted to be cleaved off before or during secretion from cells between the conserved residues R98-T99. The N-terminal propeptide differs from the human homologue by an apparent deletion of four amino acid residues (12 nucleotides) corresponding to the human amino acid residues H88-E91 (Fig. 1B).

The overall homology between the mouse and human prepro-VEGF-C open reading frames is 85%. The VEGF-homologous region is 94% identical between the mouse and human VEGF-C; the carboxyterminal region is 85% and the N-terminal propeptide 79% identical (Fig. 1B). Notably, residues 100-103 in the N-terminus of the mature, proteolytically processed VEGF-C differ between the two sequences. In addition, there are only two amino acid substitutions (A142V and G175S) in the mouse VEGF-C sequences. In addition, there are only two amino acid substitutions (A142V and G175S) in the mouse VEGF-C sequences. In addition, there are only two amino acid substitutions (A142V and G175S) in the mouse VEGF-C sequences. In addition, there are only two amino acid substitutions (A142V and G175S) in the mouse VEGF-C sequences.

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Identification and activity of mouse recombinant VEGF-C protein

The mouse VEGF-C cDNA was expressed as a recombinant protein and the secreted protein was analysed for its receptor binding properties. The binding of mouse VEGF-C to the human VEGFR-3 extracellular domain was studied by using media from BOSC23 cells transfected with mouse VEGF-C.
cDNA in a retroviral expression vector. Immunoprecipitation of VEGF-C from media of transfected and metabolically labelled cells revealed bands of approximately 30-32×10^3 M_r (a doublet) and 22-23×10^3 M_r in 12.5% SDS-PAGE. These bands were not detected in samples from nontransfected or mock-transfected cells as shown in Fig. 2A. This result indicated that the antibodies against human VEGF-C recognize the corresponding mouse ligand. The media were also incubated with VEGF-3 extracellular domain covalently coupled to sepharose and bound material was analysed by gel electrophoresis as above. As can be seen from Fig. 2A, similar 30-32×10^3 M_r doublet and 22-23×10^3 M_r polypeptide bands were obtained, thus demonstrating the binding of mouse VEGF-C to human VEGF-3. The slightly faster mobility of the mouse VEGF-C polypeptides may be caused by the four amino acid residue difference observed in sequence analysis (residues H88-E91, Fig. 1B).

In order to assay whether mouse recombinant VEGF-C is capable of inducing VEGFR-3 autophosphorylation, NIH3T3 cells expressing VEGF-3 were stimulated with media containing mouse VEGF-C, lysed, and VEGF-C was immunoprecipitated, electrophoresed, transferred to a nitrocellulose membrane and analysed by immunoblotting using antibodies against phosphotyrosine. The filter was reprobed with anti-VEGFR-3 antiserum. The results of this experiment are shown in Fig. 2B and demonstrate that mouse VEGF-C-containing culture medium stimulates the autophosphorylation of VEGF-3 polypeptides of 195×10^3 M_r and 125×10^3 M_r to a similar extent as human baculoviral VEGF-C (Michael Jeltsch, unpublished data) or the tyrosyl phosphatase inhibitor pervanadate. These polypeptides represent the uncleaved and proteolytically cleaved forms of the VEGF-3 tyrosine kinase, respectively (Pajusola et al., 1994). In contrast, the intracellular precursor of 175×10^3 M_r is tyrosyl phosphorylated only in the presence of pervanadate (a tyrosyl phosphatase inhibitor).

To check whether mouse recombinant VEGF-C can also induce VEGF-2 autophosphorylation as has been previously reported for human VEGF-C (Joukov et al., 1996), PAE cells expressing VEGF-2 were stimulated with tenfold concentrated medium from cultures transfected with mouse VEGF-C expression vector and autophosphorylation was analysed. For comparison, cells treated with tenfold concentrated medium containing human recombinant VEGF-C (Joukov et al., 1996), unconcentrated medium from human VEGF-C baculovirus infected insect cells or pervanadate were used. As can be seen from Fig. 2B, VEGF-2 was prominently phosphorylated in response to baculoviral VEGF-C as well as pervanadate treatment, whereas human and mouse recombinant VEGF-C gave a weak and a barely detectable enhancement of autophosphorylation, respectively. Media from cell cultures transfected with empty vector or VEGF-C cloned in antisense orientation did not induce autophosphorylation of VEGF-2. These results suggested that mouse VEGF-C binds to VEGFR-3 and activates this receptor at much lower concentrations than needed for the activation of VEGFR-2. We therefore next analysed the in vivo distribution of VEGF-C mRNA in comparison to that of its receptor mRNA in order to see whether the results support the hypothesis that VEGF-C acts primarily via VEGFR-3.

Expression of mouse VEGF-C mRNA

In order to assess the expression of the VEGF-C mRNA during embryonic development, we hybridized polyadenylated RNA isolated from mouse embryos of various gestational ages (7-17 day p.c.) with the mouse VEGF-C probe. These analyses showed that the amount of 2.4 kB VEGF-C
mRNA is relatively constant throughout the gestational period (Fig. 3A).

The most conspicuous signals in adult mouse tissues were obtained from the heart and lung RNA, while kidney, liver, brain and skeletal muscle had lower levels and spleen and testis had barely visible levels (Fig. 3B). Comparison with VEGFR-3 expression showed that the tissues where VEGF-C is expressed also contain mRNA for its cognate receptor tyrosine kinase, although in the adult liver VEGFR-3 mRNA was disproportionately abundant.

**Localization of VEGF-C mRNA in embryonic tissues by in situ hybridization**

To compare the distribution of VEGF-C and VEGFR-3 expression patterns during development, analysis of their RNAs was performed by in situ hybridization. Similar sections of 8.5, 12.5 and 14.5-day p.c. mouse embryos were hybridized with labeled VEGF-C and VEGFR-3 probes to localize their mRNAs in different cells and tissues.

In 8.5 day embryos, VEGF-C mRNA is detectable in the cephalic mesenchyme, along the somites and in the tail region (Fig. 4A). Extraembryonically VEGF-C mRNA was expressed in the allantois (data not shown). VEGFR-3 was expressed in the angioblasts of the head mesenchyme (Fig. 4B). VEGFR-3 was also expressed between the developing somites (data not shown) and extraembryonically a strong signal was observed in the allantois, in the giant cells partially fused to the Reichert’s membrane as well as in the endothelium of venous lacunae of the placenta. In contrast, we could not detect either VEGF-C or VEGFR-3 in the blood islands of the yolk sac.

In 12.5 day embryos, VEGF-C mRNA is particularly prominent in the mesenchyme around the developing metanephros (vm) and in the jugular area (Fig. 5A). In addition, hybridization signals can be observed between the vertebral corpuscles (vc), in the lung mesenchyme (lu), in the neck region and in the developing forehead. Strong VEGFR-3 signal was also observed in the mesenterium, the walls of the gut and the mesenchyme surrounding the metanephros, which is one of the major sites of emergence of the developing lymphatic vessels (Sabin, 1909).

The VEGF-C signal was found to originate from the anterior paravertebral and from intervertebral tissue (Fig. 6A). VEGF-C and VEGFR-3 are expressed in the anterior veins and in the intervertebral vessels, which are directly adjacent to these areas. A detailed comparison was made of the expression patterns of VEGF-C and VEGFR-3 in 12.5 day p.c. mouse embryos in the jugular region, where the developing dorsal aorta and cardinal vein are located (Fig. 6E-H). This is the area where the first
lymphatic vessels sprout from venous sac-like structures according to Sabin theory (Sabin, 1909). An intense VEGF-C signal was detected in the mesenchyme surrounding the developing jugular vessels (j), the developing metanephros (mn), and the mesenterium (m) (arrowheads). Some signal was also detected in the nasopharyngeal area, in the mesenchyme surrounding the intervertebral vessels (vc) and in the lung (lu). The VEGFR-3 (B) probe gives a signal predominantly from the vessel network in the jugular area (j) (arrowhead) and to a lesser extent from the intervertebral vessels (vc). Signal was observed in the mesenterium (m) and in the mesenchyme surrounding the metanephros (mn) (arrowheads). Control hybridization with the VEGF-C sense strand did not give any specific signal above background (C). (D) Bright-field photograph of same section. Bar, 1 mm.

At later developmental stages, the highest VEGF-C and VEGFR-3 RNA levels were observed in the mesenterium. Fig. 7 shows an example of the mesenterial in situ hybridization signals. Although the adjacent sections were not easily aligned, these sections demonstrate the intensity of the signals in the mesenterial connective tissue and in the developing vessels (arrowheads).

**DISCUSSION**

This study demonstrates a conservation of the primary structure between mouse and human VEGF-C, and suggests that VEGF-C is a high affinity ligand primarily for VEGFR-3. This conclusion is evident from the receptor stimulation experiments and in situ hybridization analysis of developing mouse embryos, where a paracrine relationship can be envisaged for VEGF-C and VEGFR-3 at several sites and time points. Although recombinant VEGF-C can also activate VEGFR-2, the in vivo significance of such an interaction requires further studies.

Four non-conservative substitutions were observed in the N terminus of mature VEGF-C and two additional amino acid substitutions in the VEGF-homology domain. Previous studies have shown that the N-terminal region of PDGF lacks an ordered structure (Oefner et al., 1992), and that this region...
of VEGF makes a good antigenic peptide for production of antibodies against VEGF (Kim et al., 1992). We have generated antibodies against a 17 amino acid peptide of the N terminus of human VEGF-C and, in this study, we show that these antibodies also recognize mouse VEGF-C. The N-terminal peptide of VEGF-C may therefore also be projecting from the very compact cysteine-bonded folding pattern of the growth factor domain typical for members of the PDGF/VEGF family (Thomas, 1996). The compact nature of the growth factor domain is also reflected by its relative resistance to proteolytic digestion by trypsin, observed in our efforts to derive internal sequences from the protein (V. J., unpublished observations). The other regions of the VEGF-C open reading frame show less conservation, except for the fourfold repeated BR3P homology motif. Notably, cysteine-rich motifs similar to those found in the carboxy terminal domain of VEGF-C are also present in VEGF- and VEGF-B amino acid sequences.

Expression of recombinant VEGF-C from a retroviral DNA construct gave rise to three secreted polypeptides, doublet of $30-32 \times 10^3 \text{M}_r$ and a $22-23 \times 10^3 \text{M}_r$ polypeptide, closely resembling the corresponding human VEGF-C polypeptides (Joukov et al., 1996). However, an additional weaker mouse VEGF-C polypeptide band appeared in the analysis of immunoprecipitates in the $38 \times 10^3 \text{M}_r$ region. This suggests that the pattern of proteolytic processing of the mouse and human forms is nearly identical in cultures of the genetically engineered BOSC23 human kidney carcinoma cell line used for transfection. Furthermore, the data suggests that both of the $30-32 \times 10^3 \text{M}_r$ and $22-23 \times 10^3 \text{M}_r$ forms are capable of binding to the human VEGFR-3 receptor, establishing a cross-species interaction that could be useful in the generation of viral vectors and transgenes.

The activity of recombinant VEGF-C was tested in vitro by stimulation of VEGFR-3- and VEGFR-2-expressing cells followed by receptor autophosphorylation analysis. Mouse VEGF-C appeared to be a potent inducer of VEGFR-3 autophosphorylation, with the $195 \times 10^3 \text{M}_r$ precursor and proteolytically cleaved $125 \times 10^3 \text{M}_r$ tyrosine kinase polypeptides of the receptor (Pajusola et al., 1994) being phosphorylated. VEGFR-2 stimulation was first tried with unconcentrated medium from cells expressing recombinant VEGF-C, but immunoblotting analysis did not reveal any receptor autophosphorylation (data not shown). When medium from VEGF-C transfected cells was concentrated tenfold, stimulation of VEGFR-2 expressing PAE cells resulted in VEGFR-2 autophosphorylation in the case of human recombinant VEGF-C. Mouse recombinant VEGF-C revealed a barely detectable autophosphorylation signal. In contrast, baculoviral VEGF-C induced autophosphorylation of VEGFR-2 at a much higher level. These data confirm that, at higher concentrations, VEGF-C is a ligand not only for VEGFR-3 but also for VEGFR-2.

As much higher amounts of VEGF-C were required to induce VEGFR-2 autophosphorylation in comparison with VEGFR-3, the interaction between VEGF-C and VEGFR-2 is probably much weaker than that with VEGFR-3. Based on our data alone, it is difficult to assess what role VEGF-C interaction with VEGFR-2 could play in vivo. It should be noted that gene targeting studies have suggested a delicate ligand dose-dependence for the effects of VEGF in early embryos (Carmeliet et al., 1996). Thus, the concentrations of VEGF factors may be critical for the specificity of their biological effects. However, it is questionable whether high concentrations of VEGF-C capable of activating VEGFR-2 occur in vivo. It is also possible that the different molecular weight forms of VEGF-C will differ in their specific activities when these purified forms are tested in receptor stimulation assays. To determine whether the pattern of VEGF-C expression is consistent with its role as a VEGFR-3 ligand and thus with the venous and lymphatic-specific pattern of expression previously described for VEGF-3 (Kaipainen et al., 1995) or whether VEGF-C mRNA is more widely expressed, suggesting also a role in embryonic angiogenesis in general, we performed northern blotting and in situ hybridization analysis of mouse embryos.

Analysis of VEGF-C expression by northern blotting and in situ hybridization showed that this growth factor is expressed both in embryonic and adult mice. The levels of the 2.4 kb VEGF-C transcript were relatively constant throughout the mouse embryonic and fetal periods. Expression on day 7 p.c. is striking, considering the appearance of VEGF-C mRNA first on day 8.5 of gestation (Kaipainen et al., 1995). However, we cannot exclude the possibility that some of the VEGF-C mRNA detected by northern blotting is actually derived from placenta/fetal membranes, where VEGF-C was also expressed according to our in situ hybridization data. This result suggests that, during early development, VEGF-C interacts with VEGFR-2, because no other known VEGF receptors are available at this time of development (Yamaguchi et al., 1993). Thus, depending on the relative concentration of VEGF-C, it could contribute to angiogenic signal transduction via VEGFR-2 in early embryos.

To investigate in more detail the possible role of VEGF-C in the early development of mouse embryos, we studied the relationship of VEGF-C and VEGF-3 via in situ hybridization at different time points of development. VEGF-C mRNA signal appeared to be strongest in the developing perinephric, mesenterial and jugular regions as well as in the non-neural gut (A,C), where VEGFR-3 decorates small developing lymphatic vessels and venules (B,D). Bar, 0.1 mm.
parts of the cephalic region. Lower levels of mRNA were detected in the lung and around and between the developing vertebrae. Our previous in situ hybridization studies showed that VEGFR-3 is confined to the venous endothelium of 8.5 day mouse embryos (Kaipainen et al., 1995). The present data show the pattern of mRNA expression for the VEGFR-3 ligand, VEGF-C at this time-point. Thus, VEGF-C may be involved in early development of venous system. Subsequently VEGF-C expression appears to be concentrated in the perinephric region and large sac-like endothelial structures in the jugular region, which become apparent on day 12.5; both are sites of sprouting of the developing lymphatic vessels, which originate from these regions according to the theory of Sabin (Sabin, 1909). VEGF-C mRNA expression follows the same pattern, being prominent in the early lymphatic structures of the axillary region and in the mesenchyme surrounding the oesophagus and the bifurcation of the trachea (our unpublished data). Thus, VEGF-C mRNA distribution correlates with that of VEGFR-3 suggestive of a paracrine relationship at several sites, with the VEGF-C ligand expressed in mesenchymal cells adjacent to the VEGF-C positive endothelia.

A strong signal for both VEGF-C and VEGF-R3 was also obtained from the mesenterium, which is rich in developing lymphatic vessels. In fact, the abundant lymphatic supply of the mesenterial tissue was the earliest observation that directed our attention to the lymphatic vessels (Kaipainen et al., 1993). VEGF-C mRNA expression in the non-neural part of the cephalic area and in the developing lung is also consistent with VEGF-C acting as a secreted ligand for the sprouting lymphatic endothelial cells expressing VEGF-R3 in these regions. This pattern is also in striking contrast to the reported expression pattern of VEGF, which is abundant in the ventricular neuroectoderm of developing embryonic brain where the endothelial cells proliferate rapidly during the ingrowth of capillaries from the perineural vascular plexus (Breier et al., 1992). The latter pattern is consistent with a role for VEGF as a major regulator of overall angiogenesis in the embryo. Studies of embryos lacking both copies of the VEGF-R2 gene or one allele of VEGF are in line with these deductions. However, the comparison of the VEGF and VEGF-R2 knockout phenotypes suggested that there may be yet another ligand for VEGF-R2 (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995). The stimulation of VEGF-R2 by VEGF-C at high concentrations and its early expression pattern make it possible that the VEGF-C/VEGF-R2 interaction is important at this early stage of development, although high ligand concentrations would probably be required. In conclusion, these considerations suggest that the VEGF-C gene may play a more specialized role in the development of the lymphatic system, which has not been the focus of developmental analysis since the early anatomical studies at the beginning of this century.

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