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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 VEGFR-3 (Flt4) binding polypeptides of 30-32×10^3 M_r and 22-23×10^3 M_r which preferentially stimulated the autophosphorylation of VEGFR-3 in comparison with VEGFR-2 (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 VEGFR-3 was also prominent. The pattern of expression of VEGF-C in relation to its major receptor VEGFR-3 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 VEGFR-3 and VEGFR-2 (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 clones from a 12 day p.c. mouse embryo cDNA library (EXlox library, Novagen # 69632-1) were screened with a radiolabeled PCR fragment of human VEGF-C cDNA containing nucleotides 495 to 1661 (sequence accession number X94216). One positive clone was isolated, a 1.3 kb *Eco*RI/*Hin*dIII fragment of the insert was subcloned to the corresponding sites of the pBluescript II SK+ vector (Stratagene) and sequenced. The cDNA sequence of this clone was homologous with the human VEGF-C sequence, except that about 710 bp of sequence present in the human clone was missing from the 5′-end. For further screening of mouse cDNA libraries, a *Hin*dIII-*Bst*XI (*Hin*dIII site is from the pBluescript II SK+ polylinker) fragment of 881 bp from the coding region of the mouse cDNA clone was used as a probe to isolate two additional cDNA clones from an adult mouse heart λZAP II cDNA library (Stratagene # 936306). Three further clones were isolated from a mouse heart 5′-stretch-plus cDNA library in λgt11 (Clontech #ML5002b). Of the latter clones, one was found to contain an insert of about 1.8 kb. The insert of this cDNA clone was subcloned into the *Eco*RI sites of the pBluescript II SK+ vector and both strands were sequenced.

Analysis of mRNA expression in tissues

Mouse embryo multiple tissue northern blot (Clontech) containing 2 μg of polyadenylated RNAs from 7, 11, 15 and 17 day postcoital (p.c.) embryos was hybridized with mouse VEGF-C cDNA fragment (base pairs 499-656) and washed in stringent conditions. A mouse adult tissue northern blot was hybridized with the same probe for VEGF-C and with a VEGFR-3 cDNA fragment (nucleotides 1-595; accession number X68203). Mouse β-actin probe (Clontech) was used as a control.

In situ hybridization of mouse embryos

In situ hybridization of tissue sections was performed as described previously (Västrik et al., 1995). The mouse VEGF-C antisense RNA probe was generated from linearized pBluescript II SK+ plasmid (Stratagene), containing an *Eco*RI/*Hin*dIII fragment corresponding to nt 558-979 of mouse VEGF-C cDNA, where the 3′ noncoding region and the BR3P repeats had been removed by exonuclease III treatment. Radiolabeled RNA was synthesized using T7 polymerase and [^{35}S]UTP (Amersham). Mouse VEGFR-3 antisense and sense RNA probes were synthesized in a similar manner from linearized pGEM-3Z(f+) plasmid containing the mouse VEGFR-3 cDNA insert described previously (Kaipainen et al., 1993). The high stringency wash was for 45 minutes at 65°C in a solution containing 30 mM DTT and 4× SSC. The slides were exposed for 28 days, developed and stained with Haematoxylin.

Expression and analysis of recombinant VEGF-C

The 1.8 kb mouse VEGF-C cDNA was cloned as an *Eco*RI fragment into the retroviral expression vector pBabe-puro (a kind gift from Dr Hartmut Land, ICRF, London) containing the SV40 early promoter region (Morgensternand and Land, 1990) and transfected into the BOSC23 packaging cell line by the calcium-phosphate precipitation method (Pear et al., 1994). For comparison, these cells were also transfected with the previously described human VEGF-C construct in the pREP7 expression vector (Joukov et al., 1996). The transfected cells were cultured for 48 hours prior to metabolic labelling. Cells were changed into DMEM medium devoid of cysteine and methionine and after 45 minutes of preincubation and medium change, about 120 μCi/ml of Pro-mix^{TM} L-[^{35}S] in vitro cell labelling mix (Amersham) in the same medium was added. After 6 hours of incubation, the culture medium was collected and clarified by centrifugation.

For immunoprecipitation, 1 ml aliquots of the media from metabolically labelled BOSC23 cells transfected with empty vector or mouse or human recombinant VEGF-C, respectively, were incubated overnight on ice with 2 μl of rabbit polyclonal antiserum against an N-terminal 17 aa peptide of mature human VEGF-C (EETIKFAAAHYNTEILK; V. J. et al., unpublished data). Incubation with protein A sepharose was carried out for 40 minutes at 4°C with gentle agitation. The sepharose beads were then washed twice with immunoprecipitation buffer, four times with 20 mM Tris-HCl pH 7.4, samples were boiled in Laemmli buffer and analysed by 12.5 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

For receptor binding experiments, 1 ml aliquots of media from metabolically labelled BOSC23 cells were incubated with VEGFR-3 extracellular domain covalently coupled to sepharose for 4 hours at 4°C with gentle mixing. The sepharose beads were washed four times with ice-cold phosphate-buffered saline (PBS) and the samples were analysed as described in (Joukov et al., 1996).

VEGFR-3 and VEGFR-2 stimulation experiments

For the VEGFR-3 receptor stimulation experiments, subconfluent NIH3T3-VEGFR-3 (Flt4) cells (Pajusola et al., 1994) were starved overnight in serum-free medium containing 0.2% BSA. The cells were stimulated with the conditioned medium from VEGF-C vector-transfected cells for 5 minutes, washed three times with cold PBS containing 200 μM vanadate and lysed in RIPA buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 0.1% SDS, 0.1 U/ml Aprotinin, 200 μM vanadate) for immunoprecipitation analysis. The lysates were centrifuged for 25 minutes at 16 000 ***g*** and the resulting supernatants were incubated for 2 hours on ice with the specific antisera, followed by immunoprecipitation using protein A-sepharose and analysis in 7% SDS-PAGE. Polypeptides were transferred onto nitrocellulose and analyzed by immunoblotting using anti-phosphotyrosine (Transduction Laboratories) and anti-receptor antibodies, as described by Pajusola et al. (1994). Stripping of the filter was carried out at 50°C for 30 minutes in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7 with occasional agitation.

VEGFR-2 stimulation was studied in subconfluent porcine aortic endothelial (PAE) cells expressing VEGFR-2 (PAE-KDR) (Waltenberger et al., 1994) which were starved overnight in serum-free medium containing 0.2% BSA. Stimulation was carried out and the lysates prepared as described above. For receptor immunoprecipitation, specific antiserum for VEGFR-2 (Waltenberger et al., 1994) was used (a kind gift from Dr Lena Claesson-Welsh, Ludwig Institute for Cancer Research, Uppsala, Sweden) and the immunoprecipitates were analyzed as described for VEGFR-3 in 7% SDS-PAGE followed by western blotting with anti-phosphotyrosine antibodies, stripping the filter and re-probing it with anti-VEGFR-2 antibodies (Santa Cruz).

RESULTS

Cloning and analysis of mouse VEGF-C cDNA

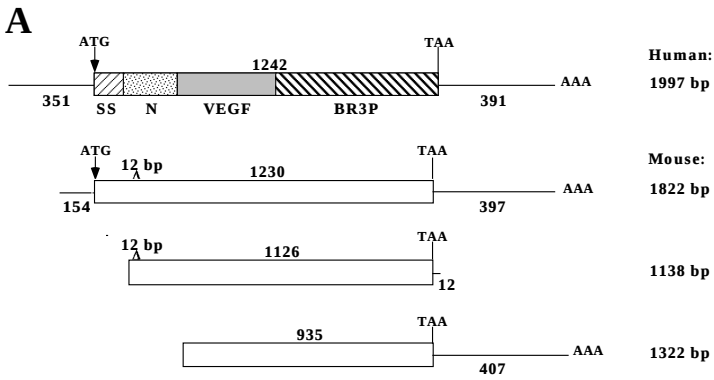
Three different cDNA libraries were screened, first using probes made from the human VEGF-C cDNA (Joukov et al., 1996) and then partial mouse VEGF-C cDNAs as hybridization probes, as detailed in the Materials and Methods section. Fig. 1A shows schematic structures of the clones obtained in comparison with human VEGF-C cDNA. Examination of the 1822 bp mouse VEGF-C cDNA and its predicted protein

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 sequence in the VEGF homology domain. The eight cysteine residues (marked above the amino acid sequence) typical for the VEGF/PDGF family and an extra cysteine residue (C133) are conserved between mouse and human sequences within the VEGF homologous region; the latter residue is not conserved in other ligands of the VEGF/PDGF family. The conserved R158 residue critical for folding of the VEGF/PDGF family ligands (Keyt et al., 1996) is also marked above the mouse sequence in bold. The C-terminal domain contains a fourfold repeated amino acid motif conforming to the spacing of cysteine residues of the Balbiani ring 3 protein (BR3P: C-10X-C-C-C; shown above the sequence). Also, certain other cysteine residues in the C-terminal tail show features of repeated motifs (C-10-11X-C-R/S-C). Similar types of motifs are also present in the C-terminal sequences of mouse and human VEGF and VEGF-B (Keck et al., 1989; Leung et al., 1989; Olofsson et al., 1996).

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



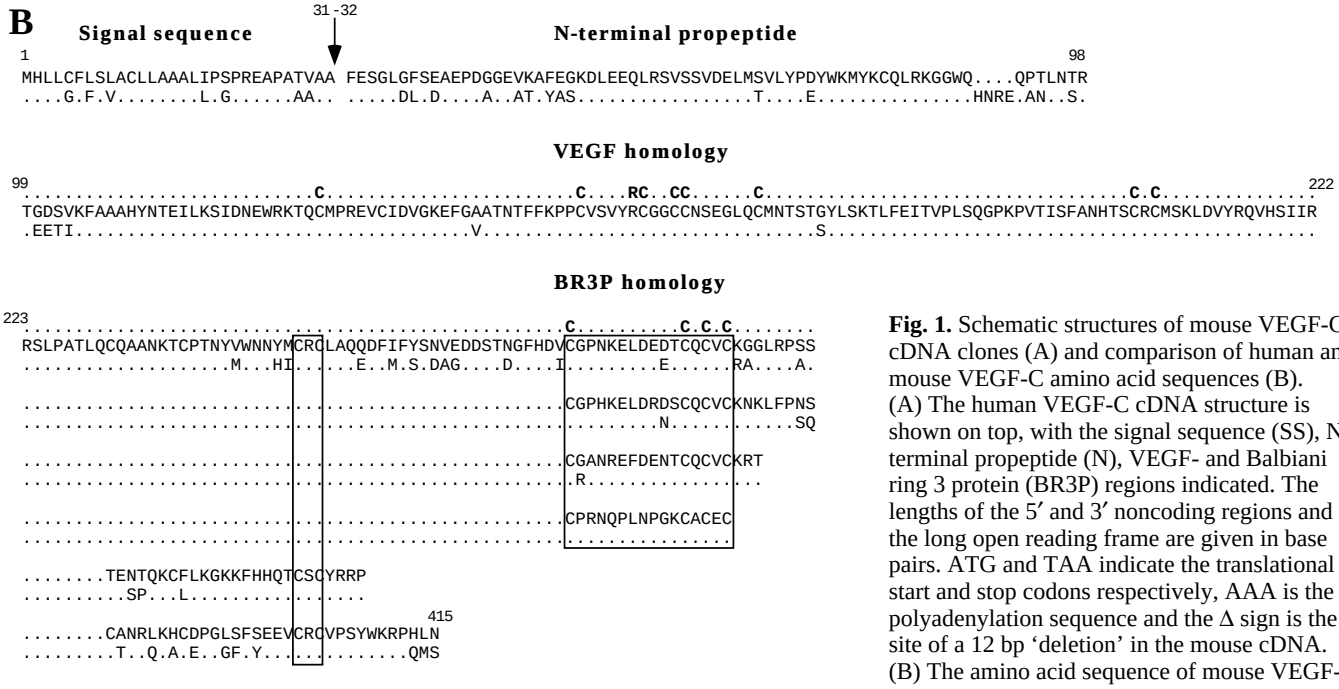


Fig. 1. Schematic structures of mouse VEGF-C cDNA clones (A) and comparison of human and mouse VEGF-C amino acid sequences (B). (A) The human VEGF-C cDNA structure is shown on top, with the signal sequence (SS), N-terminal propeptide (N), VEGF- and Balbiani ring 3 protein (BR3P) regions indicated. The lengths of the 5′ and 3′ noncoding regions and the long open reading frame are given in base pairs. ATG and TAA indicate the translational start and stop codons respectively, AAA is the polyadenylation sequence and the Δ sign is the site of a 12 bp 'deletion' in the mouse cDNA. (B) The amino acid sequence of mouse VEGF-C is given on top and only differences in the human sequence are marked below it. The sequence has been divided into the regions shown in A. The arrow indicates the putative cleavage site for the signal peptidase, BR3P motifs as well as a CR/SC motif are boxed and conserved cysteine residues are marked in bold above the sequence. Arginine residue 158 is also marked in bold. The numbering refers to mouse VEGF-C residues. The mouse VEGF-C nucleotide sequence has been deposited in Genbank (accession number U73620).

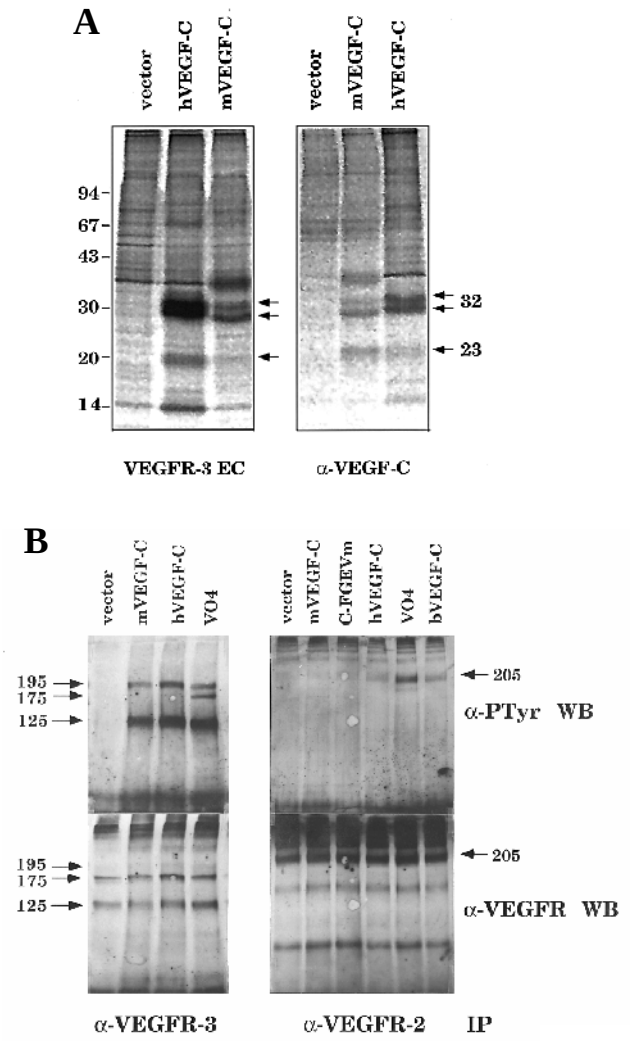


Fig. 2. Analysis of recombinant VEGF-C and its receptor binding and activation properties. (A) Recombinant mouse (mVEGF) or human VEGF-C (hVEGF) was bound to VEGFR-3 extracellular domain coupled to agarose or to antibodies made against the N terminus of human VEGF-C and analysed by immunoprecipitation and PAGE. The major previously identified 30-32×10^3 M_r and 23×10^3 M_r VEGF-C polypeptide components are indicated by arrows. Molecular mass markers are indicated on the left (M_r ×10^{-3}). (B) The VEGF-C-containing media were used for stimulation of autophosphorylation of VEGF receptors, which were then immunoprecipitated with receptor-specific antisera followed by gel electrophoresis and western blotting with anti-phosphotyrosine (α-PTyr) and anti-receptor antisera, as indicated. For comparison, receptor autophosphorylation was induced by pervanadate treatment (VO4). The arrows and numbers refer to the apparent molecular weights of the tyrosyl phosphorylated receptor polypeptide bands. bVEGF refers to the baculoviral VEGF-C protein and C-FGEVm to mouse VEGF-C cDNA cloned into the vector in antisense orientation.

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 VEGFR-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 VEGFR-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 VEGFR-3 were stimulated with media containing mouse VEGF-C, lysed, and VEGFR-3 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 VEGFR-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 VEGFR-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 VEGFR-2 autophosphorylation as has been previously reported for human VEGF-C (Joukov et al., 1996), PAE cells expressing VEGFR-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, VEGFR-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 VEGFR-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

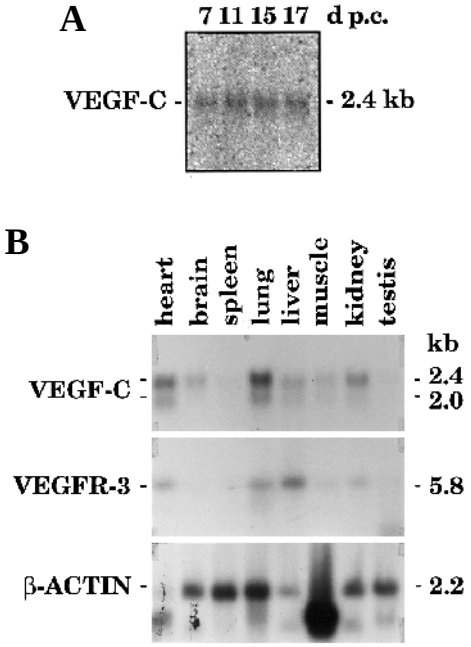


Fig. 3. Expression of VEGF-C and VEGFR-3 mRNAs in embryonic (A) and adult (B) mouse tissues. (A) Northern blotting and hybridization analysis of poly(A)+ RNA isolated from embryos of the indicated gestational ages (days post coitum, dp.c.), using the VEGF-C probe. (B) Similar analysis was carried out for adult mouse tissues. Hybridization was first carried out with the VEGF-C probe, then with the VEGFR-3 and finally with β-actin probe. Note that due to unequal loading of the samples, the signals for VEGF-C and VEGFR-3 are disproportionately weak in the heart.

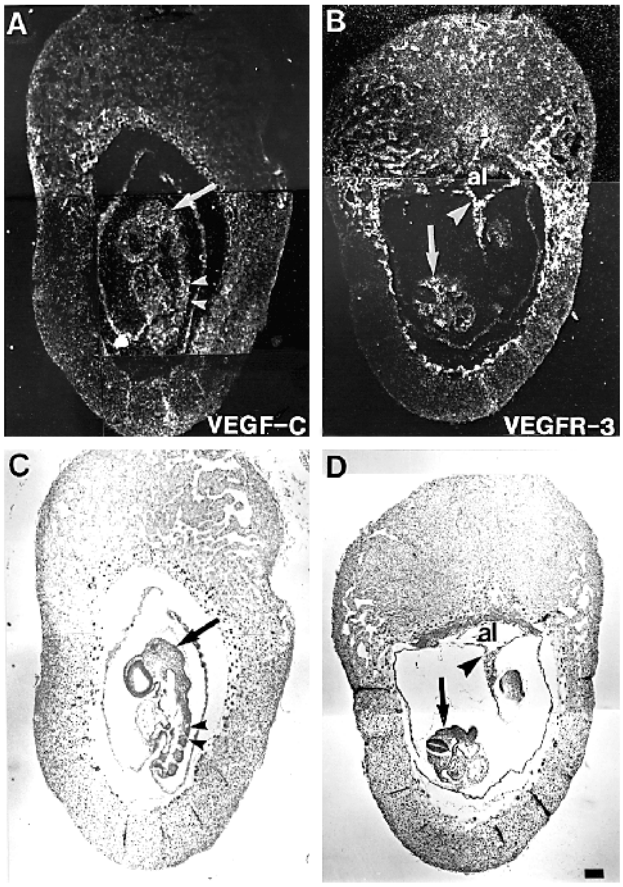


Fig. 4. Expression of VEGF-C and VEGFR-3 mRNAs in a 8.5-day embryo analysed by in situ hybridization. (A) VEGF-C signal originates from the cephalic mesenchyme (arrow) and from the developing vertebrae (arrowheads). (B) VEGFR-3 is expressed in the head mesenchyme (arrow) and extraembryonically in the allantois (al) (arrowhead). (C,D) Bright-field photographs of the same sections. Bar, 0.1 mm.

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 disproportionally 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 (mn) 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. In comparison, the expression level of VEGFR-3 appears to be especially high in the vessel network of the jugular area (Fig. 5B). 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). VEGFR-3 is 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

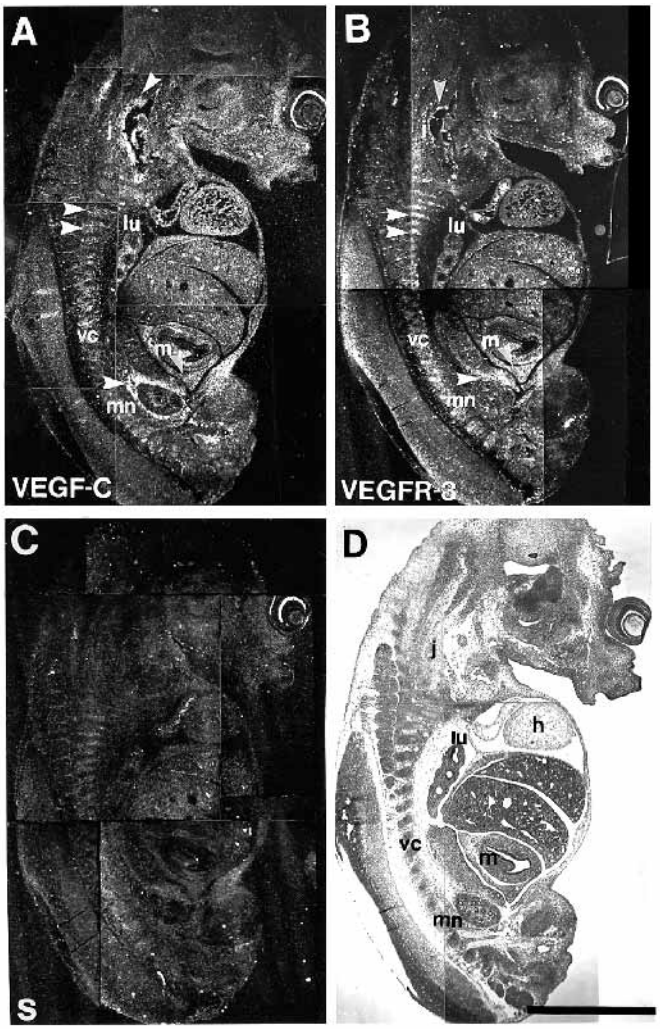


Fig. 5. Expression of VEGF-C and VEGFR-3 mRNAs in a sagittal section of a 12.5-day embryo. (A) VEGF-C mRNA is distributed in several regions of the embryo, with particularly prominent expression in the mesenchyme surrounding the jugular vessels (j), the developing metanephros (mn), and the mesenterium (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.

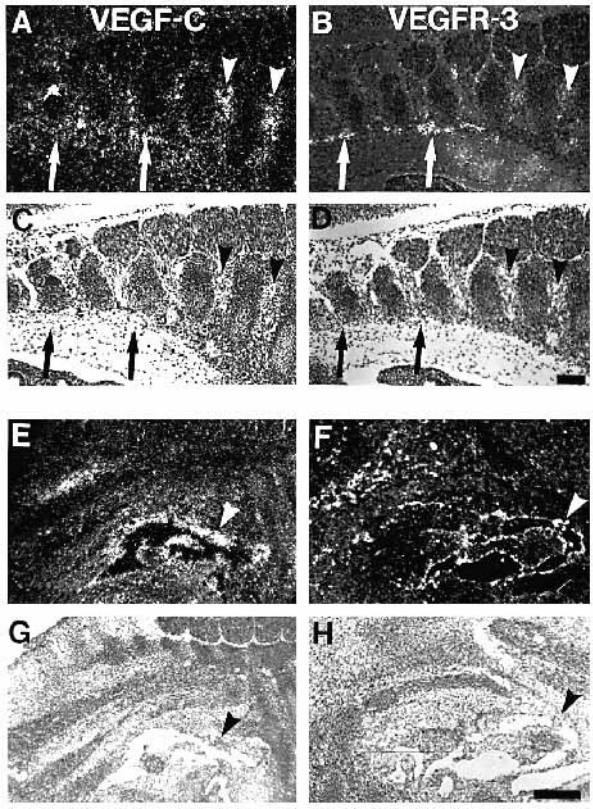


Fig. 6. VEGF-C and VEGFR-3 mRNAs in the paravertebral and jugular regions of the 12.5 d p.c. mouse embryo. (A,C) VEGF-C signal is detected in the intervertebral spaces (arrowheads) and also surrounding the anterior part of the vertebrae (arrows). (B,D) VEGFR-3 mRNA is expressed in the veins of the anterior part (arrows) of the developing vertebrae and the intervertebral vessels (arrowheads). Comparison of VEGF-C and VEGFR-3 expression in jugular vessels. (E,G) Note that the strong VEGF-C signals in the mesenchyme around the large sac-like structures in the jugular area (arrowheads). (F,H) VEGFR-3 signals are seen along the borders of the jugular venous sacs (arrowheads). Bar, 0.1 mm.

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 venous sacs (Fig. 6E,G), which were also positive for VEGFR-3 (Fig. 6F,H).

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

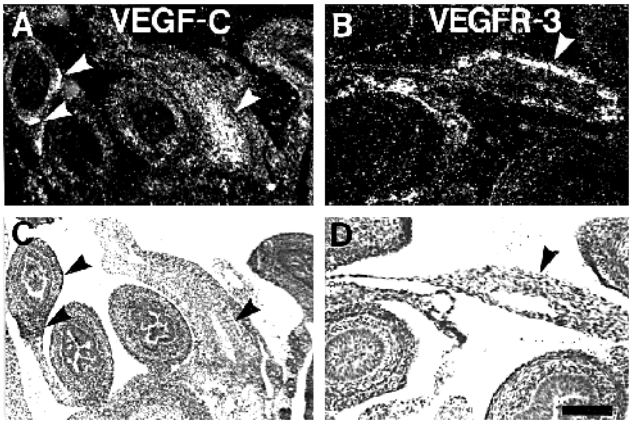


Fig. 7. VEGF-C and VEGFR-3 signals in the mesenterium. VEGF-C mRNA in the mesenteric region of a 14.5-day p.c. embryo is detected in the mesenterial connective tissue as well as around the gut (A,C), where VEGFR-3 decorates small developing lymphatic vessels and venules (B,D). Bar, 0.1 mm.

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 30-32×10^3 M_r and a 22-23×10^3 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 38×10^3 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 30-32×10^3 M_r and 22-23×10^3 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 transgenic mice and production of recombinant human factor and its testing in experimental animals.

The activity of recombinant VEGF-C was tested in vitro by stimulation of VEGFR-3- and VEGFR-2-expressing cells followed by receptor autophosporylation analysis. Mouse VEGF-C appeared to be a potent inducer of VEGFR-3 autophosphorylation, with the 195×10^3 M_r precursor and proteolytically cleaved 125×10^3 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 VEGFR-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 VEGFR-3 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 VEGFR-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

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). VEGFR-3 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 VEGFR-3 positive endothelia.

A strong signal for both VEGF-C and VEGFR-3 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 VEGFR-3 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 VEGFR-2 gene or one allele of VEGF are in line with these deductions. However, the comparison of the VEGF and VEGFR-2 knockout phenotypes suggested that there may be yet another ligand for VEGFR-2 (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995). The stimulation of VEGFR-2 by VEGF-C at high concentrations and its early expression pattern make it possible that the VEGF-C/VEGFR-2 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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