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Avian VEGF-C: cloning, embryonic expression pattern and stimulation of the differentiation of VEGFR2-expressing endothelial cell precursors

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SUMMARY

VEGF-C is a recently discovered secreted polypeptide related to the angiogenic mitogen VEGF. We have isolated the quail VEGF-C cDNA and shown that its protein product is secreted from transfected cells and interacts with the avian VEGFR3 and VEGFR2. In situ hybridization shows that quail VEGF-C mRNA is strongly expressed in regions destined to be rich in lymphatic vessels, particularly the mesenteries, mesocardium and myotome, in the region surrounding the jugular veins, and in the kidney. These expression sites are similar to those observed in the mouse embryo (E. Kukk, A. Lymboussaki, S. Taira, A. Kaipainen, M. Jeltsch, V. Joukov and K. Alitalo, 1996, *Development* 122, 3829-3837). We have observed VEGFR3-positive endothelial cells in proximity to most of the VEGF-C-expressing sites, suggesting functional relationships between this receptor-ligand couple. The comparison of the VEGF and VEGFR2 knockout phenotypes had suggested the existence of another ligand for VEGFR2. We therefore investigated the effect of VEGF-C on VEGFR2-positive cells isolated from the posterior mesoderm of gastrulating embryos. We have recently shown that VEGF binding triggers endothelial differentiation of these cells, whereas hemopoietic differentiation appears to be mediated by binding of a so far unidentified VEGFR2 ligand. We show here that VEGF-C also triggers endothelial differentiation of these cells, presumably via VEGFR2. These results indicate that VEGF and VEGF-C can act in a redundant manner via VEGFR2. In conclusion, VEGF-C appears to act during two different developmental phases, one early in posterior mesodermal VEGFR2-positive endothelial cell precursors which are negative for VEGFR3 and one later in regions rich in lymphatic vessels at a time when endothelial cells express both VEGFR2 and VEGFR3.

Key words: VEGF-C, VEGF, VEGFR2, VEGFR3, Endothelial cell, Lymphatics, Hemopoietic cell, Avian, Quail

INTRODUCTION

Vascular endothelial growth factor (VEGF) is regarded as the major inducer of angiogenesis in both normal and pathological conditions, based on its endothelial-cell-specific biological effects which include stimulation of proliferation, migration and tube formation as well as regulation of vascular permeability (Klagsbrun and D'Amore, 1996; Ferrara, 1997). Several growth factors closely related to VEGF are known, the placenta-derived growth factor (PlGF) (Maglione et al., 1991; Park et al., 1994) and the recently identified VEGF-B (Grimmond et al., 1996; Olofsson et al., 1996a), VEGF-C (Joukov et al., 1996; Lee et al., 1996) and c-fos-induced growth factor (FIGF) (Orlandini et al., 1996). PlGF has minimal angiogenic activity, but can heterodimerize with VEGF and modulate its effects (DiSalvo, 1995; Cao et al., 1996). VEGF-B has been reported to stimulate growth of human and bovine vascular endothelial cells, probably due to heterodimerisation with VEGF (Olofsson et al., 1996a,b).

VEGF-C and FIGF constitute a novel subgroup of VEGF-like growth factors, characterized by N- and C-terminal extensions flanking a VEGF homology domain. The C-terminal propeptides contain repeated motifs with a spacing of cysteine residues typical of Balbiani Ring 3 Protein (BR3P) (Joukov et al., 1996, 1997; Kukk et al., 1996; Lee et al., 1996; Orlandini et al., 1996). The VEGF-C polypeptide has been shown to be proteolytically processed by successive cleavage of the C-terminal and N-terminal propeptides, which yields mature VEGF-C containing only the VEGF-homology domain (Joukov et al., 1997). Like VEGF, mature VEGF-C stimulates vascular permeability as well as migration and proliferation of bovine capillary endothelial cells, although higher concentrations are required as compared to VEGF (Joukov et al., 1997).

The biological effects of VEGF are mediated by binding to two high-affinity receptors of the tyrosine kinase family, VEGFR1 and VEGFR2 (Mustonen and Alitalo, 1995; Shibuya, 1995 for reviews). PlGF binds only VEGFR1 (Park et al.,

1994) and the receptors for VEGF-B and FlGF are not known yet. VEGF-C binds to VEGFR2 and to a third receptor of the same group, VEGFR3 (Joukov et al., 1996, 1997). The proteolytically processed forms show increasing activity towards VEGFR3, whereas only the mature form can activate VEGFR2 (Joukov et al., 1997).

VEGFR1 and VEGFR2 are expressed exclusively by endothelial cells, and VEGFR2 also by their precursors (Mustonen and Alitalo, 1995; Shibuya et al., 1995 for reviews). VEGFR3 is also expressed by endothelial cells of the early embryo (Eichmann et al., 1993, 1996; Wilting et al., 1997), but unlike VEGFR1 and VEGFR2, it becomes largely restricted to venous and lymphatic endothelia at later developmental stages in both mouse and avian embryos (Kaipainen et al., 1995; Kukk et al., 1996; Wilting et al., 1997). These observations suggested a role for VEGF-C/VEGFR3 in the proliferation of lymphatic endothelium. Experimental support for such a role has come from overexpression of VEGF-C in the skin of transgenic mice, which was shown to induce the proliferation of lymphatic, but not vascular endothelial cells (Jeltsch et al., 1997).

The crucial role of VEGF and its receptors for the establishment of the vascular system during embryonic development has been demonstrated by targeted gene inactivation experiments. Disruption of the gene encoding VEGF is lethal at around embryonic day (E)10.5 in heterozygous embryos, due to reduced numbers of endothelial cells and abnormal blood vessel formation (Carmeliet et al., 1996; Ferrara et al., 1996). Heterozygous VEGF deficiency constitutes perhaps the most severe haploid-insufficient autosomal phenotype reported to date, indicating that reduced concentrations of VEGF are inadequate to support normal vascular development.

Targeted inactivation of the genes coding for VEGFR1 and VEGFR2 also leads to deficiencies in the formation of the vascular system and to embryonic lethality at around E9 (Shalaby et al., 1995; Fong et al., 1995). However, the phenotypes of the two mice are different: in VEGFR2/flk-1 homozygous knockout mice, development of both endothelial and hemopoietic cells is aborted (Shalaby et al., 1995). In VEGFR1/flt-1-deficient mice, endothelial as well as hemopoietic cells are present, but fail to organize proper vascular structures (Fong et al., 1995). The phenotypes of VEGF and VEGFR1 mutants are thus similar to each other, whereas the VEGFR2 phenotype appears to affect an even earlier step in development, the differentiation of the common precursor for endothelial and hemopoietic cells. These data could be explained by either the presence of maternal VEGF, which is secreted and might reach VEGFR2 in the $VEGF^{-/-}$ embryos, or by the existence of another ligand for VEGFR2, which would partially compensate for the absence of VEGF and allow the development of hemopoietic cells and to a certain extent of the vascular system in VEGF and VEGFR1 mutants.

We have recently shown that VEGFR2-positive cells isolated from the posterior mesoderm of chick embryos at the gastrulation stage were able to develop into both endothelial and hemopoietic cells in clonal cultures in vitro (Eichmann et al., 1997). Endothelial differentiation of the precursors was dependent on the presence of VEGF in the culture medium. Hemopoietic differentiation occurred in the absence of added growth factor, but was inhibited by the addition of soluble extracellular domain of VEGFR2. These experiments suggested that both endothelial and hemopoietic differentiation of the precursors was dependent on VEGFR2 and regulated by binding of two different ligands: VEGF binding triggered endothelial differentiation, whereas hemopoietic differentiation was triggered by binding of a yet unidentified ligand.

VEGF-C was a possible candidate ligand to mediate such an effect. We thus cloned avian VEGF-C, which was found to be highly homologous to the mouse and human molecules and to be expressed during development in regions rich in lymphatic vessels. Moreover, both avian and human VEGF-C were able to bind avian VEGFR3 and VEGFR2, enabling us to study the effect of VEGF-C on VEGFR2-positive cells from the posterior mesoderm of gastrulating chick embryos. We found that like VEGF, VEGF-C stimulated endothelial differentiation of the precursors. Thus, VEGF-C is a candidate growth factor that might compensate for endothelial differentiation in VEGF-deficient mice. However, hemopoietic differentiation of VEGFR2-positive cells does not appear to be mediated by VEGF-C, which therefore cannot be responsible for hemopoietic differentiation in VEGF-deficient mouse mutants.

MATERIALS AND METHODS

Isolation of quail VEGF-C cDNA clones

We used an oligo(dT) and random-primed amplified cDNA library (Invitrogen) available in our laboratory. This library was prepared from $poly(A)^+$ RNA isolated from E4 quail embryos and cloned into a pcDNA1 vector. Approximately 2×10^5 recombinant clones were screened on nitrocellulose filters (Schleicher and Schuell) with a radiolabeled PCR fragment of the human VEGF-C cDNA containing nucleotides 495-1661 (sequence accession number X94216, Joukov et al., 1996). Hybridization was performed at 42°C in buffer containing 50% formamide, followed by washing at 42°C in 2× SSC/0.1%SDS. Nucleic acid sequences were determined using the dideoxynucleotide chain termination method. Sequence comparison was performed using the Geneworks program (IntelliGenetics).

Northern blot analysis

Total RNA was isolated by the guanidinium/acid phenol extraction method (Chomczynski and Sacchi, 1987) followed by $poly(A)^+$ selection on oligo(dT)-cellulose prepacked columns (Pharmacia). 5 μg of $poly(A)^+$ RNA were loaded on a 1% formaldehyde agarose gel, electrophoresed and blotted onto nitrocellulose membrane filters (Schleicher and Schuell) as described (Maniatis et al., 1989). The filter was hybridized to a ^{32}P-labeled cDNA probe of a 2.7 kb *Eco*RI fragment of quail VEGF-C. Hybridization was done at 42°C in buffer containing 50% formamide, followed by washing at 65°C in 0.5× SSC/0.1% SDS. Exposure time was 3 days.

Expression of recombinant VEGF-C

A pcDNA1 expression vector containing the 2.7 kb full-size quail VEGF-C cDNA was transfected into 293-EBNA cells using 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) and with a human VEGF construct (Joukov et al., 1997). The transfected cells were cultured for 36 hours prior to metabolic labeling in DMEM-10% foetal calf serum (FCS), the medium was then changed to DMEM devoid of cysteine and methionine. After 30 minutes of preincubation, the medium was changed again to the same medium containing 120 μCi/ml of Promix^{TM} L-(^{35}S) in vitro cell labeling mix (Amersham) and incubated overnight. The culture medium was then collected and clarified by centrifugation.

In situ hybridization

Sense and antisense riboprobes were prepared from the 2.7 kb VEGF-

C cDNA and from a 4.2 kb VEGFR2 and 4.2 kb VEGFR3 cDNA (Eichmann et al., 1996). Whole-mount in situ hybridizations, hybridizations of cryostat sections and of dried plasma clots were performed as described by Henrique et al. (1995). For sectioning of whole mounts, embryos were embedded in gelatin-sucrose and frozen, and 20 µm cryostat sections were prepared as described in Pourquié et al. (1996). Radioactive in situ hybridizations were performed on paraffin sections (5 µm) of quail or chick embryos fixed in Carnoy's solution (60% EtOH, 30% $CHCL_3$, 10% Acetic acid). The procedures used for section treatment, hybridization and washing were described previously (Eichmann et al., 1993).

VEGFR3/Fc fusion protein

For receptor binding experiments, we prepared fusion proteins of the quail Quek1/VEGFR2 and Quek2/VEGFR3 extracellular domains (Eichmann et al., 1996) with the Fc part of human Ig as previously described for Quek1/VEGFR2 (Eichmann et al., 1997). For Quek2/VEGFR3, the extracellular domain (aa residues 1-788 of the published sequence, accession No. X83287) was amplified by PCR using primers containing suitable retriction sites for cloning into the MO90 expression vector (kindly provided by Shin-Ichi Nishikawa, Kyoto University) in frame with the Fc part of human Ig. This vector was transfected into COS cells using the DEAE dextran method and the culture medium (DMEM/F12 supplemented with 0.5% Nutridoma (Boehringer Mannheim) and antibiotics) containing the secreted VEGFR3-Fc protein was harvested after 3 and 6 days following transfection. Fusion protein was purified from the medium by Protein-A sepharose chromatography as described (Eichmann et al., 1997) and the yield and purity determined by SDS-PAGE.

Immunoprecipitations

700 µl aliquots of metabolically labeled transfected or untransfected 293 cells were incubated with 200 ng of VEGFR2/Fc or VEGFR3/Fc fusion proteins in binding buffer: Tween 0.2%, BSA 5%, heparin 10 µg/ml in phosphate-buffered saline (PBS). For immunoprecipitations of quail and human VEGF-C with antibodies we used 2 µl of rabbit polyclonal antiserum against an N-terminal 17 aa peptide of mature human VEGF-C (EETIKFAAAHYNTEILK; Kukk et al., 1996). For immunoprecipitation of human VEGF, we used 3 µl of a monoclonal IgG1 mouse anti-human VEGF antibody available commercially (R&D). After incubation for 3 hours at RT, 35 µl of protein-A or for anti-VEGF protein-G-sepharose slurry in PBS were added and incubated for 45 minutes at 4°C with agitation. This was followed by three washes on ice: (1) for receptors in binding buffer, (2) for antibodies in RIPA buffer, and (3) a final wash in 20 mM Tris-HCl pH 7.4. Samples were boiled in Laemmli buffer and analysed by 12.5% SDS-PAGE.

Cell sorting, cell culture and staining

60 outbred JA57 chick embryos were incubated for 22 hours at 38°C. Posterior mesodermal cells were dissociated by pipetting with a finely pulled pasteur pipette in Ca^{2+}/Mg^{2+}-free PBS at 25°C. Labeling with anti-VEGFR2 hybridoma supernatant and cell sorting on a FACS-Star (Beckton-Dickinson) were done as described (Eichmann et al., 1997). After sorting, cells were seeded in 0.5 ml serum-free medium prepared as described (Cormier and Dieterlen-Lièvre, 1988) and clotted by the addition of citrated bovine plasma (GIBCO, 10% v/v) and thrombin (1 IU/ml, Produits Roche, France). Human recombinant VEGF was purchased from R&D. The mature form of human recombinant VEGF-C (ΔNΔC construct, Joukov et al., 1997) was expressed in the yeast *P. pastoris* strain GS115 as described (Joukov et al., 1997), purified using a 6-His tag and protein concentrations were estimated on SDS-PAGE. Ac DiI-LDL (acetylated low density lipoprotein labelled with 1,1-dioctadecyl 1-3,3,3,3-tetramethyl-indocarbocyanine perchlorate, Biomedical Technologies Inc. Stoughton) uptake was assayed according to the manufacturer's instructions as previously described (Flamme and Risau, 1992).

RESULTS

Cloning of quail VEGF-C

A 1 kb cDNA fragment encoding the open-reading frame of human VEGF-C (Joukov et al., 1996) was used to screen an E4 quail cDNA library under low stringency conditions (see Materials and Methods). This led to the isolation of two overlapping cDNA clones of 2.7 kb which were mapped and

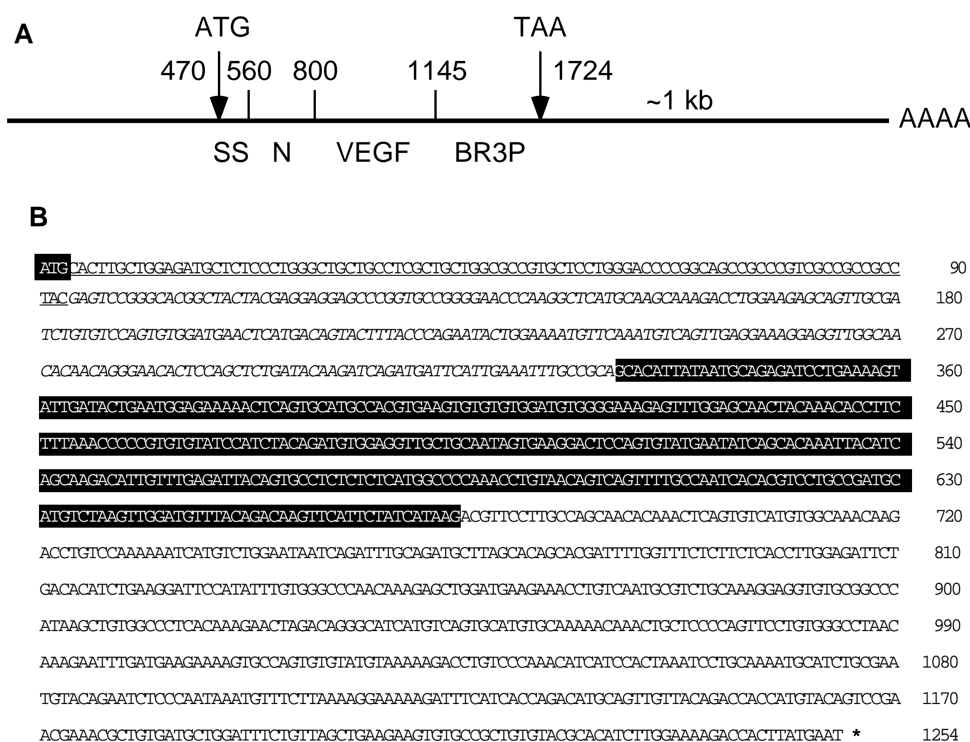


Fig. 1. Cloning of quail VEGF-C cDNA. (A) Schematic drawing of the quail VEGF-C cDNA. SS, signal sequence; N, N-terminal propeptide; VEGF, VEGF homology region; BR3P, Balbiani ring 3 protein homology region; AAA, poly(A) tail. The position of the ATG initiation codon and the stop codon are indicated (arrows). Numbers refer to the nucleotide sequence (B) and indicate the position of the homology domains as determined by analogy to the mouse and human sequences.
(B) Nucleotide sequence of the quail VEGF-C coding region. Outlined are the ATG (white on black), signal sequence (underlined), the N-terminal propeptide (italics) and the VEGF-homology domain (white on black). The remaining part of the coding region corresponds to the BR3P homology domain. Stop codon is indicated (*). The nucleotide sequence of quail VEGF-C has the accession number Y15837.

sequenced on both strands. Both clones contained a polyadenylation signal and poly(A) tail at their 3′ end, whereas the 5′ end of one clone extended 14 bp further 5′ than the other. A schematic drawing of the longer clone and the nucleic acid sequence of the coding region are shown in Fig. 1A,B, respectively. The quail VEGF-C cDNA contains a single open reading frame of 1254 bp, preceded by 470 bp of GC-rich 5′ untranslated region and followed by a 3′ untranslated region of about 1 kb. The open-reading frame (Fig. 1B) is predicted to code for a protein of 418 aa, which shows high homology to human and mouse VEGF-C (Fig. 2).

The putative initiation codon is followed by a hydrophobic signal sequence of 28 aa and the N-terminal propeptide, which by analogy to mouse and human VEGF-C is predicted to be cleaved off before or during secretion from cells between residues A111-A112 (Joukov et al., 1997). Whereas the aa identity of the signal peptide to mouse and human VEGF-C is relatively low (48%), the N-terminal propeptide shows high aa identity (71% quail-human). The aa identity of the VEGF-homology region is even higher (90% quail-human and 91% quail-mouse), notably the eight cysteine residues typical for the VEGF/PDGF family as well as an extra cysteine residue (C 136) present only in VEGF-C are conserved. In the C-terminal part of the protein, the fourfold repeated aa motif C-10X-C-X-C-X-C homologous to the Balbiani ring 3 protein (BR3P) and three other cysteine-containing repeats (C-10/11X-C-R/S-C) encountered in mouse and human VEGF-C are conserved as well. An aa identity of 71% between quail and human VEGF-C is observed in this region of the molecule.

Northern blot analysis showed that the 2.7 kb quail VEGF-C cDNA hybridized with a single transcript of about the same size present in E4 quail RNA and at lower levels in adult kidney RNA, but not in adult brain RNA (Fig. 3).

Quail recombinant VEGF-C protein binds VEGFR2 and VEGFR3

An expression vector containing the quail VEGF-C cDNA was transfected into 293-EBNA cells and the cells were metabolically labeled. The medium was analyzed for the presence of the secreted protein by immunoprecipitation with a polyclonal antibody directed against an N-terminal peptide of human VEGF-C, which reacts with both human and mouse VEGF-C (Kukk et al., 1996; Fig. 4A). Cells either untransfected or transfected with human VEGF-C cDNA were used as negative and positive controls. In both human and quail VEGF-C transfected cells, but not in untransfected cells, a doublet of 31 and 29×10^3 M_r and a band of 21×10^3 M_r were immunoprecipitated (Fig. 4A), indicating that the antibody against human VEGF-C recognized the corresponding quail ligand and that proteolytic processing mechanisms of the quail and human growth factor appear to be conserved. The 31×10^3 M_r band of human VEGF-C has been shown to correspond to the N-terminal region of secreted VEGF-C after removal of the signal peptide, whereas the 29×10^3 M_r form corresponds to the C-terminal half of the VEGF-C precursor generated by cleavage between Arg 227 and Ser 228, and the 21×10^3 M_r form is generated by precursor cleavage between Ala 111 and Ala 112 (Joukov et al., 1997).

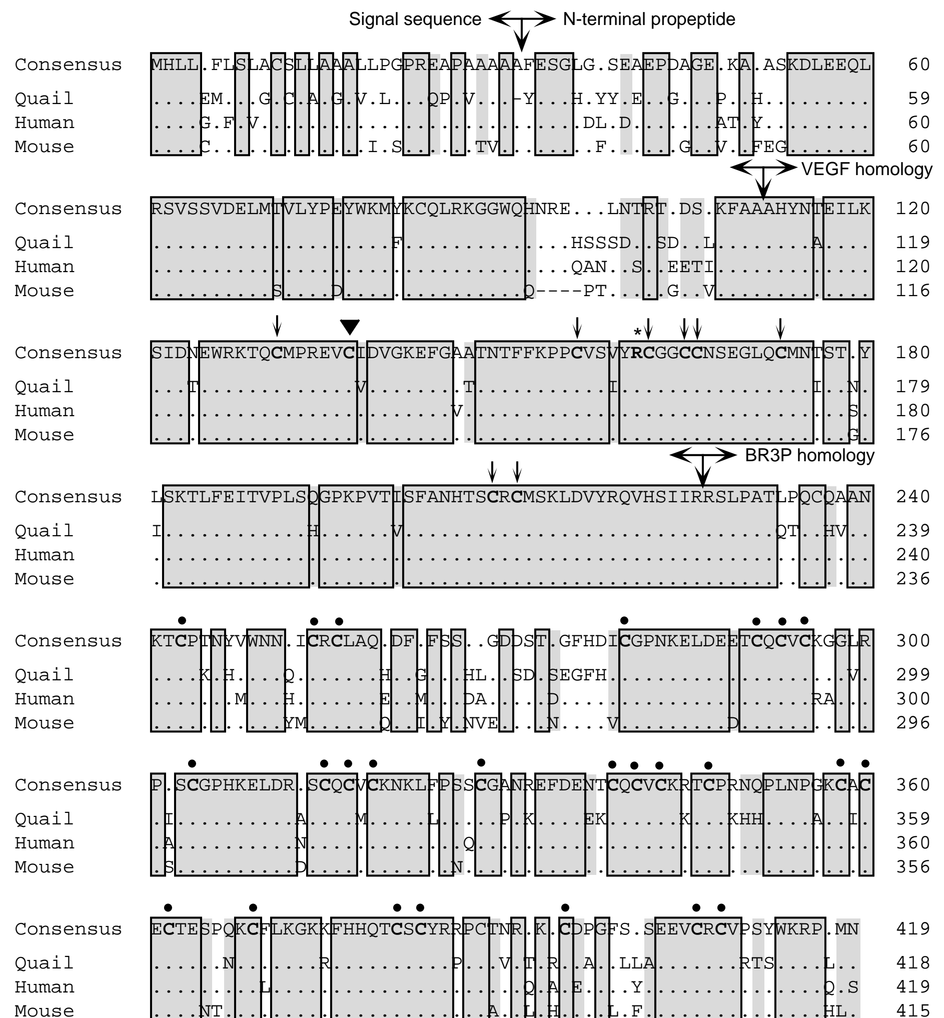


Fig. 2. Comparison of the deduced aa sequence of quail VEGF-C to mouse and human VEGF-C. The consensus sequence is given on top, identical aa residues are shown as dots and conserved substitutions are shaded. The positions of the signal sequence, N-terminal propeptide and VEGF and BR3P domains are indicated by arrows. In the VEGF homology region, the eight conserved cysteine residues conserved in VEGF and PDGF family members are marked with arrows, the cysteine residue conserved only in VEGF-C (position 137 in the consensus sequence) is marked by an arrowhead. The conserved arginine residue position 161 in the consensus sequence is marked by an asterisk. Conserved cysteine residues in the BR3P region are marked by dots.

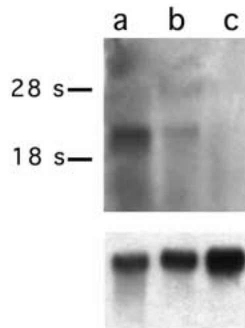


Fig. 3. Northern blot analysis of quail VEGF-C. A northern blot containing 5 µg of poly(A)$^+$RNA isolated from E4 quail embryos (a), adult kidney (b) and adult brain (c) was probed with the full-size quail VEGF-C cDNA. A single transcript of about 2.7 kb was detected. The blot was rehybridized with a probe of quail β-actin to control for the amounts of RNA loaded on the gel, as shown below. The positions of the 28s and 18s ribosomal RNAs are indicated.

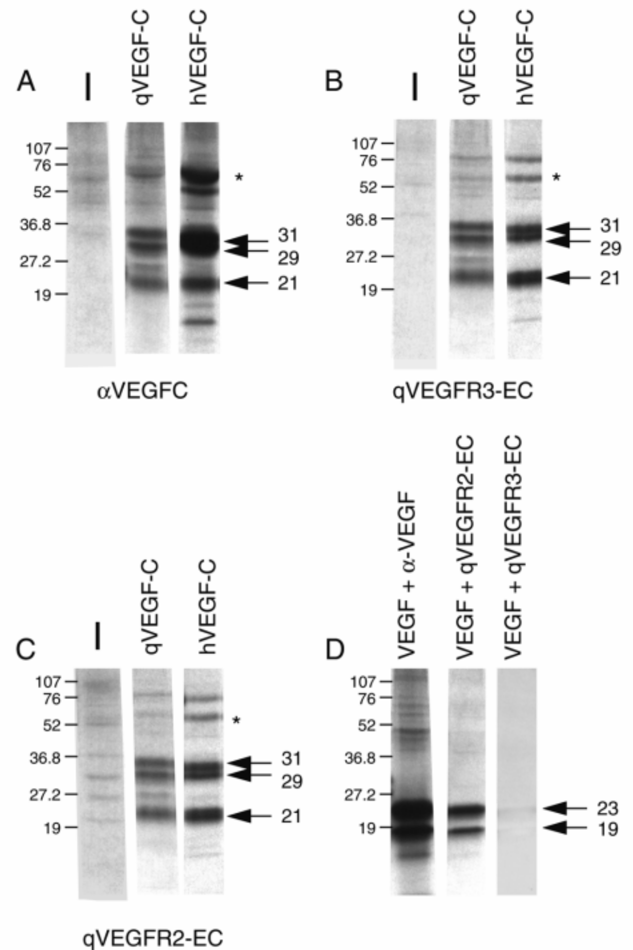


Fig. 4. Analysis of quail VEGF-C receptor binding properties. (A) Media from metabolically labeled untransfected cells (–) and from cells transfected with quail VEGF-C (qVEGF-C) or human VEGF-C (hVEGF-C) were immunoprecipitated with an antibody directed against human VEGF-C. The VEGF-C polypeptides of M_r 31 and 29×10^3 and 21×10^3 are indicated by arrows. A higher molecular mass form of about 58×10^3 M_r, corresponding to the unprocessed precursor molecule (Joukov et al., 1997), is indicated (*). (B,C) The same media were immunoprecipitated with quail VEGFR3-Fc (B) and quail VEGFR2-Fc (C) fusion proteins. The 76×10^3 M_r form immunoprecipitated with both receptors might correspond to residual dimerized forms of the VEGF-C molecule. (D) Media from metabolically labeled cells transfected with human VEGF were immunoprecipitated with a monoclonal antibody directed against VEGF (VEGF+ α-VEGF), with quail VEGFR2-Fc (VEGF+ qVEGFR2-EC) and with quail VEGFR3-Fc (VEGF+qVEGFR3-EC) fusion proteins. VEGFR2, but not VEGFR3 recognizes bands of M_r 19 and 23×10^3.

The media were subsequently analysed for receptor binding of quail VEGFR3 and VEGFR2 (Eichmann et al., 1996) (Fig. 4B,C). Similar bands were obtained, indicating that quail and human VEGF-C can bind both avian receptors. In contrast, human VEGF binds only quail VEGFR2 but not quail VEGFR3 (Fig. 4D), as shown previously for the human receptors: human VEGFR3 does not bind VEGF (Pajusola et al., 1994), whereas human VEGFR2 binds both VEGF and VEGF-C (Joukov et al., 1996).

Expression of quail VEGF-C during embryonic development

The expression of VEGF-C during the establishment of the avian vascular system was examined by in situ hybridization. Embryos between E1 and E5 were studied, corresponding to the developmental period when the cells of the vascular system proliferate and differentiate into blood-vascular and lymphatic vessel endothelia and when the expression of VEGFR2 and VEGFR3 is at maximal levels in avian embryos (Eichmann et al., 1993, Wilting et al., 1997). Quail and chick embryos were analyzed and no difference between the two species was observed.

VEGF-C expression was first detected at the 19-somite stage in two locations: the roof of the diencephalon (Fig. 5A) and the splanchnic mesoderm surrounding the omphalomesenteric vein, close to its connection with the heart, a zone that will subsequently form the mesocardium (Fig. 5C).

At later stages (E3 onward), expression in the roof of the cephalic neural tube extended caudally to the hindbrain and was also observed ventrally (see below). Moreover, branchial arch mesoderm surrounding the foregut was VEGF-C positive (Fig. 5D) and labeling was also observed in the mesenchyme surrounding the hindgut and the omphalomesenteric and vitelline veins (Fig. 5E,F). In the kidney, VEGF-C expression was detected in the glomerular region (Fig. 5H,I). In addition, endothelial cells of the aorta appeared weakly VEGF-C positive at that stage (Fig. 5C,D,H,I).

From E4, we compared the patterns of expression of VEGF-C and VEGFR3 (Fig. 6). In the diencephalon, VEGF-C was still expressed dorsally as can be seen in Fig. 6A at the base of the pineal gland. Ventrally, VEGF-C was observed in the future hypothalamus (Fig. 6A) and in the Rathke's pouch. In the rhombencephalon, VEGF-C was expressed in groups of cells of the basal plate and in the lateral part of the choroid plexus. VEGF-C was furthermore expressed in parts of the pigmented, but not in the neural retina (not shown) and in the optic nerve (Fig. 6A), as well as in different placodes (Fig. 6C). VEGFR3 was expressed in the perineural vascular plexus covering the entire neural tube and the eyes (Fig. 6B,D). At the level of the spinal cord, VEGF-C was expressed in the ventricular zone of

the neural tube (Fig. 6C), where VEGFR3-expressing cells could be seen invading it (Fig. 6D). The respective patterns of VEGF-C and VEGFR3 during these early stages of the development of the neural tube thus appeared, as a general rule, complementary. They suggest a paracrine mode of VEGFR3 activation by VEGF-C.

As mentioned above for the aorta at E3, endothelial cells could be found expressing VEGF-C transcripts at E4, for example in the wall of the anterior cardinal vein, where the receptor was no longer present (Fig. 6A,B). Disseminated capillaries invading the neuroepithelium also expressed VEGF-C (Fig. 6A). Although double-labeling with the VEGF-C and VEGFR3 probes has not been performed, coexistence of the factor and the receptor (Fig. 6B) in the same endothelial cells cannot be ruled out for the cells observed in the cephalic neural tube.

At the level of the heart, VEGF-C was still strongly expressed in the mesocardium, in splanchnic mesoderm adjacent to the pericardial epithelium (Fig. 6C) and in the branchial arch mesenchyme. VEGFR3 was strongly expressed in vessels of these regions (Fig. 6D). VEGFR3 was also strongly expressed in two parallel streams of vessels located on each side of the myotome (Fig. 6D,F), which itself expressed VEGF-C (Fig. 6C,E). In some sections, VEGFR3-expressing cells could be seen to branch from the anterior cardinal vein, supporting the theory of a venous origin of lymphatic vessels (Sabin, 1909). The VEGFR3-positive cells do not at this stage form channels, but are likely to represent the primordia of subcutaneous, periaortic and splanchnic lymphatic vessels. In the case of the dorsal root ganglia (DRG), however, expression of the receptor in the lateroventral region of the ganglia was not accompanied by the presence of the factor (Fig. 6C,D). The latter was in fact produced by the closely apposed neural tube. Developmental dependance of the developing DRG with respect to the neural tube has been previously documented (Kalcheim and Le Douarin, 1986).

VEGF-C promotes endothelial differentiation of VEGFR2-positive mesodermal precursors

The results of the receptor interaction experiments described above suggested that human VEGF-C could interact with avian VEGFR3, but also with VEGFR2. We therefore wanted to test the effect of human recombinant VEGF-C produced in a yeast expression vector on VEGFR2-positive avian mesodermal cells isolated from the posterior mesoderm of chick embryos

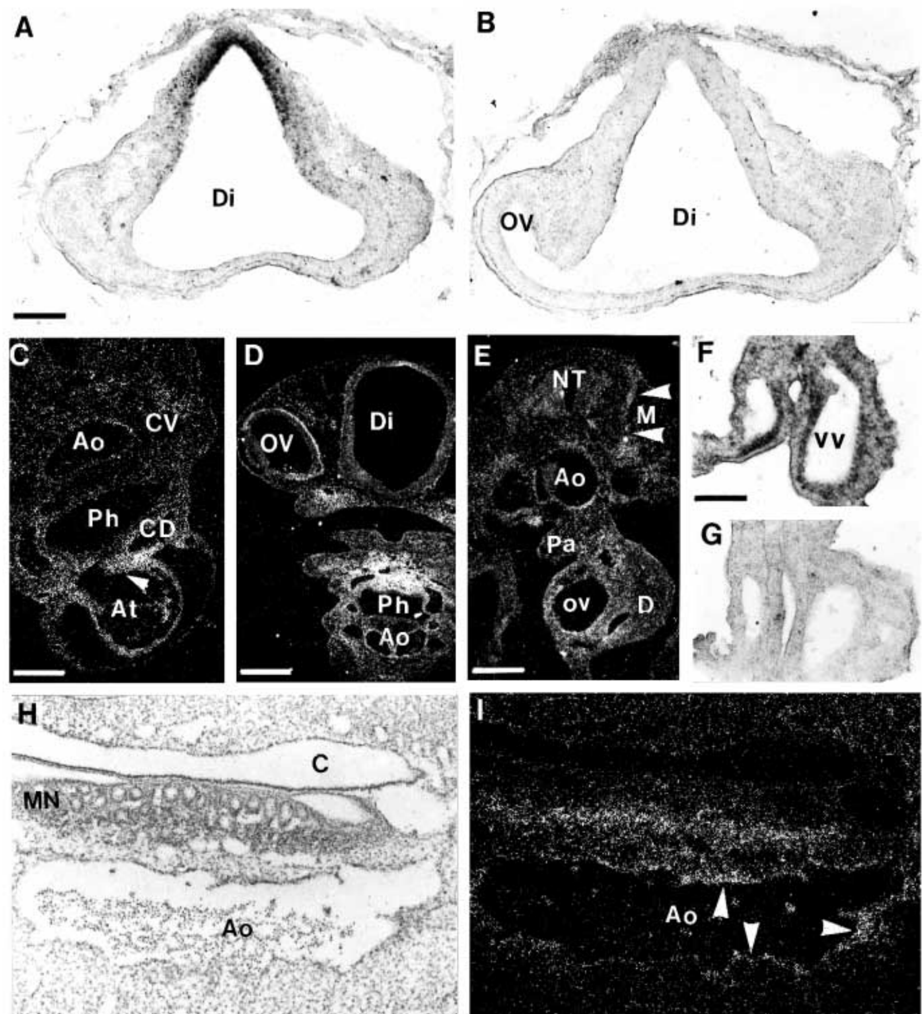


Fig. 5. VEGF-C expression during embryonic development. (A) Onset of expression. Transverse cryostat section of a 19 somite quail embryo at the level of the diencephalon (Di) hybridized with a digoxigenin-labeled VEGF-C antisense riboprobe. VEGF-C is expressed in the roof of the diencephalon. (B) Adjacent section hybridized with a sense riboprobe. OV, optic vesicle. Bar in A,B,H,I, 120 µm. (C-E) Dark-field images of radioactive in situ hybridizations with VEGF-C antisense riboprobes on paraffin-sections. (C) Expression in the mesocardium (arrowhead). Section of an E2.5 quail embryo at the level of the atrium (At). Ao, Aorta; Ph, pharynx; CV, cardinal vein; CD, Cuvier duct. Bar, 160 µm. (D) Expression in branchial arch mesenchyme. Section of an E3 quail embryo at the level of the diencephalon and branchial arches 1-3. VEGF-C is strongly expressed in the mesenchyme underneath the pharynx and persists in the roof of the diencephalon. Note also expression in the aortic endothelium. Bar, 380 µm. (E) Transverse section at the level of the limb bud of an E3.5 quail embryo. VEGF-C is expressed in the mesenchyme surrounding the omphalomesenteric vein (ov) at the level of the duodenum (D). Pa, pancreas; NT, neural tube. Note also beginning expression in the myotome (M) (arrowheads). Bar, 90 µm. (F) Hybridization of digoxygenin-labeled antisense VEGF-C to the mesenchyme surrounding the vitelline veins (vv) of an E2.5 quail embryo. (G) Sense control. Bar, 100 µm. (H,I) Bright-field (H) and Dark-field (I) images of a section through the caudal region of an E3.5 quail embryo. VEGF-C is expressed in the glomerular region of the mesonephros (MN). No expression is observed in kidney tubules. C, coelom. Note also expression in the aortic endothelium (arrowheads).

at the gastrulation stage (presomitic until 3 somite stage). We have recently shown that the VEGFR2-positive population isolated from this region can give rise to both endothelial and hemopoietic cells in vitro (Eichmann et al., 1997). In situ hybridization with antisense riboprobes for both receptors to embryos at gastrulation stage showed that a significant proportion of cells of the posterior mesoderm express VEGFR2 (Fig. 7A). In contrast, VEGFR3 expression occured only in Hensen's node and notocord at these stages (Fig. 7B). Given the absence of expression of VEGFR3 in the VEGFR2-positive cells, an effect of VEGF-C would therefore probably be mediated by VEGFR2.

Cell suspensions prepared from posterior mesodermal cells (see Fig. 7A) were labeled with a monoclonal antibody directed against the extracellular domain of quail VEGFR2 and sorted by FACS as previously described (Eichmann et al., 1997). VEGFR2-positive and -negative cells were then cultured for 72 hours in semi-solid medium containing human recombinant VEGF or increasing doses of VEGF-C (5-400 ng/ml).

As observed previously (Eichmann et al., 1997), hemopoietic differentiation from cultured VEGFR2-positive cells ocurred in the absence of VEGF/VEGF-C, whereas very few endothelial colonies developed (Fig. 8A). In the presence of 50 ng/ml of VEGF, hemopoietic differentiation was reduced and endothelial cell colonies formed (Fig. 8A).

In the presence of increasing concentrations of VEGF-C, VEGFR2+ precursors gave rise to progressively increasing numbers of endothelial colonies. As observed for VEGF (Eichmann et al., 1997), the hemopoietic response of the cells was reduced by increasing VEGF-C concentrations (Fig. 8A). The behaviour of posterior mesodermal VEGFR2-positive cells in the presence of VEGF-C was therefore very similar to the one observed with VEGF, except for the concentrations of growth factor required: VEGF has previously been shown to induce endothelial colonies from concentrations of 5 ng/ml (Eichmann et al., 1997), whereas 50 ng/ml of VEGF-C are required to obtain similar responses. The maximal number of endothelial cell colonies was obtained in the presence of 200 ng/ml of VEGF-C, whereas only 50 ng/ml of VEGF were required. Thus, compared to VEGF, VEGF-C produces a similar cellular response, but at five to ten times higher concentrations. VEGFR2-negative cells did not respond to VEGF-C (200ng/ml) (ec colony number: 6±2.5, hc colony

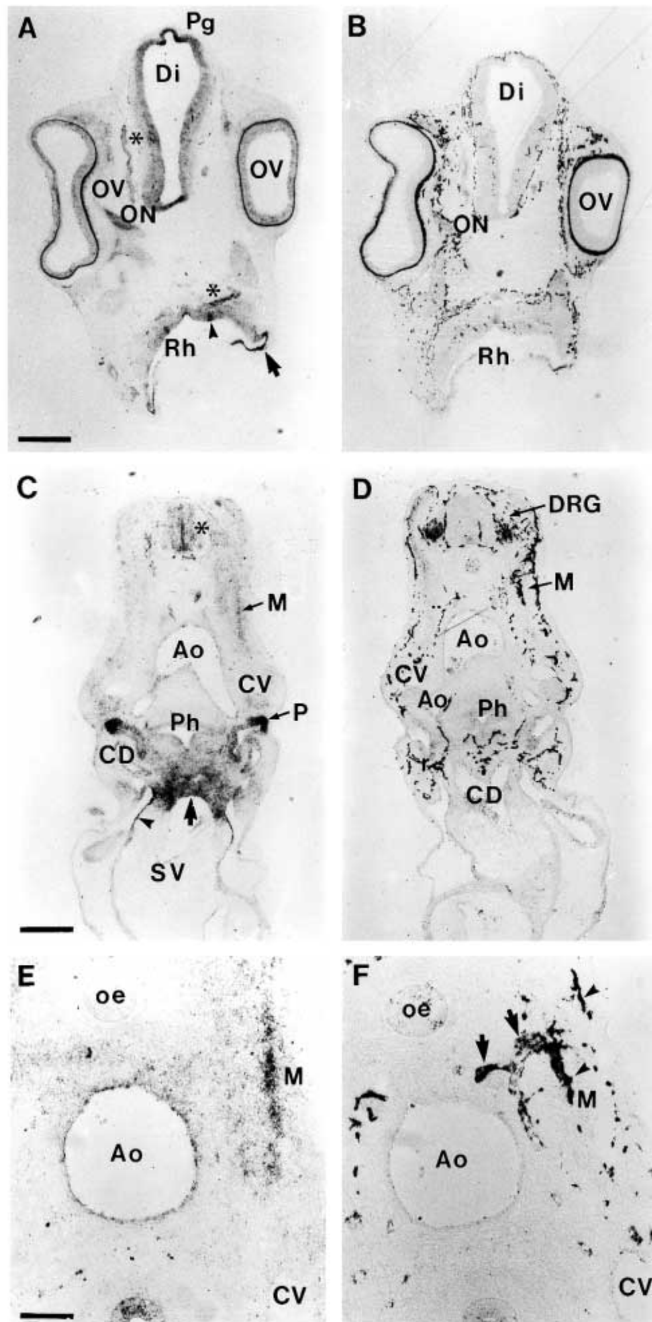


Fig. 6. Comparison of VEGF-C and VEGFR3 expression. Adjacent cryostat sections from an E4 quail embryo hybridized with digoxigenin-labeled quail VEGF-C (A,C,E) and VEGFR3 (B,D,F) antisense riboprobes. (A,B) Transverse sections through the diencephalon (Di) and rhombencephalon (Rh) at the level of the pineal gland (Pg). (A) Note VEGF-C expression at the base of the pineal gland and in the ventral diencephalon, the region of the future hypothalamus. In the rhombencephalon, VEGF-C is expressed in the rhombic lip (arrow) and in groups of neurons of the basal plate (arrowhead). VEGF-C expression is found in the optic nerve (ON) and in endothelial cells of the lateral diencephalon and ventral rhombencephalon (asterisks). (B) VEGFR3 is expressed in the perineural vascular plexus surrounding the brain vesicles and the eyes. Bar, 240 μm. (C,D) Sections through the neural tube, root of dorsal aorta (Ao) and sinus venosus (SV) of the heart. VEGF-C (C) is highly expressed in the mesocardium (arrow) and splanchnic mesoderm adjacent to the pericardial epithelium (arrowhead). Note expression in the epibranchial placode IX/X (P) and in the ventricular region of the neural tube (asterisk), as well as in the myotome (M). CV, cardinal vein; CD, cuvier duct; Ph, pharynx. (D) VEGFR3 is expressed in vessels of the mesocardial and branchial arch regions which strongly express VEGF-C. Note absence of VEGFR3 expression in the endothelium of the aorta, cardinal veins and cuvier duct as well as in the endocardium. In the dorsal region, VEGFR3 is highly expressed in forming vessels of the dorsal root ganglia (DRG). Note also expression in two parallel streams of capillaries on each side of the myotome. Bar, 300 μm. (E,F) Cross-section through the aorta at the thoracic level. Note significant VEGF-C expression (E) in the myotome and VEGFR3 expression (F) in the two parallel streams of capillaries (arrowheads) branching towards the location of the future lymphatic thoracic duct (arrows). oe, oesophagus. Bar, 100 μm.

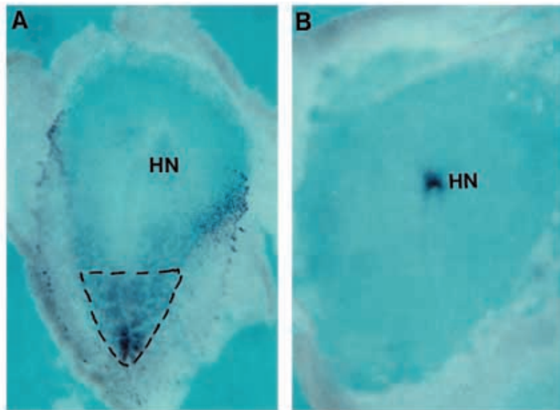
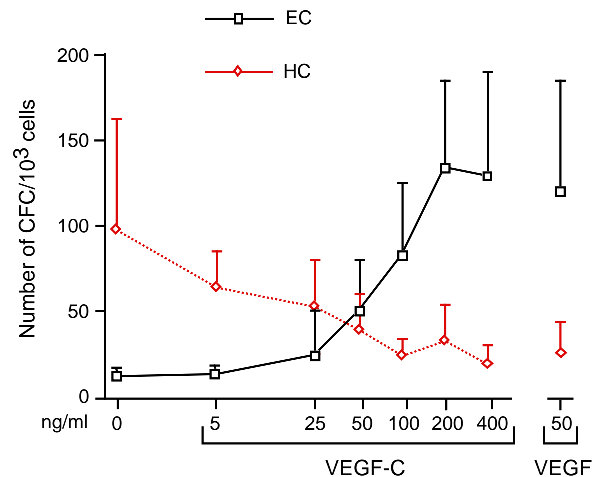


Fig. 7. VEGFR2 and VEGFR3 expression in gastrulating embryos. Whole-mount in situ hybridization using VEGFR2 (A) and VEGFR3 (B) antisense riboprobes on quail embryos at stage 4+ (A) and 4 (B) of Hamburger and Hamilton (1951). Note abundant VEGFR2 expression in posterior mesodermal cells, whereas VEGFR3 is expressed in Hensen's node (HN). Tissue from the area surrounded by a dotted line in A was used for the cell sorting experiments.

number: 2±1.5; mean ±s.d. calculated for 1×10^3 cells from three independent experiments performed in duplicates)

Endothelial cell colonies induced by VEGF-C or VEGF were indistinguishable by phase-contrast microscopy (not shown) or May-Grünwald Giemsa staining (Fig. 8B,C). To examine further their phenotypes, they were tested with a panel of markers specific for chick endothelial cells: the colonies were positive for VEGFR2 and VEGFR3 expression as determined by in situ hybridization with avian receptor probes (not shown) and incorporated DiI-Ac-LDL (Fig. 8D,E). VEGF-C induced endothelial cell colonies obtained from posterior mesodermal VEGFR2+ cells thus expressed the same markers as did colonies obtained after VEGF stimulation (Eichmann et al., 1997).



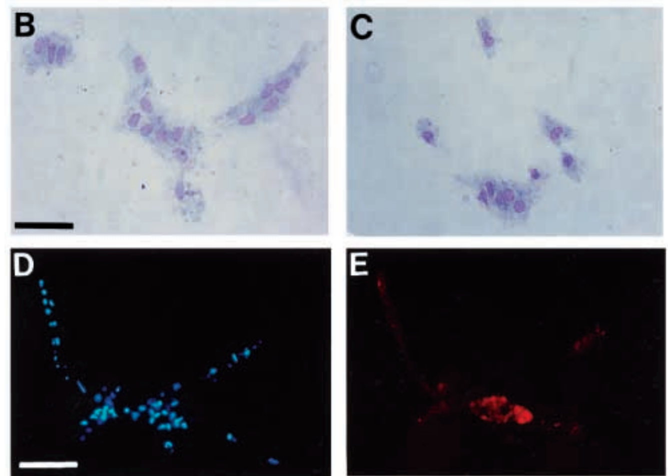


Fig. 8. VEGF-C activity on VEGFR2+ cells from the posterior mesoderm of gastrulating embryos in vitro. (A) VEGF-C stimulates endothelial differentiation of VEGFR2+ cells and reduces their hemopoietic differentiation. Each value represents the mean number ± s.d. of colony-forming cells (CFC) calculated per 1×10^3 cells obtained in four independent experiments performed in duplicates, except for VEGF-C 100 and 400 ng/ml, three experiments. Growth factor additions are indicated**.** (B,C) May-Grünwald-Giemsa stain of typical endothelial colonies induced by VEGF-C (B) and VEGF (C). Bar, 50 µm. (D) Hoechst nuclear stain and DiI-Ac LDL uptake (E) of a VEGF-C-induced endothelial cell colony. Bar, 125 µm.

DISCUSSION

In this work, we report the cloning of quail VEGF-C, interaction with avian VEGFR2 and VEGFR3, and biological activity on VEGFR2-positive cells isolated from the posterior mesoderm of embryos at the gastrulation stage.

VEGF-C was found to be highly conserved between the species where it has so far been identified, i.e mouse, human and quail. Highest sequence conservation (90% quail-human and 91% quail-mouse) is observed in the VEGF-homology domain, which constitutes the mature form of the protein (Joukov et al., 1997). Within this domain, the eight cysteine residues typical for the VEGF/PDGF family and an extra cysteine residue (position 137 in the consensus sequence) found only in VEGF-C and FlGF (Orlandini et al., 1996) are conserved in the quail sequence. High sequence conservation is also found in the N- and C-terminal propeptides, notably, the spacing of cysteine residues characteristic for BR3P protein is conserved between quail, mouse and human VEGF-C.

The deduced protein sequence of quail VEGF-C is one aa shorter than its human counterpart. The missing amino acid in the quail sequence is located at the site of cleavage of the signal sequence in human VEGF-C, which is thought to occur at pos. 31 between a stretch of alanine residues and a phenylalanine residue (Joukov et al., 1997). The quail sequence contains one less alanine residue and the phenylalanine is replaced by a tyrosine residue. Thus, cleavage of the signal sequence might occur at a different site in the quail protein. In contrast, the proteolytic cleavage sites of the N- and C-terminal propeptides as determined by Joukov et al. (1997) for human VEGF-C are conserved in the quail sequence. In the case of the N-terminal cleavage site, only conservative amino acid substitutions are found, whereas the C-terminal cleavage site is fully conserved. Thus, proteolytic processing mechanisms analogous to those described in humans might exist in the avian species.

The different VEGF-C forms generated by proteolytic processing were reported to show increasing affinity towards

VEGFR3, whereas VEGFR2 could be activated only by the mature form of VEGF-C (Joukov et al., 1997). The receptor binding data presented here indicate that, like their human homologues, avian VEGFR3 binds VEGF-C, but not VEGF, whereas avian VEGFR2 binds to both growth factors. However, human VEGFR2 binds only the mature form of VEGF-C (Joukov et al., 1997), whereas quail VEGFR2 binds both the mature and the unprocessed forms of VEGF-C. Receptor activation experiments have thus to be performed to examine if avian VEGFR2 can be activated by both forms.

The biological activity of the mature form of VEGF-C has been tested on bovine capillary endothelial (BCE) cells (Joukov et al., 1996; 1997). VEGF-C was found to stimulate proliferation and migration of these cells, albeit at around 50-fold higher concentrations when compared to VEGF. The VEGF-C action on BCE cells is probably mediated by VEGFR2, since these cells express very little VEGFR1 or VEGFR3 (Joukov et al., 1997). These data indicated that VEGF-C could induce biological responses similar to those elicited by VEGF. We thus decided to examine the biological activity of VEGF-C on VEGFR2-positive cells isolated from the posterior mesoderm of embryos at the gastrulation stage. At the time that they are isolated from the embryo, these cells do not express VEGFR3. VEGF-C does not bind to VEGFR1 (Lee et al., 1996, Joukov et al., 1997), thus the biological activity of VEGF-C on posterior mesodermal cells is probably mediated by VEGFR2. VEGF-C stimulation of VEGFR2-positive cells from posterior mesoderm resulted in the formation of endothelial cell colonies. As in the case of VEGF, increasing concentrations of VEGF-C augmented the number of endothelial colonies to reach a maximum of about 1 colony for 10 precursors seeded. The VEGF-C doses necessary to elicit this effect were five to ten fold higher when compared to VEGF: VEGF had a maximal effect at a concentration of 50 ng/ml (Eichmann et al., 1997), whereas the maximal VEGF-C effect was obtained at a concentration of 200 ng/ml. Thus, VEGF-C and VEGF both induce endothelial differentiation of early mesodermal VEGFR2-positive cells.

In the absence of VEGF, cultured posterior mesodermal VEGFR2+ cells give rise to hemopoietic cells (Eichmann et al., 1997). This process appears to be mediated by binding of a VEGFR2 ligand, since hemopoietic differentiation can be inhibited by the addition of soluble VEGFR2 extracellular domain to the culture medium. Addition of VEGF-C to cultured VEGFR2+ cells results, however, in the inhibition of hemopoietic differentiation and the stimulation of endothelial differentiation, as observed for VEGF.

Using in situ hybridization, we have detected VEGF-C expression from E2 onward in the developing avian embryo. These results raise the question as to whether the observed VEGF-C activity on posterior mesodermal cells isolated from E1 embryos in vitro takes place in vivo. One possibility is that low levels of VEGF-C, undetectable by in situ hybridization, are present in the avian embryo. Preliminary results using RT-PCR analysis indicate that VEGF-C mRNA is detectable in the embryo from early gastrulation stages onwards (Eichmann et al., unpublished results). In the mouse embryo, VEGF-C has been detected from E7.5 onward by northern blot analysis (Kukk et al., 1996). Thus, VEGF-C interaction with VEGFR2 might occur in the mouse embryo at early stages of differentiation of endothelial cells. VEGF-C might therefore be the growth factor responsible for the observed endothelial differentiation in VEGF-deficient mice (Carmeliet et al., 1996; Ferrara et al., 1996). In contrast, our results suggest that yet another growth factor is responsible for the observed hemopoietic differentiation resulting from VEGFR2 activation in $VEGF^{-/-}$ mice.

VEGF-C expression during early stages of avian embryonic development is observed mainly in ectoderm- and mesoderm-derived structures. In the nervous system, the growth factor is expressed in the dorsal and the ventral side of the brain, the ventricular zone of the spinal cord, the pigmented retina, the optic nerve and the placodes. Both VEGFR3 and VEGFR2 are expressed in the perineural vascular plexus adjacent to VEGF-C-expressing cells, suggesting a paracrine mode of growth factor action. However, expression in the dorsal side of the brain precedes the formation of the intraneural vascular plexus and merits further study. For example, proteolytic processing mechanisms might regulate the bioavailability of VEGF-C in these regions.

In the mesoderm, VEGF-C appears to be expressed by some endothelial cells (M. Aitkenhead, B. Christ, A. Eichmann, M. Feucht, D. J. Wilson and J. Wilting, unpublished results), raising the possibility of an autocrine activation mechanism of VEGF receptors by VEGF-C. VEGF expression has also been observed in some embryonic endothelial cells, suggesting that autocrine stimulation of angiogenesis might be a mechanism utilized by several angiogenic growth factors.

In the E12.5 mouse embryo, VEGF-C was found to be expressed in the mesenchyme surrounding the jugular vessels, the mesenteries and the developing kidney (Kukk et al., 1996). Weaker expression was found in branchial arches and intervertebral spaces. Our observations of VEGF-C expression in the mesoderm of the avian embryo suggest that the expression pattern of this growth factor during embryonic development is conserved between species.

VEGF-C is found mainly in regions that also express VEGFR3, including the branchial arches, mesocardium and intestinal regions. Although no account of the presence of lymphatic vessels at this stage of development is available in the avian embryo, these regions are rich in lymphatic vessels later in development, suggesting that VEGFR3 might label lymphatic endothelium from a very early developmental stage onward. In transgenic mice overexpressing VEGF-C in the skin, hyperplasia of lymphatic, but not vascular endothelial cells was induced (Jeltsch et al., 1997). Moreover, in the avian embryo, VEGF-C application to the chorioallantoic membrane was shown to selectively induce proliferation of lymphatic endothelium (Oh et al., 1997). Thus, VEGF-C clearly plays a role in the proliferation and perhaps also in the differentiation of lymphatic endothelium. However, the endothelial cells targeted by VEGF-C express both VEGFR3 and VEGFR2. It remains to be determined which of these receptors mediates VEGF-C action in vivo.

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REFERENCES

Carmeliet, P., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435-439.

Cao, Y., Chen, H., Zhou, L., Chiang, M. K., Anand-Apte, B., Weatherbee, J. A., Wang, Y., Fang, F., Flanagan, J. G. and Lik-Shing Tsang, M. (1996). Heterodimers of placenta growth factor/vascular endothelial growth factor. Endothelial activity, tumor cell expression and high affinity binding to Flk-1/KDR. *J. Biol. Chem*. **271**, 3154-3162.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*. **162**, 156-159.

Cormier, F. and Dieterlen-Lièvre, F. (1988). The wall of the chick embryo aorta harbours M-CFC, G-CFC, GM-CFC and BFU-E. *Development* **102**, 279-285.

DiSalvo, J., Bayne, M. L., Conn, G., Kwok, P. W., Trivedi, P. G., Soderman, D. D., Palisi, T. M., Sullivan, K. A. and Thomas, K. A. (1995). Purification and characterization of a naturally occuring vascular endothelial growth factor-placenta growth factor heterodimer. *J. Biol. Chem*. **270**, 7717-7723.

Eichmann, A., Marcelle, C., Breant, C. and Le Douarin, N. M. (1993). 2 molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development. *Mech. Dev*. **42,** 33-48.

Eichmann, A., Marcelle, C., Breant, C. and Le Douarin, N. (1996) Molecular cloning of Quek 1 and 2, two avian vascular endothelial growth factor (VEGF) receptor-like molecules. *Gene* **174,** 3-8.

Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Bréant, C. and Le Douarin, N. M. (1997). Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc. Natl. Acad. Sci. USA* **94**, 5141-5146.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380,** 439-442.

Ferrara, N. (1997) The biology of vascular endothelial growth factor. *Endocrine Rev*. **18**, 4-25.

Flamme, I. and Risau, W. (1992). Induction of vasculogenesis and hematopoiesis *in vitro*. *Development* **116**, 435-439.

Fong, G. H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66-70.

Grimmond, S. et al. (1996). Cloning and characterization of a novel human gene related to vascular endothelial growth factor. *Genome Res.* **6,** 124-131.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49-92.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995) Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K. and Alitalo, K. (1997). Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* **276**, 1423-1425.

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkinnen, N. and Alitalo, K. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt-4 (VEGFR3) and KDR (VEGFR2) receptor tyrosine kinases. *EMBO J*. **15**, 290-298.

Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N. and Alitalo, K. (1997). Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J*. **16**, 3898-3911.

Kaipainen, A., Korhoonen, J., Mustonen, T., van Hinsbergh, V., Fong, G. H., Dumont, D., Breitman, M. L. and Alitalo, K. (1995) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc. Natl. Acad. Sci. USA* **92***,* 3566-3570.

Kalcheim, C. and Le Douarin, N. M. (1986). Requirement of a neural tube signal for the differentiation of neural crest cells into dorsal root ganglia. *Dev. Biol*. **116**, 451-466.

Klagsbrun, M and D'Amore, P. (1996). Vascular endothelial growth factor and its receptors. *Cytokine Growth Factor Rev.* **7**, 259-270.

Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V. and Alitalo, K. (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development* **122**, 3829-3837.

Lee, J., Gray, A., Yuan, J.,Luoh, S. M., Avraham, H. and Wood, W. I. (1996). Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor flt-4. *Proc. Natl. Acad. Sci. USA* **93**, 1988-1992.

Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P. and Persico, M. G. (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc. Natl. Acad. Sci. USA* **88**, 9267-9271.

Maniatis, T., Sambrook, J. and Fritsch, E. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press.

Mustonen, T. and Alitalo, K. (1995) Endothelial receptor tyrosine kinases involved in angiogenesis. *J. Cell. Biol.* **129***,* 895-898.

Oh, S. J., Jeltsch, M. M., Birkenhäger, R., McCarthy, J. E. G., Weich, H. A., Christ, B., Alitalo, K. and Wilting, J. (1997). VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev. Biol*. **188**, 96-109.

Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Petterson, R. F., Alitalo, K., and Eriksson, V. (1996a). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc. Natl. Acad. Sci. USA* **93**, 2576-2581.

Olofsson, B., Pajusola, K., von Euler, G., Chilov, D., Alitalo, K. and Eriksson, U. (1996b). Genomic organization of the mouse and human genes for vascular endothelial growth factor B (VEGF-B) and characterization of a second splice isoform. *J. Biol. Chem.* **271**, 19310-19317.

Orlandini, M., Marconcini, L., Ferruzzi, R. and Oliviero, S. (1996). Identification of a c-fos induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. *Proc. Natl. Acad. Sci. USA* **93**, 11675-11680.

Pajusola, K., Aprelikova, O., Pelicci, G., Weich, H., Claesson-Welsh, L. and Alitalo, K. (1994). Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene* **9**, 3545-3555.

Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D. (1994). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* **90***,* 8392-8396*.*

Park, J. E., Cjen, H. H., Winer, J, Houck, K. A. and Ferrara, N. (1994). Placenta growth factor. *J. Biol. Chem*. **269,** 25646-25654.

Pourquié, O., Fan, C. M., Coltey, M., Hirsinger, E., Watanabe, Y., Bréant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996) Lateral and axial signals involved in avian somite patterning: A role for BMP4. *Cell* **84**, 461-471.

Sabin, F. R. (1909). The lymphatic system in human embryos, with a consideration of the morphology of the system as a whole. *Am. J. Anat*. **9**, 43-91.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X-F, Breitman, M. L. and Schuh, A. C. (1995). Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. *Nature* **376***,* 62-66.

Shibuya, M. (1995). Role of VEGF-Flt receptor system in normal and tumor angiogenesis. *Adv. Cancer Res*. **67,** 281-316.

Wilting, J., Eichmann, A. and Christ, B. (1997). Expression of avian VEGF receptor homologues Quek1 and Quek2 in blood-vascular and lymphatic endothelial and non-endothelial cells during quail embryonic development. *Cell Tissue Res.* **288**, 207-223.