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Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation

GEORG BREIER, URSULA ALBRECHT, SYLVIA STERRER* and WERNER RISAU

Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18A, 8033 Martinsried, FRG

*Present address: Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, 3400 Göttingen, FRG

Summary

Vascular endothelial growth factor (VEGF) is a secreted angiogenic mitogen whose target cell specificity appears to be restricted to vascular endothelial cells. Such factors are likely candidates for regulatory molecules involved in endothelial growth control. We have characterized the murine VEGF gene and have analysed its expression pattern in embryogenesis, particularly during brain angiogenesis. Analysis of cDNA clones predicted the existence of three molecular forms of VEGF which differ in size due to heterogeneity at the carboxy terminus of the protein. The predicted mature proteins consist of 120, 164 or 188 amino acid residues. Homodimers of the two lower molecular weight forms, but not of the higher molecular weight form, were secreted by COS cells transfected with the corresponding cDNAs and were equally potent in stimulating the growth of endothelial cells. During brain development, VEGF transcript levels were abundant in the ventricular neuroectoderm of embryonic and postnatal brain when endothelial cells proliferate rapidly but were reduced in the adult when endothelial cell proliferation has ceased. The temporal and spatial expression of VEGF is consistent with the hypothesis that VEGF is synthesized and released by the ventricular neuroectoderm and may induce the ingrowth of capillaries from the perineural vascular plexus. In addition to the transient expression during brain development, a persistent expression of VEGF was observed in epithelial cells adjacent to fenestrated endothelium, e.g. in choroid plexus and in kidney glomeruli. The data are consistent with a role of VEGF as a multifunctional regulator of endothelial cell growth and differentiation.

Key words: angiogenesis, brain, endothelial cells, vascular endothelial growth factor, vascular permeability factor.

Introduction

The growth of blood vessels during embryogenesis and in the adult organism is tightly controlled. Some developing organs like brain or kidney are vascularized by sprouts branching from preexisting blood vessels (Bär, 1980; Ekblom et al., 1982). This process, termed angiogenesis, is thought to be regulated by the action of soluble factors. Several polypeptide growth factors have been identified based on their ability to stimulate the proliferation of endothelial cells (For reviews see Folkman and Klagsbrun, 1987; Risau, 1990; Klagsbrun and D'Amore, 1991). The acidic and basic fibroblast growth factors, aFGF and bFGF, are potent endothelial cell mitogens both in vitro and in vivo (For review see Klagsbrun, 1989). They therefore seemed to be likely candidates for general mediators of angiogenesis. However, several observations cast doubt on this proposed role in embryonic angiogenesis; first, aFGF and bFGF lack a hydrophobic signal sequence needed for secretion and the possible mode of release from the producer cells is unknown; second, there is no good correlation between the temporal and spatial expression of aFGF and bFGF mRNA and blood vessel growth during brain angiogenesis (Emoto et al., 1989; Schnürch and Risau, 1991, and unpublished results); and third, there is no evidence that FGF receptors are expressed by endothelial cells in vivo (Heuer et al., 1990; Wanaka et al., 1991).

Recently, two angiogenic factors have been characterized whose mitogenic activities appear to be restricted to endothelial cells, a platelet-derived endothelial cell growth factor (Pd-ECGF) and a vascular endothelial growth factor (VEGF). Pd-ECGF is an inducer of angiogenesis in vivo and has chemotactic activity for endothelial cells (Miyazono et al., 1987; Ishikawa et al., 1989). The presence of the factor in human platelets has led to the suggestion that it might have a role in maintaining the integrity of blood vessels. However, Pd-ECGF also lacks a typical hydrophobic signal sequence for secretion and it is not known by which mechanism this factor is released in vivo. In contrast, VEGF is a secreted endothelial cell mitogen (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989; Levy et

al., 1989; Conn et al., 1990a). This factor has also been termed vascular permeability factor (VPF) because of its ability to induce vascular leakage in vivo (Senger et al., 1983; Keck et al., 1989). Other effects of VEGF include the induction of the procoagulant activity of monocytes and endothelial cells and the stimulation of monocyte migration in vitro (Clauss et al., 1990). VEGF is a dimeric glycoprotein composed of presumably identical disulfide-linked subunits and is structurally related to platelet-derived growth factor (Leung et al., 1989; Keck et al., 1989; Tischer et al., 1989; Conn et al., 1990b). Analysis of human cDNA clones predicted three different forms of the growth factor which differ in size due to insertions at the carboxy terminus (Leung et al., 1989). The different coding regions arise by alternative splicing of mRNA (Tischer et al., 1991). VEGF probably exerts its action via cell surface receptors which have been identified on endothelial cells (Plouet and Moukadiri, 1990; Vaisman et al., 1990; Connolly et al., 1989).

The properties of VEGF as a secreted endothelial-cell-specific mitogen prompted us to investigate this factor in more detail. We have characterized the murine VEGF gene, and we have investigated the secretory behaviour and the mitogenic activity of the various VEGF forms. To investigate its possible function in an intact organism, we have analysed its expression pattern during embryonic angiogenesis. The spatial and temporal expression pattern of VEGF during brain development correlated well with the ingrowth of blood vessels into the developing neuroectoderm. In addition to the transient VEGF expression in ventricular neuroectoderm, a constitutive VEGF expression was observed in epithelial cells adjacent to fenestrated endothelium of choroid plexus and of kidney glomeruli. This constitutive expression suggests that VEGF might have a role in establishing or maintaining the fenestration of organotypic endothelium. The results support the hypothesis that VEGF might be a regulator of neovascularization and of endothelial cell differentiation.

Materials and methods

Cell culture, transfection and bioassays

COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were transfected using the modified calcium phosphate method (Chen and Okayama, 1987). Conditioned media were collected 48-72 hours after transfection. Bovine aortic endothelial (BAE) cells were prepared as described by Schwartz (1978) and grown in DMEM supplemented with 5% FCS. Assays for mitogenic activity were performed as described (Risau et al., 1988). In short, BAE cells (passages 9-12) were seeded in 24-well dishes at a density of 10 000 cells per well the day before addition of the COS cell-conditioned media. After three days, the cells were dissociated with trypsin and counted in a Coulter counter.

Preparation of antisera and immunoblot analysis

A synthetic oligopeptide comprising 20 amino-terminal residues of the mature murine VEGF protein (see Fig. 1) was synthesized on a peptide synthesizer (Applied Biosystems), coupled to keyhole limpet hemacyanin (KLH) and was used to immunize rabbits. For the immunoblot analysis, the conditioned media from COS cells were concentrated by acetone precipitation and redissolved in buffer containing 2-mercapto-ethanol. Extracts were prepared by repeated freeze-thawing of the cell suspension. Samples were run in 12.5% polyacrylamide-SDS gels and transferred to nitrocellulose membrane. The filters were incubated with polyclonal anti-VEGF antiserum and subsequently with peroxidase-conjugated goat anti-rabbit secondary antibodies (Dianova). Non-immune serum and serum raised against the amino-terminal residues of human VEGF which were used as controls did not detect the murine VEGF polypeptides.

DNA amplification, cloning and sequence analysis

For cDNA cloning, 5 μg of poly (A)+ RNA was reverse transcribed (Krug and Berger, 1987). 20% of the cDNA were subjected to 35 rounds of amplification using a PCR reagent kit (Perkin-Elmer Cetus). Cycles were 1 minute at 94°C, 2 minutes at 55°C, 3 minutes at 72°C in a temperature cycler (Coy). Synthetic oligonucleotide primers containing in addition *Bam*HI or *Eco*RI restriction enzyme recognition sites were used. The nucleotide sequences of the primers were 5'-TGGATCCATGAACTTTCTGCT (5' oligonucleotide) and 5'-GAATTCACCGCCTCGGCTTGTC (3'oligonucleotide). Following digestion with restriction endonucleases, the amplification products were purified and cloned into pBluescript vector DNA (Stratagene) cut with the same restriction enzymes. The resulting plasmids, pVEGF-1, pVEGF-2 and pVEGF-3, were sequenced using Sequenase (USB).

For the analysis of RNA samples, the cDNA synthesis and amplification was performed in a one tube reaction essentially as described by Zafra et al. (1990) with modifications described below. The specific primers used for reverse transcription were those used for cDNA cloning. Aliquots of poly (A+) RNA (100 ng) were used in a 50 μl reaction containing 100 ng each of oligonucleotide primers, 2 U human placental RNAse inhibitor (Pharmacia), 2 U AMV reverse transcriptase (Pharmacia), 2 mM dNTP, 1 U Amplitaq DNA polymerase and buffer supplied by the manufacturer (Perkin Elmer Cetus). As an internal standard, 1 ng of RNA obtained by in vitro transcription of a truncated VEGF cDNA clone using T7 RNA polymerase (Stratagene), was added to the reaction. This clone was obtained by deleting a 249 bp restriction fragment from pVEGF-2. After 30 minutes at 40°C, 20 cycles of DNA amplification were carried out as described above. Samples containing 20% of the products were separated in an agarose gel, blotted onto GeneScreen Plus membrane and hybridized with ^{32}P-labelled VEGF-1 cDNA.

For expression in COS cells, VEGF cDNAs were cloned into pCMV5 vector DNA (Andersson et al., 1989). Plasmid DNAs were purified on Qiagen columns (Diagen).

Isolation of RNA and northern blot hybridisation

RNA was prepared using the guanidinium thiocyanate method (Chirgwin et al., 1979) and purified by centrifugation through a CsCl cushion. Poly (A)+ RNA was selected by retention on oligo(dT) columns. Aliquots of poly(A)+ RNA were fractionated on 1% agarose gels containing 6% formaldehyde and blotted onto GeneScreen Plus membrane (DuPont). Filters were hybridized at 42°C in 50% formamide, 5 × SSC (750 mM sodium chloride, 75 mM sodium citrate), 5 × Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA), 1% SDS, 10 μg/ml yeast tRNA and 10 ng random-primed [^{32}P]DNA probe. Washes were twice in 2 × SSC at

room temperature, once in 2 × SSC, 1% SDS at 42°C and finally twice in 0.1 × SSC at 42°C.

In situ hybridization

The techniques used for in situ hybridization were essentially those described by Hogan et al. (1986) with modifications described by Dressler et al. (1990). Mouse embryos or organs (Balb/c) were embedded in Tissue-Tek medium (Miles) and frozen in liquid nitrogen. Sections were cut in a cryostat at −20°C, dried on subbed slides and fixed in 4% paraformaldehyde. Sections were digested with Pronase (40 μg/ml) for 10 minutes at room temperature. The digestion was stopped in 0.2% glycine. Slides were rinsed with PBS, fixed in 4% paraformaldehyde and rinsed again with PBS. Sections were acetylated with acetic anhydride diluted 1:400 with 0.1 mM triethanolamine for 10 minutes and rinsed with PBS. Prehybridization was performed in buffer containing 50% formamide, 10% dextran sulfate, 10 mM Tris-HCl pH 7.5, 10 mM sodium phosphate pH 6.8, 2 × SSC, 5 mM EDTA, 150 μg/ml yeast tRNA, 0.1 mM UTP, 1 mM ADPβS, 1 mM ADPγS, 10 mM DTT and 10 mM 2-mercapto-ethanol for 1 hour at 48°C. Sections were dehydrated in graded ethanol and stored at −70°C.

Single-stranded [^{35}S]RNA probes were generated by in vitro transcription of a truncated pVEGF-2 cDNA clone. This clone contained 310 bp of the 3′ coding region of the VEGF-2 cDNA. In one experiment, a cDNA clone that contained a 140 bp sequence from the 5′ coding region was used a control. Both probes detected the same pattern of transcript distribution. The RNA probes were generated using 100 μCi [^{35}S]UTP and T3 or T7 RNA polymerases essentially as described by the manufacturer (Stratagene). RNA probes were purified by ethanol precipitation in the presence of ammonium acetate, dissolved in 50% formamide, 10 mM DTT and stored at −20°C.

Hybridization was performed in prehybridization buffer supplemented with 5×10^4 cts/min/μl ^{35}S-labelled RNA probe. Sections were hybridized overnight in a humid chamber at 45-48°C. Depending on the size of the coverslip, approximately 20 μl buffer/section was used. Slides were washed in 2 × SSC, 50% formamide for 2 hours at 45-48°C followed by RNAse digestion (20 μg/ml) for 15 min. Slides were washed in 2 × SSC, 50% formamide overnight and dried at room temperature. Following dehydration in graded ethanol, slides were coated with Kodak NTB-2 emulsion diluted 1:1 with water and exposed for 10-14 days. Slides were developed and stained with Giemsa.

Results

Isolation and characterization of murine VEGF cDNAs

Murine cDNA clones encoding VEGF sequences were obtained by using the polymerase chain reaction (PCR) technique after reverse transcription of poly (A)+ RNA from 14-day post-coital (p.c.) mouse embryos. The nucleotide sequence of the primers for the amplification reaction was derived from the human cDNA sequence (Leung et al., 1989). The codons for the translational start and stop signals were included in order to obtain the complete coding region. Three different products were obtained, a 580 bp VEGF-1, a 450 bp VEGF-2 and a 650 bp VEGF-3 cDNA (data not shown). In contrast to the VEGF-1 and VEGF-2 PCR products, the VEGF-3 cDNA was of very low abundance. DNA sequencing of all three cDNAs revealed a sequence homologous to human, bovine and rat VEGF (Leung et al., 1989; Conn et al., 1990b). They were identical in sequence to one another except that insertions were present in the 3′ region of the VEGF-1 and VEGF-3 cDNAs (Fig. 1). Thus, the cDNAs predict the existence of three different VEGF forms, which, after removal of a 26-residue amino-terminal hydro-

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                a  a                    c
  1  gcc tcc GGA TCC ATG AAC TTT CTG CTG TCT TGG GTG CAC TGG ACC CTG GCT TTA CTG CTG
-26                met asn phe leu leu ser trp val his trp thr leu ala leu leu leu

 61  TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ACG ACA GAA GGA GAG CAG AAG TCC
-10  tyr leu his his ala lys trp ser gln ala ala pro thr thr glu gly glu gln lys ser

121  CAT GAA GTG ATC AAG TTC ATG GAC GTC TAC CAG CGA AGC TAC TGC CGT CCA ATT GAG ACC
 11  his glu val ile lys phe met asp val tyr gln arg ser tyr cys arg pro ile glu thr

181  CTG GTG GAC ATC TTC CAG GAG TAC CCC GAC GAG ATA GAG TAC ATC TTC AAG CCG TCC TGT
 31  leu val asp ile phe gln glu tyr pro asp glu ile glu tyr ile phe lys pro ser cys

241  GTG CCG CTG ATG CGC TGT GCA GGC TGC TGT AAC GAT GAA GCC CTG GAG TGC GTG CCC ACG
 51  val pro leu met arg cys ala gly cys cys asn asp glu ala leu glu cys val pro thr

301  TCA GAG AGC AAC ATC ACC ATG CAG ATC ATG CGG ATC AAA CCT CAC CAA AGC CAG CAC ATA
 71  ser glu ser asn ile thr met gln ile met arg ile lys pro his gln ser gln his ile

361  GGA GAG ATG AGC TTC CTA CAG CAC AGC CGA TGT GAA TGC AGA CCA AAG AAA GAC AGG ACA
 91  gly glu met ser phe leu gln his ser arg cys glu cys arg pro lys lys asp arg thr

421  AAG CCA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGT CAA AAA CGA AAG CGC AAG AAA
111  lys pro glu lys lys ser val arg gly lys gly lys gly gln lys arg lys arg lys lys
                asn

481  TCC CGG TTT AAA TCC TGG AGC GTT CAC TGT GAG CCT TGT TCA GAG CGG AGA AAG CAT TTG
131  ser arg phe lys ser trp ser val his cys glu pro cys ser glu arg arg lys his leu

541  TTT GTC CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC AAG
151  phe val gln asp pro gln thr cys lys cys ser cys lys asn thr asp ser arg cys lys

601  GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC AGA TGT GAC AAG CCG AGG CGG TGAATTC
171  ala arg gln leu glu leu asn glu arg thr cys arg cys asp lys pro arg arg
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Fig. 1. DNA sequence and predicted translation products of murine VEGF cDNAs. The locations of the oligonucleotide primers that were used for the PCR are overlined. The putative signal sequence and a potential glycosylation site at Asn 74 are underlined. The complete sequence of the VEGF-3 cDNA is given. Boxed regions represent the sequences which are absent from the shorter VEGF-1 or VEGF-2 forms (see Fig. 2). VEGF-1 lacks a stretch of 24 amino acid residues from position 115 to 138, VEGF-2 lacks additional 44 residues up to position 183. In both cases, Lys 114 is replaced by Asn. The cDNA sequence is shown in capital letters. Nucleotide sequences presented in lower case were obtained from a genomic DNA clone (G. Breier, unpublished results) and differ at the indicated positions from the sequence of the 5′ PCR primer.

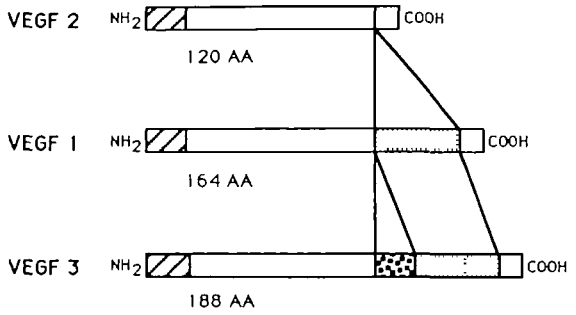

Fig. 2. Structure of murine VEGF proteins as deduced from cDNA sequences. The cross-hatched box at the amino terminus represents the putative 26-amino acid signal peptide for secretion. Additional domains in VEGF-1 or VEGF-3 are represented as shaded boxes. The numbers of amino acids refer to the length of the mature proteins.

phobic signal sequence, would consist of 120 (VEGF-2), 164 (VEGF-1) or 188 (VEGF-3) amino acid residues (Fig. 2). The direct protein sequence analysis of the amino-terminal end of the putative murine VEGF (Plouet et al., 1989) is in agreement with the sequence obtained from the cDNA clones. However, the amino acid sequence differs at position 10 which might be due to strain polymorphism. A potential N-linked glycosylation site is present at asparagine 74. The inserted protein domain in the long VEGF form (VEGF-3) is highly basic, 11 of 24 amino acid residues being lysine or arginine. This region is homologous to a stretch of amino acids encoded by the sixth exon of the human PDGF A-chain gene (Betsholtz et al., 1990). Interestingly, this exon is present only in an alternatively spliced mRNA variant and encodes a nuclear targeting signal (Maher et al., 1989). The homology between VEGF and the PDGF A- and B-chains extends to eight cysteine residues, suggesting a similar secondary structure of the proteins (Fig. 3; Leung et al., 1989). VEGF proteins of different mammalian species are highly homologous (Fig. 3). The mature murine VEGF-1 protein showed the highest degree of sequence identity (98%) with the rat VEGF protein. The amino acid sequence identities between the mature murine VEGF-1 and the corresponding human and bovine proteins were 88% each. The amino-terminal sequences of the mature proteins showed a considerable degree of sequence diversity.

Secretion and mitogenic activity of recombinant murine VEGF

The VEGF cDNAs were cloned into a mammalian expression vector which contained the transcriptional control elements of the human cytomegalovirus. The resulting plasmids were then transfected into COS cells. Culture supernatants and extracts prepared from the transfected cells were assayed by immunoblot analysis for the presence of VEGF protein. The polyclonal antibodies used had been raised against a synthetic amino- terminal 20-mer oligopeptide. Homodimers of apparent molecular weights of 20×10^3 M_r or 23×10^3 M_r were detected in the conditioned media, corresponding to VEGF-1 or to VEGF-2 polypeptide chains, respectively (Fig. 4). In contrast, the VEGF-3 chains were retained in the cells. Two major bands corresponding in size to 27×10^3 M_r and 24×10^3 M_r, were detected in the extract, but not in the conditioned medium of COS cells transfected with VEGF-3 cDNA. This size heterogeneity possibly reflects differences in posttranslational processing or proteolytic degradation. The cell culture supernatants were then assayed for mitogenic activity on bovine aortic endothelial (BAE) cells. The conditioned media which contained homodimers of VEGF-1 or VEGF-2 stimulated the proliferation of BAE cells to a similar extent (Fig. 5). Media from cells transfected with VEGF-3 cDNA had no significant mitogenic effect. Whether VEGF-3 protein can be as active as VEGF-1 or VEGF-2 in stimulating the proliferation of endothelial cells remains to be determined.

Expression of VEGF during murine development

The source of the VEGF cDNAs was RNA from 14-day

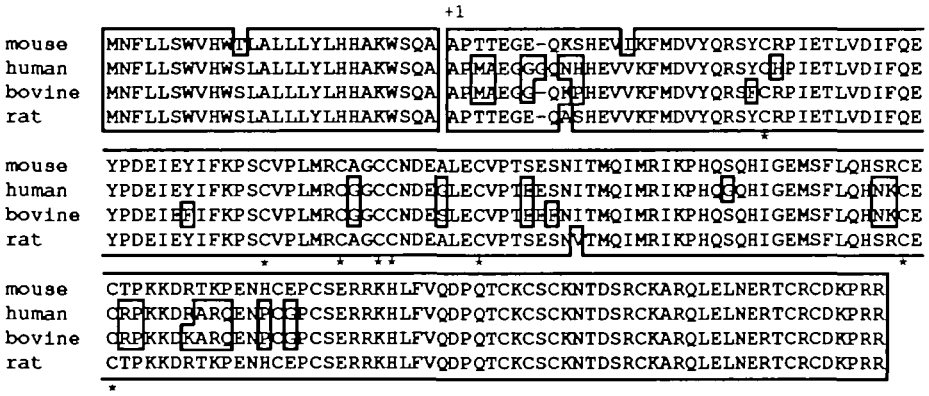

Fig. 3. Comparison of VEGF protein sequences from mouse, human, bovine and rat. Only the intermediate VEGF form (=VEGF-1) was used for the comparison because the sequences of the small and large forms have not been published for all species. The one-letter amino acid code is used. The complete sequence of the precursors are shown, the amino-terminal alanine of the mature proteins is indicated by +1. Asterisks indicate the location of conserved cysteine residues that are also shared by the PDGF-A and PDGF-B chains. The human and bovine sequences were taken from Leung et al. (1989), the rat sequence is from Conn et al. (1990b). It should be noted that the five carboxy-terminal amino acids of the murine VEGF were derived from a PCR primer containing the human sequence and do not necessarily represent the actual mouse sequence. The amino-terminal sequence of the signal peptide has been confirmed by a partial sequence of a genomic DNA clone (see Fig. 1).

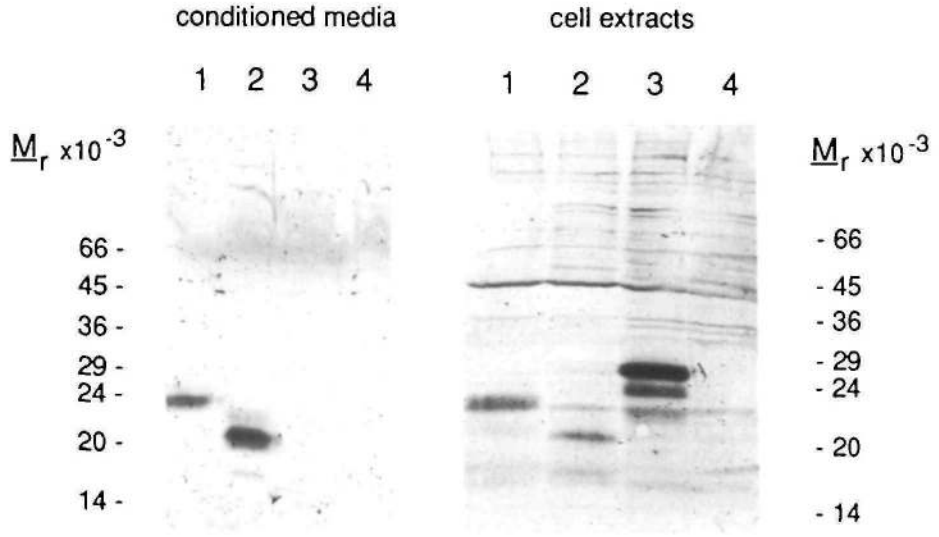

Fig. 4. Immunoblot analyses of the conditioned media (left panels) and of extracts (right panels) of COS cells expressing recombinant VEGF proteins. COS cells were transfected with eucaryotic expression vectors that contained the different VEGF cDNAs. 5% of the protein present in culture supernatants as compared to 60% of the protein of extracts, obtained from cells of two petri dishes (diameter 35 mm), were used for the analysis. The blot was stained using a polyclonal serum raised against a synthetic peptide of the VEGF amino terminus. Lane 1=VEGF-1, lane 2=VEGF-2, lane 3=VEGF-3, lane 4=untransfected COS cells.

p.c. mouse embryos. This already indicated that the VEGF gene is transcriptionally active during embryogenesis. Therefore, we performed northern blot analyses with RNA from 14-day p.c. and 16-day p.c. embryos. RNA from adult brain was also examined. The blots were hybridized with a ^{32}P-labelled VEGF-1 cDNA. Several transcripts ranging in size between 2.7 kb and 4 kb were detected in RNA from mouse embryos and from adult brain (Fig. 6). However, this type of analysis did not distinguish between the mRNAs coding for the different VEGF forms. In order to compare the relative abundance of these transcripts, we performed a semiquantitative PCR analysis. Firstly, single-stranded cDNA was generated by using VEGF-specific primers. PCR was carried out under conditions

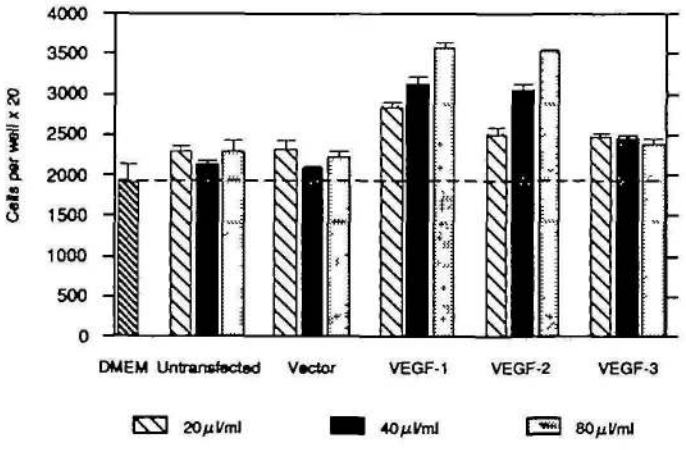

Fig. 5. Mitogenic activity of recombinant VEGF. Bovine aortic endothelial cells were cultured in the presence or absence of cell culture supernatants from transfected COS cells (see Fig. 4). DMEM supplemented with 10% FCS and the conditioned media from untransfected COS cells or from COS cells that were transfected with the vector alone were used as a negative control. After three days, the cells were dissociated with trypsin and counted in a cytometer. Values are means ± s.e.m. of double determinations.

that did not limit the DNA synthesis. The products were separated by agarose gel electrophoresis and analysed by Southern blot hybridization using ^{32}P-labelled VEGF-1 cDNA as a probe. The 580 bp VEGF-1 cDNA and the 450 bp VEGF-2 cDNA were detected both in 16-day p.c. mouse embryos and in adult brain (Fig. 6). In 16-day p.c. embryos, VEGF-2 transcripts were slightly more abundant than VEGF-1 mRNA, while in the adult brain the VEGF-1 mRNA was of the highest abundance. In both RNA samples, VEGF-3 transcripts were not detectable.

The spatial distribution of VEGF transcripts in 15-day p.c. and 17-day p.c. embryos was investigated by in situ hybridization. 6-day postnatal brain, adult brain and adult kidney were also examined. Frozen tissue sections were hybridized with single-stranded ^{35}S-labelled RNA probes corresponding to the sense or antisense strand of a VEGF cDNA fragment. This probe contained sequences common to all three VEGF forms. In addition, a probe derived from the murine homeobox gene *Hox 3.1* (Breier et al., 1988), which is also expressed during embryogenesis, was used as a positive control. VEGF transcripts were detected in various organs of developing embryos. In the brain of 17-day p.c. embryos, VEGF transcripts were observed in choroid plexus epithelium and in the ventricular layer (Fig. 7). The signal in the adjacent neural tissue was significantly lower. The pattern of VEGF transcript distribution in the ventricular layer of 6-day postnatal brain was similar to the pattern in embryonic brain (Figs 7, 8). In adult brain, the VEGF mRNA levels in the choroid plexus appeared slightly reduced. No specific labelling was observed in the ependyme, which forms the inner lining of the ventricle (Fig. 7). In the newborn and adult brain, VEGF transcripts were also detected in other brain regions in which single, unidentified cells were labelled. VEGF transcripts were also observed in the meningeal layer and in the area postrema of adult brain.

In the developing kidney of 15-day p.c. embryos, VEGF transcripts accumulated in glomerular epi-

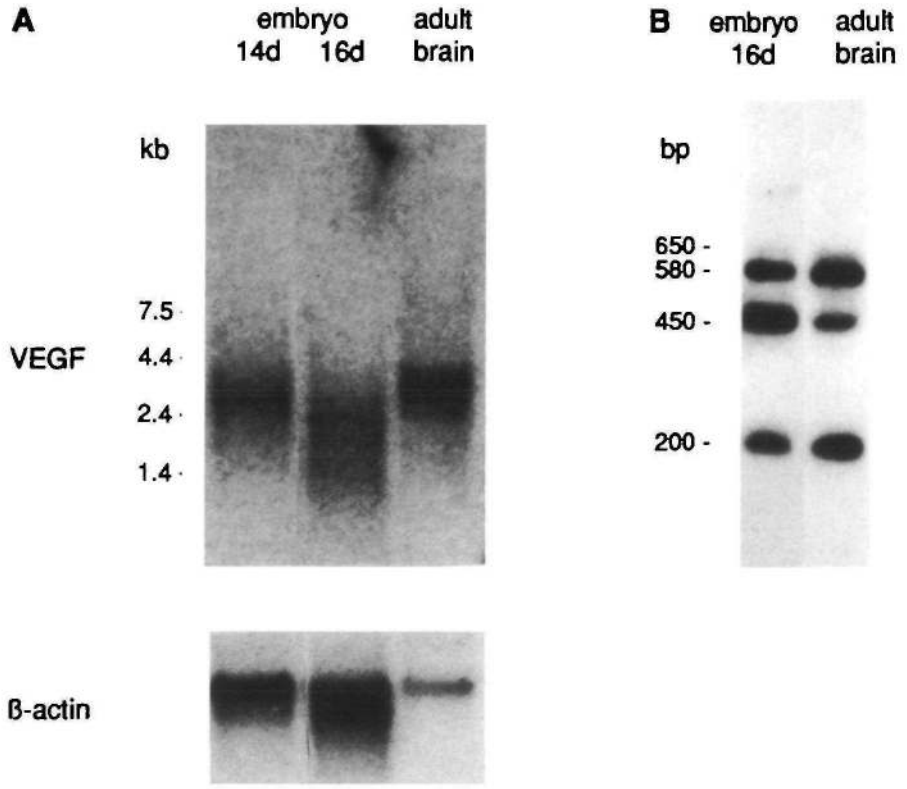

Fig. 6. (A) Northern blot hybridization of VEGF transcripts in embryonic and adult tissues. Samples of poly A+ RNA (5 μg) from 14-day p.c. and 16-day p.c. mouse embryos and from adult brain were hybridized with ^{32}P-labelled VEGF-1 cDNA and subsequently with β-actin cDNA. Exposure times were 6 days for the VEGF probe and 1 day for the β-actin probe. (B) Semiquantitative PCR analysis of VEGF transcripts. cDNA was synthesized from poly (A)+ RNA samples (100 ng) from embryonic and adult tissues using specific oligonucleotide primers. VEGF sequences were amplified by 20 cycles of PCR and analyzed by Southern blot hybridization with VEGF-1 cDNA probe. In vitro synthesized transcripts from a truncated VEGF cDNA were included in the reaction to serve as an internal standard and resulted in the synthesis of a 200 bp cDNA fragment. Exposure time was 16 h.

thelium (Fig. 9). This expression persisted during development and was still high in the adult glomerulus. No specific labelling was observed in blood vessels and in mesangium in the glomerulus. Glomerular capillaries are derived from outside blood vessels (Ekblom et al., 1982; Sariola et al., 1983) and are of the fenestrated type (Rhodin, 1962). Thus, VEGF is expressed at the time of embryonic kidney angiogenesis and continues to be expressed in glomerular epithelium which is adjacent to glomerular fenestrated endothelium. Strong expression of VEGF was observed in the 17-day p.c. placenta (Fig. 10) and was most prominent in the labyrinth region of the placenta. VEGF transcripts were localized over clusters of cell nuclei in the labyrinth layer, most likely of the multinucleated trophoblast cells (Hogan et al., 1986; Theiler, 1972). In the labyrinth, an intimate contact between the embryonic trophoblast and maternal blood space is formed. In this region, fetal blood vessels develop which are separated by trophoblast cell layers from the maternal blood sinuses (Björkman et al., 1989; Kirby and Bradbury, 1965). Endothelial cells of the labyrinth are also characterized by frequent fenestrations (Jollie, 1964). In several other embryonal organs VEGF transcripts were also detected, e.g. in bronchial epithelium of the lung, in adrenal gland and in seminiferous tubules of testis (data not shown).

Discussion

VEGF has important properties that are characteristic of a potential regulator of embryonic angiogenesis and of endothelial cell functions. It is a secreted growth factor whose mitogenic activity is apparently restricted to vascular endothelial cells. In addition, it may induce vascular permeability and is able to attract monocytes. As a step towards the elucidation of its physiological function in the developing organism, we have cloned the murine VEGF gene and analysed its expression pattern during mouse embryogenesis, particularly during brain angiogenesis.

Analysis of the cDNA clones obtained from 14-day p.c. mouse embryos predicted the existence of three different VEGF polypeptides. These differed by the insertion of one or two protein domains near the carboxy terminus. Similar forms occur in bovine and human (Leung et al., 1989; Tischer et al., 1989), and the various human VEGF coding regions result from alternative splicing of mRNA (Tischer et al., 1991). It is probable, based upon Southern blot analysis of genomic DNA, that the different murine VEGF transcripts are also derived from a single gene. We are presently characterizing genomic DNA clones in order to determine the structure of the murine VEGF gene. The three VEGF forms differed with respect to their secretory behaviour. Homodimers of the two lower molecular weight forms (VEGF-1 and VEGF-2), but not of the large form (VEGF-3), were secreted after transfection of the corresponding cDNAs into COS cells. The retention of VEGF-3 in these cells was due to the insertion of a highly basic protein domain. Homologous domains are present in the PDGF B-chain and in a variant form of the PDGF A-chain (Betsholtz et al.,

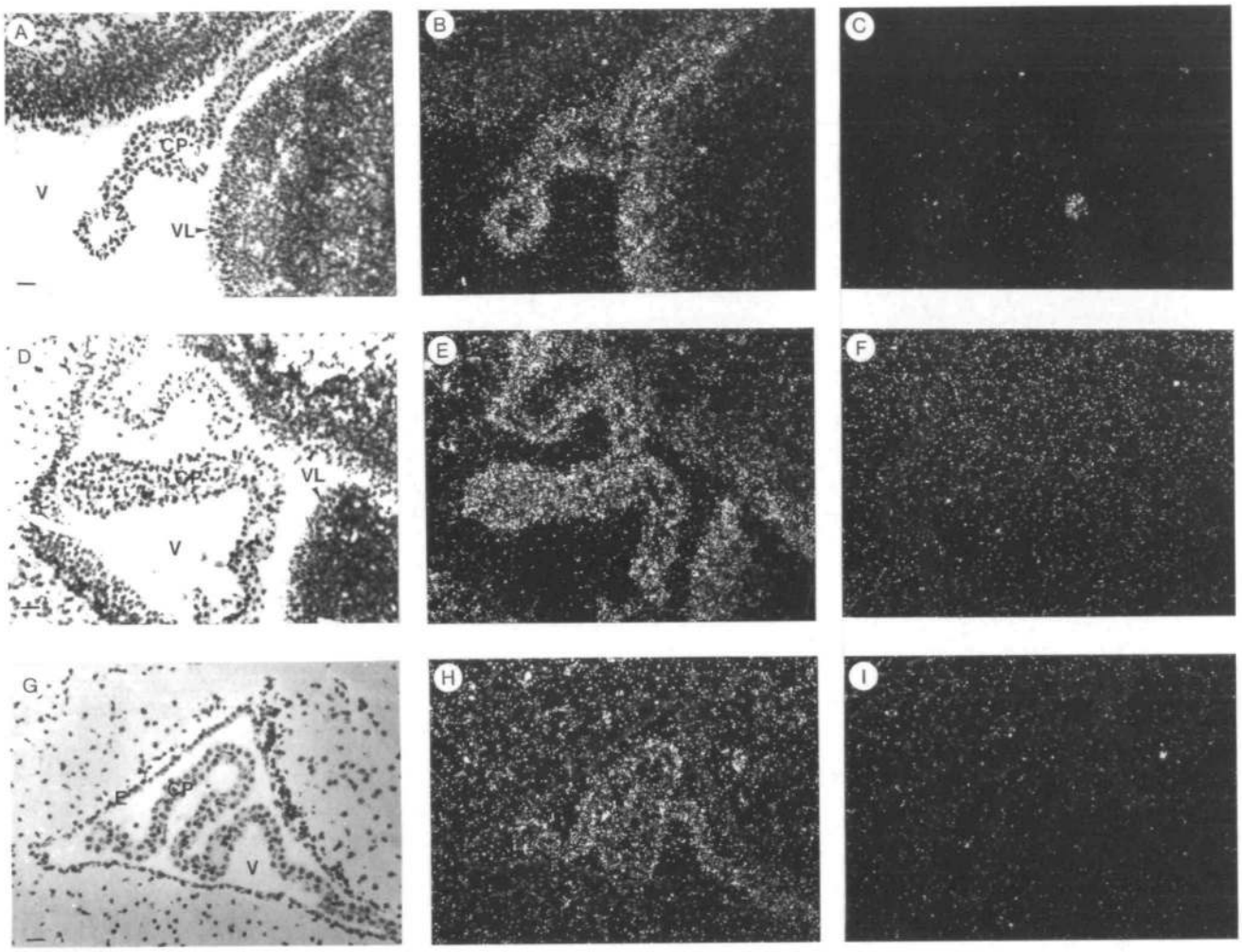

Fig. 7. Localisation of VEGF mRNA in embryonic, 6-day postnatal and adult brain. Serial brain sections were hybridized in situ with ^{35}S-labelled RNA probe. (A) Sagittal section of a 17-day p.c. embryo hybridized with antisense RNA probe. The image shows choroid plexus (CP) and ventricular layer (VL) of the third ventricle (V); (B) dark-field image of A; (C) control hybridization of a serial section with sense RNA probe. (D) Sagittal section of 6-day postnatal brain hybridized with antisense RNA probe, showing the choroid plexus (CP) and ventricular layer (VL) of the third ventricle (V); (E) dark-field image of D; (F) control hybridization with sense RNA. (G) Parasagittal section of adult brain hybridized with antisense RNA probe. The choroid plexus (CP) and ependyme (E) of a lateral ventricle (V) is shown; (H) dark-field image of G; (I) control hybridization with sense RNA probe. Bar represents 25 μm.

1990) and directed the protein to the nucleus if the signal sequences for secretion were deleted (Lee et al., 1987; Maher et al., 1989). In good correlation with our results, these basic domains function as a cell retention signal in COS cells (Östmann et al., 1991). Thus, these conserved domains of PDGF and VEGF are not only structurally, but also functionally similar. At present, we cannot rule out the possibility that the lack of secretion of VEGF-3 by COS cells is due to restrictions in the secretory pathways of these cells and that the natural producer cells are able to secrete this form. Alternatively, VEGF-3 might be integrated into the plasma membrane and might be involved in local cell-cell interactions. It is possible that VEGF-3 would interact only with closely adjacent cell layers, whereas the secreted forms, VEGF-1 and VEGF-2, might function as diffusible factors. As a precedent, it has been shown that the membrane-bound TGF-α precursor is able to bind to its receptor on adjacent cells and may lead to signal transduction (Wong et al., 1989). Although transcript levels specific for VEGF-3 were very low in embryos, preliminary analyses revealed elevated transcript levels in adult liver, lung and heart, suggesting that the large VEGF form might have a specific function in these organs. It will therefore be important to determine whether the structural diversity of three VEGF forms reflects a heterogeneity in function. The only direct effect of VEGF on endothelial cells described so far is its mitogenic activity. At present, one has to consider the possibility that the mitogenic activity of VEGF is mediated by an indirect mechanism that involves the production of a different endothelial mitogen by endothelial cells. The two secreted VEGF forms, VEGF-1 and VEGF-2, were

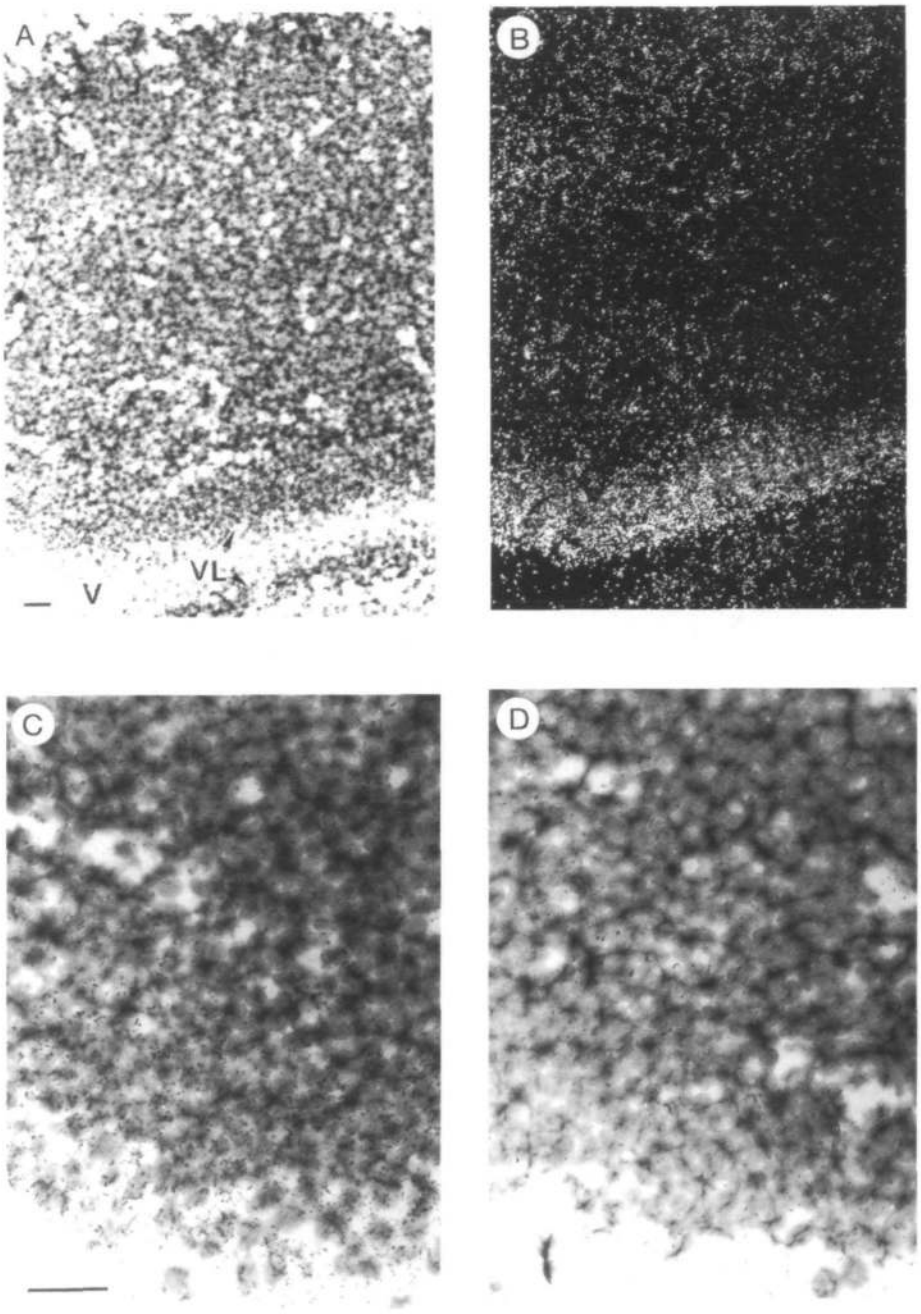

Fig. 8. Detection of VEGF mRNA in the ventricular layer of 6-day postnatal brain. (A) The ventricular layer (VL) lining the third ventricle (V) shows VEGF mRNA accumulation, whereas specific labeling in the adjacent neuroectoderm is significantly lower; (B) dark-field image of A. (C) Higher magnification of the ventricular layer. The ventricle is at the bottom; (D) control hybridization of a serial section with a sense RNA probe. Bar represents 25 μm.

equally potent in stimulating the proliferation of endothelial cells, while a mitogenic activity has not been demonstrated yet for the long variant. It will also be of interest to analyse whether the various VEGF forms differ in other functions, such as the induction of vascular permeability or the ability to attract monocytes.

The complex expression pattern of VEGF that was observed in mouse embryos argues in favour of a multifunctional role of the growth factor. The temporal pattern of VEGF expression in the ventricular neuroepithelium of the brain suggests a role of VEGF as an angiogenesis factor. The vascular system of the brain is derived from the perineural vascular plexus that covers the neural tube (Bär, 1980). Vascular sprouts originating from this perineural plexus grow radially into the neuroectoderm, toward the ventricular layer, and branch there. In rodents, the neovascularization of the developing brain starts approximately at embryonic day 11 and maximal endothelial cell proliferation is observed at postnatal day 6 (Robertson et al., 1985). In the adult organism, the vascularization of the brain, as well as of other organs, is complete and the rate of endothelial cells proliferation is very low (Engerman et al., 1967). Thus, the temporal and spatial expression of VEGF in the developing brain correlates with brain vascularization and endothelial cell growth. It is therefore conceivable that VEGF is released by cells of the ventricular layer during brain development and promotes angiogenesis by initiating an angiogenic

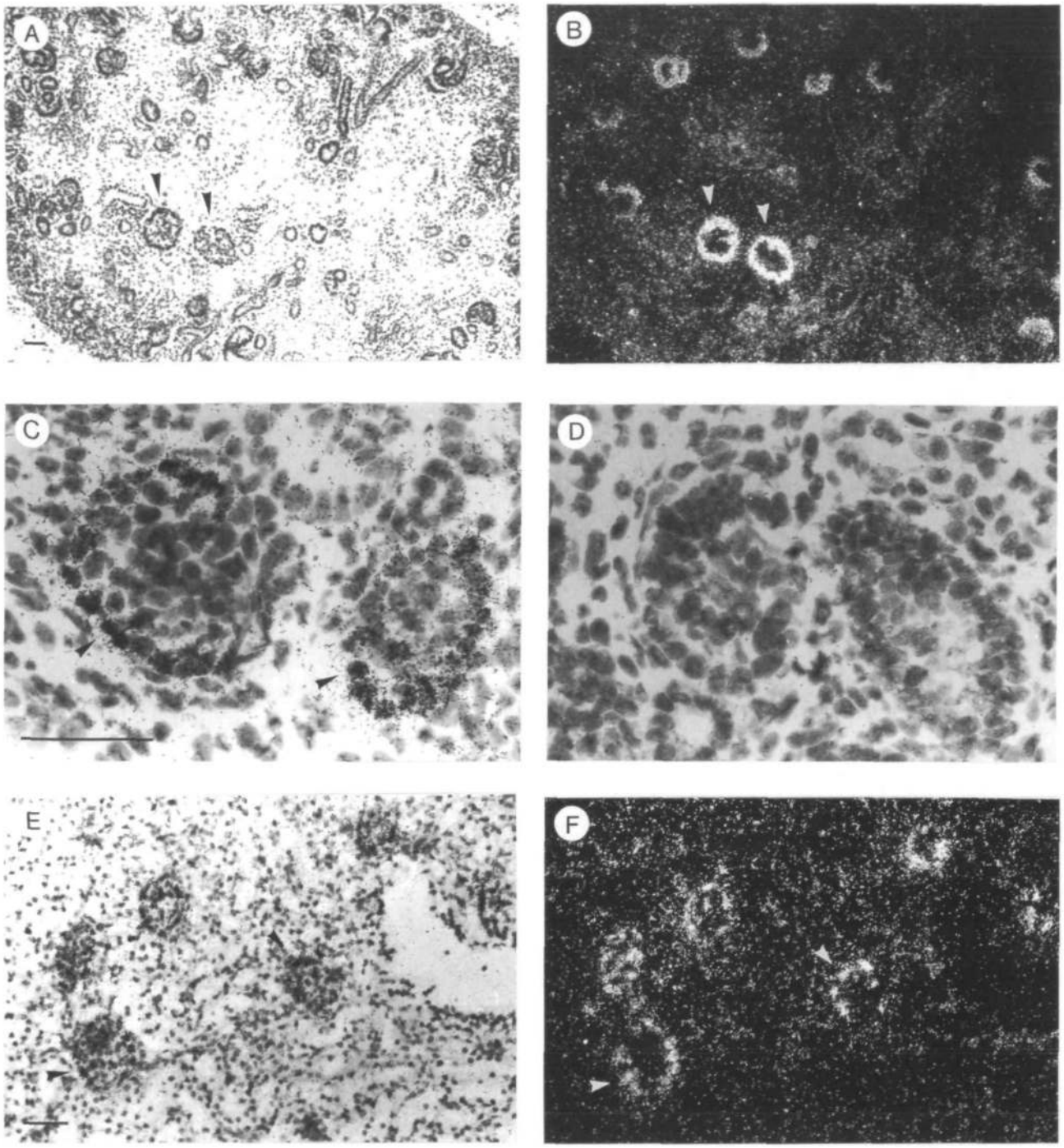

Fig. 9. Localisation of VEGF transcripts in the developing and adult kidney. (A) Parasagittal section of a 15-day p.c. embryo, hybridized with antisense RNA probe. Arrows point to glomerular epithelium; (B) dark-field image of A. (C) higher magnification of glomeruli; D control hybridization with VEGF sense RNA. (E) Section of adult kidney, hybridized with antisense RNA probe; (F) dark-field image of E. Bar represents 50 μm.

response of endothelial cells from the perineural vascular plexus. A concentration gradient of the diffusible angiogenic factor may cause capillaries to grow in direction to the angiogenic stimulus. The hypothesis that VEGF may function as an angiogenic growth factor for fetal blood vessels, is further substantiated by the high levels of VEGF mRNA in the early embryonic kidney, in the highly vascularized placenta, and by the correlation between VEGF expression and vascularization in rat corpus luteum (Phillips et al., 1990).

In contrast to its transient expression in the ventricular layer of the brain, VEGF expression in kidney glomeruli and in choroid plexus of the brain persisted in

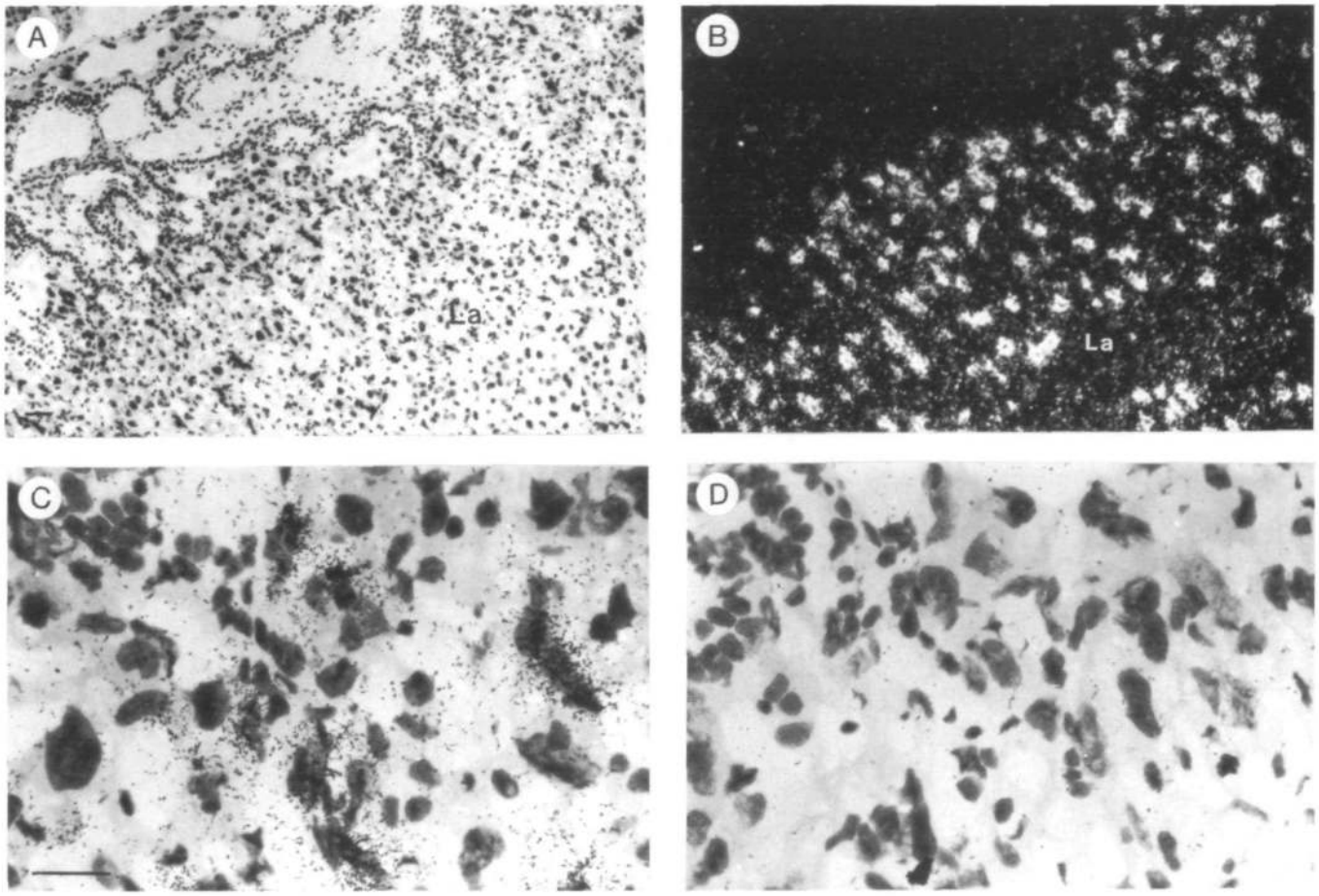

Fig. 10. Expression of VEGF in 17-day p.c. placenta. (A) In situ hybridization showing VEGF transcript accumulation in the labyrinth layer (La) whereas no specific labeling is observed in the adjacent chorionic plate; (B) dark-field image of A. (C) Higher magnification of VEGF expressing cells in the labyrinth layer. (D) control hybridization. Bar represents 25 μm.

the adult, when the vascularization of these structures is complete. This indicates that in the adult organism, VEGF has an additional function which is different from a role as mediator of neovascularization. As the expression of VEGF in these structures was confined to epithelial cells that are in close contact to fenestrated endothelium, it is tempting to speculate that VEGF may be involved in the establishment or the maintenance of fenestrated endothelia. We are currently investigating whether VEGF has a direct role in the induction of fenestrae in cultured endothelial cells. As the endothelium of the placental labyrinth is also fenestrated, VEGF might have a dual role in this organ, as an endothelial cell growth factor, but also as a factor affecting the differentiation of endothelial cells. The fenestration of endothelium reflects an increased permeability of endothelial cells for low molecular weight substances (Levick and Smaje, 1987), thus facilitating the exchange of these substances in structures such as kidney glomeruli, choroid plexus and placenta. This is consistent with the permeability-inducing properties of VEGF in vivo (Keck et al., 1989), although it is not known whether this was a direct effect of VEGF on endothelial cells or whether other factors contributed to the observed vascular permeability.

Several lines of evidence, including protein secretion, an apparently restricted target cell specificity and region-specific expression, suggest that VEGF may function as a regulator of embryonic angiogenesis and of endothelial cell differentiation. A complete understanding of its functions in endothelial cell growth and differentiation, however, will require a systematic dissection of its properties in in vitro systems, as well as genetic evidence based on induced mutations or altered embryonic expression patterns.

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