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Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium

(vascular development/signal transduction/*in situ* hybridization/*Xenopus laevis*)

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ABSTRACT **Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, induces endothelial proliferation *in vitro* and vascular permeability *in vivo*. The human transmembrane c-fms-like tyrosine kinase Flt-1 has recently been identified as a VEGF receptor. Flt-1 kinase has seven immunoglobulin-like extracellular domains and a kinase insert sequence, features shared by two other human gene-encoded proteins, kinase insert domain-containing receptor (KDR) and FLT-4. In this study we show that the mouse homologue of KDR, Flk-1, is a second functional VEGF receptor. Flk-1 binds VEGF with high affinity, undergoes autophosphorylation, and mediates VEGF-dependent Ca^{2+} efflux in *Xenopus* oocytes injected with Flk-1 mRNA. We also demonstrate by *in situ* hybridization that Flk-1 protein expression in the mouse embryo is restricted to the vascular endothelium and the umbilical cord stroma. VEGF and its receptors Flk-1/KDR and Flt-1 may play a role in vascular development and regulation of vascular permeability.**

The vascular endothelial growth factor (VEGF) family is composed of four dimeric polypeptides that induce endothelial proliferation *in vitro* and angiogenesis *in vivo* (1–3). VEGF was independently identified on the basis of its ability to induce vascular permeability and was termed vascular permeability factor (4). Alternative splicing of the human VEGF RNA transcript generates four isoforms containing 121, 165, 189, and 206 amino acids (5, 6). The VEGF isoforms have different properties *in vitro*, which may determine their functions *in vivo*. The two shorter isoforms are readily secreted from cells transfected with cDNAs and are mitogenic, whereas the two longer isoforms remain cell-associated (5). All isoforms can induce vascular permeability. In addition, VEGF promotes monocyte migration *in vitro* (7), induces intracellular Ca^{2+} shifts in endothelial cells (8), induces plasminogen activator and plasminogen activator inhibitor 1 synthesis in endothelial cells (9), and stimulates glucose transport into endothelial cells (10).

VEGF probably acts by binding to endothelial cell-surface receptors, which then initiate an intracellular signaling cascade. Early studies using radioligand binding identified high-affinity endothelial-specific binding sites, and cross-linking studies showed receptor–ligand complexes of 270, 225, 195, and 170 kDa (11–13). Recently the human transmembrane c-fms-like tyrosine kinase (Flt-1) was identified as a receptor for VEGF, capable of specific, high-affinity binding and able to induce Ca^{2+} efflux in *Xenopus* oocytes (14). Flt-1 is a member of a class of receptor tyrosine kinases that differ from previously described class III receptor tyrosine kinases in that they possess seven rather than five immunoglobulin-like extracellular domains (15). Two other human seven immunoglobulin-like domain tyrosine kinases have been identified, Flt-4 and kinase insert domain-containing receptor (KDR). Flt-4 was isolated from a human erythroleukemia cell cDNA library, and by *in situ* hybridization does not appear to be expressed in human fetal endothelium (16, 17). KDR was isolated from a human umbilical vein endothelial cell library, and by RNA blot analysis is expressed in bovine aortic endothelium but is not expressed in vascular smooth muscle (18). Flk-1 is the mouse homologue of KDR, with 85% identical amino acid sequence (19, 20). Given the structural homology between the *Flk-1/KDR* and *Flt-1* genes, we hypothesized that Flk-1 may be a VEGF receptor and could potentially play a role in vascular development. We report that Flk-1 is a functional receptor for VEGF and is expressed in the vascular endothelium of the mouse embryo.

MATERIALS AND METHODS

Construction of Flk-1 Expression Plasmids. Full-length Flk-1 cDNAs, with and without an added C-terminal sequence expressing the influenza virus hemagglutinin (HA) epitope (amino acid sequence: SSYPYDVPDYASLGGPSR) for use in immunoprecipitation, were constructed by ligation of four PCR-derived Flk-1 fragments. Total mouse lung RNA was isolated and reverse-transcribed into cDNA by using standard protocols. PCR was then done, using oligonucleotide primers corresponding to the published Flk-1 sequence, to generate four fragments spanning the Flk-1-coding sequence: fragment 1 (nt 148–968), 5′ primer GATTCTAGAGGACGGAGAAGGAGTCTGTGCCTGA and 3′ primer AGGTGGAGAGTGCCAGGTGAAATCA; fragment 2 (nt 969–2623), 5′ primer GTACAGCGAGAACAGAGCTCAATG and 3′ primer ATCTGGATCCATGACAATAGACAAGTAG; fragment 3 (nt 2624–3333), 5′ primer CTATTGTCATGGATCCAGATGA and 3′ primer CGAAGTCACAGATCTTAACCACA; and fragment 4 (nt 3334–4309), 5′ primer CGGACTCGAGTAAGATCTGTGACTT and 3′ primer CGGCTCTAGATCAAGCAGCACCT. For the Flk-1/HA construct the fragment 4 5′ primer was used, and the 3′ primer was CGCGAATTCAACAGGAGGTGAGCGCAGTGTGGTCCCTGAGTCAGC. Full-length Flk-1 was assembled by digestion with appropriate restriction enzymes and ligation of the PCR fragments into the simian virus 40-based mammalian expression vector pSV7d. Flk-1/HA was assembled by in-frame ligation of full-length Flk-1 into a

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Abbreviations: Flk-1, fetal liver kinase 1; Flt-1, c-fms-like tyrosine kinase 1; VEGF, vascular endothelial growth factor; KDR, kinase insert domain-containing receptor; PDGF, platelet-derived growth factor; HA, hemagglutinin.

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vector containing the HA sequence. DNA sequencing of both constructs was done by the dideoxynucleotide chain-termination method in both directions, and two differences were found between our Flk-1 sequence and the original published sequence. (*i*) In two independent PCR clones nt 2957 and 2958 are GC, rather than CG, and therefore amino acid 917 is cysteine rather than serine. We believe our sequence to be correct because cysteine is present at this site in KDR, the human homologue of Flk-1, and in the related receptor tyrosine kinases c-kit, c-fms, and the β-platelet-derived growth factor (PDGF) receptor. (*ii*) The adenine at position 4229 of the published sequence is not present in our PCR clones, and additional evidence supports our sequence as correct. (*i*) Translation of the Flk-1 sequence without adenine at position 4229 reveals a stop codon at nt 4239–4241, thereby shortening and changing the predicted C-terminal amino acid sequence of Flk-1 to better match its homologues KDR and Flt-1: published Flk-1 C terminus is . . . RGAA; Flk-1 without adenine 4229 is . . . SPPV; KDR is . . . SPPV; and Flt-1 is . . . TPPI. (*ii*) We have found that the influenza virus HA epitope is not expressed when subcloned onto the 3′ end of Flk-1 in the published reading frame. The epitope is expressed when subcloned onto the 3′ end in the reading frame without adenine at position 4229. Flk-1/HA was subcloned into the cytomegalovirus-based retroviral vector LNCX (21) for use in making stable cell lines.

COS Cell Culture and Transfection. COS 6M cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum. Cells were transfected by the DEAE-dextran method. Approximately 5×10^5 cells in a 35-mm plate were incubated for 3 hr in 5 ml of DMEM, DEAE at 0.4 mg/ml, 100 mM chloroquine phosphate, and pSV7d/Flk-1 expression plasmid at 0.5 μg/ml. The transfection medium was then replaced by DMEM with 10% fetal bovine serum.

VEGF Binding Assays. The 165-amino acid isoform of human VEGF was used in all assays (1). Seventy-two hours after transfection with pSV7d/Flk-1 expression vector, COS cells were washed twice with binding medium (DMEM/25 mM Hepes/1% bovine serum albumin). Binding was done for 2 hr at 4°C in 1 ml of binding medium containing 15,000 cpm of ^{125}I-labeled VEGF (2800 Ci/mMol; 1 Ci = 37 GBq) and the indicated concentration of unlabeled ligand. The cells were then washed three times with unlabeled binding medium and lysed with 0.5 ml of lysis buffer [20 mM Tris (pH 8.0)/137 mM NaCl/Triton X-100 1% (vol/vol)/10% (vol/vol) glycerol/1 mM sodium vanadate/1 mM phenylmethylsulfonyl fluoride/aprotinin at 0.15 units/ml/0.02 mM leupeptin]. The soluble lysates were counted in a γ counter.

Receptor Phosphorylation Assay. NIH 3T3 cells stably expressing Flk-1/HA were created by retroviral transfection and selection in G418. PA317 packaging cells were transfected with LNCX/Flk-1/HA by the calcium phosphate precipitation method. Two days later the supernatant was collected, filtered, and added with Polybrene (8 μg/ml) to NIH 3T3 cells. Colonies were selected in G418, stimulated with VEGF (2 nM) for 5 min at 37°C, and lysed in lysis buffer described above. Lysates were immunoprecipitated with antiphosphotyrosine antibody, separated by SDS/PAGE, immunoblotted with murine monoclonal anti-HA antibody (22), and developed with alkaline phosphatase-conjugated anti-mouse antibody.

***Xenopus* Oocyte Injection and Ca^{2+} Efflux Assays.** Full-length Flk-1 cDNA was subcloned into pBluescript (Stratagene). *In vitro* transcripts containing a 5′ GpppG cap (Pharmacia) were prepared from the linearized plasmid template using T7 RNA polymerase as described (23). Fully grown oocytes (Dumont stage VI) were obtained from *Xenopus laevis*. The oocytes were defolliculated with collagenase (Sigma type IA, 1 mg/ml) and maintained at 19°C in modified Barth's medium with Hepes (MBSH)/bovine serum albumin at 1 mg/ml/penicillin G at 100 units/ml/streptomycin at 100 μg/ml. The oocytes were injected with 50 nl of mRNA (0.5 mg/ml) or sterile water. Twenty-four hours after injection, the oocytes were incubated with $^{45}Ca^{2+}$ (100 μCi/ml) in 0.4 ml of Ca^{2+}-free MBSH for 3 hr at 19°C. The oocytes were then washed with MBSH, and groups of four oocytes were placed in 24-well tissue culture plates. The medium was collected and replaced every 10 min, and the radioactivity in the medium was determined by liquid scintillation counting. After 30 min VEGF (10 nM) or BB PDGF (10 nM) was added to the medium for 10 min only. These experiments were done at room temperature.

***In Situ* Hybridization.** Fragment 4 of Flk-1 (nt 3334–4309) was subcloned into pBluescript SK and linearized. Radiolabeled sense and antisense transcripts were generated by *in vitro* transcription in the presence of ^{35}S-labeled UTP (1200 Ci/mMol, Amersham) using Sp6 or T7 RNA polymerase. *In situ* hybridization of paraffin-embedded sections was done as described (24) with modifications (25). After hybridization the slides were coated with photographic emulsion and exposed for 5 weeks. Sections were counterstained with hematoxylin/eosin.

RESULTS

VEGF Binds with High Affinity and Specificity to COS Cells Transfected with Flk-1 cDNA. We examined binding of VEGF to Flk-1 using intact COS 6M cells transiently transfected with full-length Flk-1 cDNA. Untransfected COS 6M cells did not bind VEGF (data not shown). To investigate the affinity and specificity of VEGF binding to Flk-1, binding of human ^{125}I-labeled VEGF was assayed in the presence of increased concentrations of unlabeled human VEGF or unlabeled BB PDGF. VEGF and B-chain PDGF share sequence homology, including eight conserved cysteines (1). The apparent K_d for VEGF binding to Flk-1 was 0.6 nM, ≈20 times the K_d for VEGF binding to Flk-1 (Fig. 1) but comparable to the affinities of the PDGF and fibroblast growth factor ligands for their receptors. BB PDGF did not block VEGF binding, showing the specificity of Flk-1 for VEGF. Similar results (data not shown) were obtained when COS cells were trans-

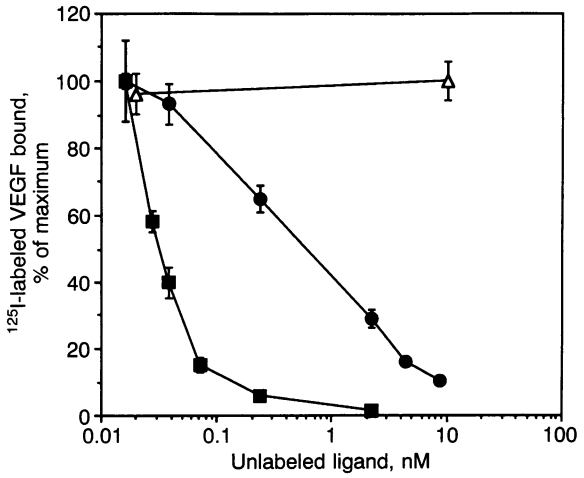

FIG. 1. Specific binding of ^{125}I-labeled VEGF to Flk-1 and Flt-1 expressed in COS cells. COS cells were transfected with the expression vector pSV7d containing the Flk-1 cDNA or the Flt-1 cDNA. Binding of ^{125}I-labeled VEGF was done in the presence of the indicated concentrations of unlabeled ligand. Receptor–ligand combinations are as follows: Flt-1 and VEGF (■), Flk-1 and VEGF (●), and Flk-1 and BB PDGF (△). Each point represents the mean ± SEM of three determinations. For some points error bars are smaller than symbols.

fected with a truncated Flk-1 cDNA encoding the extracellular domain, transmembrane region, and 21 amino acids of the cytoplasmic domain.

VEGF Induces Flk-1 Autophosphorylation. Signal transduction by receptor tyrosine kinases is initiated by ligand-induced receptor autophosphorylation. To determine whether VEGF induces Flk-1 autophosphorylation, NIH 3T3 cells expressing Flk-1/HA were exposed to VEGF (2 nM) for 5 min, lysed, and immunoprecipitated with antiphosphotyrosine, and lysates were blotted with anti-HA antibody. As seen in Fig. 2, VEGF induced phosphorylation of a 210-kDa HA-tagged protein. Untransfected cells (data not shown) did not show VEGF-dependent phosphorylation.

VEGF Induces Rapid Ca^{2+} Efflux from *Xenopus* Oocytes Injected with Flk-1 mRNA. VEGF stimulation of endothelial cells induces a rapid rise in cytosolic free Ca^{2+} (8), and we have previously shown that VEGF stimulation of *Xenopus* oocytes expressing Flt-1 induces a Ca^{2+} efflux (14). To investigate whether binding of VEGF to Flk-1 activates an intracellular signaling pathway, *Xenopus* oocytes were injected with 25 ng of Flk-1 mRNA and loaded with $^{45}Ca^{2+}$. As seen in Fig. 3, transient exposure to VEGF (10 nM) induced a rapid Ca^{2+} efflux from Flk-1 cDNA-injected oocytes but not from water-injected control oocytes. BB PDGF at the same concentration did not induce Ca^{2+} efflux from Flk-1-injected oocytes.

Flk-1 Is Exclusively Expressed in Mouse Embryonic Vascular Endothelium and Umbilical Cord Stroma at Day 12.5 Postcoitum. *In situ* hybridization studies of VEGF expression in the mouse embryo suggest that VEGF may play a role in embryonic angiogenesis (26). To investigate whether Flk-1 receptor is expressed in embryonic endothelium and, therefore, potentially a mediator of VEGF actions, *in situ* hybridization was done on mouse embryos at day 12.5 postcoitum. The sense and antisense probes corresponded to the C tail of Flk-1 from nt 3334–4309. As seen in Fig. 4 *A* and *B*, Flk-1 was expressed in the umbilical cord, liver, ventricle, endocardium, endothelium of the cardinal veins and aortae, limbs, and in the perineural plexus surrounding the neural tube. Higher-power views (*C–F*) revealed that Flk-1 was expressed exclusively in vascular endothelium and umbilical cord stroma. For example, Flk-1 was expressed in the endothelium of the aorta and cardinal vein (Fig. 4*C*) but was not expressed in the underlying cells. In the heart, expression was seen in the endocardium but was not seen in the myocardium (Fig. 4*D*). In the developing limb (Fig. 4*E*) Flk-1 was expressed in the endothelium of small vessels and in isolated single cells that may be angioblasts. Expression was found in the endothelium of the umbilical arteries and vein

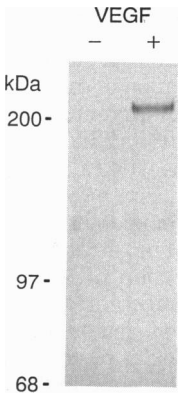

FIG. 2. VEGF-dependent phosphorylation of Flk-1. NIH 3T3 cells expressing Flk-1/HA were stimulated with VEGF (2 nM) for 5 min, lysed, immunoprecipitated with antiphosphotyrosine antibody, separated by SDS/PAGE, and immunoblotted with anti-HA antibody.

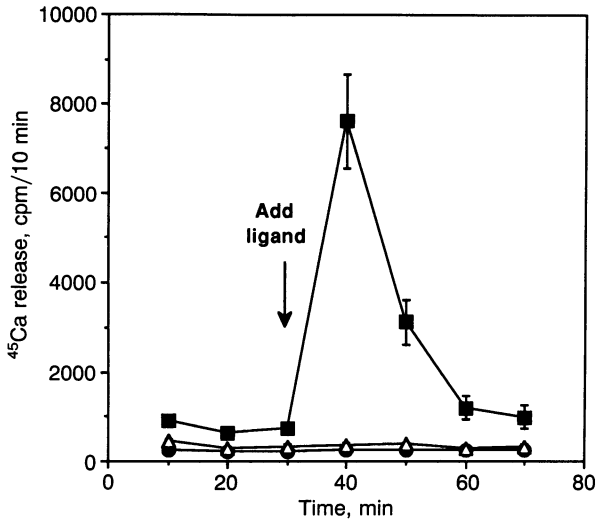

FIG. 3. Induction of Ca^{2+} efflux by VEGF from *Xenopus* oocytes expressing Flk-1. Oocytes were injected with 25 ng of Flk-1 mRNA per oocyte or water. Twenty-four hours later the oocytes were loaded with ^{45}Ca, and Ca^{2+} efflux was measured at 10-min intervals, as described in text. At the indicated time VEGF (10 nM) (■) or BB PDGF (10 nM) (●) was added to the oocytes injected with Flk-1. As a control, VEGF (10 nM) was added to water-injected oocytes (△). Each point represents the mean ± SEM of at least three determinations. For some points error bars are smaller than symbols. The experiment was repeated with similar results.

(Fig. 4*F*), but very little, if any, signal was present in the cells immediately beneath the artery endothelium, presumably vascular smooth muscle. However, Flk-1 was highly expressed in the stroma of the umbilical cord, tissue generally termed Wharton's jelly, which is not vascular endothelium. In other sections not shown, Flk-1 expression was seen in vascular sprouts invading the neuroectoderm from the perineural plexus, in the adult renal glomerulus, and in the embryonic day 7.5 yolk-sac mesoderm and intraembryonic mesoderm.

DISCUSSION

This study shows that the transmembrane tyrosine kinase Flk-1 is a functional receptor for VEGF. Terman *et al.* (20) showed that KDR, the human homologue of Flk-1, binds VEGF, but evidence of signal transduction was not presented. We show that Flk-1 not only binds VEGF with high affinity but transduces signals of two types. (*i*) VEGF induces autophosphorylation of Flk-1, which is an essential step in signal transduction by receptor tyrosine kinases. (*ii*) We demonstrate that Flk-1, like Flt-1, mediates VEGF-dependent Ca^{2+} efflux from *Xenopus* oocytes expressing the receptor.

Flk-1 and Flt-1 also share a specific structural feature: both have seven immunoglobulin-like extracellular domains instead of the five-domain structure present in the c-kit, c-fms, PDGF, and Flt-3 receptors. In light of the structural and functional similarities shared by these two receptors, and to simplify the nomenclature, we suggest that Flt-1 be designated VEGF receptor 1 (VEGFR1) and Flk-1/KDR be designated VEGF receptor 2 (VEGFR2).

Our *in situ* hybridization experiments showed that Flk-1 is an endothelial-specific receptor tyrosine kinase in the mouse embryo at day 12.5 postcoitum. Although Flk-1 is expressed in many organs, including the developing brain, the perineural vascular plexus, the heart, aortae and cardinal veins, liver, lung, intestine, and limb mesenchyme, in all of these organs expression is restricted to the vascular endothelium

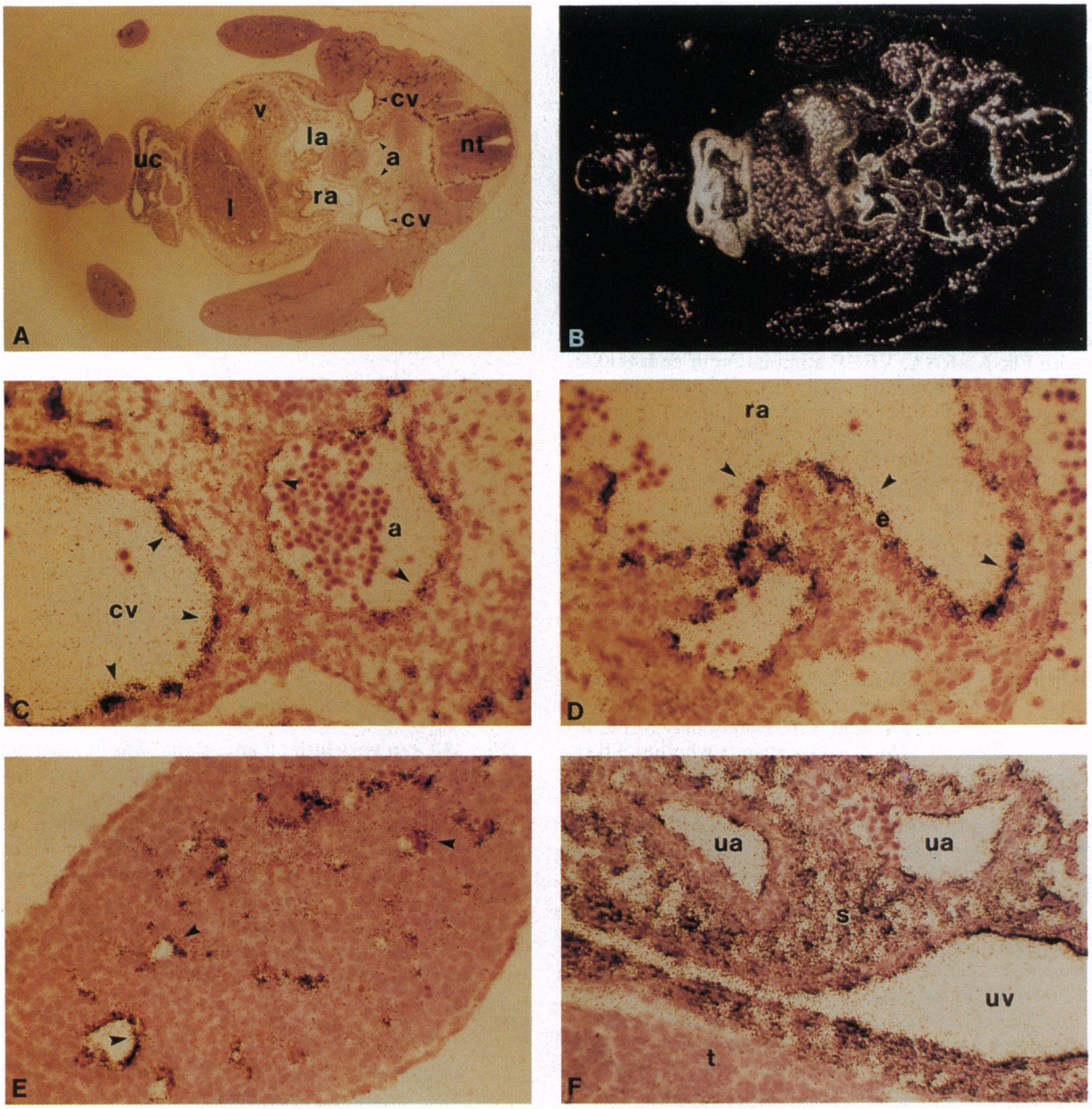

FIG. 4. *In situ* hybridization of Flk-1 antisense probe to mouse embryo day 12.5 postcoitum. Transverse section at the level of the heart in bright-field (*A*) and dark-field (*B*) illumination. uc, Umbilical cord; l, liver; v, left ventricle; la, left atrium; ra, right atrium; a, aortae; cv, cardinal veins; nt, neural tube. Flk-1 expression is present in the endothelium of cardinal vein and aorta (*C*), in endocardium (e) of right atrium (*D*), and in endothelium of small vessels in the developing limb (*E*). (*F*) Probe is present in the endothelium of the umbilical arteries (ua) and umbilical vein (uv), as well as in the stroma (s) of the umbilical cord but is not present in adjacent tail (t). (*A* and *B*, ×45; *C–F*, ×360.)

and endocardium. The only nonendothelial cells expressing Flk-1 are present in the stroma of the umbilical cord. The high level of expression in the cord stroma raises the possibility that other factors use the Flk-1 receptor. Placental growth factor (27), which shares 53% amino acid sequence identity with the PDGF-like region of VEGF, is one such candidate.

Receptor tyrosine kinases mediate diverse ligand-dependent cellular responses, including mitogenesis and differentiation. The identification of endothelial-specific receptor tyrosine kinases, such as Flk-1/KDR, as reported here, and Flt-1 (K.G.P., C.D.V., and L.T.W., unpublished work), and others with unknown ligands such as tie (tyrosine kinase with immunoglobulin-like and epidermal growth factor homology domains) (28) and tek (29) suggests that cellular functions specific to endothelium require signaling through these receptors. The embryonic vascular system develops by differentiation of mesodermal cells into angioblasts, which then form vessels by the processes of angiogenesis (sprouting from existing vessels) and vasculogenesis (invasion of angioblasts and *in situ* formation of endothelial vesicles) (30). The expression of Flk-1 in embryonic day 7.5 mesoderm suggests that Flk-1 may regulate the differentiation of mesoderm to form angioblasts. The isolated single cells expressing Flk-1 in the embryo limb bud (Fig. 4*E*) may represent invasion or local differentiation of angioblasts, and Flk-1 might regulate either process. Embryonic angiogenesis appears to be stimulated by local production of VEGF (26), suggesting that Flk-1/KDR or Flt-1 receptors may transduce signals for endothelial proliferation and migration. A role for Flk-1/KDR in mediating vascular permeability is suggested by the finding that Flk-1 is expressed in the adult renal glomerulus and the observation that VEGF expression is highest in the adult glomerulus and choroid plexus (26), where regulation of permeability is critical to homeostasis. Further information on the developmental expression of VEGF ligand isoforms and receptors and identification of the downstream intracel-

lular signaling pathways will be needed to establish the roles of Flk-1/KDR and Flt-1 in vascular development and function.

Note Added in Proof. After submission of this manuscript similar results were reported by Millauer *et al.* (31).

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