Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8915–8919, October 1993
Developmental Biology

Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth

(angiogenesis/endothelium/embryo/wound healing/tyrosine kinase)

KEVIN G. PETERS*, CARLIE DE VRIES†, AND LEWIS T. WILLIAMS‡

Program of Excellence in Molecular Biology, Cardiovascular Research Institute and the Howard Hughes Medical Institute, University of California, San Francisco, CA 94143

Communicated by Judah Folkman, June 24, 1993

ABSTRACT **Vascular endothelial growth factor (VEGF) is a polypeptide mitogen that stimulates the growth of endothelial cells *in vitro* and promotes the growth of blood vessels *in vivo*. We have recently shown that the fms-like receptor tyrosine kinase (flt) is a receptor for VEGF. Here we used *in situ* hybridization to show that, in adult mouse tissues, the pattern of *flt* expression was consistent with localization in endothelium. We also show that *flt* was expressed in endothelium during neovascularization of healing skin wounds and during early vascular development in mouse embryos. Moreover, *flt* was expressed in populations of embryonic cells from which endothelium is derived such as early yolk sac mesenchyme. The expression of *flt* in the endothelium of both developing and mature blood vessels suggests that VEGF might regulate endothelial differentiation, blood vessel growth, and vascular repair.**

Vascular endothelial growth factor (VEGF) was originally isolated by several different groups as both an endothelial cell mitogen and a vascular permeability factor (refs. 1–4; for review, see refs. 5 and 6). Peptide sequencing and cDNA cloning subsequently demonstrated that both of these activities were mediated by the same protein (7–10). Although VEGF is a potent mediator of vascular permeability, its highly specific mitogenic activity for vascular endothelial cells is remarkable. Furthermore, VEGF has been shown to promote angiogenesis in rat cornea and chicken chorioallantoic membrane assays (2, 7, 11). These properties suggest that VEGF could play a role in blood vessel growth and development.

We have recently shown that flt (fms-like tyrosine kinase; refs. 12 and 13), a receptor tyrosine kinase closely related to the platelet-derived growth factor receptor, is a receptor for VEGF. We found that flt, when expressed in COS cells, bound VEGF with high affinity and, when expressed in *Xenopus* oocytes, mediated calcium efflux in response to VEGF (14). However, whether flt mediates vascular permeability and endothelial growth in response to VEGF is not known. To determine whether flt might mediate the endothelial response to VEGF *in vivo* and to determine whether VEGF might play a role in vascular growth and development, we studied the expression of flt in adult mouse organs, in mouse embryos, and in healing skin wounds.

MATERIALS AND METHODS

Probe Generation. A murine *flt* cDNA clone was amplified by PCR from cDNA made by reverse transcription of total RNA from an adult BALB/c mouse brain. Using the murine cDNA as a template, a cDNA fragment encoding the transmembrane and juxtamembrane portions of flt was generated by PCR (5′ primer, GTCACAGAAGAGGATGAAGGTGTCTAT; 3′ primer, CCCAGCCACAGTCCGGCACGTAGGTGATT). The PCR fragment was then subcloned into pGEM-4Z for sequencing and for *in vitro* transcription of RNA probe. To generate radiolabeled antisense and sense transcripts, the plasmid was linearized and transcribed using Sp6 or T7 RNA polymerase (Promega). For RNase protection assays, transcription reactions were done in the presence of [^{32}P]UTP (800 Ci/mmol; 1 Ci = 37 GBq; Amersham) and for *in situ* hybridization transcripts were labeled using UTP[^{35}S] (1200 Ci/mmol; Amersham).

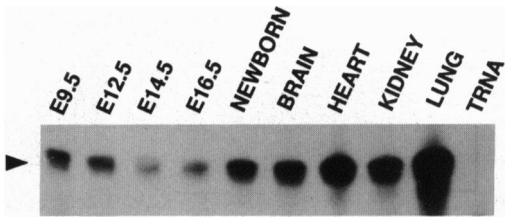

FIG. 1. RNase protection assay showing expression of *flt* during mouse embryonic development and in adult mouse organs. ^{32}P-labeled *flt* antisense transcript was hybridized to 50 μg of total RNA from mouse embryos (E9.5, E12.5, E14.5, and E16.5), newborn mice, and adult mouse organs (brain, heart, kidney, and lung). For a negative control, the *flt* probe was hybridized to 50 μg of tRNA. After RNase digestion, the protected fragments were separated by polyacrylamide gel electrophoresis and the gel was exposed for 3 days to Hyperfilm-MP (Amersham).

RNase Protection Assays. Swiss–Webster mice were sacrificed by cervical dislocation at various stages of gestation (9.5, 10.5, 12.5, 14.5, 16.5, and 18.5 days postcoitus) and the embryos were dissected from the uterus. Newborn and adult mice were killed by cervical dislocation. Adult mouse organs were dissected and washed briefly in ice-cold phosphate-buffered saline (PBS). All tissues were frozen immediately in liquid nitrogen and stored at −70°C until used for RNA isolation. Total cellular RNA was isolated by established techniques. Fifty-microgram samples of total RNA were hybridized with 1×10^5 cpm of ^{32}P-labeled antisense RNA probe at 42°C overnight and then digested with RNase A and T1 for 40 min at 30°C (15). The digests were resolved on 5% polyacrylamide gels and visualized by autoradiography.

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Abbreviations: VEGF, vascular endothelial growth factor; E, embryonic day.

*Present address: Duke University Medical Center, Box 3623, Durham, NC 27710.
†Present address: Netherlands Institute for Developmental Biology, 3584 CT Utrecht, The Netherlands.
‡To whom reprint requests should be sent at: University of California, Cardiovascular Research Institute, 505 Parnassus Avenue, Box 0130, San Francisco, CA 94143-0130.

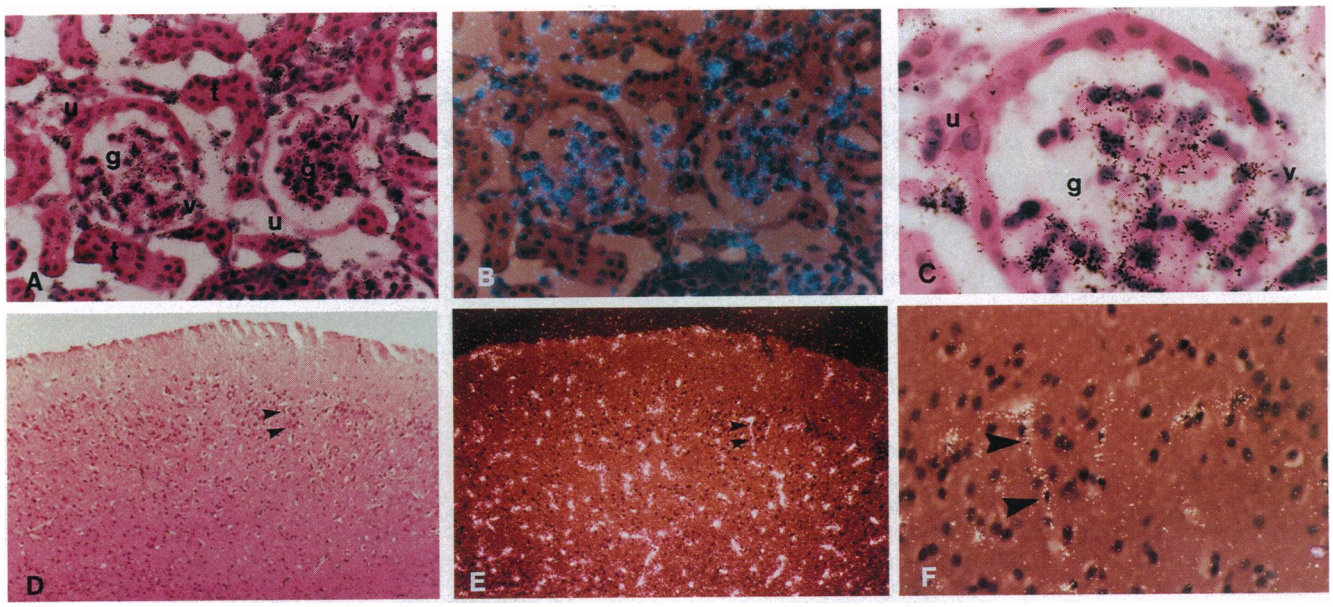

FIG. 2. Expression of *flt* in adult mouse organs. Sections of adult mouse kidney (*A–C*) and brain (*D–F*) were hybridized with radiolabeled antisense *flt* transcripts. (*A–C*) Expression of *flt* in the capillary tufts (g) and in the vascular pole (v) of two adjacent glomeruli. Note the absence of *flt* expression in the urinary pole of the glomeruli (u) and renal tubule cells (t). (*D–F*) Expression of *flt* in vascular profiles (arrowheads) in the cerebral cortex. (*A*, *C*, and *D*) Bright-field micrographs. (*E*) Dark-field micrograph. (*B* and *F*) Epiillumination micrographs to visualize silver grains. (*A*, *B*, and *F* ×370; *C*, ×930; *D* and *E*, ×95.)

***In Situ* Hybridization.** Full thickness skin wounds were created along the backs of adult CF1 mice; the wounds were then harvested at 3 and 7 days after wounding. Swiss–Webster mouse embryos and adult mouse organs were collected as described above. After dissection all tissues were immersed overnight in 4% paraformaldehyde/PBS at 4°C. After fixation, the embryos were dehydrated in an ethanol series, cleared in toluene, and embedded in paraffin and stored at room temperature until sectioned. The adult tissues, including wounds, were fixed as described above and then cryoprotected by immersion in 15% sucrose/PBS at 4°C, embedded in OCT (optimal cutting temperature compound) and stored at −70°C until cryosectioned. *In situ* hybridization on paraffin sections was performed as described (16) with modifications described by Frohman *et al.* (17). *In situ* hybridization on frozen sections was performed as described by Wilcox *et al.* (18). After hybridization, the slides were coated with photographic emulsion and exposed for 5–7 weeks. Sections were counterstained with hematoxylin/eosin.

RESULTS

We first used RNase protection assays to determine the relative abundance of *flt* mRNA in total RNA isolated from mouse embryos (E9.5, E12.5, E14.5, and E16.5; E, embry-

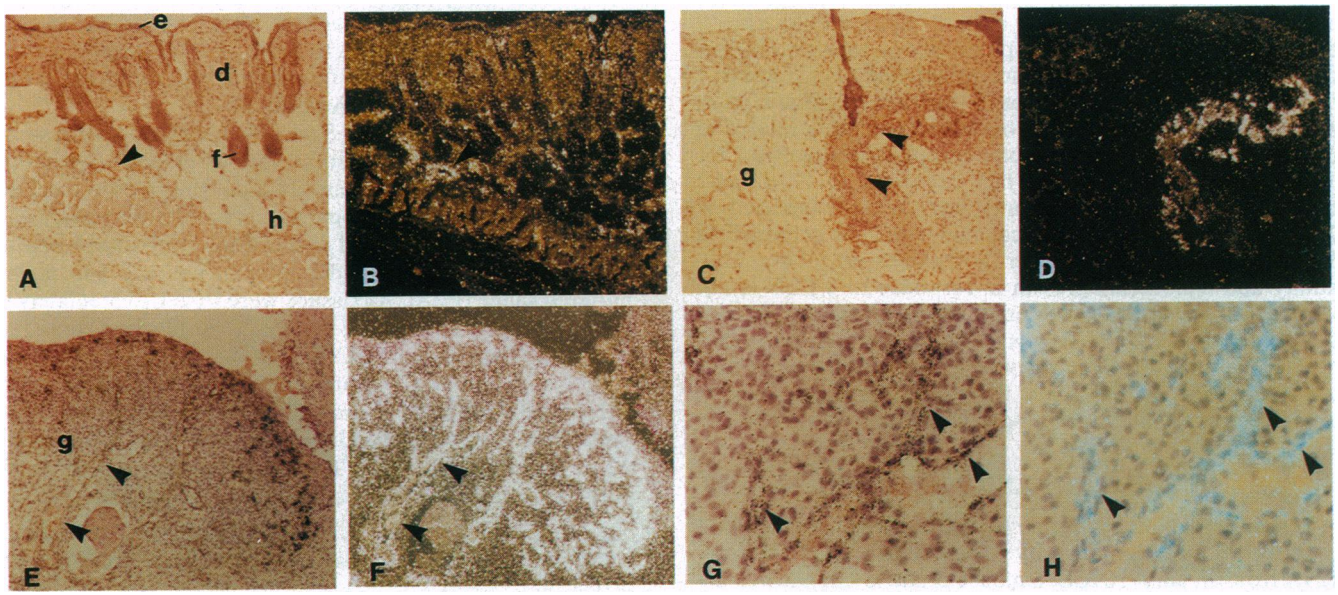

FIG. 3. Expression of *flt* during wound healing in the adult mouse. Sections through an excision skin wound were hybridized with radiolabeled antisense *flt* transcripts. (*A* and *B*) Expression of *flt* in a small vessel (arrowhead) in the hypodermis (h) of normal skin adjacent to a 7-day-old wound. (*C* and *D*) Expression of *flt* in large, ectatic vessels (arrowheads) at the wound edge after 3 days. (*E–H*) Marked expression of *flt* in the wound neovasculature after 7 days. Arrowheads in *E–H* show expression of *flt* in the endothelium of a larger feeder vessel as well as in the vascular sprouts that originate from it. e, epidermis; d, dermis; f, hair follicle; g, granulation tissue. (*A*, *C*, *E*, and *G*) Bright-field micrographs. (*B*, *D*, and *F*) Dark-field micrographs. (*H*) Epiillumination micrographs. (*A–F*, ×95; *G* and *H*, ×370.)

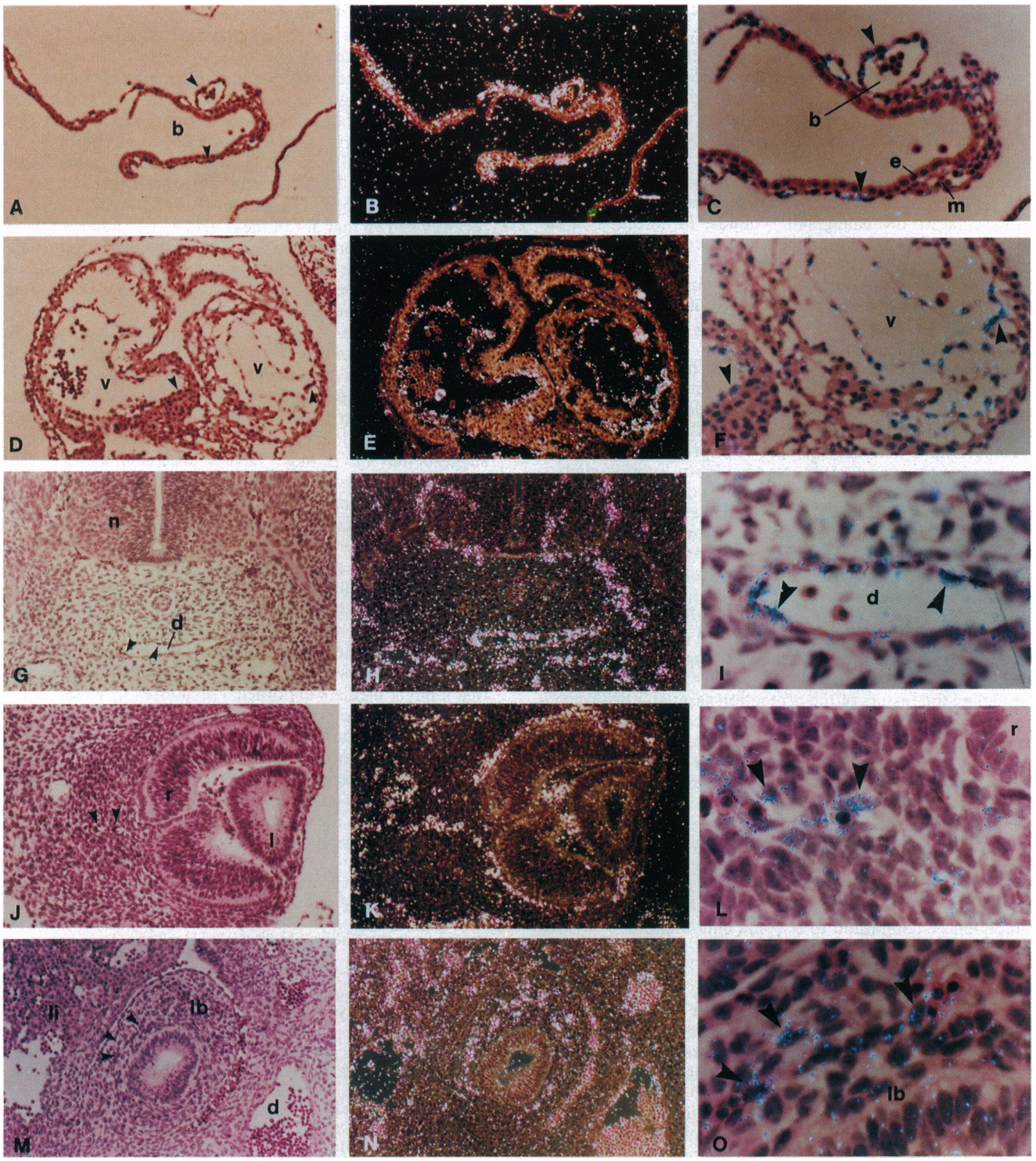

FIG. 4. Expression of *flt* in embryonic mouse tissues. Sections from mouse embryos were hybridized with radiolabeled antisense *flt* transcripts. (*A–C*) *flt* expression in an E9.5 yolk sac fragment; arrowheads indicate endothelium surrounding a blood island (b) and cells in the yolk sac not obviously associated with vessels or blood islands. (*D–F*) *flt* expression in the endocardium (arrowheads) lining the ventricle (v) of an E9.5 heart. (*G–I*) Expression of *flt* in the endothelium (arrowheads) of the dorsal aorta (d) and in the smaller vessels surrounding and penetrating the neural tube (n) of an E10.5 embryo. (*J–L*) Expression of *flt* in the endothelium of the ophthalmic artery (arrowheads) and in the vascular networks surrounding the retina (r) and lens. (*M–O*) Expression of *flt* in endothelium of the E10.5 embryonic liver (li) and lung bud (lb). Arrowheads in *M–O* show presumptive endothelial cells or angioblasts expressing *flt* mRNA. (*A, D, G, J,* and *M*) Bright-field micrographs. (*B, E, H, K,* and *N*) Dark-field micrographs. (*C, F, I, L,* and *O*) Epiillumination micrographs. (*A, B, D, E, G, H, J, K, M,* and *N,* ×185; *C, F, I, L,* and *O,* ×370.)

onic day), newborn mice, and adult mouse organs (Fig. 1). The RNase protection assays demonstrated a bimodal expression pattern of *flt* with a moderate level of expression in embryos during organogenesis (E9.5–E12.5), a low level of expression during fetal growth (E14.5–E16.5), and a high level of expression in newborn mice and in all adult mouse organs studied (brain, heart, kidney, and lung).

Next, we used *in situ* hybridization to study the cellular distribution of *flt* mRNA in adult mouse lung, heart, kidney, and brain. In all tissues examined, *flt* mRNA was distributed

in a pattern consistent with expression in endothelium and was not detected in other cell types (Fig. 2). In the kidney, for example, *flt* was expressed in the capillary tuft and the vascular pole of the glomeruli but was not detected in renal tubule cells (Fig. 2 *A–C*). In the brain, *flt* was expressed in the endothelium of microvessels creating a pattern of vascular profiles (Fig. 2 *D–F*). No *flt* mRNA was detected in neurons or glia.

Since *flt* was expressed in the endothelium of normal adult tissues, we wondered whether it would be expressed in the neovasculature of healing skin wounds. In normal skin adjacent to the wound, *flt* mRNA was detected in the endothelium of vessels in the dermis and the hypodermis (Fig. 3 *A* and *B*). At 3 days after wounding, before neovascularization had occurred, *flt* was not detected within the wound but was detected in ectatic vessels bordering the wound (Fig. 3 *C* and *D*). At 7 days after wounding, when substantial neovascularization had occurred, *flt* was highly expressed in endothelium of large vessels bordering the wound as well as in the small vessels of the neovasculature that course throughout the substance of the wound (Fig. 3 *E–H*).

To determine whether *flt* might be expressed in the developing embryonic vasculature, we used *in situ* hybridization to examine *flt* expression in mouse embryos. At E7.5–E12.5 when the embryonic vasculature is being established, *flt* was detected in most if not all endothelia and was not detected in other populations of cells (Fig. 4). *flt* was detected in the endothelium of large vessels such as the dorsal aorta as well as in the endothelium of small vessels such as the ophthalmic artery and the vascular networks surrounding the neural tube and retina (Fig. 4 *G–L*). More specialized endothelia such as the endocardium and the endothelial lining of the embryonic liver also expressed *flt* mRNA (Fig. 4 *D–F* and *M–O*). Paralleling the results of the protection assays, little or no *flt* mRNA was detected in mouse embryos during the fetal growth stages (E14.5–E16.5; data not shown).

In yolk sac and lung bud, endothelial cells lining blood islands or nascent blood vessels expressed *flt* mRNA (Fig. 4 *A–C* and *M–O*).

Interestingly, the yolk sac and lung buds also contained isolated cells expressing *flt* mRNA that were not obviously associated with blood islands or blood vessels. Thus, in embryonic tissues such as the lung bud and yolk sac, it is possible that *flt* is expressed in endothelial precursor cells competent or committed to become endothelium.

DISCUSSION

Previously, we have shown that flt is a receptor tyrosine kinase specific for VEGF (14). In this study, we have shown that in adult and embryonic tissues, expression of *flt* mRNA was localized to endothelium and to populations of cells that give rise to endothelium. Since VEGF is a mitogen for endothelial cells in culture and is angiogenic *in vivo*, expression of *flt* in embryonic endothelium suggests a role for VEGF in embryonic vascular growth and development. Expression of *flt* in the neovasculature of healing skin wounds also supports a role for VEGF in vascular growth and repair. The recent study by Brown *et al.* (19) showing that VEGF is expressed in the epithelium overlying healing skin wounds suggests a paracrine mechanism for stimulation of *flt* in the wound neovasculature.

Peptide growth factors, such as fibroblast growth factor, epidermal growth factor, and nerve growth factor, interact with receptor tyrosine kinases and can influence cell differentiation (20–22). In two embryonic structures that are known sites of endothelial differentiation, the yolk sac and the lung bud, we detected *flt* expression in cells, perhaps angioblasts, that were not associated with blood vessels (23–25). Based on these findings, we hypothesize that VEGF is not only involved in blood vessel growth, but that it may also be important for endothelial differentiation.

During fetal development (E14.5–E16.5), a time when endothelial differentiation might be complete but blood vessel growth is ongoing, *flt* expression markedly decreased. This could mean that factors other than VEGF drive blood vessel growth during fetal development or, alternatively, that a different VEGF receptor is expressed during this stage of development. In fact, receptor tyrosine kinases closely related to *flt* have been cloned (26–28) and we and others have recently shown that one of these genes, the human *KDR* gene and its mouse homologue *flk-1*, encode high-affinity VEGF receptors that are expressed in endothelium during the fetal growth stage as well as during earlier embryonic development and in adult tissues (29–31).

By protection assay the highest level of *flt* expression was in adult organs and by *in situ* hybridization *flt* mRNA was detected only in endothelium. The pattern of *flt* mRNA expression in endothelium was identical to the previously described pattern of VEGF binding in adult tissues (32). Expression of *flt* in the quiescent endothelium of adult organs suggests that VEGF might have a function in mature vessels other than mediating vascular growth. Since VEGF is a potent mediator of vascular permeability, perhaps *flt* mediates VEGF-induced changes in the permeability of the adult vasculature. VEGF mRNA has been localized to the choroid plexus and the glomerular epithelium, both sites where control of vascular permeability is particularly important (33). In other adult vessels, it is possible that VEGF is important for the maintenance and repair of the endothelium.

In summary, the *flt* receptor tyrosine kinase, a receptor for VEGF, is expressed by vascular endothelium, suggesting that it might mediate endothelial responses to VEGF *in vivo*. The expression of *flt* in embryonic structures from which endothelial cells arise and in the endothelium of developing embryonic vessels suggests that VEGF might play a role in endothelial differentiation and in embryonic vascular growth. Expression of *flt* in endothelium of adult tissues suggests a role for VEGF in the regulation of vascular permeability and in vascular repair and maintenance.

This work was supported by the National Heart Lung and Blood Institute Program of Excellence at the University of California, San Francisco. K.G.P. was also supported by a Physician Scientist Award from the National Institutes of Health.

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