VEGF and KDR Gene Expression during Human Embryonic and Fetal Eye Development

Karin Gogat,1,2 Laurence Le Gat,1,2 Loïc Van Den Berghe,1 Dominique Marchant,1 Alexandra Kobetz,1 Stéphanie Gadin,1 Bernard Gasser,5 Isabelle Quéré,4 Marc Abitbol,1 and Maurice Menasche1

PURPOSE. It is important to understand the development of the normal retinal vascular system, because it may provide clues for understanding the mechanisms underlying the neovascularization associated with several retinopathies of infancy and adulthood. However, little is known about normal human ocular vascularization. VEGF is a key growth factor during vascular development and one of its receptors, KDR, plays a pivotal role in endothelial cell proliferation and differentiation. The purpose of this study was to analyze VEGF and KDR gene expression patterns during the development of the human eye during the embryonic and fetal stages.

METHODS. The gene expression of VEGF and KDR was analyzed by in situ hybridization in 7-week-old embryos and in 10- and 18-week-old fetuses. In addition, we performed VEGF and KDR immunohistochemistry experiments on 18-week-old fetus tissue sections.

RESULTS. These results clearly demonstrated that the levels of VEGF and KDR transcripts are correlated during the normal development of the ocular vasculature in humans. The complementarity between the patterns of VEGF and KDR during the early stages of development suggests that VEGF–KDR interactions play a major role in the formation and regression of the hyaloid vascular system (HVS) and in the development of the choriocapillaris. In later stages (i.e., 18-weeks-old fetuses), the expression of KDR seems to be linked to the development of the retinal vascular system. VEGF and KDR transcripts were unexpectedly detected in some nonvascular tissues—that is, in the cornea and in the retina before the development of the retinal vascular system.

CONCLUSIONS. The expression of VEGF and KDR correlates highly with the normal ocular vascularization in humans, but VEGF may also be necessary for nonvascular retinal developmental functions, especially for the coordination of neural retinal development and the preliminary steps of the establishment of the definitive stable retinal vasculature. (Invest Ophthalmol Vis Sci. 2004;45:7–14) DOI:10.1167/iovs.02-1096

Blood vessels develop by vasculogenesis, angiogenesis, or intussusception. The term vasculogenesis describes the de novo formation of vessels from vascular endothelial cells (angioblasts). Angioblasts migrate to and differentiate at the location of future vessels, coalesce into cords, differentiate into endothelial cells, and ultimately form patent vessels.1 The term angiogenesis describes a different process of blood vessel formation in which proliferating endothelial cells from preexisting blood vessels extend the vascular network.2 The term intussusception describes the remodeling and expansion of new vessels by the insertion of interstitial tissue columns into the lumen of preexisting vessels. Most organs are vascularized by vasculogenesis, but the central nervous system (CNS) and kidneys are vascularized by angiogenesis. However, the retina is vascularized by a combination of vasculogenesis and angiogenesis.3

The retina, which is embryologically an extension of the telencephalon,4 is an excellent model for studying vascular development in the CNS. Retinal blood vessels are restricted to the inner two-thirds of the retina. To accommodate the visual function, the outer retina is completely avascular and receives oxygen and nutrients from the choroidal vessels.5 To enhance transport, there is a large collection of fenestrated choroidal capillaries beneath the retina, known as the choriocapillaris. During vascular development, superficial inner retinal vessels form by vasculogenesis, starting at the optic nerve and developing along a gradient from the posterior to the anterior retina. Vessels then sprout from superficial retinal vessels and invade the retina where they form the intermediate and the deep capillary beds by angiogenesis. This process takes approximately 20 weeks in humans,6 beginning in about the middle of the second trimester of pregnancy. However, a network of capillaries, called the hyaloid vascular system (HVS), forms transiently before the definitive retinal vasculature. This network regresses during the later stages of ocular development. Indeed, during the first 3 or 4 weeks of embryological development, the hyaloid artery, a branch of the dorsal ophthalmic artery, enters the developing eye through the fetal fissure7 and gives off branches that form the tunica vasculosa lenti (TVL) around the developing lens. The TVL nourishes the immature lens, retina, and vitreous body and may be involved in the formation of the primary vascular vitreous body.8

VEGF is an important stimulatory factor during retinal vascularization. VEGF was first identified as a vascular permeabil-
ity factor (VPF) and as a vascular endothelial cell-specific growth factor. The VEGF gene consists of eight exons. Alternative splicing can generate several VEGF isoforms. Four main isoforms have been described in humans: VEGF121, VEGF165, VEGF189, and VEGF206. The VEGF121 and VEGF165 appear to be the most abundant forms. Although all VEGF isoforms are synthesized with a signal peptide, their secretion profiles differ. The long isoforms of VEGF can be associated with heparan sulfate proteoglycans (HSPG) on the extracellular matrix (ECM) and on the cell surface. The VEGF121 isoform is a freely diffusible protein, whereas VEGF189 and VEGF206 are almost completely sequestered in the ECM. The VEGF165 isoform is also secreted, although a significant fraction remains bound to the cell surface and the ECM. The various VEGF isoforms bind two type 1 transmembrane protein-tyrosine kinase receptors, Flt-1 (fms-like tyrosine kinase)17 and KDR (kinase domain region),18 the human homologue of Flk-1. The Flt-1 and KDR/Flk-1 genes are both expressed in endothelial cells, but have somewhat different functions. Knockout experiments have shown that KDR/Flk-1 plays a central role in endothelial cell proliferation and differentiation. Recent studies have revealed that neuropilin 1 (NP-1), a semaphorin receptor, plays an important role in VEGF differentiation.20

Tissue Preparation

The sections were hybridized with the probes and incubated in a solution in acetone. Sections were then used to expose x-ray film by exposure to photographic emulsion. The 60-mer oligonucleotide probes were synthesized and purified by Genset, Evry, France. The oligonucleotides were-3' dATP (NEN) using terminal deoxynucleotidyl transferase (15 U/ml; Invitrogen-Gibco, Cergy Pontoise, France) to a specific activity of approximately 7 × 10^6 cpm/mg as described by Abitbol et al. The probes were purified on biopin columns (BioRad, Ivory-sur-Seine, France) before use. The VEGF probes were chosen according to the human VEGF cDNA sequence (GenBank accession number: NM_003576; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The sequences of the KDR sense probes were KDR1: 5'-CATGCCACTTCCAA-3' (positions 1216-1275). The sequences of the VEGF probes were ASVEGF1: 5'-ACAGCCTCGGGATCTGGTGAAGGCAGACGTGT-3' (positions 1125-1184), and ASVEGF2: 5'-GCAAGGGAGGACGCTGTAGTTAAACGACTGTCAGTGAAGCAGCCAGGGGT-3' (positions 1216-1275). The sequences of the VEGF antisense probes were ASVEGF1: 5'-ACAGCCTCGGGATCTGGTGAAGGCAGACGTGT-3' (positions 1125-1184), and ASVEGF2: 5'-ACAGCCTCGGGATCTGGTGAAGGCAGACGTGT-3'. The sequences of the KDR antisense probes were ASKDR1: 5'-TTGGAAGATGGCATTGTTGCCAGAAGGACAGTGCACAGCAAA-3' (positions 356-415), and ASKDR2: 5'-TTGGAAGATGGCATTGTTGCCAGAAGGACAGTGCACAGCAAA-3'. The antisense probes were ASVEGF1: 5'-ACAGCCTCGGGATCTGGTGAAGGCAGACGTGT-3' (positions 1125-1184), and ASVEGF2: 5'-ACAGCCTCGGGATCTGGTGAAGGCAGACGTGT-3'. The sequences of the KDR probes were 3' week-old fetuses (n = 2) that had been fixed in formalin and embedded in paraffin. Sections were cut at 5-μm intervals, mounted on glass slides (Superfrost plus; Fisher Scientific, Illkirch, France), dried overnight at 37°C, and stored at room temperature until use.

DNA Probes for In Situ Hybridization

The 60-mer oligonucleotide probes were synthesized and purified by Genset, Evry, France. The oligonucleotides were-3' dATP (NEN) using terminal deoxynucleotidyl transferase (15 U/ml; Invitrogen-Gibco, Cergy Pontoise, France) to a specific activity of approximately 7 × 10^6 cpm/mg as described by Abitbol et al. The probes were purified on biopin columns (BioRad, Ivory-sur-Seine, France) before use. The VEGF probes were chosen according to the human VEGF cDNA sequence (GenBank accession number: NM_003576; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The sequences of the KDR sense probes were KDR1: 5'-CATGCCACTTCCAA-3' (positions 1216-1275). The sequences of the VEGF probes were ASVEGF1: 5'-ACAGCCTCGGGATCTGGTGAAGGCAGACGTGT-3' (positions 1125-1184), and ASVEGF2: 5'-GCAAGGGAGGACGCTGTAGTTAAACGACTGTCAGTGAAGCAGCCAGGGGT-3' (positions 1216-1275). The sequences of the VEGF antisense probes were ASVEGF1: 5'-ACAGCCTCGGGATCTGGTGAAGGCAGACGTGT-3' (positions 1125-1184), and ASVEGF2: 5'-ACAGCCTCGGGATCTGGTGAAGGCAGACGTGT-3'. The sequences of the KDR antisense probes were ASKDR1: 5'-TTGGAAGATGGCATTGTTGCCAGAAGGACAGTGCACAGCAAA-3' (positions 356-415), and ASKDR2: 5'-TTGGAAGATGGCATTGTTGCCAGAAGGACAGTGCACAGCAAA-3'. The specificity of the probes used was confirmed by Northern blot analysis in human tissues during fetal development.

In Situ Hybridization

The sections were hybridized with the probes and incubated in a humidified chamber at 43°C for 20 hours, as described previously. The sections were then used to expose x-ray film (Hyperfilm Betamax; Amersham, Orsay, France) for 4 days and then photographed emulsion.
7-Week-Old Embryos

Distribution of VEGF and KDR mRNA in 7-Week-Old Embryos

In 7-week-old embryos, VEGF and KDR transcripts were present in the two proliferating neuroblastic layers (Figs. 1a, 2a). Examination at a higher magnification confirmed the presence of VEGF transcripts in the retina (Fig. 1b), but also allowed us to detect them in the retinal pigment epithelium (RPE). As it is very difficult to distinguish the silver grains from the melanosomes, we decided to use bright-field illumination and to focus on the silver grains present in the RPE (Fig. 1c).

Examination at a high magnification also confirmed the presence of KDR transcripts in the proliferating layers of retina (Fig. 2b), although the KDR labeling was very faint compared with the VEGF labeling. No KDR transcripts were detected in the RPE; however, they were present in the pericellular mesenchyme, including a layer of cells adjacent to the RPE (Fig. 2c). Finally, KDR transcripts were observed in the blood vessels entering the vitreous space through the optic stalk—that is, in the posterior and lateral TVL (Fig. 2d). It is noteworthy that no VEGF transcripts were detected in the TVL (Fig. 1d).

The specificity of the VEGF and KDR mRNA hybridization signals obtained with the antisense probes was further confirmed by the use of a sense probe (Figs. 1f, 2f, respectively), which did not reveal any specific signal.

Distribution of VEGF and KDR mRNA in 10-Week-Old Fetuses

At this stage, VEGF mRNA was still detected in the neuroretina but exclusively in the inner neuroblastic layer (Figs. 3a, 3b).

Immunohistochemistry

Before use, the paraffin-embedded sections of 18-week-old fetuses were dehydrated through a graded alcohol series and cleared in xylene. The sections were labeled using the detection kit (ChemMate; Dako, Trappes, France) according to the manufacturer’s instructions. The following primary antibodies were used: a rabbit anti-human VEGF polyclonal antibody (diluted 1:100), a mouse anti-human KDR monoclonal antibody (diluted 1:40), and a rabbit anti-human platelet endothelial cell adhesion molecule (PECAM)-1 (CD31) polyclonal antibody (all from Santa Cruz Biotechnology, Tebu, France; diluted 1:20). The secondary antibody was a biotinylated antibody (ChemMate detection kit; Dako), with diaminobenzidine (DAB) as the substrate. After DAB staining, tissue sections were counterstained with hematoxylin.

RESULTS

Distribution of VEGF and KDR mRNA in 7-Week-Old Embryos

In 7-week-old embryos, VEGF and KDR transcripts were present in the two proliferating neuroblastic layers (Figs. 1a, 2a). Examination at a higher magnification confirmed the presence of VEGF transcripts in the retina (Fig. 1b), but also allowed us to detect them in the retinal pigment epithelium (RPE). As it is very difficult to distinguish the silver grains from the melanosomes, we decided to use bright-field illumination and to focus on the silver grains present in the RPE (Fig. 1c).

Examination at a high magnification also confirmed the presence of KDR transcripts in the proliferating layers of retina (Fig. 2b), although the KDR labeling was very faint compared with the VEGF labeling. No KDR transcripts were detected in the RPE; however, they were present in the pericellular mesenchyme, including a layer of cells adjacent to the RPE (Fig. 2c). Finally, KDR transcripts were observed in the blood vessels entering the vitreous space through the optic stalk—that is, in the posterior and lateral TVL (Fig. 2d). It is noteworthy that no VEGF transcripts were detected in the TVL (Fig. 1d).

The specificity of the VEGF and KDR mRNA hybridization signals obtained with the antisense probes was further confirmed by the use of a sense probe (Figs. 1f, 2f, respectively), which did not reveal any specific signal.
Detection of VEGF mRNA and Protein in 18-Week-Old Fetuses

VEGF and KDR mRNA were detected in the inner nuclear layer and in the differentiating ganglion cell layer of the neuroretina. The VEGF labeling (Fig. 5a) seemed to be stronger than the KDR labeling (Fig. 6a). At this stage, VEGF transcripts were not detected in the RPE, and KDR transcripts were not detected in the choroid layer (Figs. 5c, 6c, respectively). The same amount of silver grains were present as in the sense control sections (Figs. 5d, 6d, respectively). The specificity of the VEGF and KDR mRNA hybridization was confirmed by the use of sense probes (Figs. 5b, 6b, respectively) that did not reveal any specific signal.

This last result differs from the finding that although the level of VEGF mRNA decreases with age after the formation of the choriocapillaris, it is still present in rodents. To complete these initial results, we performed an immunohistochemical analysis on paraffin-embedded sections of eyes of the same age. It was very difficult to distinguish the labeling from the retinal pigmented epithelium, but we observed a strong VEGF expression in the choroid layer (Figs. 7a, 7b) compared with a control section (Figs. 7c, 7d). This suggests that the VEGF

However, no KDR mRNA (Figs. 4a, 4b) were detected in this layer. VEGF mRNA was still detected in the RPE (Fig. 3c) and KDR mRNA was still detected in the cell layer adjacent to the RPE, which may correspond to the choriocapillaris layer (Fig. 4c). We also detected VEGF mRNA in the primary lens fibers (Fig. 3d) originating from the posterior wall of the lens and in the epithelial cells of the anterior wall of the lens behind the pupillary membrane (Fig. 3e). Examination at a higher magnification allowed us to visualize the anterior wall of the lens and the pupillary membrane more clearly (Fig. 3f). KDR mRNA was not detected in the lens but was detected around it, in the hyaloid system—that is, in the posterior and lateral TVL (Fig. 4d). KDR was also present in the pupillary membrane (Figs. 4e, 4f), whereas VEGF transcripts were detected in the wall of the lens. Finally, we detected KDR mRNA in the corneal stroma, just below the corneal epithelium (Fig. 4c). The specificity of the VEGF and KDR mRNAs detection was confirmed by use of sense probes (Figs. 5g, 4g, respectively), which did not reveal any specific hybridization signal of VEGF or KDR mRNAs.

**FIGURE 3.** VEGF expression in the developing eye at 10 weeks of gestation. Expression was assayed by in situ hybridization with a 35S-labeled probe. (a) VEGF transcripts were detected in the inner neuroblastic layer of the retina and in the primary lens fiber. (b) Confirmation that VEGF was present in the inner neuroblastic layer but not in the outer neuroblastic layer. (c) Bright-field image at high magnification makes it possible to distinguish the silver grains (arrowheads) from the melanosomes in the retinal pigment epithelium. (d) Confirms the presence of VEGF mRNA in the primary lens fiber and shows its absence in the TVL (white arrows). (e) Specific labeling of VEGF transcripts in the epithelial cells of the lens (arrowheads) and the absence of labeling in the cornea. (f) High-magnification image of (e) showing the labeling in the epithelial cells of the lens (arrowhead) in front of the pupillary membrane (white arrow). (g) Use of a sense probe did not reveal any specific signals for VEGF. C, cornea; Chr, choroid; EL, epithelial cells of the lens; INbL, inner neuroblastic layer; LE, lens; ONbL, outer neuroblastic layer; PLF, primary lens fiber; PM., pupillary membrane.

**FIGURE 4.** KDR expression in the developing eye at 10 weeks of gestation. Expression was assayed by in situ hybridization with a 35S-labeled probe. (a) Global view of the eye at this stage, showing specific labeling of KDR in the TVL (arrowhead). (b) No KDR transcripts were visible in the neuroretina, but they were still present in the choroid (Chr). (c) High-magnification image confirming the heavy labeling of the choroid (arrowheads). (d) Confirms the presence of KDR in the TVL (arrowheads). (e) Specific labeling of KDR in the pupillary membrane and in the corneal stroma (arrowheads). (f) High-magnification image of (e) showing labeling in the pupillary membrane (arrowhead) in front of the epithelial cells of the lens (white arrow). (g) Use of a sense probe did not reveal any KDR mRNA hybridization signal. C, cornea; EL, epithelial cells of the lens; INbL, inner neuroblastic layer; LE, lens; ONbL, outer neuroblastic layer; PLF, primary lens fiber; PM., pupillary membrane; RPE, retinal pigment epithelium; TVL, tunica vasculosa lentis.
mRNA is produced from RPE cells and VEGF protein is secreted in the choroid. In parallel, we can also observe a specific but faint labeling of KDR protein in the choroid in contrast to control sections (Figs. 7e, 7f and 7g, 7h, respectively). This immunohistochemical experiment also confirms that the VEGF and KDR proteins are present in the ganglion cell layer (Figs. 7a–h). This pattern was consistent with the localization of well-formed endothelial cells in the choriocapillaris and of the endothelial cells of the developing primary retinal plexus in the ganglion cell layers, as shown by the specific labeling observed with a CD-31 antibody (Figs. 7i, 7j), compared with a control section (Figs. 7k, 7l).

**DISCUSSION**

A large number of studies have focused on the development of the retinal vasculature—in particular, in the context of eye diseases. Although animal studies have provided some insights into the development of the retinal vasculature in many species, we do not fully understand the normal development of the human retinal vasculature. The importance of studying the human retina is highlighted by comparative data showing that although retinal vascularization in humans resembles that observed in other mammals, there are significant differences. The developing retinal vasculature of premature infants is extremely vulnerable, and perturbations of the normal developmental processes can result in retinopathy of prematurity (ROP), which is still a major cause of infantile blindness in the world. Persistent hyperplastic primary vitreous (PHPV) is another human developmental eye disease that results from the failure of the embryonic primary vitreous and hyaloid vasculature to regress. Without treatment, PHPV can result in recurrent intraocular hemorrhage, secondary glaucoma, and eventually enucleation. A better understanding of how the expression of particular genes relates spatially to the whole vascular network may provide further insights into disease processes. The molecular mechanism underlying ROP was elucidated and vascular endothelial growth factor (VEGF) was shown to be the major protagonist.

**FIGURE 5.** The distribution of VEGF expression transcripts in the developing eye at 18 weeks of gestation. Expression was assayed by in situ hybridization with a ^35^S-labeled probe. (a) VEGF transcripts were detected in the inner nuclear layer (INL) and in the differentiating ganglion cell layer (Gg). (b) Use of a sense probe did not reveal any specific signal for VEGF. (c) VEGF transcripts in the RPE were not detected by any of the antisense probes used. (d) No difference was observed with the control sense probe. Chr, choroid; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

**FIGURE 6.** KDR expression in the developing eye at 18 weeks of gestation. Expression was assayed by in situ hybridization with a ^35^S-labeled probe. (a) KDR transcripts were detected in the inner nuclear layer (INL) and in the differentiating ganglion cell layer (Gg). (b) Use of a sense probe did not reveal any specific signals for KDR. (c) Bright-field view at a high magnification shows no significant amount of silver grains, both in the choroid (Chr) and the RPE. (d) Confirms the absence of any significant hybridization signal detected by a sense probe. ONL, outer nuclear layer; RPE, retinal pigment epithelium.

**FIGURE 7.** (a, b) VEGF, (c, d) KDR, and (e, f) CD-31 protein expression in the developing eye at 18 weeks of gestation. Proteins were detected by immunohistochemistry and counterstained with 3% hematoxylin. VEGF and KDR were both detected in the choriocapillaris and in the ganglion cell layer (a and e, respectively). This pattern is consistent with the localization of endothelial cells selectively labeled with CD-31 antibody (e). The retinas from control sections for VEGF, KDR, and CD-31 show no background (b, d, and f, respectively). Chr, choroid; Gg, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.
larly interested in the distribution of VEGF and of one of its receptors, KDR, the activity of which is fundamental to the biological effects mediated by VEGF. Thus, we studied the distribution of VEGF and KDR mRNA on adjacent retinal sections during the development of the different vascular systems of the eye, and in other nonvascular structures.

Our results clearly demonstrate that VEGF and KDR transcripts are temporally and spatially correlated with the normal development of ocular vasculature in humans.

Before the definitive retinal vascular system is established, a temporary one, the HVS, develops. The HVS then gives rise to the TVL, composed by the posterior, lateral, and anterior TVL. The anterior TVL is also called the pupillary membrane.\(^5\) The HVS nourishes the developing lens, the retina, and the primary vitreous.\(^6\) This vascular system starts to develop from the head of the optic disc in the fifth week of gestation (5th WG) and begins to regress during the fourth month. This regression coincides with the development of the retinal vascular system.

We detected KDR transcripts in cells adjacent to the lens structure and also on the vitreous side of the retina, which is consistent with the localization of the TVL. In parallel, we detected VEGF transcripts within the lens. This pattern is consistent with a study performed in developing mice.\(^3\) These results suggest that in humans the VEGF secreted by the lens is the main factor inducing vasculogenesis. It may act by stimulating the proliferation and migration of angioblasts, which are at the origin of the future TVL, through KDR.

Other investigators have studied the role of VEGF in the regression of the HVS. In the mice and humans, the HVS regresses due to the apoptosis of endothelial cells and pericytes.\(^5\) In humans, after the 6th WG, the cells of the posterior wall of the lens vesicle, which is completely surrounded by the TVL,\(^7\) start to become elongated and narrow and completely separate the lens vesicle from the primary lens fibers, and a thick lenticular capsule is formed.\(^7\) Thus, some researchers have proposed that the endothelial cells of the TVL that express KDR are progressively separated from the ligand (VEGF), leading to the loss of the critical juxtacrine survival signal and apoptosis. However, in humans, the differentiation into primary lens fiber cells occurs during the 6th WG, and the HSV begins to regress during the fourth gestational month. Thus, it seems unlikely that the induction of apoptosis is linked to the physical separation of KDR and its ligand, because this takes place several weeks before the HVS regresses. Moreover, this hypothesis is nonviable for the anterior TVL—that is the pupillary membrane, because we clearly demonstrated the spatiotemporal complementarity between the distribution of KDR in the endothelial cells of the pupillary membrane and that of its ligand (VEGF) in the epithelial cells of the anterior lens, which are definitively formed. Thus, our data suggest that the apoptotic regression of the membrane cannot be induced by the distance between VEGF sources and KDR cellular sites. However, given the crucial role of VEGF in survival,\(^3\) we cannot exclude the possibility that unknown regulators downregulate the production of VEGF. This decreased secretion of VEGF may promote HVS regression and prevent PHPV. This hypothesis is supported by very recent results showing that the different VEGF isoforms play different roles in retinal vascularization in mice and highlighting the role of the VEGF164 isoform in the regression of the hyaloid vessels.\(^8\)

Our results concerning the development of the choroidal vasculature indicate that during the two first developmental stages studied (i.e., 7th and 10th WG), VEGF mRNA was present within retinal pigment epithelial (RPE) cells. At the same time, KDR was found in a subset of pericellular mesenchymal cells adjacent to the RPE that were probably endothelial cells originating from the developing choroidal vasculature. Our results are consistent with results obtained in rodents\(^25\) and with the hypothesis that the VEGF secreted by the RPE could play a paracrine role in the development of the choriocapillaris through KDR. Indeed, high levels of both VEGF and KDR were found at the stage at which the choriocapillaris develops (i.e., between the 6th WG and the 4th month). In the 18th WG we were unable to detect VEGF or KDR mRNA in the RPE or choriocapillaris, although we did detect the VEGF and KDR proteins. These results confirm the decrease in VEGF mRNA levels already observed in rodents species,\(^24,25\) but the total absence of mRNA may be due to differences in the sensitivity of the immunohistochemistry and in situ hybridization methods used to examine VEGF and KDR protein and mRNA levels, respectively. Alternatively, both VEGF and KDR gene expression might genuinely decrease at this particular developmental stage of the retina, associated with the increased stability or decreased degradation of VEGF and KDR transcripts, thus leading to the persistence of a significant rate of synthesis of the corresponding proteins.

Finally, it is noteworthy that VEGF and KDR were expressed in the same layers of the retina at the 18th WG. At this developmental stage, and since the fourth month, the retinal vascular system is forming. Thus, these data are consistent with those previously described,\(^39,40\) according to which, at this time, expressions of VEGF and KDR in retina are linked to the development of the retinal vasculature.

These data are particularly interesting, as many studies have shown that there is a link between VEGF expression, ischemic ocular conditions, and ocular neovascularization, both during development\(^41\) and in disease states.\(^42\) Thus, a better understanding of the developmental mechanisms controlled by the different VEGF isoforms would help to elucidate the mechanisms responsible for many ocular diseases.

The distribution of VEGF and KDR in nonvascular structures raises several other intriguing issues. Indeed, we observed VEGF and KDR transcripts in the human neuroretina in the 7th WG, when the retina is still an avascular tissue. At this stage, the sensory retina differentiates and forms an outer and an inner neuroblastic layer. We observed the same KDR and VEGF distributions in the two neuroblastic layers of the retina in the 7th WG. These data are surprising, as VEGF is generally thought of as being a vascular endothelial cell-specific mitogen and as VEGF receptors are considered to be endothelial cell specific. Moreover, other groups failed to detect VEGF mRNA in the human neuroretina before the 20th WG.\(^35\) However, our data are consistent with those obtained in mice.\(^43,44\) The identical cellular neuroretinal distribution of VEGF and KDR mRNAs at this stage could be consistent with their being expressed in Müller cells that span the entire retina. However, many other cells (e.g., nonendothelial neuroretinal cells) could also contain VEGF and/or KDR transcripts. In the 10th WG, we detected no KDR in the neuroretina. However, VEGF was still present, with a specific distribution in the internal layer and a particularly strong signal in the ganglion cell layer, which subsequently migrates to form an isolated layer. The absence of KDR in the neuroretina at this stage, during which the neuroretina continues to mature, can be explained by the completion of the differentiation of the VEGF-dependent cells, leading to the downregulation of this receptor in the mature cells.

The presence of KDR in the avascular retina suggests that VEGF acts as a neurogenic factor for progenitors and newly postmitotic cells, before acting as an angiogenic chemotactic-factor–promoting blood vessel network formation.\(^41\) In summary, VEGF and its receptor are critical for normal retinal development, coordinating neural and vascular development.

Another surprising result was the presence of KDR in the corneal stroma. However, these results are consistent with those described in the developing mouse.\(^45\) To allow good
vision, the cornea has to be transparent—that is, a nonvascular tissue—and it is therefore difficult to link the presence of KDR receptors in the cornea with the possible formation of physiological vessels. One explanation, if we do not link the presence of the KDR in the cornea with the formation of blood vessels, is provided by some observations of the destiny of embryonic stem cells expressing Flk-1 during mouse development. Indeed, this study shows that VEGF is required for the maintenance of Flk-1 expression and for differentiation into endothelial cells. In the absence of VEGF, Flk-1 is not expressed, and cells proliferate and differentiate into mural cells (pericytes and vascular smooth muscle) through mainly platelet-derived growth-factor BB (PDGF-BB) signaling. However, the presence of other growth factors, such as basic fibroblast growth factor, causes cells expressing Flk-1 to differentiate into cells that are neither endothelial nor mural cells. Thus, cells expressing Flk-1 can also differentiate into nonvascular lineages, which could explain the presence of KDR in corneal stroma cells.

VEGF and KDR are good examples of a receptor and its ligand that participate in multiple, distinct biological processes during development. To date, KDR has been observed in two progenitor cell pools during human development: initially in the common progenitors of the hematopoietic and endothelial lineages and subsequently in a neural progenitor pool that gives rise to neurons and glia. However, other roles of KDR cells in embryogenesis should be explored and may provide insights into additional capabilities of KDR cells for engineering nonvascular tissue.

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