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VEGF₁₆₅b in the developing vasculatures of the fetal human eye

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Abstract

VEGF₁₆₅b is an anti-angiogenic form of VEGF₁₆₅ produced by alternative splicing. The localization of pro-angiogenic VEGF₁₆₅ and anti-angiogenic VEGF₁₆₅b was investigated during development of the vasculatures in fetal human eyes from 7 to 21 weeks gestation (WG). The fetal vasculature of vitreous, which includes tunica vasculosa lentis (TVL), had moderate VEGF₁₆₅ immunoreactivity at 7WG and very little VEGF₁₆₅b. Both forms were elevated at 12WG. VEGF₁₆₅ then decreased around 17WG when the TVL regresses but VEGF₁₆₅b remained elevated. In choroid, VEGF₁₆₅ was present in forming choriocapillaris (CC) and retinal pigment epithelium (RPE) at 7WG while VEGF₁₆₅b was present in CC and mesenchymal precursors within the choroidal stroma. By 21WG, both forms were elevated in RPE and choroidal blood vessels but VEGF₁₆₅b was apical and VEGF₁₆₅ basal in RPE. Diffuse VEGF₁₆₅ immunoreactivity was prominent in 12WG innermost retina where blood vessels will form while VEGF₁₆₅b was present in most CXCR4⁺ progenitors in the inner neuroblastic layer and migrating angioblasts in the putative nerve fiber layer. By 21WG, VEGF₁₆₅ was present in nerve fibers and VEGF₁₆₅b in inner Muller cell process. The localization of VEGF₁₆₅b was distinctly different from VEGF₁₆₅ both spatially and temporally and it was often associated with nucleus in progenitors.

Keywords

angioblasts; choroid; endothelial cells; pericytes; retina; vasculogenesis; vascular endothelial growth factor

Introduction

The fetal human eye has several unique vascular systems that form during different stages of development. The three major systems are the hyaloid or fetal vasculature in vitreous, the choroidal vasculature, and the retinal vasculature. During embryonic and early fetal development, the fetal vasculature in vitreous supplies the lens and avascular inner retina with oxygen and nutrients from within the vitreous cavity while the choroidal vasculature supplies the outer retina from the scleral aspect of the eye.

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The fetal vasculature of vitreous (FVV) begins forming around 5WG, and consists of the hyaloid artery, which enters from the optic fissure and extends into the primitive vitreous. Its capillaries branch freely to form the vaso hyaloidea propria (VHP) in preretinal vitreous and the tunica vasculosa lentis (TVL) adjacent to lens. After the lens has developed, this vascular system begins to regress around 13WG (Zhu et al., 2000). This regression coincides with the initiation of retinal vascularization (Zhu et al., 2000).

The choroidal vasculature begins to develop around 5-6WG by a process called hemovascuogenesis, the formation of hematopoietic and erythropoietic cells from a common precursor, the hemangioblast (Hasegawa et al., 2007). The primitive choroidal vasculature forms initially in the posterior region and extends anteriorly with continued development. As it matures, three layers of vessels are established by angiogenic and anastomotic processes (Lutty et al., 2010). Fenestrations, which allow transport of small molecules and solutes between choriocapillaris and RPE, begin to develop around 16WG (Baba et al., 2009).

Primitive retinal vessel development begins around the optic nerve head around 14-16 WG when the FVV starts to regress. The superficial or primary retinal vascular system forms first by a process of vasculogenesis (McLeod et al., 2006; Hasegawa et al., 2008) followed by the development of the deep capillary network by sprouting from pre-existing vessels or angiogenesis. The factors involved in retinal vascular development have been intensely investigated. Vascular endothelial growth factor (VEGF) is one of the major pro-angiogenic factors and its importance during vascular development has been well documented (Ferrara et al., 2003). VEGF production by astrocytes and Muller cells is regulated by hypoxia-induced factor-1 (HIF-1), which is elevated in response to hypoxia. VEGF levels are elevated in avascular retina due to physiological hypoxia (Chan-Ling et al., 1995).

There is considerable evidence that VEGF plays an important role in normal and pathologic vascular development. Recently, it has been reported that VEGF-A has two isoforms VEGF_{xxx} and VEGF_{xxxb} in addition to the known isoforms with varying heparin binding affinities and molecular sizes: VEGF₁₂₁, 145, 165, 189, 206 (Bates et al., 2002; Lodomery et al., 2007; Qiu et al., 2009). These isoforms are alternatively spliced from one VEGF-A gene. The VEGF gene consists of eight exons and exon 8a can be alternatively spliced generating the VEGF_{xxxb} family. The number of exons (exon 1 to 7b) spliced results in the amino acid number xxx of the VEGF isoforms. VEGF_{121b}, VEGF_{165b}, and VEGF_{189b} have been reported so far. These VEGF_{xxxb} family members are anti-angiogenic and the splicing is stimulated by TGF beta through splicing factor SRp55 (serine/ arginine protein 55) (Nowak et al., 2008). On the contrary, VEGF_{xxx} is pro-angiogenic and splicing to form it is upregulated by IGF1 and TNF alpha through ASF/SF2 (alternative splicing factor/ splicing factor 2) (Nowak et al., 2008; Nowak et al., 2010).

Previous immunohistochemical studies involving VEGF localization in the developing eye may have employed antibodies that recognize both 165 isoforms (David Bates, personal communication). Because VEGF_{xxx} and VEGF_{xxxb} have opposite effects on blood vessels, this study was initiated to demonstrate localization of VEGF_{xxx} versus VEGF_{xxxb} and perhaps suggest the role of both VEGF forms in human ocular vascular development. In this study, we used an antibody specific for the VEGF_{165b} to examine the spatial and temporal

localization of this anti-angiogenic isoform of VEGF in the developing fetal human eye. We compared the pattern of VEGF_{165b} staining with VEGF₁₆₅, which is known to be dominant in most angiogenic states (Bates et al., 2002). Our data show a molecular shift from VEGF₁₆₅ to VEGF_{165b} in the developing ocular vessels and provides insights regarding expansion and regression of blood vessels during vascular development.

Results

Fetal Vasculature of Vitreous (FVV)

The FVV and TVL were observed in eyes from 7-17WG in this study. It was difficult to see the regressing FVV at 21WG because the size of the eyes was relatively large and the posterior eyecup had to be cut into pieces. Histologically, highly cellular blood vessels with poorly defined lumens were observed at 7WG (Figure 1). The endothelial cell marker CD31 was expressed in 7WG FVV endothelium and labeling was more intense at 10-17WG (Figure 1) but von Willebrand factor (vWf) was not prominent until 12WG (Figure 2). Meanwhile, alpha smooth muscle actin (SMA), which is a marker for pericytes and smooth muscle cells in capillaries and arteries respectively, was positive at 10WG, suggesting mural cell differentiation in hyaloid vessels at that time. Alpha SMA antibody clearly labeled actin filaments in pericytes covering the entire vasculature at 17WG implying pericyte maturation (data not shown). VEGF_{165b} was present in both endothelial cells and pericytes (Figure 2). VEGF₁₆₅ was present diffusely in blood vessels at 7WG, increased in staining until 12WG, and decreased by 17WG when FVV was undergoing regression (Figure 1). Conversely, VEGF_{165b} was barely detectable at 7WG, increased steadily thereafter and was associated with nuclei of endothelial cells and pericytes (Figure 1). Double labeling with VEGF₁₆₅ and VEGF_{165b} antibodies clearly showed different localization of these two VEGF₁₆₅ forms in the fetal vasculature and that both endothelial cells and pericytes had both forms (Figure 3). While VEGF₁₆₅ levels were decreased in the fetal vasculature at 17WG, VEGF_{165b} remained elevated (Figure 1). The apparent nuclear localization of the VEGF_{165b} in pericytes and endothelial cells was quite striking at 12-17 WG (Figures 1-3).

Choroidal vasculature

At 7WG, a single layer of choroidal blood vessels labeled with anti CD31 antibody was observed at the level of the choriocapillaris (CC). VEGF₁₆₅ staining was present diffusely around the CC at this age and there was no detectable VEGF_{165b} present (data not shown). VEGF₁₆₅ was also observed around CC at 10WG. On the other hand, VEGF_{165b} was barely detectable around CC and weakly positive in mesenchymal progenitors in choroid (Figure 4).

At 12WG, a second layer of choroidal vessels had formed extending from the CC to choroidal stroma (Figure 4). VEGF₁₆₅ labeling increased around CC but weak staining was associated with the deeper choroidal vessels. VEGF₁₆₅ was also associated with the basal portion of the RPE. VEGF_{165b} was weakly positive in the CC and mesenchymal cells in choroid at 12WG (Figure 4 and 5).

At 17WG, the deeper layer of choroidal vessels were more mature (Hasegawa et al., 2007) and showed prominent CD31 immunoreactivity (Figure 4). The choroidal vessels and CC had more intense VEGF₁₆₅ surrounding them as did the basal portion of the RPE. VEGF_{165b} labeling was seen in the CC but deeper vessels were unstained.

At 21WG, a third layer of choroidal vasculature was present in presumed submacular choroid (Figure 4) and both VEGF₁₆₅ and VEGF_{165b} were prominent in and around all choroidal vessels. Immunofluorescence showed double labeling of all choroidal vessels with both VEGF₁₆₅ splice variants (Figure 5). Double labeling with alpha SMA and VEGF_{165b} showed choroidal arterioles and arteries had VEGF_{165b} expression as well as choroidal venules/ CC (data not shown). In arteries, the localization of VEGF_{165b} appeared to be nuclear in endothelial cells and smooth muscle cells as well. At this age, there was a distinctly different localization of these two VEGF isoforms in RPE cells. VEGF₁₆₅ was localized at the basal portion of the RPE, while VEGF_{165b} was in RPE nuclei and the apical portion of the cells (Figure 5).

Retinal vasculature

VEGF₁₆₅ was diffusely distributed in innermost retina at 7WG and VEGF_{165b} weakly labeled progenitor cells in the inner neuroblastic layer (data not shown). At 10WG, VEGF_{165b} positive cells were observed migrating inwardly from the inner neuroblastic layer (INL) to the putative nerve fiber layer (NFL). While VEGF_{165b} staining was mainly cell-associated, VEGF₁₆₅ immunoreactivity was diffuse throughout the inner retina. This was similar to retina at 12WG (results not shown).

At 12WG, regional staining patterns showed the diffuse distribution of VEGF₁₆₅ in NFL and INL and VEGF_{165b} was associated with inwardly migrating cells from the progenitor pool in the INL, similar to the localization at 7 and 10WG (Figure 6). Migrating cells were most often observed in the peripapillary or posterior pole of retina where blood vessels develop first. Double-labeling and confocal microscopy showed diffuse VEGF₁₆₅ labeling in NFL and INL and cell-nuclei associated VEGF_{165b} in migrating cells within the NFL and progenitors in the INL (Fig. 7). In mid peripheral retina where the NFL is thinner and the INL is thicker, the localization pattern of VEGF₁₆₅ was similar to that observed in the posterior pole (Figure 6). In midperiphery, VEGF_{165b} was present in migrating progenitors in the nerve fiber layer, which we previously identified as angioblasts (McLeod et al., 2006; Hasegawa et al., 2008), but was diffuse throughout the inner neuroblastic layer (Figure 6-7).

At 17WG, VEGF₁₆₅ immunoreactivity was diffuse in the nerve fiber and putative ganglion cell layer (GCL). Nerve fibers were prominently labeled in midperipheral retina as well. Cell-associated VEGF_{165b} was in ganglion cells in avascular retina (Figure 8H). VEGF_{165b} was weak around developing vasculature in peripapillary retina (Figure 8G), but many cells were positive in the NFL in the periphery (Figure 8I).

VEGF₁₆₅ immunoreactivity at 21WG was weaker in the nerve fiber layer and GCL and it had decreased as vascularization progressed in inner retina. Cell-associated VEGF_{165b} staining was intense in GCL throughout retina (Figure 9). Double-labeled 21WG mid peripheral retina showed VEGF₁₆₅ labeling of NFL and a few cells in GCL. VEGF_{165b} was

more prominent in the GCL than in NFL. Double labeling using endothelial cell markers (vWf or CD31) and VEGF₁₆₅ splice variants revealed that the developing retinal vessels were associated with VEGF₁₆₅ and weakly with VEGF_{165b} (data not shown).

Retinal progenitor cell markers and VEGF_{165b}

The association of VEGF_{165b} with progenitors was striking in retina. VEGF_{165b}⁺ / CXCR4⁺ progenitors migrating from the neuroblastic layer into the inner retina represented two distinct populations at 10 WG. Some cells were CD39⁺ angioblasts, and others were ganglion cell precursors expressing Brn-3B in their nuclei. Double labeling using CD39 and CXCR4 antibodies showed that some of the migrating (VEGF_{165b}⁺) progenitors were angioblasts (CD39⁺ / CXCR4⁺) (Figure 10). Also of note was the apparent nuclear localization in retinal progenitors.

By 21WG, VEGF_{165b}⁺ / CXCR4⁺ progenitors have migrated from the neuroblastic layer into the formed GCL and NFL. In regions where vascularization had occurred, CD39⁺ blood vessels, CD39⁺ angioblasts and Brn-3B⁺ ganglion cells were evident. Some undifferentiated CXCR4⁺ progenitors (CD39⁻ / Brn-3B⁻) still existed in these regions (Figure 11).

Quantitative real time PCR (qRT-PCR) of VEGF splice variants in the fetal vasculature of vitreous

qRT-PCR was performed to assess VEGF_{165b} and VEGF₁₆₅ expression in 8WG and 11WG fetal vasculature. VEGF_{165b} RNA was increased 32 fold in the 11WG fetal vasculature compared to that detected in the 8WG fetal vasculature. The fold change was calculated using the C_t method after normalizing to β -actin. VEGF₁₆₅ RNA levels, however, were comparable at 8 and 11WG in the fetal vasculature.

Discussion

The localization of pro-angiogenic VEGF₁₆₅ and anti-angiogenic VEGF_{165b} was investigated in the developing fetal human eye. VEGF₁₆₅ in the fetal vasculature of vitreous was expressed early during embryonic developmental and declined after 12 WG while VEGF_{165b} expression appeared after blood vessels had formed and increased with gestational age when this vasculature is regressing. In choroid, VEGF₁₆₅ was expressed early and throughout development while VEGF_{165b} expression appeared after blood vessels formed and it steadily increased with age. In RPE, VEGF₁₆₅ was expressed in the basal portion of the RPE throughout development while VEGF_{165b} expression in the nuclei and apical RPE was prominent at 21WG. In retina, VEGF₁₆₅ was diffusively expressed in inner retina and was most prominent in advance of forming blood vessels. VEGF_{165b} was expressed in nuclei or possibly the nuclear envelope of cells in the INL progenitor pool (CXCR4⁺) and cells migrating into inner retina from the pool, which were predominantly CD39⁺/CXCR4⁺ vascular progenitors or angioblasts (Lutty et al., 1993a; McLeod et al., 2006; Hasegawa et al., 2008). In terms of expression, there appeared to be a distinct balance between VEGF₁₆₅ and VEGF_{165b} during vascular development. Developing vessels had more VEGF₁₆₅ associated with them and little or none of the VEGF_{165b} isoform. When vessels had formed and were becoming mature, the intensity of VEGF₁₆₅ immunoreactivity

declined and VEGF_{165b} was upregulated suggesting that VEGF_{165b} is involved in maintaining the established vascular system and possibly preventing further angiogenesis. This was particularly evident in the transient fetal vasculature of the vitreous where the trend was confirmed with qRT-PCR.

VEGF₁₆₅ and VEGF_{165b} are generated by alternative splicing of the same gene encoding VEGF-A. This splicing system in RPE is known to be regulated by growth factors (Nowak et al., 2008; Nowak et al., 2010). TGF-beta activates p38/MAPK and subsequent Clk/sy kinases. Clk phosphorylates SR protein splicing factors such as SRp55 which is a key regulatory splice factor binding to VEGF pre-RNA in the exon 8b region. SRp55 splices at DSS (distal splice-site selection) and produces VEGF_{165b}. On the other hand, IGF (Insulin-like growth factor 1) activates PKC (protein kinase C) and phosphorylate ASF/SF2 (alternative splicing factor/ splicing factor 2) through the SR protein kinase SRPK1 (serine/ arginine domain protein kinase 1). ASF/SF2 can bind to VEGF pre-RNA around PSS (proximal splice-site selection) and results in VEGF₁₆₅ expression (Nowak et al., 2008; Nowak et al., 2010). IGF-1 has reported to control VEGF expression via HIF1 alpha (hypoxia induced factor-1 alpha) dependent and independent pathways (Slomiany and Rosenzweig, 2006). Vasculatures develop in physiologically hypoxic tissue where HIF 1 alpha is abundant (Ozaki et al., 1999). Collectively, the growing vessels need VEGF₁₆₅ and once vessels have developed and oxygen levels are increased, the switch in splicing might occur in response to reduced HIF-1 alpha/IGF-1 and result in increased VEGF_{165b} expression. TGF-β is produced by pericytes and smooth muscle cells in mature blood vessels to maintain endothelial cells in a quiescent state (Orlidge and D'Amore, 1987; Walshe et al., 2009). The fact that differentiated but not dedifferentiated podocytes in human glomerulae secrete significant amounts of VEGF_{165b} protein could underpin this idea (Cui et al., 2004) since podocytes are similar to pericytes in their relationship with endothelial cells.

The down-regulation of VEGF_{165b} has been reported in angiogenic pathologies such as tumor (Bates et al., 2002; Woolard et al., 2004; Pritchard-Jones et al., 2007; Varey et al., 2008), diabetic retinopathy (Perrin et al., 2005) and experimental oxygen-induced retinopathy (Konopatskaya et al., 2006). Those studies showed increased VEGF₁₆₅ expression and less VEGF_{165b} expression compared to normal conditions where VEGF_{165b} is the dominant splice variant. For example, 64% of total VEGF in the vitreous was VEGF_{xxx}b (this includes VEGF_{165b}, VEGF_{121b}, VEGF_{145b}, VEGF_{183b}, VEGF_{189b}) in non-diabetic patients, whereas only 12.5% of total VEGF was VEGF_{xxx}b in diabetic patients (Perrin et al., 2005). That study showed the switch in splice variants from anti-angiogenic to pro-angiogenic in diabetic retinopathy and suggested that VEGF_{xxx}b might be a potential therapeutic modality for diabetic retinopathy. Hua et al demonstrated that intravitreal administration of VEGF_{165b} inhibited laser-induced choroidal neovascularization in the mouse eye (Hua et al., 2010). Another study using an oxygen-induced retinopathy model as an experimental retinal neovascularization model has shown more direct evidence of anti-angiogenic effect of VEGF_{165b} (Konopatskaya et al., 2006). In that model, intraocularly administered VEGF_{165b} peptide significantly inhibited the area of retinal neovascularization from 23% of retina to 12% and, interestingly, increased normal vascular area from 62% to 74%. In our study, the developing vasculature in its formative state expressed more VEGF₁₆₅ and the developed vasculature in stable or anti-angiogenic stage

had prominent VEGF_{165b}. This data is coincident with previous reports about decreased VEGF_{165b} in angiogenic status in pathologic condition.

Both VEGF₁₆₅ and VEGF_{165b} were often associated with endothelial cells in developing blood vessels. In retina, there is substantial VEGF₁₆₅ diffusely throughout inner retina, including in blood vessels at the older time points. It is not clear from immunohistochemistry, which cells produce it and whether it is bound or diffusable VEGF₁₆₅. However, in the fetal vasculature of vitreous, VEGF₁₆₅ and VEGF_{165b} are clearly associated with endothelial cells as well as pericytes (Fig. 2-3) and there is only one non-vascular cell type in close proximity to some vessels, the hyalocyte or dendritic cell of vitreous. So it is possible that the VEGF₁₆₅ and VEGF_{165b} observed in this vasculature were autocrine in nature, made and used by endothelial cells of the fetal vasculature, or paracrine, produced by pericytes and targeting adjacent endothelial cells. There is precedent for autocrine production of VEGF₁₆₅ by endothelial cells in vitro (Yamagishi et al., 1999; Bocci et al., 2001) and for pericytes inducing endothelial cell production in vessel fragments (Franco et al., 2011). However, there have been no localization studies of VEGF_{165b} during development so autocrine versus paracrine production of this splice variant is still not known.

VEGF₁₆₅ in our study was present in the basal portion of the RPE throughout fetal development. Conversely, VEGF_{165b} was not detected in RPE at early ages but was prominent in the apical portion of RPE and their nuclei after the choriocapillaris had formed. Choriocapillaris homeostasis is dependent on VEGF secreted from the basal RPE that development and maintenance of fenestrations in the CC relies on the availability of RPE-derived VEGF (Marneros et al., 2005; Saint-Geniez et al., 2009). Our findings of VEGF₁₆₅ in the basal portion of the RPE may function to stimulate fenestration formation during development. We have observed contiguous fenestrations in CC at 21WG (Baba et al., 2009). Meanwhile, VEGF_{165b} immunoreactivity in the apical RPE might be important in preventing the choroidal vasculature from invading the subretinal space. Surprisingly, both forms of VEGF₁₆₅ were seen in 21WG RPE in the same cells. It may suggest the polarity-dependent expression of VEGF splice variants in RPE cells, namely VEGF_{165b} in apical and VEGF₁₆₅ in basal RPE. However, how two splicing systems can function simultaneously in the same cell is not known. In adult human RPE cells, using the same APase technique used in the current study, we found VEGF₁₆₅ most prominently in white blood cells in choroid and did not observe it in RPE cells (Bhutto et al., 2006), although Saint-Geniez and associates found VEGF₁₆₅ mRNA in adult human RPE (Saint-Geniez et al., 2006). VEGF_{165b} also was not associated with RPE in the adult human by APase immunohistochemistry, but was associated with choroidal blood vessels (unpublished data).

The localization of VEGF_{165b} and not VEGF₁₆₅ to nucleus is also unknown. The alternative splicing does not generate or expose a nuclear translocation sequence (unpublished data). VEGFR-1 is translocated to nucleus in some circumstances (Cai et al., 2006), but both splice variants bind to VEGFR-1 (Bates et al., 2003). It is also not known why the nuclear localization of VEGF_{165b} is so prominent in progenitor cells in retina while it had nuclear localization in fully differentiated endothelial cells and pericytes in established blood vessels in vitreous. VEGF_{165b} from pericytes may serve to stabilize these developed vessels. Recent

studies have demonstrated the neuroprotectant potential of VEGF_{165b} (Hua et al, submitted) and VEGF₁₆₅ is a neuroprotectant as well (Saint-Geniez et al., 2008). Perhaps the presence of diffuse VEGF₁₆₅ in the inner neuroblastic layer and nuclear VEGF_{165b} in neuronal progenitors in that layer are necessary for survival of neurons during the remodeling stage in retinal development.

In conclusion, this study demonstrates for the first time the localization of VEGF_{165b} spatially and temporally in developing vasculatures of the fetal human eye. VEGF_{165b} and VEGF₁₆₅ often had distinctly different localizations from each other in the tissues studied: fetal vasculature of vitreous, retina, and choroid. In retina, it appeared that VEGF₁₆₅ was both extracellular (diffuse) and cell associated in both the confocal (Fig. 7) and APase analysis (Fig. 8-9), while VEGF_{165b} seemed mostly cell associated. We focused on anti-angiogenic form of VEGF, VEGF_{165b}, and found a distinct change in expression during vasculogenesis and hemo-vasculogenesis. VEGF₁₆₅ was prominently expressed during vascular development and VEGF_{165b} expression was most prominent in developed vasculature. This dramatic molecular shift in splicing of VEGFA from VEGF₁₆₅ to VEGF_{165b} during vascular development provides insights into a novel system for formation and maintenance of vascular systems by VEGF_{165b}.

Materials and Methods

Age determination and preparation of human fetal tissue

Fetal human eyes from 7 to 21 WG were included in this study. Tissues were provided by Advanced Bioscience Resources Inc. (Alameda, CA) and Stem Express (Placerville, CA) after aspiration abortions in accordance with the guidelines set forth in the Declaration of Helsinki and with the approval by the Joint Commission for Clinical Research at the Johns Hopkins University School of Medicine. The age of each fetus was determined using last menstruation date and/or ultrasonography and fetal foot length as a reliable indicator of gestational age (Mhaskar et al., 1989). The eyes were then fixed within an hour of harvest in 2% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.2 with 5% sucrose at room temperature for 60 min. The eyes then were washed in 0.1M sodium phosphate buffer with 5% sucrose and shipped overnight at 4°C in the same buffer. For older cryopreserved eyes (14 WG), the anterior segments were removed and eye cups were washed in 0.1M sodium phosphate buffer with increasing concentration of sucrose: a 2:1, 1:1, and 1:2 mixture of 5% sucrose: 20% sucrose (Lutty et al., 1993b). The eyecups were kept at room temperature for 2 hrs in 0.1 M sodium phosphate buffer with 20% sucrose and then dissected into four blocks, each containing an arcade of retinal blood vessels in 16 WG and older eyes. For younger eyes (12 WG), whole eyes were cryopreserved and frozen by the same method. All tissue was infiltrated at room temperature for 30 min and then embedded in a solution consisting of a 2:1 mixture of 20% sucrose in 0.1 M phosphate buffer: OCT compound (TissueTek, Baxter Scientific Columbia, MD), frozen and stored at -80°C (Lutty et al., 1993a; Hasegawa et al., 2007). Eight µm thick cross sections were cut on Microm HM500M cryostat (Global Medical Instrumentation, Ramsey, MI) at -25°C.

Hematoxylin and eosin staining

After treating cryosections in absolute methanol for 5 min at -20°C and allowing them to air dry, sections were stained with Harris' hematoxylin for 20 seconds. After washing in distilled water, the sections were blued in lithium carbonate, rinsed in distilled water, and then stained in 0.5% alcoholic eosin (Polysciences, Warrington, PA). After dehydration to xylene, the sections were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ).

Streptavidin alkaline phosphatase (APase) immunohistochemistry on cryopreserved tissue

APase immunohistochemistry was performed on sections of cryopreserved tissue using a nitroblue tetrazolium (NBT) development system that we described previously (Bhutto et al., 2004). In brief, 8-um-thick cryopreserved sections were permeabilized with absolute methanol and blocked with 2% normal goat serum in Tris-buffered saline (TBS; pH 7.4) with 1% BSA. After washing in TBS, the sections were also blocked with avidin-biotin complex (ABC) blocking kit (Vector Laboratories, Inc., Burlingame, CA). Tissues were then incubated overnight at 4°C with one of the primary antibodies listed in Table 1 at the stated titer. Mouse monoclonal IgG1 VEGF_{165b} antibody (Abcam clone MRVL56/1) has been reported previously using immunohistochemistry (IHC) (Pritchard-Jones et al., 2007). Woolard et al (2004) previously demonstrated the specificity of this antibody for VEGF_{165b} in cell lysates using Western blots and more recently Zhao et al (2011) also used Western blots of mouse retinal tissue to show that it recognizes VEGF_{165b} and not VEGF₁₆₅. After washing in TBS, sections were incubated for 30 minutes at room temperature with the biotinylated secondary antibodies diluted 1:500 (KPL, Gaithersburg, MD). Finally, sections were incubated with streptavidin-alkaline phosphatase (APase)(KPL) and APase activity developed using a BCIP-NBT kit (Vector Laboratories) with the addition of 1 mM (-) Tetramisole HCL (Sigma), yielding a blue reaction product at sites of antibody binding. Melanin in RPE was bleached as previously reported (Bhutto et al., 2004) and coverslips were applied with Kaiser's glycerogel.

Double label Immunohistochemistry on cryopreserved tissue

Sections were treated with absolute methanol to increase permeability for 5 minutes at -20°C. After washing with TBS, tissues were blocked with 2% goat or rabbit serum for 20 minutes at room temperature. Sections were then incubated with two of primary antibodies (Table 1) for 3 hours at room temperature. After washing, the sections were labeled with one of these combinations, goat anti mouse Cy3 (Jackson Immuno Research, West Grove, PA) 1:500 and goat anti rabbit Cy5 (JIR) 1:500, or rabbit anti goat Cy3 (JIR) and rabbit anti mouse Cy5 (JIR) for 30 minutes. Sections were coverslipped with Dakocytomation (Dako, Carpinteria, CA) and observed using Zeiss confocal microscope meta510 LSM (Carl Zeiss Inc., Thornwood, NY).

Laser capture of fetal vasculature, RNA preparation and qRT-PCR

Fetal human eyes (three 8WG; three 11WG) were quickly cryopreserved in an increasing gradient of sucrose in 0.1M phosphate buffered saline (prepared in DEPC-treated water) then infiltrated and frozen in a 1:2 mixture of 20% sucrose to OCT. Eight µm sections were

cut until the fetal vasculature was visible. At this point, sections were collected on 2.0 μ m PEN-Membrane slides (*Leica* 11505189) and immediately frozen. Sections were brought to room temperature in a closed chamber containing desiccant, rehydrated in ethanol gradients, stained with cresyl violet, and dehydrated in ethanol gradients (all solutions were prepared in DEPC-treated water). Air-dried slides were placed on the *Leica* LMD6000 Laser Capture Micro-dissection microscope. The fetal vasculature was captured from each section and collected in tubes containing RNAlater. The fetal vasculature captured from a minimum of 25 sections was pooled for each eye. RNA was isolated according to the manufacturer's instructions using the RNeasy Plus micro kit (*Qiagen*, Valencia, CA). Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer. So that amplification was not necessary, RNA from the three eyes at each age was pooled to perform qRT-PCR.

PCR amplification was performed utilizing Brilliant II SYBR^(R) Green QRT-PCR Master Mix Kit (*Stratagene*) according to the manufacturer's instructions. Beta-actin was run as the loading control. A two-step cycling protocol included: 30 min at 50°C, 10 min at 95°C, followed by 40 cycles of denaturation for 30 seconds at 95°C combined with annealing/extension at 55°C for 1 min. Primers were obtained from Integrated DNA Technologies (Coralville, Iowa). VEGF_{165b} was amplified with primers VEGF_{165b}F: CAAGATCCGCAGACGTGTAA and VEGF_{165b}R: TCCTGGTGAGAGATCTGCAA. VEGF₁₆₅ was amplified with primers VEGF₁₆₅F: GAGCGGAGAAAGCATTGTGTT and VEGF₁₆₅R: CTCGGCTTGTCACATCTGC. Beta-actin was amplified with primers Actinb250F: CATGTACGTTGCTATCCAGGC and Actinb250R: CTCCTTAATGTCACGCACGAT. All data were analyzed in Mx300P real time PCR system using the data assist software (*Stratagene*) and graphs were plotted using Microsoft Excel. The fold change was calculated using the C_t method after normalizing to β -actin as we have reported previously (Ma et al., 2011; Zigler et al., 2011).

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Figure 1.

Localization of VEGF₁₆₅ and VEGF_{165b} in the human fetal vasculature of vitreous at 7, 10, 12 and 17WG human eyes. Hematoxylin and eosin staining shows the highly cellular and poorly defined luminal structure of blood vessels at 7 WG which mature into well developed blood vessels by 12 WG. CD31 is expressed until vessels have fully regressed by 21 WG. VEGF₁₆₅ was present diffusely in blood vessels at 7 WG, increased in staining until 12 WG and decreased by 17 WG. Conversely, VEGF_{165b} was undetectable at 7 WG, increased steadily thereafter, and was associated with nuclei of endothelial cells (e) and pericytes (p) (insets 12 WG). (arrows= blood vessels, L = lens)(APase reaction product)

Figure 2.
Expression of VEGF₁₆₅ b (green) in endothelial cells (red, vWf) and pericytes (red, αSMA) in the FVV at 12WG. VEGF165b was associated with both cell types.

Figure 3.

VEGF₁₆₅ (red) and VEGF₁₆₅ b (green) in the human fetal vasculature of vitreous at 7 and 12 WG. At 7 WG, moderate VEGF₁₆₅ immunostaining was present diffusely in blood vessels while very little VEGF₁₆₅b staining was detected. By 12 WG, VEGF₁₆₅ immunostaining had increased in endothelial cells and pericytes and VEGF₁₆₅b labeling was strong and localized to endothelial cell and pericyte nuclei. (arrows indicate blood vessels).

Figure 4.

Localization of VEGF₁₆₅ and VEGF_{165b} in the human choroid at 10, 12, 17 and 21 weeks gestation (WG) eyes. Hematoxylin and eosin staining shows the structure of the choroid and the presence of mesenchymal precursors throughout the forming choroid (single cells at the bottom of the micrograph). CD31 is used as an endothelial cell marker, which demonstrates that only a capillary system is forming at 10WG. VEGF₁₆₅ staining was present diffusely around the capillaries at 10 WG and increased around all vessels with age. VEGF_{165b} was barely detectable at 10 WG and increased in endothelial cell and mesenchymal precursor nuclei steadily with development. (small white arrowhead = RPE, arrows = choroidal capillaries, large black arrowhead = large vessels)(APase reaction product)

Figure 5.

VEGF₁₆₅ (red) and VEGF_{165b} (green) localization in the human RPE/Bruch's membrane/choriocapillaris complex at 12 and 21WG. At 12 WG VEGF₁₆₅ immunostaining was present diffusely in the choriocapillaris (arrows) and weakly associated with the basal portion of the RPE (arrowhead). VEGF_{165b} appeared to have a nuclear association in the choriocapillaris with very little RPE staining. At 21 WG, the choriocapillaris (single arrows), larger choroidal vessels (paired arrows) and RPE all had increased expression of both VEGF₁₆₅ and VEGF_{165b}. VEGF_{165b} in RPE appeared apical and nuclear while VEGF₁₆₅ was predominantly basal

Figure 6.

VEGF₁₆₅ and VEGF_{165b} localization in different regions of a 12 WG human retina. Structure of developing inner retina at 12 WG showing the nerve fiber layer (NFL= }) and inner neuroblastic layer (INL= [) and cells migrating inwardly (arrows). Both ganglion cells and blood vessels will occupy the inner retina. Regional staining patterns show the diffuse distribution of VEGF₁₆₅ in both layers while VEGF_{165b} is cell-associated. (APase reaction product)

Figure 7.

VEGF₁₆₅ (red) and VEGF_{165b} (green) in mid peripheral and peripapillary 12WG retina. There is diffuse VEGF₁₆₅ labeling in the nerve fiber layer (NFL= }) and inner neuroblastic layer (INL= [). VEGF_{165b} is associated with migrating cells of the NFL and progenitors in the INL.(DAPI nuclear stain)

Figure 8.

Three regions in a 17 WG Retina. The inner retina at 17 WG has nerve fiber layer ({}), with blood vessels (arrowhead), a putative ganglion cell layer ({}), and an inner neuroblastic layer ([]). Regional expression patterns show the diffuse distribution of VEGF₁₆₅ in the nerve fiber and putative ganglion cell layer. Nerve fiber VEGF₁₆₅ is apparent in (E). Cell-associated VEGF_{165b} is more intense in advance of forming blood vessels and weakest in the vascularized peripapillary area. (APase reaction product)

Figure 9.

VEGF₁₆₅ and VEGF_{165b} localization in different regions of a 21 WG retina. The developing inner retina at 21 WG has a nerve fiber layer (}), blood vessels (arrowhead), a definitive ganglion cell layer (}) and inner neuroblastic layer (I). VEGF₁₆₅ immunoreactivity in the nerve fiber and ganglion cell layer has decreased as vascularization has occurred in inner retina. Cell-associated VEGF_{165b} immunoreactivity is prominent in the ganglion cell layer (}) throughout retina. (APase reaction product)

Figure 10.

Markers for angioblasts/endothelial cells (CD39, red) and ganglion cells (Brn3B, green) in a 10 WG retina. VEGF₁₆₅^b/CXCR4⁺ progenitors migrating from the neuroblastic layer into the inner retina represent two distinct populations. Some cells are CD39⁺ angioblasts, and others are ganglion cell precursors expressing Brn3. The migrating VEGF₁₆₅^b progenitors (arrows) were mostly angioblasts (CD39⁺).

Figure 11.

Angioblasts and endothelial cells ($CD39^{+}$, red) and ganglion cells ($Brn3B^{+}$) in inner retina at 21 WG. $VEGF_{165b}^{+}$ (green)/ $CXCR4^{+}$ (red) progenitors have migrated from the neuroblastic layer into the formed ganglion cell layer and nerve fiber layer. In regions where vascularization has occurred, $CD39^{+}$ blood vessels (large arrow), $CD39^{+}$ angioblasts (small arrows), and $Brn-3B^{+}$ ganglion cells (arrowheads) are evident. Some undifferentiated $CXCR4^{+}$ progenitors ($CD39^{-}/Brn-3B^{-}$) still exist in these regions.

Table 1
A summary of antibodies for immunohistochemistry

Protein	Function	Marker for	Antibody, manufacturer, titer (APase/Immunofluorescence)	Reference
VEGF ₁₆₅	Pro angiogenic factor		Rabbit anti VEGF Ab-1, Thermo, Waltham, MA, 1:800/ 1:500	(Bates et al., 2002)
VEGF _{165b}	Anti angiogenic factor		Mouse anti VEGF _{165b} , Abcam, Cambridge, MA, 1:500/ 1:400	(Bates et al., 2002)
CD31	Adhesion molecule	Hematopoietic cells and endothelial cells	Mouse anti CD31, Dako, Carpinteria, CA, 1:100/ 1:100	(Sheibani and Frazier, 1998)
vWf	Glycoprotein in plasma, endothelium, megakaryocytes	Vascular endothelial cells or megakaryocytes	Rabbit anti vWf, Dako, Carpinteria, CA, -/ 1:2500	(Sadler, 1998)
αSMA	Actin isoform	Smooth muscle cells and pericytes	Rabbit anti αSMA, Abcam, Cambridge, MA, -/ 1:400	(Herman, 1993; Tomasek et al., 2006)
CD39	Ectoenzyme controlling the extracellular ADP level	Angioblast and endothelial cells	Mouse anti CD39, Ancell, Bayport, MN, -/ 1:400	(McLeod et al., 2006)
CXCR4	Alpha chemokine receptor for SDF-1	Angioblasts, embryonic stem cells, hematopoietic progenitors	Rabbit anti CXCR4, Novus, Littleton, CO, -/ 1:250	(Kucia et al., 2004; McLeod et al., 2006)
Brn-3B	Transcription factor	Sensory neural precursors	Goat anti Brn-3B, Santa Cruz, Santa Cruz, CA, -/ 1:250	(Bain et al., 1995; Fedtsova and Turner, 1995)