Role of Vascular Endothelial Growth Factor and Placental Growth Factors During Retinal Vascular Development and Hyaloid Regression

Susan A. Feeney, David A. C. Simpson, Thomas A. Gardiner, Cliona Boyle, Pamela Jamison, and Alan W. Stitt

**PURPOSE.** Vascular endothelial growth factor (VEGF)-A and placental growth factor (PlGF) are members of a large group of homologous peptides identified as the VEGF family. Although VEGF-A is known to act as a potent angiogenic peptide in the retina, the vasoactive function of PlGF in this tissue is less well defined. This study has sought to elucidate the expression patterns and modulatory role of these growth factors during retinal vascular development and hyaloid regression in the neonatal mouse.

**METHODS.** C57BL/6J mice were killed at postnatal days (P)1, P3, P5, P7, P9, and P11. The eyes were enucleated and processed for in situ hybridization and immunocytochemistry and the retinas extracted for total protein or RNA. Separate groups of neonatal mice were also injected intraperitoneally daily from P2 through P9 with either VEGF-neutralizing antibody, PlGF-neutralizing antibody, isotype immunoglobulin (Ig)-G, or phosphate-buffered saline (PBS). The mice were then perfused with fluorescein isothiocyanate (FITC)-dextran, and the eyes were subsequently embedded in paraffin wax or flat mounted.

**RESULTS.** Quantitative (real-time) reverse transcription–polymerase chain reaction (RT-PCR) demonstrated similar expression patterns of VEGF-A and PlGF mRNA during neonatal retinal development, although the fluctuation between time periods was greater overall for VEGF-A. The localization of VEGF-A and PlGF in the retina, as revealed by in situ hybridization and immunohistochemistry, was also similar. Neutralization of VEGF-A caused a significant reduction in the hyaloid and retinal vasculature, whereas PlGF antibody treatment caused a marked persistence of the hyaloid without significantly affecting retinal vascular development.

**CONCLUSIONS.** Although having similar expression patterns in the retina, these growth factors appear to have distinct modulatory influences during normal retinal vascular development and hyaloid regression. (*Invest Ophthalmol Vis Sci.* 2003;44: 839–847) DOI:10.1167/iovs.02-0040

The hyaloid system and developing retinal vasculature of the neonatal mouse provide a useful model to investigate physiologically relevant angiogenesis and vascular remodeling. During the first two postnatal weeks, the murine intraretinal vasculature develops through vasculogenesis and angiogenic outgrowth from preexisting vessels.1,2 This culminates in the formation of two distinct capillary plexi in the inner retina.3,5 Vascularization is coordinated with development of the neural retina and is a response to increasing metabolic demands as neural growth and differentiation proceed and synaptic connections are achieved. In unison with maturation of the retina, lens, vitreous, and optic nerve head, the hyaloid vascular system in the vitreous, consisting of vasa hyaloidea propria (VHP), tunica vasculosa lentis (TVL), and pupillary membrane (PM), progressively undergo vasoregressive events involving apoptosis and necrosis.

The oxygen demands of the developing retina are considerable, and during ontogeny its component tissues require an extremely rich vascular supply to differentiate and function normally. The vasculature develops or regresses in a highly regular, organized manner, with the angiogenic and vaso-obliterative components being modulated by growth factors in the tissue microenvironment.6–8

Vascular endothelial growth factor (VEGF)-A and placental growth factor (PlGF) are members of a large group of homologous peptides (the VEGF family) that share many biochemical and molecular characteristics.9,10 VEGF-A has been well characterized as an endothelial cell mitogen and survival factor,11,12 with a central role in angiogenesis, neovascularization, and vasopermeability.13–15 Although the importance of this growth factor in hypoxia-induced angiogenic mechanisms is unquestioned, the role of PlGF in such processes is less well understood. However, recent findings suggest PlGF may specifically influence the angiogenic response to VEGF-A by acting through flt-1 (VEGF-R1).10 PlGF has previously been shown to activate VEGF-R1.17,18 Several studies suggest that PlGF can dimerize with VEGF-A, forming heterodimers that limit ligand interactions with the KDR tyrosine kinase receptor (VEGF-R2) and thereby attenuate VEGF-A’s activation of mitogenic, migratory, and proliferative responses.19,20 Alternatively, Carmeliet et al.16 and Park et al.21 suggest PlGF may actually have the greatest stimulatory effect on angiogenesis by preferentially occupying VEGF-R1 binding sites and thereby freeing VEGF-A to activate VEGF-R2.16–21 It has also been shown in various tissues, including retina, that PlGF and VEGF have distinct expression patterns during angiogenesis22,23 and display differential responses to hypoxia.24,25

The precise roles and complex interplay between VEGF-A and PlGF in the developing retinal vasculature remain ill defined. In the present study, we examined the expression of these growth factors. During retinal developmental angiogenesis and hyaloid regression. Selective neutralization of these growth factors at critical stages of vascular development and regression by using specific antibodies also provided an insight into the roles of VEGF-A and PlGF during angiogenesis and vaso-obliteration.
METHODS

Retinal Vascularization in the Murine Model

To study the developing murine retinal vasculature and hyaloid system, neonatal C57BL/6j mice were killed on postnatal days (P)1, P3, P5, P7, P9, and P11 after birth. Before death, some mice were heavily anesthetized (Hypnorn; Janssen-Cilag, High Wycombe, UK; 0.01 ml/g intraperitoneally; fentanyl, 0.315 mg/mL; fluanisone, 10 mg/mL; and midazolam, 5 mg/mL) and perfused with fluorescein isothiocyanate (FITC)-dextran (molecular weight $2 \times 10^5$; Sigma Chemical Co., Poole, UK), dissolved in phosphate-buffered saline (PBS), to assess the extent of retinal vascular development. After enucleation, the eyes were fixed in paraformaldehyde (PFA) for 4 hours and then placed in PBS at 4°C. The anterior segment, lens, vitreous, and hyaloid were removed, and the posterior eye cup was subjected to four radial full-thickness cuts to facilitate flat mounting. FITC-dextran-perfused eyes and labeled eyes were viewed by confocal scanning laser microscopy (CSLM; MicroRadiance; Bio-Rad, Herts, UK) after they were flat mounted onto microscope slides. Eyes to be processed for immunohistochemistry and in situ hybridization were enucleated in mice under terminal anesthesia and fixed in 4% PFA for 2 hours. After fixation, the eyes were washed in PBS before dehydration and embedding in wax for standard histologic sectioning.

Treatment of the mice throughout this study was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and according to British governmental guidelines.

Treatment of Neonatal Mice with PlGF and VEGF Antibodies and Quantification of Vascular Development and Regression

Groups of neonatal mice were used in PlGF- and VEGF-neutralization studies. Other investigators have effectively used intraperitoneal delivery of VEGF-A-neutralizing antibodies to manipulate retinal peptide bioavailability. In the present study, age-matched mice from three different litters were randomized among four nursing dams, to arrange them in litter groups of eight pups. Between P2 and P9, each mouse was injected intraperitoneally with either an anti-mouse PlGF polyclonal antibody (5 mg/kg; R&D Systems, Ltd., Oxon, UK), anti-mouse VEGF-A polyclonal antibody (5 mg/kg; R&D Systems, Ltd.), isotypic nonimmune IgG (5 mg/kg; Sigma Chemical Co.), or sterile PBS. Twenty-four hours after the final injection, the mice were killed with terminal anesthesia and the eyes placed in 4% PFA. After fixation, the eyes were washed in PBS and either prepared for flat mounting (as outlined earlier) or subjected to dehydration and embedding in wax for standard histologic sectioning.

Eyes in wax blocks were trimmed to the level of the optic nerve head, and from there onward sections were cut every 30 μm until the optic nerve disappeared from the block. The sections were then coded and examined by an investigator who was unaware of the identity of each eye. During the analysis, vascular cell nuclei juxtaposed to the lens (TVL) and those associated with the hyaloid membrane were counted and recorded separately. Single nuclei, probably representative of hyalocytes, were also counted. Single nuclei, probably representative of hyalocytes, were also counted.

For quantification of intraretinal vessels, flatmounted retinas were imaged on the CSLM with a ×4 plan-apochromatic objective. For comparative analysis, the retinal angiographic images were always oriented with the optic nerve at the center of the field of view. The analysis was conducted according to a novel method that displayed each digital angiographic image with a superimposed 64-square grid (8 × 8 squares) corresponding to a real area of 9.95 mm². Each grid square, equivalent to 0.155 mm² of retinal area, was analyzed and annotated with on-screen letters that specifically recorded and quantified normally vascularized retina and residual ischemic retina at P9. A computer program using the following classification was designed to assist with the analysis of retinal angiograms. Vessels were designated according to the following classes: E, empty; N, normally vascularized retina; I, ischemic, nonperfused retina; U, unidentifiable. Empty representations those areas of an image corresponding to the expansion of the four radial cuts applied in the flatmounting procedure. An operator familiar with the relevant angiographic morphology applied the letter codes. The annotation procedure allowed for the recording of different features within any given grid square—that is, coding a square by more than one letter, which was usually necessary, because a retinal area of 0.155 mm² was characterized by several angiographic features. The program was able to quantify all possible letter combinations and calculate the total retinal areas displaying the particular morphologies. The total areas characterized by each of the designated features were expressed in square millimeters and as percentages of a total analyzed retinal area. The program performed simple summary statistics of an analyzed image and the data files were transferred to other programs for further analysis.

A one-way analysis of variance (ANOVA) was conducted between the various groups and subgroups, and significance was taken to be above the 95% confidence limits.

Immunolocalization of PlGF in Retina

Sections of mouse eyes were dewaxed and rehydrated in PBS, and endogenous peroxidase activity was quenched in 3% hydrogen peroxide. Sections were washed in PBS and blocked with 5% normal goat serum (NGS), 1% BSA, 0.01% Triton X-100, and mouse-on-mouse blocking reagent (Vector Laboratories, Peterborough, UK) to neutralize cross-reactivity with endogenous mouse immunoglobulins. Murine PlGF monoclonal antibody (R&D Systems, Ltd.) or control mouse IgG (Sigma) was added to the sections overnight at 4°C in a humidified chamber at 1:200 dilution. After the sections were washed, a 1:200 dilution of biotinylated goat anti-mouse antibody (Dako Ltd.) was added, followed by streptavidin in the form of the avidin-biotin complex (ABC; Vector Laboratories). Detection was performed by addition of 3-amin-9-ethylcarbazole (AEC; Vector Laboratories), to yield a red reaction product. Immunolocalization was also performed on whole retinas prepared as just described. The posterior eye cups were permeabilized, and nonspecific immunoreactive sites blocked for 16 hours at 4°C in PBS containing 0.5% Triton X-100 (TX-100), 5% normal goat serum, and mouse-on-mouse reagent (Vector Laboratories). Murine PlGF monoclonal antibody (R&D Systems, Ltd.) or control mouse IgG (Sigma) was added to the retinas overnight at 4°C at 1:100 dilution in PBS containing 0.5% TX-100. The retinas were then blocked in 5% NGS in permeabilizing buffer, washed extensively, and exposed to anti-mouse Alexa 488 (Molecular Probes Inc., Eugene, OR), diluted 1:500 in PBS containing TX-100 for 3 hours at 4°C. The retinas were then washed extensively, mounted (Citifluor; Agar Scientific Ltd., Essex, UK) on microscope slides, and the immunofluorescence detected by CSLM.

Quantitative PCR

Freshly dissected mouse retinas (at least six to eight retinas per sample) were snap frozen in liquid nitrogen. RNA was extracted with a kit (RNeasy Mini Kit; Qiagen, Crawley, UK). The quantity of RNA in each sample was determined spectrophotometrically (U 1100 model, Hitachi Ltd., Tokyo, Japan), and the purity and quality of each RNA sample was estimated by visualization of clear 18S and 28S ribosomal RNA bands after electrophoresis of 1 μg of each sample on a 1% agarose gel.

RNA samples were reverse transcribed into cDNA using a first-strand cDNA synthesis kit (Life Technologies, Paisley, UK) and random hexamer primers (Roche Molecular Biochemicals, Mannheim, Germany). Real-time PCR was conducted for quantitative analysis of mRNA expression. A 200-bp fragment of murine PlGF cDNA was amplified with murine sequence-specific primers (forward: 5’ CAC TTG CTT CCT ACA GGT CC 3’; reverse: CAC CTC ATC AGG GTA TTC AT 3’). Murine VEGF-A primers (forward: 5’T TA CTG CTG TAC CTC CAC C 3’; reverse: 5’ ACA GGA CGG CCT GAA GAT G 3’) were used to amplify a 189-bp fragment. Primers used to amplify further murine genes were: acidic ribosomal phosphoprotein PO (ARP), 109-bp fragment (forward: 5’ CA CCT AGT CCA ACT AC 3’; reverse: 5’ ATC TGC TGC TGC 3’).
TGC TTG 3', von Willebrand factor (vWF), 127-bp fragment (forward: 5' CAC TGA TAT TTG TCC CAC CT 3' /H11032, reverse: 5' AAA TTT TAG AAA TGG GCT CC /H11032), preproendothelin-1 (PPE-1), 142-bp fragment (forward: 5' GAT GGA CAA GGA GTG TGT CT 3' /H11032, reverse: 5' GGC CTT ATT GGG AAG TAA GT /H11032), and cGMP phosphodiesterase (PDE), 161-bp fragment (forward: 5' CAT CAA AGT CAT CCC GAA CC 3' /H11032, reverse: 5' TCA TCC ACC CAG ACT CAT CC 3'). Real-time PCR was performed with a rapid thermal cycler system (LightCycler; Roche Molecular Biochemicals), according to protocols outlined by Simpson et al.28 Briefly, PCR was performed in glass capillary reaction vessels (Roche Molecular Biochemicals) in a 20-μL volume with 0.5 μM primers. Reaction buffer, 2.5 mM MgCl₂, dNTPs, Taq DNA polymerase (Hot-start), and green fluorescent dye (SYBR Green I) were included in a kit (QuantiTect LightCycler, SYBR Green PCR Master Mix; Qiagen, Crawley, UK). Amplification of cDNAs involved a 15-minute denaturation step followed by 40 cycles with a 95°C denaturation for 15 seconds, 55°C to 58°C annealing for 20 seconds, and 72°C for an appropriate extension time (5–25 seconds). Fluorescence from the green dye that bound to the PCR product was detected at the end of each 72°C extension period. The specificity of the amplification reactions was confirmed by melting-curve analysis and subsequently by agarose gel electrophoresis.28 The quantification data were analyzed with the analysis software that accompanied the thermal cycler (LightCycler; Roche Molecular Biochemicals), as described previously.29 The base-
line of each reaction was equalized by calculating the mean of the five lowest measured data points for each sample and subtracting this value from each reading point. Background fluorescence was removed by setting a noise band. The number of cycles at which the best-fit line through the log-linear portion of each amplification curve intersects the noise band is inversely proportional to the log of copy number.19 A dilution series of a reference cDNA sample was used to generate a standard curve against which the experimental samples were quantified. For each gene, PCR amplifications were performed in triplicate on at least two independent RT reactions.

In Situ Hybridization
Riboprobes were prepared from PCR products derived from murine VEGF-A and PlGF retinal RNA. In situ hybridization was performed according to the protocol outlined previously.24,31 Briefly, sections of eyes were dewaxed, rehydrated, and postfixed in 4% PFA. The proteins were denatured in hydrochloric acid, and the sections were then treated with protease K for 30 minutes at 37°C. Digoxigenin-labeled riboprobes were then hybridized to the sections for 18 hours at 42°C in a humidified chamber. After hybridization, the sections were washed in decreasing concentrations of SSC buffer and then incubated with an anti-digoxigenin alkaline phosphatase antibody (Roche Molecular Biochemicals) for 2 hours. Hybridized probe was detected with nitroblue tetrazolium solution and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP; 75 mg/mL, in dimethylformamide; Roche Molecular Biochemicals) and sections counterstained with 0.02% methyl green. Before viewing, the slides were washed and mounted (Glycergum; Dako Ltd., Glostrup, Denmark).

RESULTS
Histology and Fluorescein Angiography
Sections of murine eyes from P1 to P11 showed the typical neural retina and vascular developmental stages previously described.1 The neural retina underwent considerable remodeling during this developmental program, manifested by progressive reduction in the number of cells, cleavage of the inner and outer nuclear layers, and associated neuronal and glial differentiation events (data not shown). As previously described, in the murine eye, intraretinal vessels were observed spreading outward from the optic disc toward the retinal periphery at P1. At P3 and P5, the larger vessels continued to extend toward the retinal periphery where capillary growth was progressive reduction in the number of cells, cleavage of the inner nuclear layer (Fig. 2A). At P3 to P5 there was an increase in VEGF mRNA that had started to infiltrate the areas between the larger vessels, whereas at P7 to P9 the superficial capillary plexus was beginning to form. At P9, the superficial capillary network was becoming remodeled, and capillary density of the deep plexus had increased. At P11, the superficial capillary plexus was complete, and the deep capillary network was also well formed. The hyaloid vasculature was extensive at P1 and was progressively reduced in vessel density and complexity, concurrent with retinal development (as outlined by Ashton1).

Real-Time PCR
Quantitative PCR revealed similar trends in expression for VEGF-A and PlGF mRNA during postnatal development from P1 to P9 (Fig. 1). Both decreased from P1 to P3 and then increased to higher levels at P7 and P9, before declining to significantly lower levels on P11 (VEGF: \(P < 0.05\); PlGF: \(P < 0.01\)). However, the variation was more pronounced with VEGF. Also, unlike PlGF, the expression of VEGF-A mRNA was markedly higher (\(P < 0.01\)) at postnatal day 1 (P1) than at all other developmental stages. The retinal RNA samples were normalized by mass, and, to demonstrate that the variations in VEGF and PlGF mRNA expression were not due to nonspecific variations in RNA or cDNA quality, a series of other genes were shown to have alternative expression patterns. These included a housekeeping gene (ARP) and two vascular-specific genes (vWF and PPE-1). In addition, a photoreceptor-specific gene (PDE) was not evident until the later stages of development (from P5 onward), demonstrating a peak at P9 (\(P < 0.03\)) before a significant reduction at P11 (\(P < 0.01\)). It should be noted that the arbitrary scales used facilitate comparison of changing expression levels for individual genes throughout the time course but cannot be used to infer relative absolute expression levels between genes.

In Situ Hybridization
Localization of mRNAs was determined by in situ hybridization. VEGF-A expression was only evident in the innermost layers of the developing neural retina, at the putative ganglion cell layer (A, arrow), whereas at P5 the mRNA appears more abundant and is localized to the inner retina, especially around the forming inner nuclear (arrows) and ganglion cell layers (B). Sense probes were negative for all retinas (C) through P11. At the later developmental stages, VEGF-A mRNA was expressed largely by the ganglion cells (arrow), although there was some labeling in neurons at the inner and outer aspects of the inner nuclear layer (D, arrowhead). Original magnification, \(\times 250\).

Immunolocalization of PlGF in Retina
Sections of retina showed intense PlGF immunoreactivity in the retinal vasculature, with the most intense staining evident in the large vessels at the inner limiting membrane (Fig. 3E). On examination at higher magnification, this immunoreactivity
was localized to the walls of these vessels (Fig. 3F). Control experiments using nonimmune serum produced negative immunoreactivity.

Retinal flatmounts were also examined by CSLM for PlGF immunofluorescence. In some flatmounts the hyaloid membrane was not removed during the initial dissection, and in these preparations, this vascular system showed intense immunoreactivity for PlGF, especially in the early developmental stages (Fig. 3G). On examination of the retinal vasculature, PlGF was localized to both the large vessels and capillary networks, especially in the inner plexi. As observed in the sections, immunolocalization of PlGF occurred largely in the walls of the vasculature (Fig. 3H).

Effect of Neutralization of VEGF-A and PlGF on Retinal Vascular Development

When flatmounts of FITC-dextran-perfused retinal vasculature at P9 were examined by confocal microscopy, it was clear that the superficial vascular plexus had developed extensively, and this layer was used for evaluation (Fig. 4). The normal and IgG-treated retinas showed extensive intraretinal vascular formation with highly developed capillary beds (Figs. 4A, 4B). Mice treated with VEGF-neutralizing antibody showed a reduction in the density of the superficial retinal capillary plexus in comparison with the control (Fig. 4C, 4E; P < 0.05). PlGF-antibody–treated animals showed a qualitative increase in cap-
illary density (Fig. 4D) although this was not statistically significant in comparison with the control (Fig. 4E).

In the sections, the various cell types within the vitreous body were clearly evident (Figs. 5A, 5B). During quantification of the hyaloid system in control and growth factor-depleted mice, several clear responses were noted. VEGF neutralization caused a significant reduction in hyalocytes (P < 0.002; Fig. 5C) and hyaloid vascular cell nuclei (P < 0.0001; Fig. 5D) when compared with normal and IgG-treated control mice. VEGF had no discernible influence on the TVL (Fig. 5E). Treatment with PlGF antibody caused a significant increase in vascular cell density in the hyalocytes and hyaloid vasculature (P < 0.01 and P < 0.001 respectively; Figs. 5C, 5D). This treatment caused a decrease in nuclei representative of the TVL when compared with VEGF-treated or control animals (Fig. 5E).

**DISCUSSION**

VEGF-A is produced by neurons and glia in response to retinal ischemia and has been shown to be a potent angiogenic agent. This growth factor has been heavily implicated in the pathogenesis of proliferative retinopathies, although it also...
plays key roles in vasopermeability (including breakdown of
the blood-retinal barrier)\textsuperscript{54,55} and retinal vascular cell survi-
av.\textsuperscript{12} The current investigation has demonstrated that expres-
sion of VEGF-A and PlGF is regulated during retinal vascular
development. In addition, when bioactivity of these peptides is
reduced by using neutralizing antibodies, regression of the
hyaloid system is markedly disrupted. Neutralization of VEGF-A,
but not of PlGF, appeared to attenuate retinal vascular devel-
oping. It is evident that these growth factors, although they
are close in sequence homology, are regulated differently and
have very distinct roles in retinal development.

VEGF-A has been shown to be vital for angiogenic processes
during ontogeny\textsuperscript{56,57} and is induced by hypoxic stimuli from
growing organs. This peptide is necessary for the vasculariza-
tion of the embryonic lens and formation of the hyaloid sys-
tem\textsuperscript{58} and is also essential in retinal vascular development.\textsuperscript{59,60}
Indeed, VEGF-A, induced by oxygen demands of active cells, is
vital for the eventual formation of two distinct capillary plexi to
meet the metabolic requirements of the inner retinal neuro-
pile.\textsuperscript{7,8}

In the present study, we examined expression of VEGF-A
during key stages of retinal vascular development and quanti-
fi ed changes in mRNA during formation of the inner capillary
plexus (P1–P5) and outer capillary plexus (P9). In situ hybrid-
ization has demonstrated that the differentiating inner neural retina is the major source of VEGF-A. High expression of
VEGF-A correlates both temporally and spatially with high
angiogenic activity. Although PlGF is also synthesized in the
inner portion of the neural retina, it exhibits a distinct tempo-
ral expression pattern when compared with VEGF-A.

Dense capillary networks form during retinal vascular de-
velopment, and these can undergo remodeling in parallel with
differentiation of the neural retina.\textsuperscript{1,3,40} VEGF-A has been
shown to be a potent survival factor for the mature retinal vas-
culature\textsuperscript{12} and the thinning of the dense capillary plexi is
probably initiated by subtle withdrawal of VEGF from the
hyperoxygenated microenvironment, leading to apoptotic
death of superfluous vascular cells.\textsuperscript{12} It is significant that treat-
ment of developing mice with VEGF-neutralizing antibodies,
which probably equates with withdrawal of VEGF, leads to
reduced vessel density in the inner retinal capillary plexus. It
seems likely that similar events are invoked in the hyaloid
system during its regression in concomitance with retinal vas-
ularization. Indeed, the neutralization of VEGF-A in the mouse
vitreous results in a significant acceleration of hyaloid regres-
sion in comparison with normal control mice. TVL degenera-
tion was unexpectedly uninfluenced by treatment with a
VEGF-A antibody, which suggests that programmed regression
is independent of VEGF-A or may indicate that development
and maturation of the lens had gone beyond the point of
plasticity and susceptibility to certain growth factors.

In a recent study involving oxygen-induced retinopathy
(OIR) in the mouse, it was shown that several other members
of the VEGF family are expressed in retina, and many of these
(and their splice variants) are subject to altered expression
during the vaso-obliterative and hypoxic phases characteristic
of this model.\textsuperscript{24} PlGF showed an expression pattern that dif-
fered from that of VEGF-A and was not induced by hypoxia but
was highest during hyperoxia-induced vaso-obliteration.\textsuperscript{24} The
regulation of PlGF gene expression has also been shown to
diverge from that of VEGF-A in other tissues and cell types
where it is not induced by hypoxia\textsuperscript{25,41,42} and may actually
serve to inhibit endothelial cell proliferation.\textsuperscript{41} In the present
study, expression of PlGF correlated with vaso-obliteration
(during hyaloid regression), and neutralization of this peptide
can significantly attenuate hyaloid degeneration.

The basis of the diversity of PlGF and VEGF-A probably lies
in the activation of common and distinct receptors. VEGF-A

\begin{figure}[h]
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\caption{Quantification of vascular regression in vitreous of neonatal mice. (A) Lens and surrounding area at P9 (IgG-treated mouse). The vessels of the TVL were identifiable by their juxtaposition to the lens (\textit{small arrow}). A remnant of the hyaloid can be seen in the section (\textit{large arrow}). (B) Section through the hyaloid at the optic disc (P9, PBS-treated mouse). The nuclei of the major hyaloid vessels can be clearly observed (\textit{arrow}). (C) When hyalocytes were quantified at P9, it was apparent that VEGF-A neutralization caused a significant reduction in the number of cells, whereas neutralization of PlGF significantly increased the number of hyalocytes persisting in the vitreous. (D) Quantification of the nuclei associated with the hyaloid showed a significant reduction in VEGF-A antibody-treated mice when compared with control and nonimmune IgG-treated mice. By contrast, PlGF antibody treatment caused a significant persistence of the hyaloid vascular cell nuclei. (E) The TVL was unaffected by treatment with a VEGF-A antibody, although neutralization of PlGF caused a significant reduction in the vascular profiles around the lens. \textit{aP} < 0.01; \textit{**P} < 0.001; \textit{***P} < 0.0001. Magnification, \times 250.}
\end{figure}
bonds primarily to VEGF-R1 and VEGF-R2, with the latter receptor being necessary for mitogenic and proliferative responses in endothelial cells.15 PIGF is unable to activate tyrosine phosphorylation of VEGF-R2, and this is reflected in its inability to induce angiogenesis.16,17 PIGF can activate VEGF-R1, which may induce monocyte recruitment and procoagulant activity in endothelial cells17 and has recently been shown to inhibit VEGF-R2-mediated effects.15–17 PIGF (VEGF-100 isoform) and PI GF-2 have high affinity for neuropilin-1, which acts as a dominant-negative coreceptor with VEGF-R1.17–19 VEGF-PIGF heterodimers are readily formed,19 and it is now evident that such peptide associations have significantly less affinity for VEGF-R2 than VEGF homodimers.20 PI GF, through the formation of heterodimers with VEGF-A, may modulate angiogenesis by reducing activation of VEGF-R2.19

PIGF may attenuate VEGF-A’s vascular cell survival potential within the context of hyaloid regression. Our data suggest that neutralization of PI GF in the vitreous body results in significant persistence of the hyaloid system. Reduced PI GF-induced activation of VEGF-R1 may promote vascular cell survival mediated by VEGF-R2. A reduction in the PI GF-VEGF-A heterodimer formation may also contribute to enhanced VEGF-A-mediated cell survival.

The differential expression patterns and distinct modulatory influence of peptide neutralization suggest that VEGF-A and PI GF are important for retinal vascular development and appropriate regression of the hyaloid system. Patterns invoked in developmental systems such as the mouse retina may provide the basis for further studies in manipulating retinal angiogenic mechanisms and vascular cell survival with implications for proliferative and vasodegenerative retinopathies.

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**References**


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