Purpose. Disruption of the normally antiangiogenic environment of the retina leads to aberrant angiogenesis, the major cause of vision loss throughout the world. Prolactin (PRL), the hormone originally associated with milk production, can be proteolytically processed to 16K-PRL, a 16 kDa N-terminal PRL fragment with potent antiangiogenic and vasocostrictive actions. This study was conducted to determine whether 16K-PRL is found naturally in the retina and plays a role in angiogenesis and vasodilation.

Methods. Expression of PRL mRNA in rat retina was determined by RT-PCR and in situ hybridization. The role of PRL and 16K-PRL in the retina was studied by intravitreal injection of either antibodies used to examine the expression of PRL and derived fragments.]}

Results. Rat retina expressed PRL mRNA in the outer nuclear, outer plexiform, inner nuclear, and ganglion cell layers. Both full-length PRL and N-terminal 16K-PRL were detected in retinal homogenates by polyclonal and monoclonal antibodies. Western blot was used to examine the expression of PRL and derived fragments in retinal homogenates. The role of PRL and 16K-PRL in the retina was studied by intravitreal injection of either antibodies against PRL or small interfering RNAs (siRNA), to suppress expression of retinal PRL mRNA.

Conclusions. These results demonstrate that PRL is synthesized and cleaved to antiangiogenic 16K-PRL by retinal tissue and that these molecules play a key role in preventing angiogenesis in the healthy retina. (Invest Ophthalmol Vis Sci. 2005;46: 2947-2953) DOI:10.1167/iovs.05-0173

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Angiogenesis, the growth of new blood vessels from preexisting vessels, is highly restricted in most healthy tissues, in part by the action of naturally occurring antiangiogenic factors that prevent new vessel growth.1 Disruption of antiangiogenic environments underlies diseases such as cancer, rheumatoid arthritis, and vasoproliferative retinopathies.2 In the healthy adult eye, blood vessels are excluded from various compartments, including the cornea, the lens, and the vitreous. Also, in the adult retina, vessels are confined to the inner half, as the outer half never becomes vascularized.3 Failure to inhibit blood vessel growth can result in reduced visual acuity, opacification, and abnormal healing,4 whereas excessive retinal neovascularization underlies premature retinopathy, diabetic retinopathy, and age-related macular degeneration, the leading causes of blindness throughout the world.5,6 Whereas ocular angiogenesis can result from an increase in the local concentration of angiogenic factors, a reduction in the levels of angiogenesis inhibitors is also necessary.5-7 The importance of such reduction is indicated by the fact that, in the healthy eye, vessel growth is quiescent, despite the presence of many stimulators of angiogenesis.5,5-10 Several inhibitors of ocular angiogenesis have been identified, including pigment epithelium-derived growth factor, transforming growth factor-β, angiostatin, endostatin, thrombospondin-1, and tissue inhibitors of matrix metalloproteinases.7 Attention is now focused on the characterization of these inhibitors and the discovery of new ones, with the final goal of using these factors to treat neovascular eye diseases.

One potential inhibitor of ocular angiogenesis is the N-terminal 16-kDa fragment of prolactin (16K-PRL). PRL is a pleiotropic protein that acts as a hormone or cytokine with effects on multiple processes, ranging from reproduction and osmoregulation to immunomodulation and angiogenesis.9,10 The full-length 23-kDa PRL protein can be cleaved in vivo to yield 16K-PRL, which exerts antiangiogenic actions not shared with the parent molecule.10 16K-PRL inhibits angiogenesis in vivo and in vitro, suppressing growth factor-induced endothelial cell proliferation11 and stimulating the expression of the type I plasminogen activator inhibitor12 and endothelial cell apoptosis.13 Evidence suggests that 16K-PRL is a natural inhibitor of ocular angiogenesis.16 16K-PRL inhibits the stimulation of corneal angiogenesis by basic fibroblast growth factor,14 and it appears to play a key role in maintaining corneal avascularity. Implantation into the cornea of a pellet releasing antibodies able to inactivate endogenous 16K-PRL resulted in vessel growth into the normally avascular cornea.14 Moreover, PRL is found in the aqueous humor of the human eye,15,16 and cultures of rat retinal capillary endothelial cells express and release PRL.17 Furthermore, 16K-PRL is detected in the eye of patients with advanced retinopathy of prematurity (ROP).18 Evidence suggests that 16K-PRL promotes apoptosis-mediated vascular regression of the newly formed blood vessels in the eye of these patients and of ocular hyaloid vessels after birth.16

In this study, we report that PRL was synthesized and cleaved by adult retina to yield antiangiogenic 16K-PRL. We
also show that blocking the local expression of PRL or the action of 16K-PRL resulted in significant retinal neovascularization and vasodilation. These data establish a role for PRL molecules as essential inhibitors of retinal angiogenesis.

**METHODS**

**Reagents**

Rat PRL (BIO grade) was obtained from the National Hormone and Pituitary Program (NHPP, Torrance, CA). The N-terminal 16K-PRL and the C-terminal 6K-PRL fragments were generated by enzymatic proteolysis of rat PRL with a particulate fraction from rat mammary glands followed by gel filtration and carbamidomethylation, as described. Anti-PRL polyclonal (C-1) and monoclonal (INN-1, INN-368) antibodies were obtained and characterized as reported. Control antibodies were purified from normal rabbit serum. Purified nonimmune mouse antibodies were from ICN Biomedicals (Aurora, OH).

**Animals**

Animal care and treatment were according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Wistar rats (200–250 g) were anesthetized with 70% ketamine and 30% xylazine (1 µL/g body weight intraperitoneally) for intravitreal injection of antibodies or small interfering (si)RNA.

**Reverse Transcription–Polymerase Chain Reaction**

The rats’ eyes were removed shortly after death, and the retinas were quickly dissected and frozen. Total RNA was extracted and quantified, and 5 µg was reverse-transcribed in a 25 µL reaction using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Two-microliter aliquots were used for cDNA amplification by PCR (30 seconds at 94°C, 45 seconds at 65°C, and 45 seconds at 72°C for 40 cycles) with two oligonucleotides specific for rat PRL (sense primer ACT TCT TCC CTA GCT ACT CCT GAA GAC and antisense primer GCA GTT GTT TTT ATG GAC AAT TTG GCA). The resultant product represents the 418-bp fragment upstream of the stop codon. The oligonucleotides correspond to exons 3 and 5 of the rat PRL gene, ensuring that amplification of cDNA could be distinguished from that of genomic DNA.

**In Situ Hybridization**

Sense and antisense PRL RNA probes labeled with digoxigenin were synthesized from a linearized plasmid containing the full open reading frame of the rat PRL cDNA. Rats were killed and the eyeballs removed and hemisected. Isolated retinas were fixed for 24 hours in 4% formaldehyde, incubated for 24 hours in RNase-free 10% saccharose and 30% xylazine (1 µL/g body weight intraperitoneally) for intravitreal injection of antibodies or small interfering (si)RNA.

**Quantification of Retinal Neovascularization**

Blood vessels were labeled using antibodies against platelet endothelial cell adhesion molecule (PECAM)-1 (anti-PECAM-1; Endogen, Woburn, MA) that specifically stain endothelial cells, followed by a biotinylated secondary antibody developed using the avidin-biotin complex (ABC)-detection kit (Vector Laboratories, Burlingame, CA). In addition, the anti-mouse antibody coupled to fluorescein isothiocyanate (Alexa Fluor 488; Molecular Probes, Eugene, OR) was used as a specific marker of blood vessels. This antibody stained blood vessels in all samples, as confirmed by dual label immunofluorescence studies with anti-PECAM-1 or antibodies against the von Willebrand protein, another specific endothelial cell marker (not shown).

Six whole retinal serial sections per eye obtained 60 µm apart were visually scanned with a high-power (40×) objective to record the stained blood vessels. Images were digitized and evaluated with image-analysis software (Image Pro-Plus; Media Cybernetics, Silver Spring, MD). Blood vessels were outlined and counted, and their area and number calculated per unit area, determined by tracing the outline of
each retinal image. The mean counts and vessel areas of six sections per eye were averaged and used in the analysis.

Measurement of Blood Vessel Dilation

The retinal vascular network was delineated in flatmount retinal preparations by staining the enclosed red blood cells using a peroxidase reaction, as described. Each preparation was digitized with a 20× objective and tiled, to create a picture of the entire preparation. Vasodilation was evaluated by recording the area and length of superficial blood vessels. Blood vessel area was divided by blood vessel length to give a mean diameter for blood vessels that could be compared across experiments.

Statistical Analysis

All results were replicated in three or more independent experiments. Data are presented as the mean ± SEM. As appropriate, Student’s unpaired t-test or one-way ANOVA followed by Tukey’s test to compare individual means was used for statistical comparisons. The significance level was set to 5%.

RESULTS

PRL mRNA in the Retina

The expression of PRL mRNA in the retina was investigated by RT-PCR (Fig. 1A). Amplification yielded a single product with the expected length of 418 bp, similar to the control PCR band from rat PRL cDNA. The distribution of PRL mRNA was determined by in situ hybridization. No labeling was evident with a sense probe (Fig. 1B). An antisense probe revealed significant retinal expression of PRL (Fig. 1C). PRL mRNA was found mostly in the outer nuclear and ganglion cell layers of the retina, with fewer labeled cells in the outer plexiform and inner nuclear layers.

PRL and 16K-PRL in the Retina

Because recent studies have shown that PRL can be cleaved to yield either antiangiogenic N-terminal 16K-PRL or a 16-kDa C-terminal fragment devoid of antiangiogenic activity, we investigated the nature of endogenous PRLs in the retina by using two different monoclonal antibodies that selectively recognize the N- or C-terminal ends of the PRL molecule (Fig. 2B). Selectivity of the monoclonal antibodies was demonstrated by comparing their binding to a mixture of full-length 23-kDa PRL, N-terminal 16K-PRL, and C-terminal 6K-PRL (Fig. 2A). The anti-PRL polyclonal antibody (C-1) recognized all three PRL isoforms. The two monoclonal antibodies reacted with full-length PRL, but only INN-1 bound to the purified N-terminal 16K-PRL standard, and only INN-368 detected the C-terminal 6K-PRL standard (Fig. 2A). Accordingly, we conclude that the epitope for INN-1 is located in the N terminus of PRL and that for INN-368 resides in the C terminus.

Induction of Retinal Angiogenesis by Immunoneutralization of PRL

To investigate whether endogenous PRLs inhibit retinal angiogenesis, the effect of the intravitreal injection of anti-PRL antibodies on retinal neovascularization was evaluated (Fig. 3).
Induction of Retinal Angiogenesis and Vasodilation by Intravitreal Injection of PRL siRNA

To further examine the antiangiogenic effect of endogenous PRLs, siRNA was injected into the vitreous to suppress retinal expression of PRL. The ability of PRL siRNA to target PRL mRNA was confirmed by RT-PCR 6 days after injection. PRL siRNA suppressed PRL mRNA expression, whereas the nonspecific GFP siRNA or the vehicle lacking siRNA had no effect (Fig. 4A). Retinas treated with PRL siRNA exhibited significant neovascularization, reflected by an up to threefold increase in both number of vessels and vascular area compared with GFP siRNA or vehicle control (Figs. 4B, 4D). In addition, vasodilation was seen in some retinal capillary vessels of PRL siRNA-treated eyes (Fig. 4C). To investigate these vasodilation changes, retinal vasculature was imaged in flatmounted preparations. Eyes injected with GFP siRNA showed normal vasculature (Fig. 5A), whereas retinal blood vessels were wider in PRL siRNA-treated eyes (Fig. 5B). This difference was significant ($P < 0.05$) and represented a 32% increase in vessel diameter (Fig. 5C).

**DISCUSSION**

Effective treatment of retinal neovascular disorders, including diabetic retinopathy, age-related macular degeneration, and ROP, remains one of the greatest challenges in ophthalmology today. The number of individuals with diabetes has increased worldwide and is projected to continue to rise, whereas ROP and age-related macular degeneration are the major causes of loss of vision in children and the elderly, respectively. Natural antiangiogenic factors within the eye are potential candidates for development of drugs to treat and prevent neovascular eye diseases. Several endogenous inhibitors of angiogenesis have been reported in the eye, some of which result from the proteolysis of larger inactive precursors. In the present study, we show that the antiangiogenic 16-kDa N-terminal fragment of PRL is expressed in the adult rat retina and is critical in maintaining the quiescent state of the local vasculature.

The highest levels of expression of PRL mRNA were seen in the outer nuclear layer, which contains the photoreceptor cell nuclei and is an avascular zone where essential angiogenesis inhibitors are concentrated. In addition, PRL expression was detected in the outer plexiform, inner nuclear, and ganglion cell layers of the vascular retina. Thus, photoreceptors synthesize PRL, and although the precise cellular distribution of PRL expression in the vascular retina remains to be determined,
another likely source is the vascular endothelium. Cultures of rat retinal capillary endothelial cells synthesize and secrete PRL, and blood vessels from retrolental fibrovascular membranes of patients with ROP express PRL mRNA.

Immunoprecipitation and Western blot analysis of retinal homogenates detected PRL-like proteins of 46, 23, and 16 kDa. Based on their apparent molecular weights, the 23-kDa protein could correspond to unmodified PRL, whereas the 46-kDa protein may represent a glycosylated form of PRL. High-molecular-weight (53–100 kDa) PRL variants resistant to β-mercaptoethanol reduction have been detected in serum and amniotic fluid, and some of these isoforms bind to concanavalin A, indicating that they are glycosylated. In addition, we conclude that the 16K-PRL species found in retina is the antiangiogenic N-terminal fragment of the hormone, as indicated by its affinity for the N-terminal specific but not for the C-terminal specific monoclonal antibodies. Identifying this PRL fragment is relevant because a 16-kDa C-terminal PRL fragment devoid of antiangiogenic activity has been reported, although its in vivo production has not been demonstrated.

The fact that PRL is synthesized and cleaved to 16K-PRL in the retina suggests that 16K-PRL is a local inhibitor of angiogenesis. Consistent with this proposal, antibodies to PRL able to neutralize the action of 16K-PRL increased by two- to threefold the number of blood vessels and the capillary area of the inner retina. This appeared to be due to the inhibition of endogenous 16K-PRL, because an anti-PRL antibody not recognizing 16K-PRL was inactive. To our knowledge, this is the first report showing that neutralizing antibodies induce neovascularization of the inner retina, and it implies that 16K-PRL is a crucial, active inhibitor of angiogenesis at this site.

The role of local PRLs in suppressing retinal angiogenesis is further supported by the observation that angiogenesis is stimulated by intravitreal injection of siRNA targeting PRL. The neovascularization response was similar to that observed with neutralizing PRL antibodies and suggests that both methods are equally effective in blocking the antiangiogenic action of endogenous 16K-PRL. However, in contrast to the antibody experiments, treatment with PRL siRNA also induced a specific increase in the diameter of both larger and smaller vessels. The increase in vessel diameter (32%) was similar to that (24%) resulting from an adenoassociated virus vector used to express vascular endothelial growth factor (VEGF) in the retina of adult mice and indicates that local PRLs exert potent vasoconstrictive actions in the retina.

It is unclear why vasoconstriction is effectively blocked by PRL siRNA and not by antibodies targeting PRL. Because the suppressive action of siRNA is pretranslational, vasodilation may reflect the collective inhibition of all forms of intraocular PRL. However, vasoconstriction can also be attributed to retinal 16K-PRL. 16K-PRL, but not PRL, inhibits the activation of endothelial nitric oxide synthase (eNOS) by bradykinin and acetylcholine, which leads to inhibition of vasodilation, since 16K-PRL inhibits acetylcholine- and bradykinin-induced endothelium-dependent relaxation of rat aortic segments and coronary vessels. Because 16K-PRL appears to inhibit eNOS activation at a lower dose than that needed to inhibit endothelial cell proliferation (0.1 nM vs. 1 nM, respectively), it is possible that only the more efficient blockage of its synthesis by PRL siRNA can reveal inhibition of vasodilation. Moreover, inhibition of vasodilation can be linked to inhibition of angiogenesis. In fact, dilation of retinal blood vessels is among the earliest and most common manifestations of diabetic retinopathy and occurs in response to VEGF, the major promoter of retinal angiogenesis. The vasodilation and proangiogenic activity of VEGF is mediated by eNOS activation, and 16K-PRL inhibits VEGF-induced eNOS activation and endothelial cell proliferation. Likewise, other antiangiogenic protein fragments, including angiotatin and endostatin, block NO-induced vaso-dilation and angiogenesis.

The present findings indicate that 16K-PRL occupies a key position in preventing angiogenesis in the healthy adult retina. This crucial antiangiogenic function is consistent with the observation that 16K-PRL is effective against various inducers of ocular angiogenesis, including VEGF and basic fibroblast growth factor. Moreover, 16K-PRL gene transfer via an adenoviral vector results in significant inhibition of retinal angiogenesis in the mouse model of ischemia-induced retinopathy. In addition, PRL expression may be a contributing factor in the progression of vasoproliferative retinopathies. Most ocular angiogenesis is driven by ischemia (low oxygen), which is known to upregulate and downregulate angiogenic stimulators and inhibitors, respectively. Recently, hypoxia was shown to decrease PRL synthesis and to suppress its cathepsin D-mediated conversion to 16K-PRL in rat pituitary tumor cells. Furthermore, 16K-PRL has been detected in the eye of patients with advanced ROP, where it appears to promote apoptosis-induced vascular regression. 16K-PRL can halt angiogenesis by inducing apoptosis in endothelial cells, and endothelial cell apoptosis mediates the regression of blood vessels in ROP, leading to resolution of the disease. Intravitreal administration of anti-PRL antibodies in neonatal rats causes a significant reduction in apoptosis in the hyaloid vasculature, a transient network of fetal intraocular vessels that normally regresses after birth in the rat.

Ocular 16K-PRL also can be derived from the cleavage of systemic PRL internalized into the eye by active uptake. Iodinated PRL injected intracardially is incorporated into ocular tissues, including the retina, choroids, and ciliary body, and PRL is high in the circulation of patients with advanced...
ROP. In this regard, human milk feeding reduces ROP, which may relate to the findings that high PRL concentrations are found in milk, and PRL in circulation can reach ocular fluids where it is cleaved to antiangiogenic, proapoptotic 16K-PRL.

In conclusion, the present study demonstrates that 16K-PRL occurs naturally in the adult retina and exerts a tonic inhibition of both local angiogenesis and vasodilation. Identification of the mechanisms mediating 16K-PRL inhibition of angiogenesis in the retina, as well as the identification of the functional role of PRL in the eye under both physiological and pathologic conditions, warrants further investigation.

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