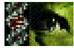
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Transgenic expression of leukemia inhibitory factor (LIF) blocks normal vascular development but not pathological neovascularization in the eye

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Purpose: Vascular development in the eye has been described as a complex process involving both vasculogenesis and angiogenesis. Multiple cell types are involved in the process including angioblasts, vascular endothelial cells, astrocytes, pericytes, and Müller glial cells. This suggests that multiple growth factors and cytokines are required to regulate retinal vascular development. Leukemia inhibitory factor (LIF) is a member of the interleukin 6 family of cytokines. LIF is expressed during inflammation and has been reported to affect vascular development in culture; however, its effects in vivo have not been demonstrated. The purpose of this study was to determine how LIF could regulate ocular vascular development.

Methods: We have analyzed ocular vascular development in transgenic mice that express LIF in the ocular lens from embryonic day 11.

Results: In transgenic mice, LIF reduced development of embryonic vasculature in the eye, and inhibited retinal vascular development. Inhibition in vivo was independent of vascular endothelial cell growth factor (VEGF) expression. In older transgenic mice, the absence of a retinal vasculature resulted in retinal ischemia and elevated VEGF levels. The upregulation of VEGF resulted in the proliferation of pathological vascular membranes in the vitreous and neovascularization penetrating the retina, which in turn resulted in tractional retinal detachment.

Conclusions: LIF is a potent inhibitor of retinal vascular development. These transgenic mice will be useful as a model of persistent fetal vasculature and to study the mechanism for the development of neovascular membranes in the vitreous and could be used to develop inhibitors of tractional detachment.

During human and mouse fetal development, transient blood vessels form in the posterior and anterior poles of the eye. This fetal vasculature consists of a hyaloid system, which traverses the vitreous to form the posterior tunica vasculosa lentis, a vasculature covering the posterior surface of the lens. Additionally, branches from the hyaloid artery form the vasa hyaloidea propria (VHP), an umbrella shaped system of capillaries that contributes to the primary vitreous and lie immediately anterior to the sensory retina. In humans, these intraocular vessels begin to develop as early as the first month of gestation, reach maturity between the second and third months, and begin to regress by the fourth gestational month. At birth they have generally disappeared in humans. In mice, the hyaloid vascular system continues to grow during the first five days after birth, but regresses quickly at the end of the first postnatal week and is essentially gone by the end of the second postnatal week. In both mice and humans the hyaloid vascular system regresses due to the apoptosis of endothelial cells and pericytes [1]. This regression coincides with the development of the retinal vascular system. Persistent fetal vasculature (PFV) is a term used to describe a congenital condition where the one or more components of the fetal intraocular vasculature fail to regress by birth [2]. This pathological condition can cause ocular complications including detached retina, cataract, angle closure, recurrent intraocular hemorrhages, and chronic uveitis. The presence of abnormal vessels in PFV is the major cause of complications in this disease. Pathological vessels in the vitreous is also a serious complication and a major cause of blindness in a number of other ocular diseases including sickle cell retinopathy, diabetic retinopathy, branch vein occlusion retinopathy, and retinopathy of prematurity. Combined, they are a leading cause of blindness in the United States. Consistent among these diseases, retinal blood vessel circulation is compromised resulting in poor blood supply to the retina. Hypoxia ensues, causing the expression of angiogenic growth factors, including vascular endothelial cell growth factor (VEGF) in the retina [3-5]. VEGF is expressed in the retina and it is a potent mediator of blood vessel growth, but it does not reinitiate blood vessel growth within the retina to replace the lost vessels. Over time, the elevated VEGF results in the growth of abnormal blood vessels located in membranous structures in the vitreous. As the disease progresses, the blood vessels in the vitreous acquire contractile cells (myofibroblasts) that pull the retina from the supporting pigmented epithelium, which results in vision loss [6-8].

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Leukemia inhibitory factor (LIF) is a member of the interleukin 6 (IL-6) family of cytokines, which also includes oncostatin-M, ciliary neurotrophic factor, interleukin-11, and cardiotrophin-1. Members of this family are grouped together based on activation of a common tyrosine kinase receptor, gp130 [9]. The high affinity receptor for LIF is a heterodimer between a low affinity LIF receptor (LIFRβ) and the tyrosine kinase gp130 [10]. On the surface of target cells LIF binds to and dimerizes the LIFRβ with gp130. Active gp130, phosphorylates itself and LIFRβ, which recruits and activates the Janus kinases (JAK2, and Tyk2), which then phosphorylate members of the signal transducer and activator of transcription (STAT) family, STAT1, and STAT3 [11,12]. Upon phosphorylation, the STATS dimerize and migrate to the nucleus where they activate transcription [13]. In addition, gp130 also signals through the mitogen activated protein kinase pathway (Erk1/2), and the phosphatidylinositol 3 kinase (PI3K) pathway [14,15].

LIF was first identified as a factor from conditioned media that could induce the differentiation of murine myeloid leukemia cells in culture, and was initially cloned from a murine T cell library [16,17]. LIF has a low basal expression but has been shown to be up regulated at focal sights of inflammation and increased systemically in serum following septic shock [18,19]. The expression in the immune system has been localized to activated T-cells, monocytes, and macrophage [20,21]. This expression pattern suggests that LIF may play a role during acute inflammatory stress. LIF is also expressed in the central nervous system (CNS), where it appears to serve a role in astrocyte differentiation, and is a protective cytokine during the inflammatory stress response [22-26].

LIF expression is clearly linked to inflammatory responses, which suggests that LIF may also have an affect on vascular endothelial cells. In several studies, LIF has been shown to alter vascular growth and differentiation in vitro. For example, LIF has been identified as a protein factor secreted by pituitary follicular cells, which acts as an inhibitor of bovine aortic endothelial cell proliferation [27]. However, the effect is dependent on cell type because LIF does not inhibit the proliferation of adrenal cortex capillary endothelial cells [27]. Previous studies have suggested that LIF could block arterial angiogenesis but not microvascular angiogenesis. Other studies have shown that LIF in combination with basic fibroblast growth factor (bFGF) could program an immortalized embryonic cell line to form primitive blood vessels in vitro, and it was found that the reprogrammed cells could participate in blood vessel development in vivo [28]. These studies suggest that LIF can inhibit some angiogenesis and possibly promote vasculogenesis. However, they have not shown how LIF would affect vascular development in vivo.

To study how LIF regulates vascular development in vivo, we have utilized the αA-crystallin promoter to drive lens specific expression of LIF in transgenic mice. This study demonstrates that the expression of LIF can block both vasculogenic and angiogenic vascular development in the eye. Interestingly, the inhibitory effects of LIF appear to be partially overcome by increased expression of VEGF, which leads to extensive neovascularization within a persistent fetal vasculature in the eye. This caused the formation of a contractile vascular membrane and subsequent tractional retinal detachment.

METHODS

Animal breeding: The αA-LIF transgenic mice were generated in the FVB mouse strain. The FVB/N mice are homozygous for the $Pde6b^{rd1}$ mutation (formerly) in the cGMP-phosphodiesterase β-subunit. As a result, these mice undergo rapid photoreceptor degeneration beginning at postnatal day 9 [29]. To avoid this complication, our transgenic mice were crossed with Balb/C mice to produce F1 offspring that are heterozygous for the $Pde6b^{rd1}$ mutation. $Pde6b^{rd1}$ is an autosomal recessive disorder; therefore, the F_1 heterozygotes will not undergo retinal degeneration. All animal work was in strict accordance with both the NIH Guide for the Care and Use of Laboratory Animals and the animal use guidelines of Association for Research in Vision and Ophthalmology (ARVO). The animal protocols were approved by the IACUC of the University of Oklahoma Health Sciences Center and the Dean A. McGee Eye Institute.

Screening of transgenic mice: Newly weaned mice were genotyped by collecting 2 mm diameter tissue samples from the tip of the left ear during the placement of a sequentially numbered tag for permanent identification of each mouse. Genomic DNA was isolated from ear punches by incubating in 15 μl of proteinase K buffer (100 mM EDTA, 50 mM Tris-HCl (pH 8), 0.5% SDS, 1.3 mg/ml proteinase K), for 20 min at 55 °C. We then added 300 μl of water to each sample and boiled them for 5 min to inactivate proteinase K. To identify transgenic mice, PCR was carried out on 1 μl of genomic DNA using a sense primer (5'-CTC ATG AAC CAG ATC AGG AGC CAA-3') and an antisense primer (5'-TTG ACA GCC CAG CTT CTT CTT CTG-3'). These primers are specific to the human LIF cDNA (BC069540). They will amplify LIF between nucleotides 180 through 608. Since the site of integration can influence transgene expression, two independent transgenic families were analyzed for this study, and both had identical changes in retinal vascular development.

Dissecting whole retinas: Mice were euthanized by CO_2 inhalation and the eyes enucleated. The cornea was pierced using a scalpel to relieve ocular pressure. Dumont number 5 forceps were then used to grasp the cornea, and the anterior chamber was removed using Vannas spring scissors. The lens was removed and the eyecup was fixed in either cold 2% paraformaldehyde in cacodylate buffer overnight or 4% paraformaldehyde-phosphate buffered saline (PBS) for 30 min, according to either the ADPase or the lectin staining procedures, respectively.

ADPase enzyme histochemistry of whole retinas: ADPase enzyme histochemistry of whole retinas Adenosine diphosphatase (ADPase) activity is highly specific to the retinal vasculature and is also expressed in retinal angioblasts in dog and humans [30-32]. Prior to retinal dissection the fixed eye cups were washed three times (10 min each) in 15% sucrose/0.1 M cacodylate buffer, then three times (10 min each) in 10% sucrose/0.1 M cacodylate buffer. The retinas were dis-

sected and washed overnight in 10% sucrose/0.1 M cacodylate buffer at 4 °C. The next morning retinas were washed three times (10 min each) in 5% sucrose/0.1 M cacodylate and 1% Triton X-100. ADPase enzyme histochemistry was performed by incubating the retinas at 37 °C for one h in 0.2 M Tris-maleate (pH 7.2), 3 mM lead nitrate, 6 mM magnesium chloride, 1% Triton X-100, and 1 mg/ml ADP. The retinas were then washed once in 5% sucrose, 0.1 M cacodylate, with 1% Triton X-100, then washed twice in 5% sucrose in 0.1 M cacodylate buffer before they were flat mounted onto glass slides for microscopy. In flatmounts, ADPase positive cells appeared white under dark field illumination due to the lead ADPase reaction product. The stained retinas were then flat embedded in glycol methacrylate. After imaging the vasculature, the areas of embedded tissue of interest were sectioned at 2 μm, and ADPase activity was developed in 2% ammonium sulfide for 30 s [31]. Sections were then counterstained with toluidine blue and viewed by light microscopy. In the sections, stained angioblasts and endothelial cells appeared brown/purple following counterstaining.

Griffonia simplicifolia (GS) lectin staining: For GS lectin staining, retinas were dissected from the fixed eye cups in cold PBS, then stored in (4:1) methanol:dimethyl sulfoxide (DMSO) overnight at -20 °C [33]. The retinas were incubated with methanol:DMSO:H_2O_2 (4:1:1), to inactivate endogenous peroxidases. The retinas were rehydrated by washing them three times in PBS for 10 min each. The retinas were incubated overnight at 4 °C with a biotinylated GS lectin (Vector Labs, B-1025, Burlingame, CA) diluted 1:200 in PBS. The next day the retinas were washed 3 times in PBS (10 min each wash) and incubated 4 h at room temperature in a solution containing a peroxidase avidin biotin complex (Vector Laboratories). The retinas were washed 3 times in PBS (10 min each wash), and the blood vessels were detected by incubation in a solution containing diaminobenzidine-H_2O_2 (DAB) and nickel (Vector laboratories). The retinas were then washed in PBS and flat mounted on glass slides in PBS:glycerol 1:1 for image analysis. The retinas are stable for viewing and imaging for 1 month.

Immunostaining: To detect laminin and VEGF, whole eyes were processed and paraffin embedded for sectioning. Sections on glass slides were deparaffinized and rehydrated. Tissue sections were then pre-digested with Ficin diluted 1:200 in PBS (Sigma; F-4125) for 30 min at 37 °C to unmask the antigen [34]. Sections were washed three times in PBS then incubated in blocking solution (10% horse serum in PBS) for one h at room temperature. Sections were then incubated overnight at 4 °C with a rabbit anti-laminin antibody (Sigma St. Louis, MO; catalog number L9393) diluted 1:30, or with rabbit anti-VEGF antibody (Santa Cruz, Santa Cruz, CA; catalog number sc-7269) diluted 1:500 in blocking solution as above. Sections were washed three times in PBS and then incubated with the appropriate secondary antibody. To detect laminin, sections were incubated for 1 h at room temperature with an FITC labeled anti-rabbit secondary antibody (Sigma) diluted 1:200 in blocking solution. After the final wash all slides were mounted in 50% glycerol-PBS containing 2 μg/ml DAPI (Sigma) to label nuclei. The fluorochromes were observed using a fluorescence microscope (Nikon, Lewisville, TX, model number E800). To detect VEGF, sections were incubated with a biotinylated anti-rabbit antibody (Vector Labs) diluted 1:200 in blocking solution as above. The sections were washed in PBS, then VEGF expression was detected by incubating in a solution containing diaminobenzidine-H_2O_2 (DAB, Vector Laboratories).

In situ hybridization: In situ hybridization was performed on tissue sections to measure VEGF mRNA expression. Tissues were fixed overnight in 10% neutral buffered formalin, and then dehydrated in a graded ethanol series prior to paraffin embedding. Tissue sections (5 μm thick) were dried on poly-lysine coated slides, and deparaffinized prior to rehydration in a graded ethanol series. Sections were pretreated with 20 μg/ml proteinase K to permeabilize tissue. RNA probes were generated using T3 RNA polymerase to transcribe a linearized plasmid that contained a 450 bp fragment of the mouse VEGF cDNA (obtained from Dr. Warner Risau). The probes were radiolabeled using ^{35}S-UTP (Amersham, Piscataway, NJ). Hybridization and washing were performed as described previously [35]. After the final wash, slides were dipped in Kodak NTB-2 nuclear emulsion diluted 1:1 with 0.6 M ammonium acetate and exposed at 4 °C for one to two weeks. Slides were lightly counterstained with hematoxylin. Additional hybridizations were carried out with sense probes as a control for background. The darkfield images were captured using a Roper digital camera (Roper Scientific, Trenton, NJ), and Metamorph image analysis software version 5.0r1 (Universal Imaging Corporation, Downingtown, PA).

RESULTS

Transgenic expression: To study the effects of LIF during early retinal development, the αA crystallin promoter was used to drive the expression of human LIF. This promoter drives expression specifically in the ocular lens from embryonic day 10 and continues throughout the life of the mouse [35,36]. Five transgenic mouse lines were generated that had ocular phenotypes, designated OVE-736, -774, -775, -773 and, -777. Eyes from each of the five families were analyzed by histology at three weeks of age, and all had dramatic changes in retinal vascular development. To determine the effects of LIF on retinal vascular development, we have performed detailed analysis of two transgenic families designated 736 and 774. We have confirmed that transcription of transgenic LIF is restricted to the lens by in situ hybridization (data not shown). To demonstrate that LIF secreted by the lens is taken up by cells in the retina, we performed ELISA analysis on total proteins extracted from dissected retinas. The levels of LIF in postnatal day (P)7 retinas were measured at 91 pg LIF/mg protein in 736 and 5.3 pg LIF/mg protein in 774. However, both families had identical changes in retinal vascular development. We did not detect LIF in non-transgenic retinas. The results demonstrate that the phenotype is LIF dependent and that the lower levels of LIF in the 774 mice are sufficient to cause a vascular phenotype. The representative data in this manuscript is from the 736 line.

To determine how LIF alters retinal vascular development, we collected retinas from non-transgenic and LIF transgenic mice in a developmental series from P3 to P21, and analyzed the retinal vasculature in flatmounts using Griffonia simplicifolia lectin isotype B4 (GS lectin). In normal retinas (top row), the vessels begin to grow out from the optic nerve head (center of the retina) at birth, and by P3 they have spread to about midway to the outer margins. By P5 the vessels have nearly grown to the margin and have reached the margins by P7. In contrast, growth of the surface vessels does not occur in the LIF transgenic eyes (bottom row). The retinas from P3 to P7 demonstrated that LIF blocked the development of the

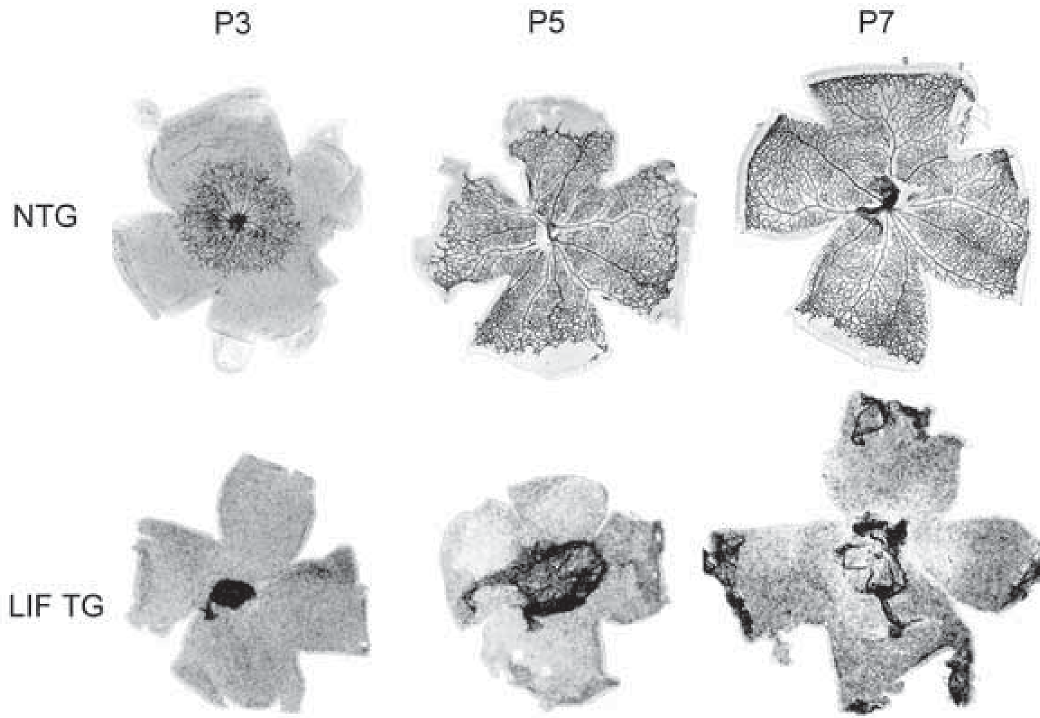

Figure 1. LIF blocks the development of the initial retinal vasculature. Retinas were dissected from the eyes and the blood vessels were stained using Griffonia simplicifolia (GS) lectin. After staining, the retinas were flat mounted on glass slides. The image shows retinas from P3, P5, and P7 mice from non-transgenic retinas (NTG), and LIF transgenic retinas (LIF TG).

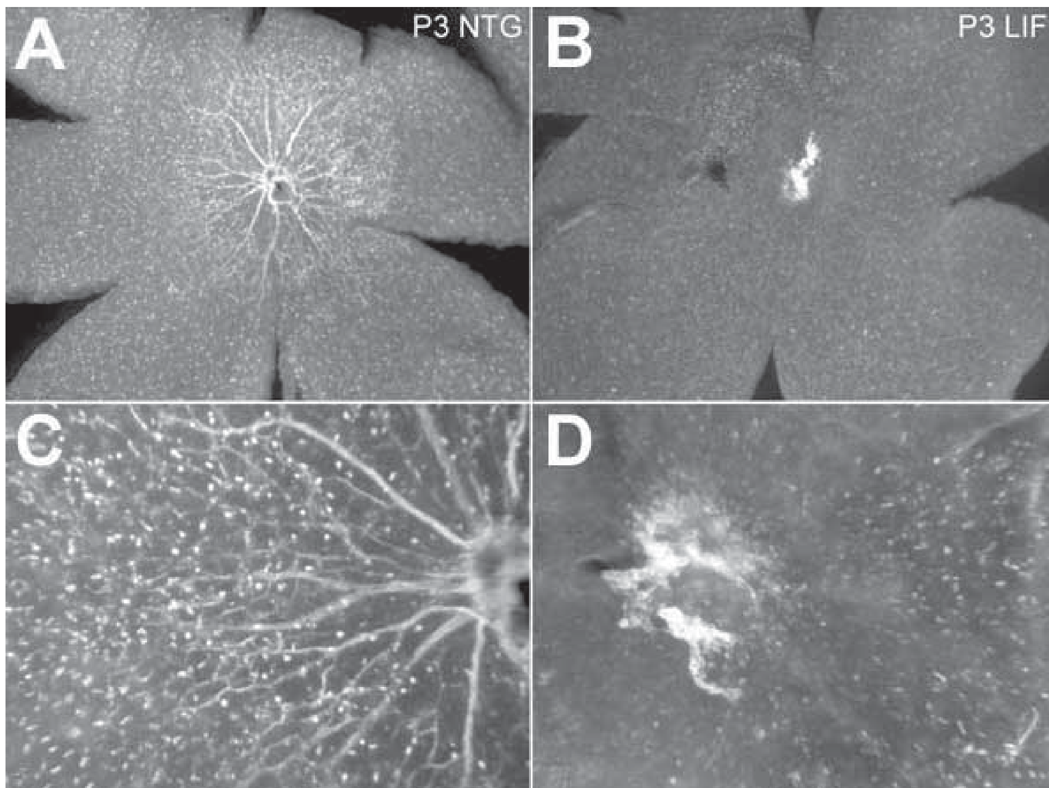

Figure 2. Reduction of ADPase positive cells in transgenic retinas. ADPase incubated retinas from non-transgenic (**A**,**C**) and LIF transgenic animals (**B**,**D**) at P3. The hyaloid vessels in the vitreous were removed to demonstrate the density of ADPase positive angioblasts and endothelial cells in the retina. Higher magnifications of the optic nerve heads are shown in the lower panels (**C**,**D**). We observed marked reduction of ADPase positive cells in the LIF transgenic retinas.

superficial blood vessels, which normally form during this developmental period (Figure 1). We have analyzed retinas from 12 different transgenic mice at P3 and all 12 retinas lacked retinal blood vessels. Also the vessels are absent in transgenic mice at P5 and P7. Growth of the superficial retinal vasculature does not occur at any age in the LIF transgenic mice (bottom row, Figure 1). After P5 there is an accumulation of vascular membranes in the vitreous, but not before. The stained blood vessels in the P7 retinas are remnants of the VHP and are located in the vitreous.

LIF is a known inhibitor of cell differentiation [37-39], and it is possible that LIF blocked the differentiation of

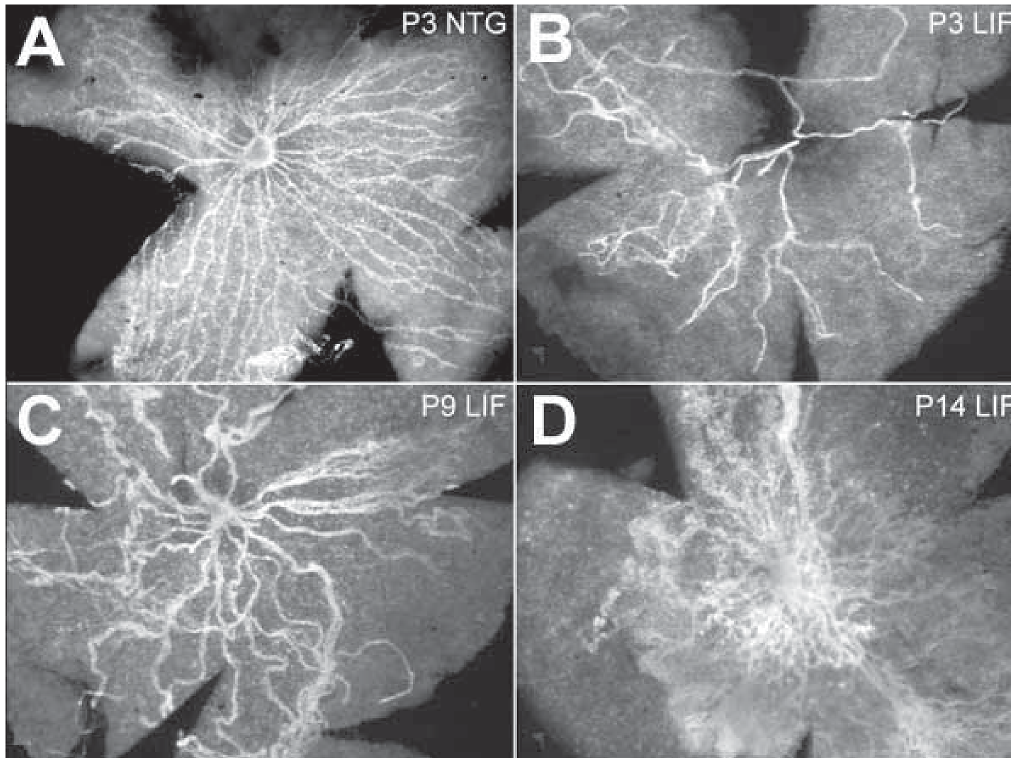

Figure 3. ADPase incubated retinas with vitreous intact. The eyes were dissected to leave the vitreous and posterior fetal vasculature intact and the retina and vitreous were stained for ADPase activity. At P3, the vasa hyaloidia propria in transgenic mice was smaller than normal; at older postnatal ages, it persisted and continued to expand. **A**: Non-transgenic P3 retina. **B**: P3 LIF transgenic retina. **C**: P9 LIF transgenic retina. **D**: P14 LIF transgenic retina.

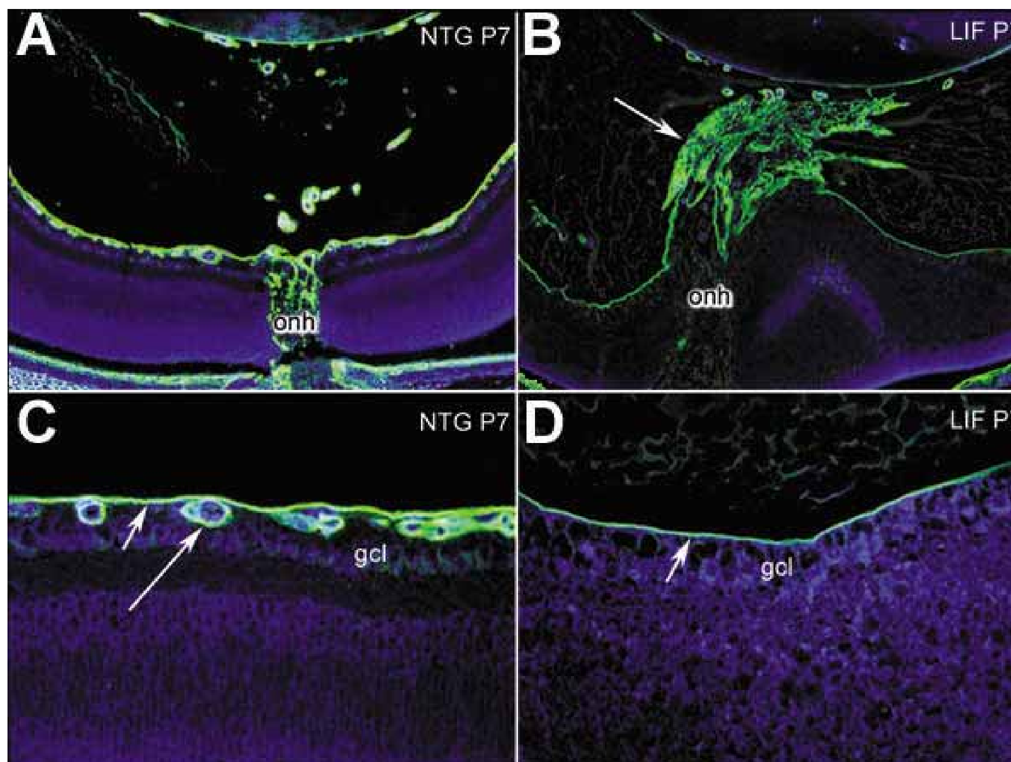

Figure 4. Retinal blood vessels are absent in transgenic mice. P7 retinas were stained by immunofluorescence using an anti-laminin antibody to label the basement membrane of the vasculature and inner limiting membrane of the retina. In non-transgenic eyes (**A**,**C**), the blood vessels (**C**, long arrow) are present in the retina just below the inner limiting membrane (**C**, short arrow). A tuft of laminin positive tissue was observed near the optic nerve head (onh), which extended into the vitreous to the lens (**B**, arrow). However, no blood vessels were found in the retina, underneath the inner limiting membrane (**D**, arrow). "gcl" refers to the ganglion cell layer in **C**,**D**.

angioblasts into vascular endothelial cells. To investigate this possibility, we analyzed P3 retinas for ADPase enzymatic activity, a cell marker that has been used to identify both angioblasts and endothelial cells [30-32]. In the ADPase stained retinas from non-transgenic control mice (Figure 2A,C), ADPase positive retinal blood vessels were located in the peripapillary region extending several disk diameters out from the nerve head. Additionally, ADPase positive solitary cells, presumably angioblasts, were observed throughout the entire extent of the inner retina within and in advance of the formed vasculature (Figure 2C). In αA-LIF transgenic mice, no ADPase positive retinal blood vessels were observed (Figure 2D,C), and fewer ADPase positive solitary cells were labeled within the retina (Figure 2D). The analysis of αA-LIF transgenic retinas from P3 to P10 (n>3 at each age) demonstrates that the superficial retinal vasculature is developmentally inhibited. LIF not only blocked the development of the retinal vessels, but also initially caused the VHP to be less extensive than normal (compare Figure 3A,B). However, in older animals, where normally the VHP has regressed by P9, whole mounts of αA-LIF transgenic mice had what appeared to be an abnormal persistence of the fetal vasculatures (Figure 3C) followed by proliferation of endothelial cells and expansion of the pre-retinal vasculature (Figure 3D). To confirm that blood vessels were in the vitreous and not in the retina, we immunostained basement membranes using an anti-laminin antibody (Figure 4). In the non-transgenic retina the laminin antibody simultaneously stained the basement membranes of blood vessels in the retina (long arrow, Figure 4C) and the inner limiting membrane of the retina (short arrow, Figure 4C). With this analysis we could clearly distinguish blood vessels located in the vitreous (vessels above the inner limiting membrane of the retina) from those located in the retina (underneath the inner limiting membrane). In the retinas from the αA-LIF transgenic mice, blood vessels were stained in the vitreous (Figure 4B), but we did not detect blood vessels underneath the inner limiting membrane in the retina (Figure 4D). This confirms that all vascular structures observed in whole mounts of the αA-LIF transgenic mice at P7 and earlier are in the vitreous and not in the retina.

In transgenic mice from P9 to P14 we observed substantial angiogenesis within the vitreous (Figure 3). We observed many mitotic endothelial cells (data not shown), demonstrating that neovascularization is due to endothelial cell proliferation of a persistent fetal vitreous vasculature (Figure 3D). The observation of pre-retinal vessels by ADPase staining is consistent with the data from retinas stained with GS lectin (Figure 1) and the sections stained with the laminin antibody (Figure 4). Expansion of the blood vessels in the vitreous continued over time, and by P21 neovascular membranes were prominent features in the vitreous of transgenic mice (Figure 5B). In contrast, no membranes are present in non-transgenic mice (Figure 5A). The membranes are reminiscent of contractile epiretinal membranes (ERMs) that form during the course of proliferative vitreoretinopathy (PVR) in humans. Nearly half of the transgenic eyes had complete retinal detachment by P21 (Figure 5C,D), The contractile membranes contain numerous blood vessels and fibroblastic cells orga-

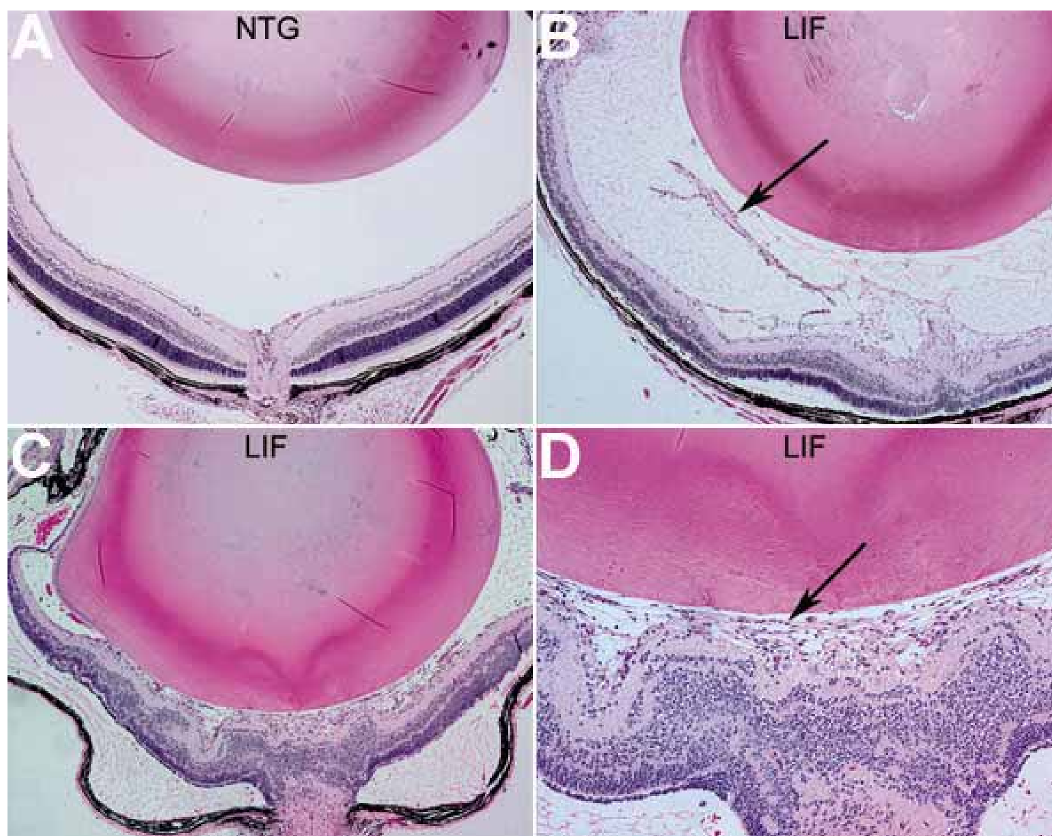

Figure 5. Transgenic eyes have vascular membranes in the vitreous. Hematoxylin and eosin stained images are shown of a non-transgenic eye (**A**), and LIF transgenic eye (**B**) at P21. In the non-transgenic eye, the hyaloid vascular system has regressed. However, blood vessels are evident in the vitreous in the transgenic eyes (**B**, arrow). Retinal detachment from the RPE and subsequent attachment to the lens was often observed in transgenic animals (**C**). In such eyes, the lens and retina are separated by blood vessels and a fibrous matrix (**D**, arrow).

nized into a membranous matrix (Figure 5D). The vascular membranes are present in adult αA-LIF transgenic mice, and retinal detachment becomes progressively more common.

Surprisingly, in P21 and older mice we frequently observe branches from the larger blood vessels in the vitreous passing through the inner limiting membrane to enter the retina (Figure 6A-C). To characterize this unusual invasive retinal vascularization, we analyzed ADPase incubated adult retinas in the flat perspective and sectioned the fellow eyes (Figure 6). Transgenic retinas had numerous blood vessels in the vitreous at P21 (Figure 6B,D-F), but they also had capillaries located deep into the retina (Figure 6E-F). The retinal capillaries were localized to the middle of the retina at P21 but were not present at or before P14. The origin of these deep capillaries was due to invasive growth of vessels from the neovascular network in the vitreous (Figure 6B,C,E,F). Identical results were obtained using lectin stained retinas (data not shown).

A likely mechanism for the explosive postnatal expansion of blood vessels in the vitreous of the αA-LIF transgenic mice is the upregulated expression of VEGF by the avascular retina. To evaluate this possibility, we analyzed the expression of VEGF by in situ hybridization and immunohistochemistry (Figure 7). In normal retinas, expression of VEGF in cells in the nerve fiber layer that appear to be astrocytes (Figure 7A) is thought to drive vascularization of the inner retina, and VEGF expression in the Müller cells located in the inner nuclear layer (Figure 7A,B) is thought to drive vascularization of the inner retina [40,41]. In the P6 non-transgenic retinas, VEGF expression was detected in the nerve fiber layer (presumed astrocytes) and in the inner nuclear layer (Figure 7A,B). In the transgenic retinas, VEGF expression was elevated in the nerve fiber layer and throughout the retina (Figure 7C,D). To quantify the relative expression levels, darkfield digital images were captured from the microscope and the signal intensities were measured using Metamorph image analysis software. Relative to the normal retina, VEGF expression in the αA-LIF transgenic mice was three fold higher prior to expansion of the fetal vasculature by neovascularization. The increased number of blood vessels in the vitreous of older αA-

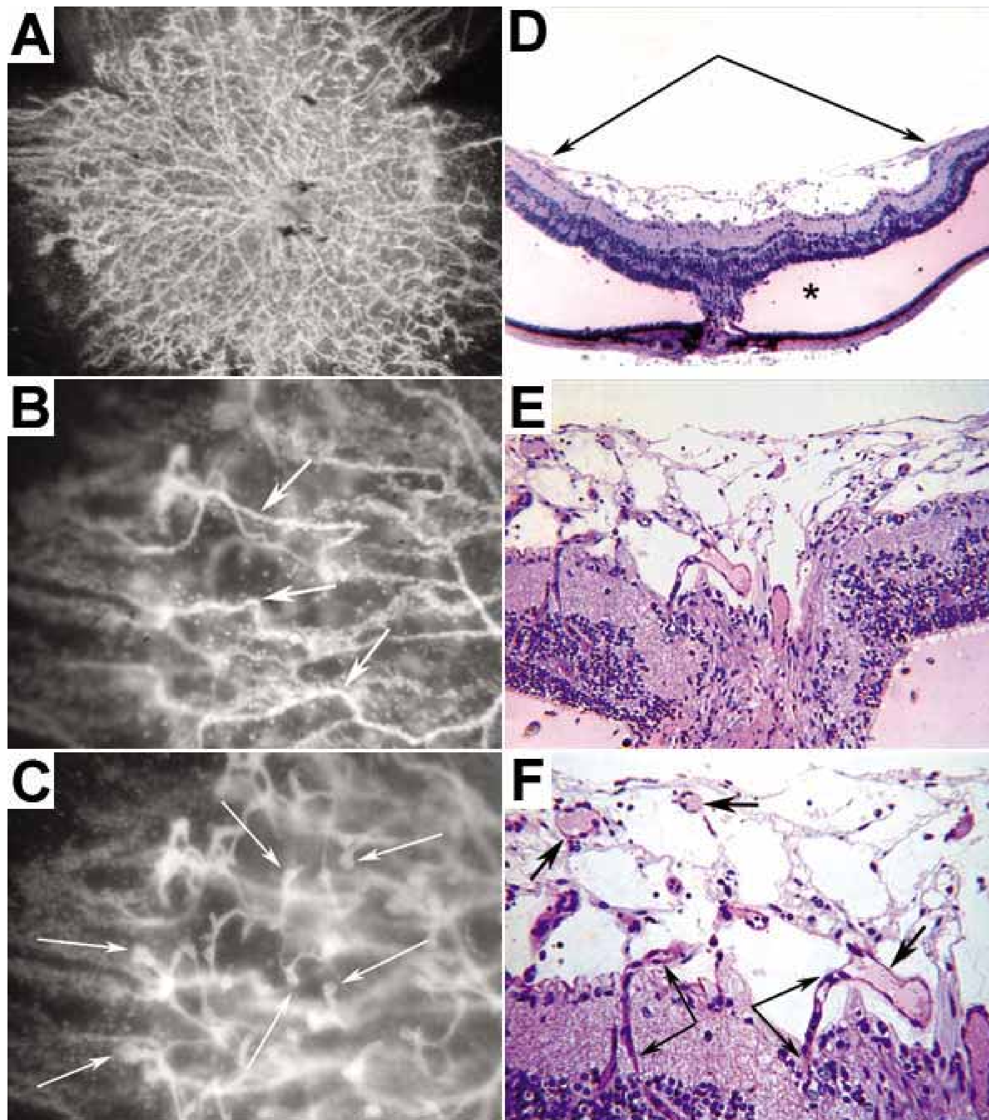

Figure 6. Proliferation of neovascular membranes in older transgenic retinas. An ADPase incubated P21 LIF Tg retina that has been flat embedded and viewed en bloc (**A**-**C**). The fellow eye was embedded whole in JB-4 and then sectioned (**D**-**F**). **A**: A dense pre-retinal neovascular membrane. **B**: Higher magnification of the neovascular membrane in **A** with focus specifically on the pre-retinal blood vessels (arrows) at the vitreoretinal interface. **C**: The same area at high magnification but focused on the budding of the vasculature and invasion into the inner retina. **D**: Retinal detachment due to traction on the retina (double arrows) by the pre-retinal membrane. There is eosinophilic subretinal fluid (*) associated with the retinal detachment. **E**-**F**: Higher magnification of a section through the optic nerve showing capillaries invading retina (double arrows) from the pre-retinal neovascular membrane (arrow).

LIF transgenic mice was consistent with elevated VEGF protein levels. To analyze VEGF protein expression, we used immunohistochemistry on tissue sections. In the non-transgenic P14 retinas, VEGF protein was found in the ganglion cell layer and in the RPE and choroid (Figure 7E). In contrast, the P14 αA-LIF transgenic retinas had high levels of VEGF protein in the inner nuclear layer, inner plexiform layer, ganglion cell layer, RPE, and choroid (Figure 7F).

DISCUSSION

We have found that transgenic expression of LIF causes a cascade of changes in retinal vascular development. The first change was a reduced hyaloid vasculature at birth and the absence of retinal blood vessels during the first two postnatal weeks. Since blood vessels were not observed in 1-week-old and 2-week-old transgenic retinas, LIF caused a failure of blood vessel development rather than induced vascular regression. Retinal vascular development did not initiate during the first postnatal week despite the expression of VEGF in Müller cells and astrocytes. At the end of the second postnatal week, we observed elevated expression of VEGF. The increase in VEGF was sufficient to cause the formation of neovascular membranes in the vitreous. The neovascular membranes were contractile, and caused retinal detachment following the third postnatal week. It is highly unlikely that the Pde6b^{rd1} mutation is impacting the phenotype in the LIF mice. Pde6b is not expressed until P7 and then only in retinal photoreceptors. The inhibition of retinal vascular development occurs from P0 to P7, before the onset of Pde6b expression.

Cells that respond to LIF: In a recent study, cells in the nerve fiber layer stain strongly with antibodies to one of the LIF receptors, gp130 [42]. This layer contains both retinal astrocytes and the superficial retinal vasculature. The signaling components JAK2, Tyk2, Erk1/2, STAT1, and STAT3 are coexpressed in cells with the gp130 receptor and can be activated by ligands of gp130 [42,43]. During the P0 to P7 ages, vascular endothelial cells, retinal astrocytes, and ganglion cells all express the gp130 receptor, which can be activated by LIF or CNTF [42,43]. Therefore, the inhibition of retinal vascular development is caused by a response of four candidate cells, retinal astrocytes, ganglion cells, vascular endothelial cells, or angioblasts. Later in development, the remaining retinal neurons and Müller glial cells also express LIF receptors and signaling components.

Anti-angiogenic and pro-angiogenic effects of LIF: The obvious suppression of vascular development in these

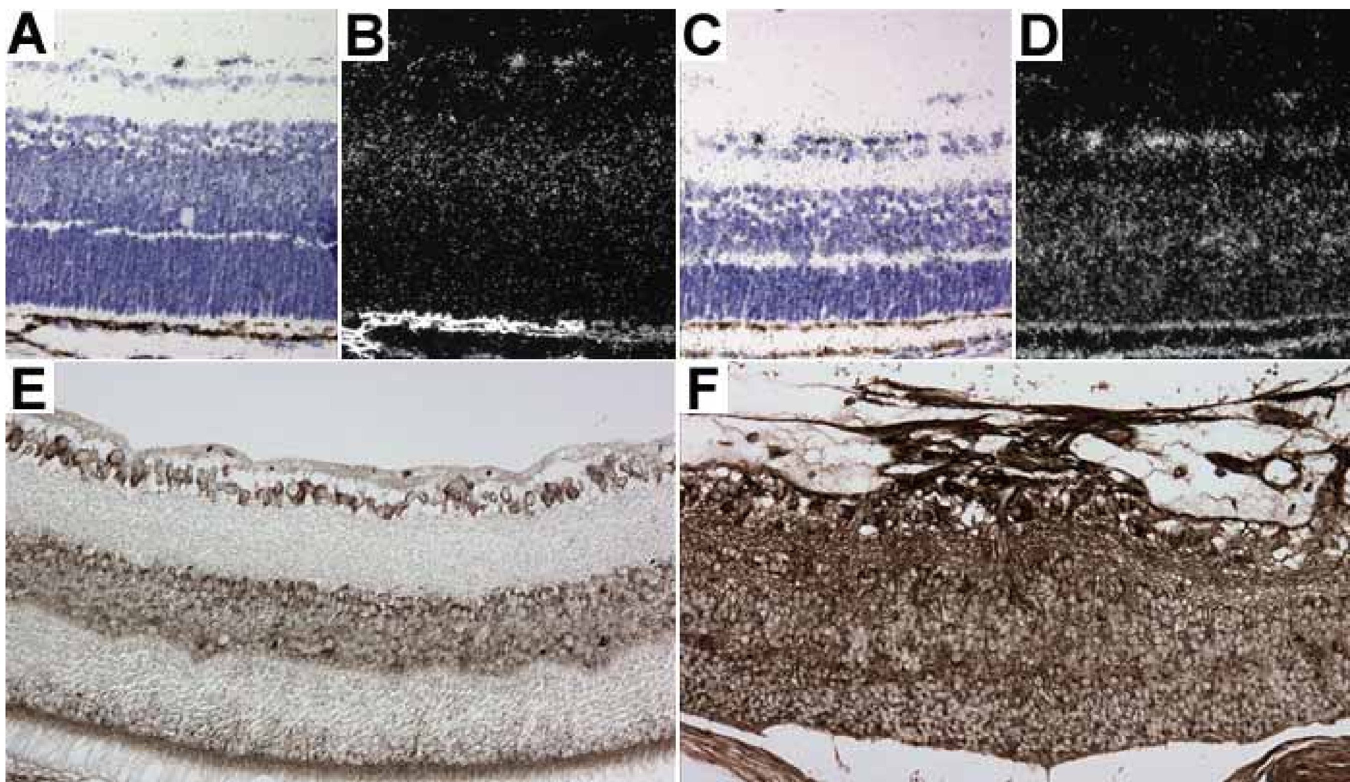

Figure 7. VEGF expression is up regulated in LIF transgenic retinas. VEGF mRNA expression was observed by in situ hybridization (**A**-**D**). Brightfield images (**A**,**C**) showing the retinal cell layers. Darkfield images (**B**,**D**) showing localization of VEGF mRNA expression. In non-transgenic P6 retinas (**A**,**B**), the astrocytes and the Müller cells located in the inner nuclear layer were positive. In the LIF transgenic P6 retina (**C**,**D**), we observed higher levels of VEGF mRNA expression in Müller cells and astrocytes, but we also observed high expression in photoreceptors and inner retinal neurons. VEGF protein was localized by immunohistochemistry. In normal P14 retinas, VEGF protein was found in the ganglion cell layer (GCL) and in the RPE and choroid (**E**). In P14 LIF transgenic retinas (**F**), VEGF protein was markedly increased throughout the retina and in the vascular membrane.

transgenic mice suggests that LIF is an anti-angiogenic factor; however, the subsequent neovascularization in the vitreous of the αA-LIF mice suggests that LIF is pro-angiogenic. We know from the ELISA data that levels of LIF in the transgenic retinas do not decrease over the first four weeks following birth. This demonstrates that the switch in vascular phenotype is not due to decreased LIF expression over time. Instead, we have observed that the neovascularization occurs after increased VEGF expression in the avascular transgenic retinas. The most plausible explanation of these combined data is that LIF is anti-angiogenic, and that elevated VEGF can partially overcome the effects of LIF. There are two possible causes of the increased VEGF. It is possible that LIF stimulation of retinal cells directly stimulates expression of VEGF, or more likely that the ischemia in the avascular retina leads to elevated VEGF expression.

It is important to note that even though elevated VEGF could override the inhibition of angiogenesis, the blood vessels that eventually form did not follow the normal temporal or spatial pattern of growth in the nerve fiber layer. Instead the vessels invaded the retina from the neovascular membranes in the vitreous and formed a secondary or capillary network in the deep retina. This suggests that elevated VEGF overrides the growth inhibition caused by LIF, but cannot correct the deficiencies of normal retinal vascular development in the nerve fiber layer.

Vascular development in the nerve fiber layer is thought to be initiated by vasculogenesis with subsequent growth by angiogenesis [24,44]. If LIF is more effective at inhibiting vasculogenesis than angiogenesis, then it is possible that LIF blocked the vasculogenic events in ocular vascular development but was less effective at blocking angiogenic growth. The fact that LIF reduced the development of the embryonic hyaloid vascular system, which may also be initiated by vasculogenesis [35], further suggests that LIF inhibits vasculogenesis. In vitro studies have suggested that LIF may promote the differentiation of embryonic stem cells to form angioblasts [28]. We did not observe this affect in our LIF transgenic mice. In the LIF retinas at P3, we had fewer ADPase positive cells compared to normal mice at P3. This suggests that LIF prevented their migration to the retina, or that LIF prevented angioblast differentiation. LIF is a known inhibitor of cellular differentiation [45]. Therefore, inhibition of angioblast differentiation is more consistent with the biological activity of LIF.

Alternatively, LIF may prevent the growth and differentiation of vascular endothelial cells during retinal angiogenesis. While there is no evidence that LIF affects quiescent endothelial cells, in vitro assays have been used to show that LIF can block VEGF or bFGF induced proliferation of bovine aortic endothelial cells or bovine microvascular endothelial cells in culture [46]. However, we could not measure an effect of LIF on endothelial cell proliferation in retina since there were no endothelial cells in the retinas of LIF transgenic mice (as shown by lectin and ADPase staining).

Retinal astrocytes in mice have been suggested to play a role in vascularization of the nerve fiber layer [47]. We have observed that LIF did not alter the early development or expression of GFAP and S100 in retinal glia (data not shown), and did not prevent astrocytes from expressing VEGF. Therefore, if the vascular defect in the LIF transgenic mice is related to astrocytes, it is independent of their migration or expression of VEGF.

αA-LIF mice as new model of persistent primary vitreous and tractional detachment: In postnatal αA-LIF mice, the attenuated hyaloid vascular system fails to regress, and eventually undergoes extensive proliferation probably in response to rising VEGF. As a result, LIF transgenic mice develop a neovascular membrane that contracts and causes retinal detachment in 100% of the adult transgenic mice. This establishes αA-LIF mice as a genetic mouse model of persistent fetal vasculature (PFV) and tractional retinal detachment. There are several possible mechanisms that could work alone or in combination to explain the persistent fetal vasculature. In normal mouse development, the retinal vasculature initiates growth at the time of birth just as the hyaloid system stops growing and begins regression [35]. It is possible that the rising VEGF levels provide a survival signal to the endothelial cells of the fetal vasculature. Persistence of the fetal vasculature has been associated with an attenuated retinal vasculature in neonatal mice exposed to hyperoxia for long periods [48]. This has been attributed to reduced blood flow through the forming retinal vasculature, which results in blood flow passing through the hyaloid vasculature. The persistent flow of blood through the hyaloid system would result in sufficient circulation to promote survival of hyaloid endothelial cells. In our LIF transgenic mice the retinal vasculature fails to grow and as a result all blood flowing into the eye circulates through the hyaloid system. As a result, the hyaloid system would receive sufficient circulation to survive. During the second week, the rising VEGF levels in the LIF mice would induce neovascularization in the vitreous causing it to expand and form a contractile membrane. Identifying the molecular mechanism underlying vascular suppression in the LIF transgenic mice will ultimately prove useful in identifying the mechanisms of normal vascular development in the mouse nerve fiber layer of the retina. These mice are, therefore, an interesting model in which to study the development of the retinal vasculature, persistence of fetal vasculature (PFV), and the mechanism for formation of contractile membranes. These mice can also be used to test therapeutics that may prevent the formation of pre-retinal membranes and initiate their contraction. At the basic science level, the LIF transgenic mice may hold a key to distinguishing vasculogenic from angiogenic processes because retinal vascular development is inhibited while angiogenic invasion of the retina occurs.

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