Developmental Biology **237,** 45–53 (2001)
doi:10.1006/dbio.2001.0360, available online at http://www.idealibrary.com on IDEAL®

Elevated TGFβ Signaling Inhibits Ocular Vascular Development

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Alterations in the ocular vasculature are associated with retinal diseases such as retinopathy of prematurity and diabetic retinopathy. Vascular endothelial growth factor (VEGF) as a potent stimulator for normal and abnormal vascular growth has been extensively studied. However, little is known about secreted factors that negatively regulate vascular growth in ocular tissues. We now report that expression of a self-activating TGFβ1 in the ocular lens of transgenic mice results in inhibition of retinal angiogenesis followed by retinal degeneration. Transgenic TGFβ1 can rescue the hyperplasic hyaloid tissue and reverse the corneal deficiency in TGFβ2-null embryos. These results demonstrate that TGFβ signaling modulates development of ocular vasculature and cornea in a dosage-dependent manner and that TGFβ1 can substitute for TGFβ2 in ocular tissues.

Key Words: **vascular development; retinal angiogenesis; hyaloid vasculature; TGFβ signaling; TGFβ2; cornea.**

INTRODUCTION

Neovascularization in the eye causes severe visual impairment in a number of diseases, including retinopathy of prematurity, diabetic retinopathy, and the wet form of age-related macular degeneration (Campochiaro, 2000). It has been proposed that normal or abnormal growth of blood vessels in the retina is driven by hypoxia and/or hypoglycemia (Stone and Maslim, 1997). The proliferative effect of hypoxia/hypoglycemia on vascular endothelial cells is thought to be mediated by VEGF (Stone and Maslim, 1997). However, whether there are secreted proteins in the eye that can counterbalance the effects of VEGF and modulate ocular angiogenesis remains unknown. Identification of such negative regulators could have implications for treatment of ocular diseases caused by abnormal angiogenesis.

Members of the transforming growth factor family (TGFβ1, TGFβ2, and TGFβ3) are multifunctional proteins that regulate cell growth, differentiation, migration, and extracellular matrix production and play important roles in embryonic development, wound healing, immune responses, and vascular development (Massague, 1998; Blobe *et al.*, 2000). TGFβ proteins are secreted from cells as high-molecular-weight latent complexes in which C-terminal mature homodimers are associated with inhibitory dimers of the N-terminal pro-region (known as the latency-associated peptide) (Khalil, 1999). Abnormalities in TGFβ function cause or contribute to a number of human diseases, including fibrosis, cancer, hereditary hemorrhagic telangiectasia, atherosclerosis, and various developmental defects (Blobe *et al.*, 2000). Animal studies have shown that TGFβ can be either angiogenic or anti-angiogenic. TGFβ can be a potent inducer of angiogenesis when administered subcutaneously to mice or rats (Roberts *et al.*, 1986; Sprugel *et al.*, 1987; Rubbia-Brandt *et al.*, 1991; Frank *et al.*, 1994), when applied to the chick chorioallantoic membrane (Yang and Moses, 1990) or the rabbit cornea (Phillips *et al.*, 1992, 1993), or in the disc angiogenesis system (Fajardo *et al.*, 1996). In contrast to the above findings, TGFβ1 has been shown to inhibit vascular tumor growth (Dong *et al.*, 1996) and FGF-induced angiogenesis (Passaniti *et al.*, 1992). Tissue-specific overexpression of TGFβ in transgenic mice does not induce angiogenesis (Nabel *et al.*, 1993; Jhappan *et al.*, 1993; Pierce *et al.*, 1993; Lee *et al.*, 1995; Sanderson *et al.*, 1995; Wyss-Coray *et al.*, 1995; Sellheyer *et al.*, 1995; Zhou *et al.*, 1996). It has been shown that all three members of the TGFβ family and their type I and II receptors are expressed in developing and adult rodent eyes (Srinivasan *et al.*, 1998; Gordon-Thomson *et al.*, 1998; Obata *et al.*, 1999).

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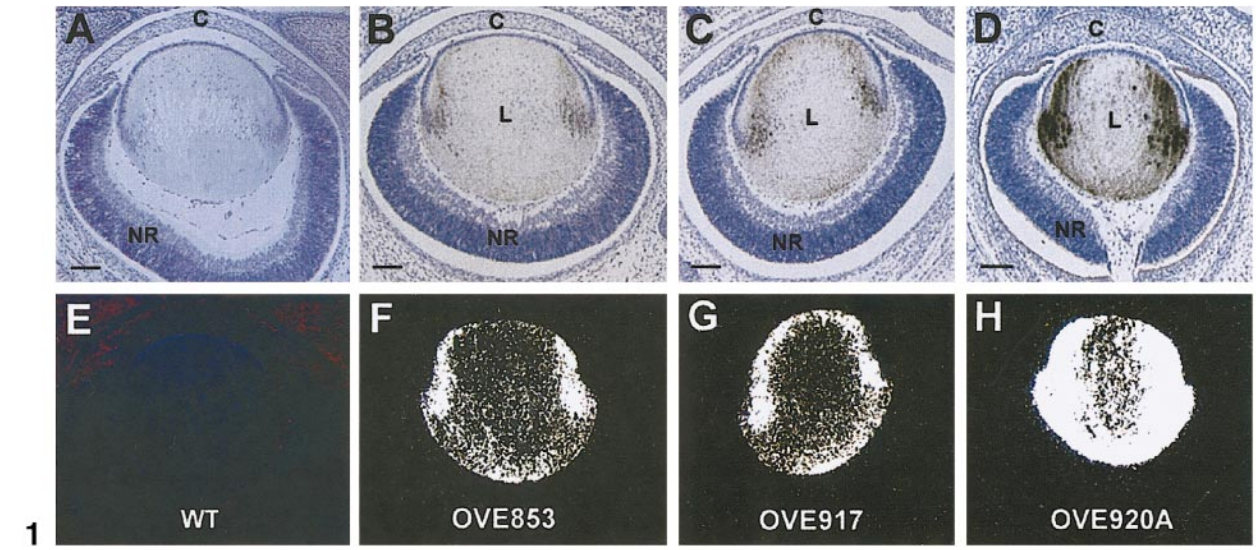


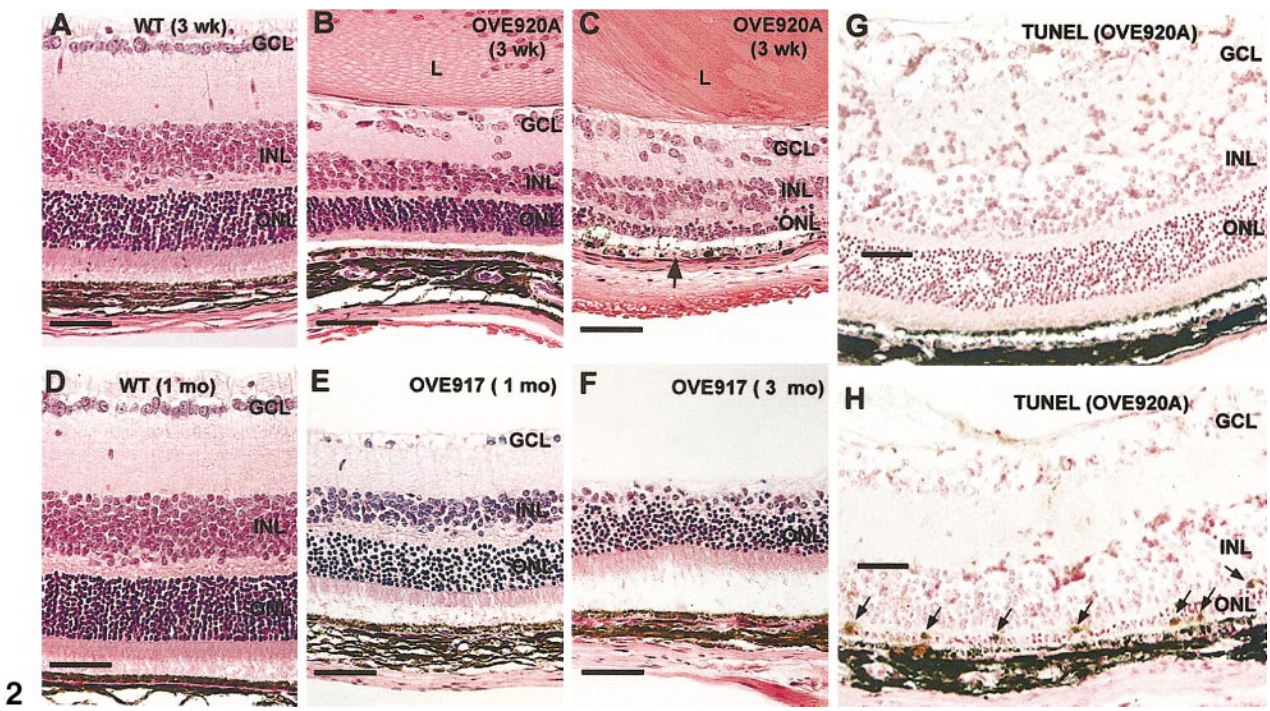
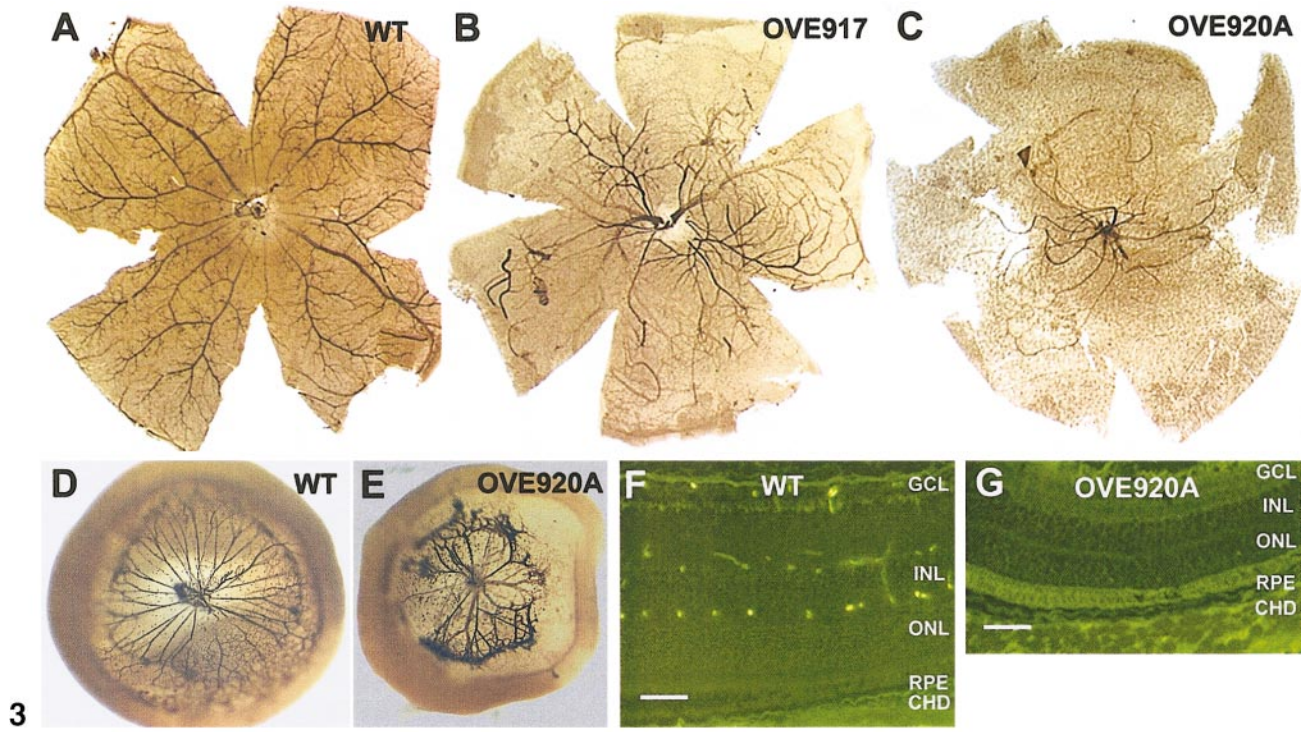


FIG. 1. Lens-specific transgene expression. An RNA probe specific to the transgenic SV40 sequences was hybridized to E16 embryonic eye sections from the wild-type (A, E) and transgenic families, OVE853 (B, F), OVE917 (C, G), and OVE920A (D, H). The upper panels are bright-field images (A–D) and the lower panels are dark-field images (E–H). Family OVE920A shows the highest levels of transgene expression. Transgene expression in all families is confined to the elongated fiber cells of the lens. The cornea in the OVE920A embryo is 2- to 3-fold thicker than in the wild-type embryo while the corneal thickness in families OVE853 or OVE917 is comparable to the wild type. Scale bars, 100 μm. Abbreviations: C, cornea; L, lens; NR, neural retina.

FIG. 2. Retinal degeneration in TGFβ1 transgenic mice (FVB/N $\times$ C57BL/6). In 3-week-old and 1-month-old wild-type mice (A, D), retinal differentiation was complete and three distinct nuclear layers can be clearly seen. In 3-week-old OVE920A transgenic mice (B, C), the retina remained attached to the lens and was significant thinner than the wild type. (B, C) Two different regions in the same retinal section. Severe photoreceptor degeneration was often associated with loss of RPE pigmentation and choroid tissues (arrow in C). At 1 month of age, reduction in retinal thickness became apparent in family OVE917 (E). By 3 months of age, significant loss of ganglion and inner nuclear layer cells was apparent in certain regions of the retina in this family (F). TUNEL assays on 3-week-old OVE920A mouse retina show no apoptotic cells in regions where the photoreceptor cell layer remained relatively intact (G) and many apoptotic cells in regions where the photoreceptor cells were nearly depleted (arrows in H). Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; L, lens; ONL, outer nuclear layer. Scale bars, 100 μm (A–F); 50 μm (G, H).



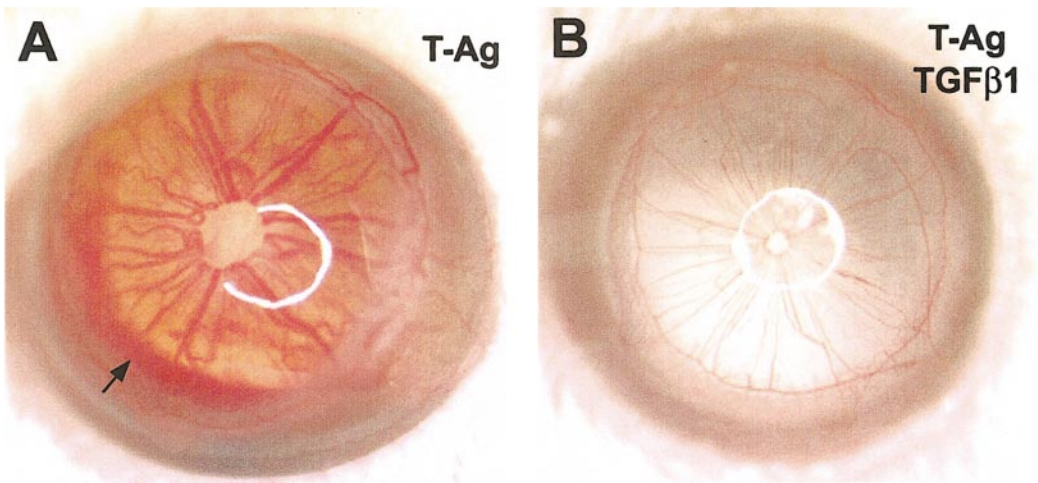


FIG. 3. Vascular deficiencies in the retina of TGFβ1 transgenic mice. Blood vessels were visualized by ADPase staining in 3-week-old flat-mount retinae (A–C) or 5-day-old retinal cups (D, E). In the 3-week-old wild-type retina, major and micro-vessels were well organized and extend to the peripheral retina (A). In the retina of 3-week-old OVE917 mice, a deficiency of microvessels was apparent, especially in the peripheral regions (B). Vasculature was more severely affected in the retina of 3-week-old OVE920A mice (C). In 5-day-old wild-type retinal cups (D), hyaloid vessels were well organized and evenly distributed. In the 5-day-old retinal cups of OVE920A mice, there are fewer hyaloid vessels with uneven spacing and poor organization (E). Immunofluorescent labeling of 3-week-old wild-type retinal sections using anti-mouse serum antibodies shows the presence of three layers of blood vessels in the normal retina (F) while in certain regions of OVE920A retinas, blood vessels were absent (G). In these regions, significant loss of retinal cells was observed (compare the retinal thickness in F to that in G). Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars, 100 μm.
FIG. 4. Reduced hypervascularization in TGFβ1 transgenic mice. Mice expressing T-Ag in the lens (A) exhibited tumor-induced hypervascularization in the iris and hemorrhage in the eye (arrow in A) at 6 weeks of age. In mice co-expressing T-Ag and TGFβ1 (B), vascularization was reduced and no hemorrhage was observed at the same age.

However, the roles of TGFβ proteins in ocular development remain unclear.

To investigate the effects of TGFβ signaling on ocular development, we generated transgenic mice expressing a self-activating form of TGFβ1 from a lens-specific αA-crystallin promoter (Srinivasan *et al.*, 1998). These mice exhibit anterior subcapsular cataracts, corneal opacities, and defects in the iris and ciliary body (Srinivasan *et al.*, 1998). We now report that these transgenic mice also exhibit a vascular deficiency in the retina and subsequent retinal degeneration. Transgenic TGFβ1 also reduced lens tumor-induced hypervascularization in the iris and inhibited the accumulation of the excessive hyaloid tissue in TGFβ2-null embryos. In addition, transgenic TGFβ1 stimulated growth of the corneal stroma and reversed the corneal phenotype in TGFβ2-null embryos. These results indicate that endogenous and ectopic TGFβ proteins negatively regulate vascular development in the eye and also influence the differentiation of other mesenchymal cells during ocular development.

MATERIALS AND METHODS

Transgenic Mice

Generation of the transgenic mice has been previously described (Srinivasan *et al.*, 1998). Briefly, a 1.5-kb cDNA encoding an active form of human TGFβ1 (Arrick *et al.*, 1992) was inserted downstream of the lens-specific αA-crystallin promoter and upstream of SV40 small t intron and polyadenylation sequences. Transgenic mice were generated by microinjection of the construct into pronuclei of one-cell stage FVB/N embryos (Taketo *et al.*, 1991). Potential transgenic mice were screened by polymerase chain reaction (PCR) using DNA extracted from mouse tails. The primers used for PCR are specific to the SV40 sequences (primer A: 5′-GTG AAG GAA CCT TAC TTC TGT GGT G-3′; primer B: 5′-GTC CTT GGG GTC TTC TAC CTT TCT C-3′). Since FVB/N mice carry an autosomal recessive mutation causing retinal degeneration (rd) (Pittler and Baehr, 1991; Bowes *et al.*, 1993), the transgenic mice were mated with pigmented inbred C57BL/6 or albino outbred ICR mice to generate offspring for further analysis. To examine whether ectopic expression of TGFβ1 can inhibit tumor-induced hypervascularization, the TGFβ1 transgenic mice were mated with transgenic mice with lens tumors induced by SV40 T antigen (T-Ag) (Mahon *et al.*, 1987). Mouse genotypes from the cross-breeding were determined by PCR using a primer pair specific to the TGFβ1 transgene (primer Tβ1b: 5′-AGC AGC TGT CCA ACA TGA TC-3′ plus primer B) and a primer pair specific to T-Ag (primer TAg1: 5′-GCT AGG AGT AGC TAT TGA CCA G-3′; primer TAg3: 5′-GAA GCA AAG CAA TGC CAC TTT G-3′). TGFβ1 transgenic mice were also mated with TGFβ2 knockout mice (Sanford *et al.*, 1997; obtained from The Jackson Laboratory) to examine whether TGFβ1 can compensate for the loss of TGFβ2 in the eye. TGFβ1 transgenic mice were first mated with $TGF\beta2^{+/-}$ mice to produce mice that were heterozygous for TGFβ2 and also carried the TGFβ1 transgene. These mice were then mated with $TGF\beta2^{+/-}$ mice. TGFβ2 mutants were genotyped by PCR (primer 1: 5′-AAT GTG CAG GAT AAT TGC TGC-3′; primer 2: 5′-AAC TCC ATA GAT ATG GGG ATG C-3′) (Sanford *et al.*, 1997). These primers produce 132-bp and 1.3-kb PCR products from the wild-type and the mutant alleles, respectively.

Histology and in Situ Hybridizations

Mouse embryos or postnatal eyes were fixed in 10% formalin at room temperature for 24 h and then rinsed in 70% ethanol for 24 h. The tissues were dehydrated in a series of increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. Sections (5 μm) were cut on a microtome for hematoxylin/eosin staining, immunohistochemistry, apoptosis assay, or for *in situ* hybridizations. *In situ* hybridizations were performed to examine transgene expression as previously described (Zhao and Overbeek, 1999). Anti-sense RNA probes specific for the SV40 sequences, mouse VEGF, or VEGF receptors (Flt-1 and Flk-1) were synthesized in the presence of ^{35}S-UTP. After hybridization to tissue sections, unhybridized probes were removed by washing and RNase treatment. Tissue sections were then dehydrated and coated with Kodak NTB-2 emulsion for autoradiography. The slides were developed, stained with hematoxylin, and mounted with a coverslip for examination of silver grains under dark-field illumination.

ADPase Staining

The vasculature in the retina was visualized by ADPase staining following previously described procedures (Lutty and McLeod, 1992). Mouse eyes were collected and fixed overnight in 10% formalin at 4°C. The neural retinae were dissected from the eyes and washed three times with tap water before being incubated in the reaction solution for 15 min at 37°C. The reaction solution contained 0.2 M Tris maleate (pH 7.2), 2 mM lead nitrate, 6 mM magnesium chloride, and 1 mg/ml ADP. The retinae were next washed five times in distilled water followed by development of the reaction product in 2% ammonium sulfide for 1 min. After washing in distilled water, the retinae were flat-mounted in 50% glycerol and photographed.

Immunohistochemistry

Tissue sections were incubated with 5% normal goat serum (NGS) in phosphate-buffered saline (PBS) at room temperature for 15 min to block nonspecific antibody binding. The tissue sections were then incubated with rabbit anti-mouse serum antibodies (Sigma, St. Louis) at 4°C overnight. After rinsing with PBS, the tissue sections were incubated with fluorescein-labeled goat anti-rabbit IgG (Sigma) at room temperature for 1 h. The slides were then rinsed with PBS and coverslipped in 50% glycerol in PBS for examination and photography under blue light illumination.

Assay for Apoptosis

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assays were carried out to detect programmed cell death as previously described (Fromm *et al.*, 1994). Tissue sections were incubated with terminal deoxynucleotidyl transferase and biotin-16–dUTP. Biotin incorporation was detected with an avidin–peroxidase complex, followed by visualization with diaminobenzidine–H_2O_2. Sections were counterstained with Nuclear Fast Red (Poly Scientific, Bay Shore, NY).

RESULTS

Transgenic Mice

Lens-specific expression of the TGFβ1 transgene was demonstrated at embryonic day 16 (E16) by *in situ* hybridizations using a riboprobe specific to the SV40 sequences (Fig. 1). Family 920A showed the highest levels of transgene expression (Figs. 1D and 1H). In all transgenic families, the overall morphology of the retina and the lens appeared normal during embryonic development, but formation of the vitreous was delayed (Figs. 1B–1D). In family OVE920A, the vitreous body never developed in the FVB/N strain and therefore these mice all had small eyes. When OVE920A transgenic mice were mated with the outbred strain ICR, approximately 50% of the transgenic offspring had nearly normal eye size. Some of the FVB/ICR transgenic mice had one normal-size eye and one small eye (data not shown). In embryos from the OVE920A family, there was a significant increase in the thickness of the corneal stroma (Fig. 1D), suggesting that TGFβ stimulates proliferation and/or migration of neural crest-derived stromal cells. In other transgenic families, changes in the cornea thickness were insignificant (compare Figs. 1B and 1C with Fig. 1A). In all transgenic families, subcapsular cataracts were observed in the lens by 3 weeks of age as previously described (Srinivasan *et al.*, 1998). In family OVE920A, the subcapsular cataracts were present in neonatal mice (data not shown), indicating that the age of onset of the phenotype is correlated with the level of transgene expression.

Retinal Degeneration

Loss of retinal cells was observed in family OVE920A by 2 weeks of age and in families OVE853 and OVE917 by 4 weeks of age. Retinal thickness was significantly reduced in OVE920A mice by 3 weeks of age (Figs. 2B and 2C). Loss of photoreceptors was more severe in certain areas (Fig. 2C) of the retina than others (Fig. 2B). Severe photoreceptor degeneration usually occurred in the peripheral retina and was sometimes associated with loss of pigmentation in the RPE and/or loss of pigmented choroidal tissues (arrow in Fig. 2C). In family OVE917, the retina was thinner than that of the wild type at 1 month of age (compare Fig. 2D with 2E). In 3-month-old OVE917 mice, the thickness of the retina was significant reduced (Fig. 2F). In general, retinal degeneration was more severe in OVE920A mice than in OVE853 or OVE917 mice in all retinal layers at comparable ages. No apparent loss of pigmentation in the RPE or the choroid was observed in families other than OVE920A. Since RPE and choroid were not exposed to the transgenic TGFβ1 produced by the lens, the defects in the RPE and/or choroid in family OVE920A were presumed to be caused by changes in biochemistry and/or blood supply in the neural retina rather than being induced directly by transgenic TGFβ1.

To determine whether retinal cells in TGFβ1 transgenic mice died through programmed cell death, TUNEL assays were carried out with 3-week-old retinal sections from OVE920A mice. No apoptotic cells were detected in regions where no severe loss of retinal cells was observed (Fig. 2G). Many apoptotic cells were detected in areas of the retina where significant loss of photoreceptors had occurred (Fig. 2H).

Vascular Deficiency

To determine whether the retinal degeneration was associated with vascular defects in the retina, ADPase staining was carried out to visualize the retinal vasculature in transgenic mice and their nontransgenic littermates. In 3-week-old wild-type mice (Fig. 3A), the retinal vasculature was well established and the major and microvessels can be clearly seen. In the transgenic mice at the same age, retinal vasculature was compromised (Fig. 3B and 3C). Family OVE920A showed the most severe vascular deficiency (Fig. 3C). To determine whether vascular development was also affected at earlier stages, vasculature in the eyecup of postnatal mice was visualized by ADPase staining. In the wild-type mice at postnatal day 5 (P5), organized and evenly spaced hyaloid vessels were observed (Fig. 3D). In the eyecup of OVE920A mice at P5, there were fewer blood vessels and they appeared abnormal in size and in distribution (Fig. 3E). The presence of retinal blood vessels was also assayed in tissue sections by immunolabeling with anti-mouse serum antibodies (Sigma). At 3 weeks of age, blood vessels were clearly identified in wild-type retina (Fig. 3F). In OVE920A mice, blood vessels were absent in certain areas of the retina (Fig. 3G). The absence of blood vessels was always associated with a significant reduction of retinal thickness (compare the retinal thickness in Fig. 3F to that in Fig. 3G).

Reduction in Tumor-Induced Hypervascularization

To test whether transgenic TGFβ1 can have any effect on neovascularization in the eye, OVE920A mice were mated with transgenic mice expressing SV40 T-Ag in the lens driven by the same αA-crystallin promoter (family OVE31) (Mahon *et al.*, 1987). The T-Ag mice develop lens tumors and show hypervascularization in the iris (Fig. 4A) and hemorrhage (arrow in Fig. 4A) in the eyes at 6 weeks of age. When TGFβ1 was co-expressed in the eye, the blood vessels in the iris were thinner and hemorrhage was not seen at this age (Fig. 4B).

Expression of VEGF and Its Receptors

VEGF is known to be a potent angiogenic factor and its expression can be elevated under hypoxic conditions. One would expect an increase in VEGF expression in the eye of transgenic mice due to their vascular deficiency. Alternatively, transgenic TGFβ1 might inhibit retinal angiogenesis by suppressing VEGF expression. We assayed VEGF mRNA expression by *in situ* hybridizations. Surprisingly, neither an increase nor a decrease in VEGF mRNA expression was

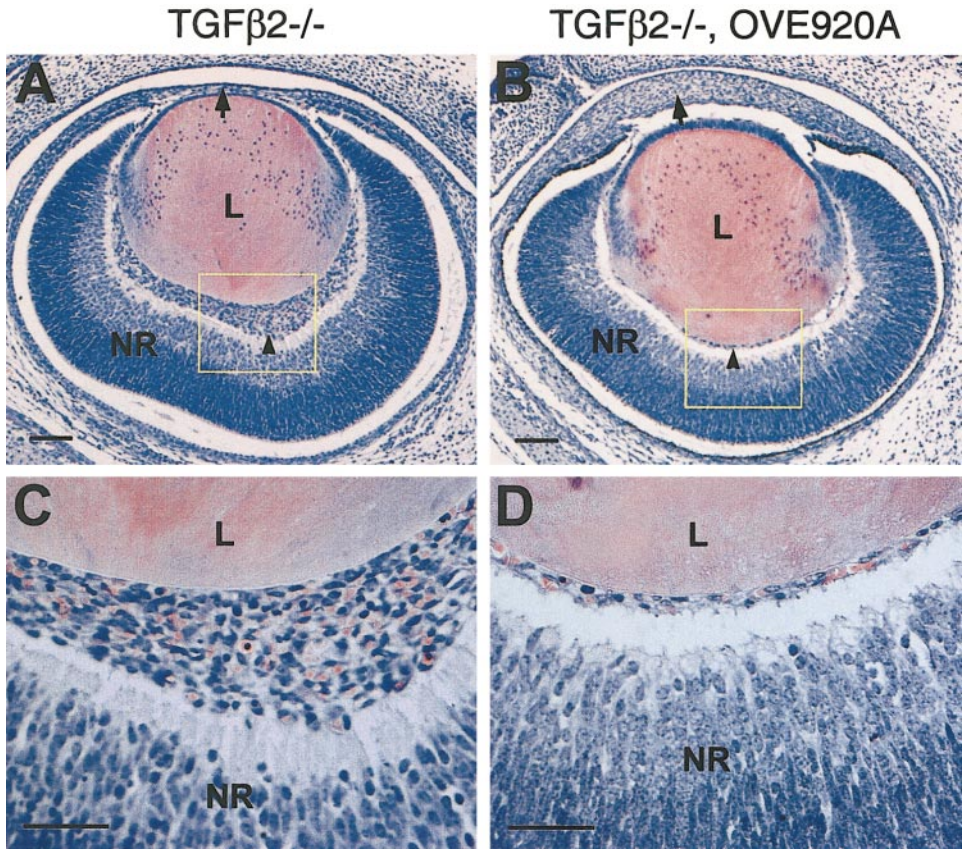


FIG. 5. Rescue of the ocular phenotypes in the TGFβ2-null embryo by transgenic TGFβ1. Hematoxylin/eosin staining of an E16 eye section from a TGFβ2-null embryo (A) shows stromal cell deficiency in the cornea (arrow) and the excessive accumulation of hyaloid cells in the vitreous (arrowhead). In the TGFβ2-null embryo expressing transgenic TGFβ1 (B), the corneal phenotype was reversed (arrow) and the excess hyaloid cells were eliminated (arrowhead). The boxed regions in (A) and (B) are shown at higher magnification in (C) and (D), respectively. The vitreous of the TGFβ2-null embryo was filled with a disorganized mixture of red blood cells, endothelial cells and other mesenchymal cells (C). The hyaloid vasculature in the TGFβ2-null embryo expressing the TGFβ1 transgene more closely resembles that of the wild-type mice (D). Abbreviations: L, lens; NR, neural retina. Scale bars, 100 μm (A, B); 50 μm (C, D).

observed in the eye of OVE920A mice compared to the wild type (data not shown). VEGF transcripts were detected in the retinal astrocytes at the vitreous surface, in the Müller cells in the inner nuclear layer, and in the RPE as previously reported (Stone and Maslim, 1997). We also examined the possibility of whether expression of VEGF receptors Flt-1 or Flk-1 could be affected. *In situ* hybridization results show that transgenic TGFβ1 had no apparent effect on mRNA levels of Flt-1 or Flk-1 (data not shown). Transcripts for Flt-1 and Flk-1 were detected in the endothelial cells of hyaloid blood vessels around the lens and near the retina. They were also detected in a subset of choroidal cells adjacent to the RPE but not RPE itself.

Compensation for TGFβ2 Loss by Transgenic TGFβ1

Mice deficient for TGFβ1, TGFβ2, or TGFβ3 have been generated by gene targeting, but only the TGFβ2-null mice exhibit ocular abnormalities (Shull *et al.*, 1992; Kulkarni *et al.*, 1993; Sanford *et al.*, 1997; Proetzel *et al.*, 1995; Kaartinen *et al.*, 1995). Disruption of TGFβ2 gene causes perinatal lethality (Sanford *et al.*, 1997). To test whether transgenic TGFβ1 can compensate for the loss of TGFβ2 function in the eye, OVE920A mice were bred onto the TGFβ2-null background. In the eyes of TGFβ2-null embryos, the cornea is deficient in stromal cells (arrow in Fig. 5A). In the vitreous, there is excessive and disorganized hyaloid tissue (Fig. 5A), which appears to consist of blood vessels containing blood cells, undifferentiated angioblasts, and perhaps other mesenchymal cells (Fig. 5C). In the TGFβ2-null embryo that expressed transgenic TGFβ1, the corneal phenotype was reversed (arrow in Fig. 5B). The corneal thickness increased significantly and was even slightly greater than the wild type (see Fig. 1). In the vitreous, the hyperplasia of the hyaloid tissue was eliminated (Figs. 5B and 5D). These results demonstrate that the ocular phenotypes

caused by loss of TGFβ2 can be, for the most part, rescued by lens-specific expression of the homologous TGFβ1 protein.

DISCUSSION

TGFβ has been shown to have angiogenic or anti-angiogenic activities in animal models (Pepper, 1997). The present study demonstrates that ectopic TGFβ1 expression can inhibit ocular vascular development and supports the notion that TGFβ is a negative regulator of angiogenesis *in vivo*. In most of the experiments showing an angiogenic effect of the TGFβ protein, marked inflammatory cell infiltration was observed (Roberts *et al.*, 1986; Sprugel *et al.*, 1987; Rubbia-Brandt *et al.*, 1991; Frank *et al.*, 1994; Yang and Moses, 1990; Phillips *et al.*, 1992, 1993; Fajardo *et al.*, 1996). In these cases, angiogenesis is likely stimulated by VEGF and other angiogenic factors released by the inflammatory cells and, therefore, a secondary effect. In cases where inflammatory reactions were not observed, TGFβ did not induce angiogenesis (Dong *et al.*, 1996; Passaniti *et al.*, 1992; Nabel *et al.*, 1993; Jhappan *et al.*, 1993; Pierce *et al.*, 1993; Lee *et al.*, 1995; Sanderson *et al.*, 1995; Wyss-Coray *et al.*, 1995; Sellheyer *et al.*, 1995; Zhou *et al.*, 1996). *In vitro* studies have shown that TGFβ can inhibit proliferation of vascular endothelial cells and smooth muscle cells (SMCs) (Orlidge and D'Amore, 1987; Sato *et al.*, 1990; Beck and D'Amore, 1997). Since vascular cells but not retinal neurons express both type I and type II TGFβ receptors (Srinivasan *et al.*, 1998; Obata *et al.*, 1999), it is likely that TGFβ proteins modulate retinal vascular development by directly acting upon vascular endothelial cells and/or pericytes. TGFβ may promote differentiation and physical interactions of endothelial cells and pericytes which leads to maturation of blood vessels. Gene-targeting studies have shown that in embryos lacking TGFβ1 or type II TGFβ receptor, contacts between vascular endothelial cells either do not form or are disrupted in the yolk sac (Dickson *et al.*, 1995; Oshima *et al.*, 1996). The TGFβ binding protein endoglin has been demonstrated to regulate differentiation of smooth muscle cells and endothelial remodeling (Li *et al.*, 1999).

Mesenchymal cell accumulation in the vitreous of TGFβ2-null embryos was previously reported (Sanford *et al.*, 1997). The authors hypothesized that the excessive number of cells in the vitreous resulted from failure in elimination of the vascular tunic and suggested that TGFβ2 plays a role in ocular tissue remodeling. However, our transgenic and cross-breeding data suggest that TGFβ signaling controls the proper growth and maturation of ocular vasculature in a dosage-sensitive manner. Although endogenous TGFβ1 is expressed in rodent eyes in a pattern similar to TGFβ2 (Srinivasan *et al.*, 1998; Gordon-Thomson *et al.*, 1998), it is apparently unable to compensate for the loss of TGFβ2 either because it activates a different signaling pathway or because its dosage is insufficient. We show that additional dosage of TGFβ1 derived from the transgene can rescue the ocular phenotypes in TGFβ2-null embryos, indicating that these two family members must be capable of stimulating similar responses in the ocular tissues and that an appropriate level of TGFβ signaling is critical for normal development of ocular vasculature.

TGFβ proteins have been implicated in ocular diseases. It has been reported that decreases in levels of active TGFβ protein in the vitreous were associated with active proliferative diabetic retinopathy and hypoxic angiogenesis in patients (Spranger *et al.*, 1999). This is consistent with our finding that TGFβ proteins can inhibit ocular angiogenesis. It is also known that the vitreous in patients with proliferative vitreoretinopathy (Gaudric *et al.*, 1990; Kon *et al.*, 1999) or primary open-angle glaucoma (Tripathi *et al.*, 1994) contains abnormally high levels of TGFβ protein. It is not yet known whether the high levels of TGFβ are a cause or a consequence of these diseases.

In summary, our present study demonstrates that TGFβ signaling plays an important role in regulation of ocular vascular development. Our results show that either overproduction or underproduction of TGFβ proteins causes ocular abnormalities, indicating that the expression and/or activation of these proteins must be tightly controlled during normal development. Since elevated TGFβ signaling does not appear to affect expression of VEGF and its receptors Flt-1 and Flk-1, it will be interesting to find out how VEGF and TGFβ signaling pathways interact at molecular levels in regulation of vascular development.

ACKNOWLEDGMENTS

We thank B. Harris and D. Liang for technical assistance. This work was supported by NIH Grant EY10448 (to P.A.O.) and the Knights Templar Foundation and Fight For Sight, Inc. (to S.Z.).

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Received for publication April 2, 2001
Revised June 7, 2001
Accepted June 7, 2001
Published online July 30, 2001