Transforming growth factor β1 induction of vascular endothelial growth factor receptor 1: Mechanism of pericyte-induced vascular survival in vivo

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Degeneration of vessels precedes and precipitates the devastating ischemia of many diseases, including retinopathy of prematurity and diabetic retinopathy. Ischemia then leads to proliferative retinopathy and blindness. Understanding the mechanisms of blood vessel degeneration is critical to prevention of these diseases. Vessel loss is associated with oxygen-induced suppression of vascular endothelial growth factor (VEGF) and with pericyte (vascular smooth muscle cell) dropout. The molecular mechanism of pericyte protection of the vasculature is unknown. We show that transforming growth factor β1 (TGF-β1)-expressing pericytes are specifically found on vessels resistant to oxygen-induced loss. TGF-β1 potently induces VEGF receptor 1 (VEGFR-1) expression in endothelial cells and thereby prevents oxygen-induced vessel loss in vivo. Vessel survival is further stimulated with a VEGFR-1-specific ligand, placental growth factor 1. TGF-β1 induction of VEGFR-1 in endothelial cells explains pericyte protection of vessels and the selective vulnerability of neonatal vessels to oxygen. These results implicate induction and activation of VEGFR-1 as critical targets to prevent vessel loss.

Angiogenesis plays a crucial role in many blinding eye diseases, including retinopathy of prematurity (ROP) and diabetic retinopathy (DR). These diseases are initiated by degeneration of the retinal vasculature. In neonates, oxygen induces vessel loss (ROP). In diabetes, vessel degeneration follows pericyte (vascular smooth muscle cell) loss. We sought to understand molecular mechanisms of vessel protection by pericytes and of oxygen-induced vascular degeneration. This would be of great clinical importance, potentially enabling early treatment to prevent the onset of ocular diseases caused by hypoxia-driven angiogenesis and alternatively to precipitate vessel degeneration in tumors.

The mechanism of blood vessel degeneration is not well understood. In neonatal animal models of ROP (but not in adults), hyperoxia causes regression of some retinal blood vessels through suppression of the survival factor vascular endothelial growth factor A (VEGF-A) (1-5). We recently identified VEGF receptor 1 (VEGFR-1) activation in endothelial cells as critical to retinal vessel survival (6). VEGFR-1 predominates in the retinal vascular cells of perinatal mice, and specific activation of VEGFR-1 with placental growth factor 1 (PIGF-1) efficiently protects retinal capillaries from oxygen-induced loss, whereas specific activation of VEGFR-2 confers no protection (6).

Blood vessels in adults are much more resistant to oxygen-induced loss (7, 8) but degenerate without pericyte protection (9, 10). Animal models have better defined the association between pericyte loss and vessel loss in diabetes. In mice lacking platelet-derived growth factor (PDGF)-B in endothelial cells, pericyte recruitment is compromised and vessel loss is similar to that of early DR (11). In PDGF-B−/− mice with pericyte deficit, vessel loss is increased further in diabetic animals (10). Pericytes are also important in the developing retina. In neonates, vessels without pericytes are more susceptible to oxygen-induced vessel degeneration (9, 10). These results suggest that pericyte support of vessel survival appears to be related to VEGF-induced survival,

and we sought to identify a molecular mechanism that rationalizes this cooperativity.

In vitro, pericyte/endothelial cell cocultures are associated with transforming growth factor (TGF)-β expression and activation (12-14), which in turn promote vascular cell differentiation and suppress proliferation (13, 15). Thus, we examined whether TGF-β1 was also involved in vessel survival in vivo. Specifically, we asked whether TGF-β1 induces VEGFR-1 expression in endothelial cells in vitro and whether this regulation is associated with increased retinal vessel survival under oxygen stress in vivo. We also sought a molecular rationale for differential susceptibility to oxygen in some neonatal vessels and resistance in adults and examined the role of TGF-β1.

Materials and Methods

VEGFR-1 Expression in Bovine Retinal Endothelial Cells (BREC). We generated BREC (80-85% confluent; Clonetics, San Diego) in room air with 5% CO2 in endothelial cell basal medium with 1% FBS for 24 h. The BREC were then treated for 8 h without additives (control) or with 1 ng/ml TGF-β1, 20 ng/ml tumor necrosis factor α (TNF-α), 20 ng/ml VEGF-A, or hypoxia (3% O2). We extracted total RNA, then quantified VEGFR-1 mRNA with real-time RT-PCR.

FITC-Dextran Perfusion and Retinal Whole-Mount. We anesthetized C57BL/6 mice with Avertin (Sigma) and killed them by intracardiac perfusion with 4% paraformaldehyde and 20 ml 2× 104 mol wt FITC-dextran in PBS (16). We enucleated and fixed eyes in 4% paraformaldehyde for 2 h at 4°C. We isolated retinas and either directly whole-mounted them with glycerol-gelatin (Sigma) onto polylysin-coated slides with the photoreceptor side up or mounted them after immunohistochemical staining. We examined retinas with a fluorescence microscope (Olympus, Tokyo), digitized images using a three-charge-coupled device color video camera (DX-950P, Sony), and processed them with NORTHERN ECLIPSE software (Empix Imaging, Toronto).

Whole-Mount Immunohistochemical Staining. We rinsed 4% paraformaldehyde-fixed retinas in PBS, blocked with PBS/0.5% Triton/1% BSA, then stained with primary antibodies against TGF-β1 (R & D Systems), α-smooth muscle actin (αSMA; cy3 conjugate; Sigma), chondroitin sulfate proteoglycan NG2 (Chemicon), or the biotin-labeled endothelial cell-specific isoelectric griffonia simplicifolia I (GSI) (Vector Laboratories) for 2 h at room temperature. The secondary reagents used were anti-murine-FITC (Sigma), anti-rabbit-cy3 (Sigma), anti-goat-

Abbreviations: PDGF, platelet-derived growth factor; αSMA, α-smooth muscle actin; PIGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; ROP, retinopathy of prematurity; DR, diabetic retinopathy; GSI, griffonia simplicifolia I; TGF, transforming growth factor; BREC, bovine retinal endothelial cell; TNF-α, tumor necrosis factor α; PN, postnatal day n.

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cy3 (Sigma), and 7-amino-4-methylcoumarin-3-acetic acid-labeled avidin (Vector).

**Whole-Mount in Situ Hybridization.** We transcribed sense (control) and antisense mRNA probes for VEGF-R1 by using a digoxigenin-UTP labeling kit according to the manufacturer’s protocol (Roche). Retinas were preincubated with 0.2 M HCl, digested with proteinase K (20 μg/ml) in PBS buffer, postfixed in 4% paraformaldehyde-PBS, and treated with 0.1 M triethanolamine containing 0.25% acetic anhydride. We prehybridized retinas in 50% formamide containing dextran sulfate, single-stranded DNA, and tRNA in phosphate buffer, pH 7.5, for 1 h at 50°C and then hybridized with 100 ng/ml digoxigenin-labeled RNA probe at 50°C overnight. Antibody was applied to digoxigenin (1:1000) (Roche) for 4 h at room temperature and color-developed with alkaline phosphatase substrate (Roche) for 10 min at room temperature. We flat-mounted retinas as described.

**RNA Isolation and cDNA Preparation.** For each of the developmental time points postnatal day 3 (P3), P5, P7, P8, P12, P15, P17, P26, and P33, total retinal RNA was extracted by RNAeasy kit with DNaseI treatment (Qiagen, Chatsworth, CA) from the retinas of one mouse from each of 12 litters and then pooled to reduce biologic variability. We converted 100 ng of purified total RNA from pooled retinas of each time point into cDNA by using murine leukemia virus reverse transcriptase (Invitrogen). We stored all cDNA samples at −80°C.

**Real-Time RT-PCR Analysis of Gene Expression.** We designed real-time PCR primers (Genemed Synthesis, South San Francisco, CA) targeting murine VEGF-R1, VEGF-R2, TGF-β1, VEGF-A, and cyclophilin using PRIMER EXPRESS software (Applied Biosystems). Cyclophilin expression was unchanged during retinal development and was used as the normalizer. We determined specificity of each primer with the National Center for Biotechnology Information BLAST module and assured efficacy of each primer set by testing amplicons for the specific melting point temperatures (PRIMER EXPRESS). We used the Prism 7700 Sequence Detection System (Applied Biosystems) and the SYBR Green master mix kit (Applied Biosystems) for detecting real-time PCR products from 0.25–2.5 ng of reverse-transcribed cDNA samples (17). To determine absolute copy numbers of murine TGF-β1 mRNA, we first cloned and isolated individual cDNA templates of TGF-β1 and cyclophilin that cover the sequences bracketed by the real-time PCR primers (17). We plotted standard curves for each gene with quantified cDNA template during each real-time PCR to determine absolute copy number. We normalized each target gene mRNA copy number to 10^6 copies of cyclophilin control.

The sequences of the PCR primer pairs (5′ to 3′) that were used for each gene are as follows: VEGF-R1, 5′-GGAGGAGGAGCAGTTGCTATAGGT-3′ (forward) and 5′-GTGATGCAGTCCAGGTGTGTTGTT-3′ (reverse); VEGF-R2, 5′-GCCCTGCTTGGTCTCCTGTC-3′ (forward) and 5′-CAAGATTGCCATTCGAT-3′ (reverse); TGF-β1, 5′-TACCTGGCAAGAACCATCATCAGAC-3′ (forward) and 5′-GGGCTCTAGTTGGGCAGAATCCGT-3′ (reverse); VEGF-A, 5′-GGGAGTCTCCCGAGGAGATCCT-3′ (forward) and 5′-GGCGAAGGTGGTGAGAG-3′ (reverse); cyclophilin, 5′-CACAGGAGCTCGTTCTGAGTG-3′ (forward) and 5′-TGATCTTGGGAAGTGGGATA-3′ (reverse).

**Effect of Systemic TGF-β1 on VEGF-R1 and Oxygen-Induced Vessel Loss in Vivo.** Dr. Steve Ledbetter (Genzyme) kindly donated active TGF-β1. We injected 400 μg of active TGF-β1 in 20 μl of PBS (n = 6 mice) or PBS (20 μl) i.p. once daily until P6 and P7 with i.p. TGF-β1 or PBS at P7 intravitreally with the specific VEGF-R1 ligand human PlGF-1 (0.01 μg per 0.5 μl of balanced salt solution) (R & D Systems) into one eye and control 0.5 μl of balanced salt solution in the contralateral eye (n = 6 for each condition). After 17 h of 75% O2 exposure, we collected eyes after FITC-dextran perfusion, whole-mounted retinas, and measured nonvascularized areas to evaluate vessel loss.

**Results**

**Active TGF-β1 Potently Induces VEGF-R1 mRNA Expression in Retinal Vascular Endothelial Cells in Vitro.** Because TGF-β1 is associated with pericyte influence on endothelial cells, we investigated in BRECs whether TGF-β1 might mediate protection through modulation of VEGFRs. In BRECs, VEGF-R1 expression increased 6.5-fold after 8 h of TGF-β1 (1 ng/ml) treatment compared with untreated control cells (Fig. 1a). This induction was much more potent than VEGF-R1 induction with 20 ng/ml TNF-α (3.8-fold), 20 ng/ml VEGF-A (2.0-fold), or hypoxia (2.1-fold). This induction was also sustained. TGF-β1 (1 ng/ml) stimulation of BRECs increased VEGF-R1 mRNA expression maximally 6.5-fold at 8 h, with a plateau continuing until at least 24 h (Fig. 1b). Retinal vessels are unique in their pericyte...
consistent with pericyte coverage of all retinal vessels. In contrast, we saw TGF-β (green) (Fig. 3c) and αSMA (red), another pericyte marker (Fig. 3d), at the growing vessel front and in larger arteries and veins but not on all GSI-positive vessels (Fig. 3e). αSMA coincided largely with cells expressing TGF-β (Fig. 3f; shown more clearly in higher magnification merged images in Fig. 3g and h). Both TGF-β-positive (green) and αSMA-positive (red) cells appear to be on the surface of GSI isolectin-positive endothelial cells (Fig. 3g and h), consistent with the position of pericytes. Both TGF-β and αSMA signals were greatly diminished in the area between the growing front and the optic nerve, the area with weak VEGFR-1 mRNA expression (Fig. 3i) and the area most susceptible to oxygen-induced vessel loss as seen with FITC-dextran perfusion of vessels after 17 h of hyperoxia treatment (Fig. 3j). At P7–P8, when superficial retinal vessels extend further to the periphery, we see TGF-β at the vessel front and on large vessels in the same pattern as P5 (data not shown). These results suggest that some but not all retinal pericytes appear to express TGF-β at P5. Those that produce TGF-β coincide with increased VEGFR-1 expression and with increased oxygen resistance. However, at P15, when all vessels are oxygen-resistant (8), we see TGF-β associated with all vessels (Fig. 3k and l) on the surface of GSI-positive endothelial cells in a pattern consistent with pericyte origin (Fig. 3m and n).

TGF-β1 Protects Retinal Capillaries from Oxygen-Induced Loss. Our recent study shows that activation of VEGFR-1 by the specific VEGFR-1 ligand PI GF-1 protects retinal vessels from hyperoxia-induced loss (6). Therefore, we tested whether the increased level of VEGFR-1 expression through TGF-β1 treatment prevents oxygen-induced vessel loss. After 17 h of hyperoxia treatment, TGF-β1-treated retinas retain more capillaries than do the PBS-treated control retinas as seen with FITC-dextran perfused whole-mounted retinas (Fig. 5a). Quantitative analysis of the nonvascularized retinal area after 17 h of oxygen exposure in Fig. 5b shows a 28.7% reduction in the nonvascularized area after TGF-β1 systemic treatment (23.9 ± 1.5%) (n = 8 eyes) compared with controls (33.5 ± 1.9%) (n = 8; P < 0.005).

TGF-β1 Plus VEGFR-1-Specific Ligand PI GF-1 Further Increases Protection from Hyperoxia-Induced Degeneration. To determine whether TGF-β1 protects against oxygen-induced vessel loss through induction of VEGFR-1, we cotreated mice with TGF-β1 and PI GF-1, a specific ligand for VEGFR-1 as well as with PI GF-1 and TGF-β1 alone (Fig. 6). PI GF-1- and TGF-β1-cotreated mice had a 56% reduction of hyperoxia-induced capillary loss compared to saline-treated controls (n = 8, P < 0.0001) (17.5 ± 3.8% versus 40.0 ± 1.6% nonvascularized area). PI GF-1 alone induced a 32% reduction in vessel loss compared with saline-treated controls (n = 8, P < 0.001) (27.1 ± 2.8% versus 40.0 ± 1.6% nonvascularized area). TGF-β1 alone induced a 17% reduction in vessel loss compared with saline-treated controls (n = 8, P < 0.05) (33.3 ± 2.1% versus 40.0 ± 1.6% nonvascularized area). These results suggest that induction of VEGFR-1 in retina by TGF-β1 protects neonatal mouse retinas from hyperoxia-induced vessel loss, and maximum activation of VEGFR-1 by its specific ligand PI GF-1 can further strengthen the protection.

Temporal Expression of TGF-β1 mRNA in Retinal Development. We examined TGF-β1 mRNA expression from whole retina during vessel development by quantitative real-time RT-PCR. At P3, when retinas are still largely avascular, we found 1,681 copies of TGF-β1 mRNA per 10^6 copies of cyclophilin from P3 to P23 (Fig. 2a). At P7, when retinal vasculature development of the superficial layer is almost complete, TGF-β1 mRNA per 10^6 copies of cyclophilin control from total retinal RNA of room air mice (Normoxia) or after O2 treatment of P7 mice (Hyperoxia), indicating a 34% reduction of TGF-β1 mRNA expression in retinas with hyperoxia treatment (n = 12, P < 0.001, Student’s t test).

In Retinal Whole Mounts, TGF-β1 Protein Coincides with Pericytes Expressing αSMA and with Vessels Resistant to Oxygen. NG2 proteoglycan is exclusively expressed by pericytes during mouse vascular morphogenesis (20). To determine the extent of total pericyte coverage at P5, we examined the pattern of NG2-expressing pericytes and endothelial cells (GSI-positive) in retinal whole mounts. At P5, NG2 (red) (Fig. 3a) was always associated with GSI-positive endothelial cells (blue) (Fig. 3b),
importance of VEGFR-1 in vessel stability. These results also emphasize the 

Discussion

We have identified a molecular pathway in vitro and in vivo that protects vessels from degeneration involving active TGF-β1 induction of VEGFR-1 in endothelial cells. This finding explains both pericyte protection of vessels in DR and VEGF protection in ROP. We find that a subset of pericytes producing TGF-β are exclusively associated with stable vessels. In normal adult retina (resistant to oxygen-induced vasoobliteration), TGF-β-producing pericytes are found on all vessels, whereas in neonates they are absent from vessels susceptible to oxygen-induced loss. In vivo, exogenous TGF-β1 protects retinal capillaries against oxygen-induced vessel degeneration through the induction of VEGFR-1, because further protection is provided with the addition of TGF-β1 ligand. These studies provide evidence that TGF-β1 induces VEGFR-1 in vascular endothelial cells in vitro and in vivo and through that induction provides protection against vessel degeneration. These results also emphasize the importance of VEGFR-1 in vessel stability.

VEGF induced by “physiological hypoxia” controls normal vessel development, whereas, in ROP, oxygen-induced loss of VEGF prevents normal vessel growth and precipitates the loss of some formed vessels. VEGF replacement prevents oxygen-induced vasoobliteration (1–5). We recently found, that in neonatal retina, VEGF protection is mediated through VEGFR-1 because PlGF-1, a specific ligand of this receptor, protects against oxygen damage but the specific ligand of VEGFR-2 (VEGF-E) does not (6).

In the normal developing retina, vessels without pericytes (as defined by αSMA expression) are more susceptible to oxygen-induced vessel loss (9). In neonatal PDGF−/− mice (with decreased pericyte number) exposed to hyperoxia, proliferative retinopathy is increased, suggesting that further pericyte loss increases susceptibility to oxygen-induced vasoobliteration leading to increased proliferative disease (10). Interestingly, in the neonate, not all pericytes (as defined by NG2) express TGF-β1. We found that specific pericytes expressing αSMA coincided with pericytes expressing TGF-β1, both in the neonatal retina and in more mature retinas. Because TGF-β induces αSMA expression in pericytes in vitro (13, 21), diminished αSMA expression may be attributable to decreased TGF-β levels. Our results suggest that in ROP there is both too little ligand (oxygen suppression of VEGF) and on some immature vessels too little VEGFR-1 because of a lack of TGF-β1-expressing pericytes. In normal mature (P15) retinas, which are resistant to oxygen-induced loss (8), we found that TGF-β1-expressing pericytes are localized on all vessels, consistent with TGF-β1 promoting survival in the presence of hyperoxia. Both TGF-β1 (Fig. 2a) and
VEGFR-1 mRNA expression in retina increases with maturation and reaches a maximum in adults (6). In contrast to the normal adult retina, in clinical diabetes there is a loss of pericytes (which precedes vessel loss). Animal models have defined a causal association between pericyte deficit and vessel loss. In mice with compromised pericyte recruitment (lacking PDGF in endothelial cells), the vessel degeneration seen is similar to that of early DR (11).

In the vitreous of patients with proliferative DR (and extensive vasoobliteration) there is a substantial decrease in activated TGF-β1 versus patients with nonproliferative disease, consistent with active TGF-β1 acting as a survival factor (22). In mice with pericyte deficiency (PDGF/SMA) made diabetic, vessel loss is increased further (10). Our studies have not specifically addressed the role of TGF-β1 and VEGFR-1 in diabetic animals. VEGFR-1 is localized in adult mice to vessels (19). In a rat model of diabetes, an increase in VEGFR-1 mRNA was seen in all retinal layers with in situ hybridization, perhaps representing mRNA for soluble VEGFR-1 (23). Further studies are warranted.

Tumor studies suggest that the same mechanism seen in retina might pertain. In tumors with conditional deactivation of VEGF, vessels without pericytes (expressing αSMA) are selectively eliminated in hyperoxia (24), suggesting that control of TGF-β and VEGFR-1 might be critical for tumor vessel survival.

TGF-β1 actions are complex and diverse. In vitro studies implicate a role for TGF-β1 in vascular cell maturation and inhibition of proliferation, perhaps through suppression of VEGFR-2 (25, 26). Cocultures of pericytes and endothelial cells activate TGF-β (12, 14), which inhibits proliferation and causes differentiation of vascular cells (13, 15, 27, 28). However, work in vivo has been more limited, except for genetic alterations in mice.

Knockout mouse studies are consistent with an important role for both TGF-β1 and VEGFR-1 in vessel survival. Overexpression of TGF-β1 in endothelial cells causes hyperplasia (29). TGF-β1−/− is embryonically lethal in ~50% of mice, secondary to cardiovas-
cular abnormalities. TGF-β1 is also critical in suppression of inflammation because those pups that survive soon die from generalized inflammatory infiltrates (30). Interestingly, early diabetic retinal vascular loss, which is associated with a loss of pericytes, is also associated with inflammatory cell infiltration (31).

VEGFR-1−/− is embryonically lethal because of a lack of vessel organization (32). Interestingly, the mutation with VEGFR-1 lacking the tyrosine kinase domain is not lethal (33), suggesting either that VEGFR-1 survival function is mediated through another mechanism or that VEGFR-1/ligand signaling is critical for endothelial cell survival only under abnormal stress situations such as hyperoxia.

In summary, we find that TGF-β expressing pericytes are found exclusively on vessels resistant to oxygen-induced obliteration. TGF-β1 increases VEGFR-1 expression in endothelial cells and, through this induction, increases retinal capillary protection in vivo. These results indicate that TGF-β1 and VEGFR-1 play a critical role in blood vessel survival, rationalize the mechanism of the protective effect of pericytes on vessel loss in both ROP and diabetes, and rationalize the selective vulnerability of neonatal retinal vessels to oxygen. VEGF-1 induction and specific activation may be a valuable strategy to prevent vessel loss in diabetes and ROP.

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