

Soluble Forms of EphrinB2 and EphB4 Reduce Retinal Neovascularization in a Model of Proliferative Retinopathy

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PURPOSE. Ephrin ligands and their Eph receptors are key regulators of endothelial cell (EC) proliferation, migration, adhesion, and repulsion during mammalian vascular development. The hypothesis was that these molecules also play a role in pathologic neovascularization (NV) in the mouse model of oxygen-induced retinopathy.

METHODS. C57BL/6 mice at postnatal day (P)7 were exposed to 75% oxygen (O₂) for 5 days (until P12) and allowed to recover in room air to induce retinal NV. Retinas from unexposed and hyperoxia-exposed mice between P7 to P24 were analyzed specifically for EphrinB2 and EphB4 transcript expression by RT-PCR. Phospho-Eph (p-Eph) receptor was evaluated during active EC proliferation at P15 and P17 by immunohistology. Some hyperoxia-exposed mice had one eye injected intravitreally with 150 ng/1.5 μ L of soluble EphrinB2/Fc or EphB4/Fc chimeras during transition from high O₂ to room air (P12) and injected again on P14. Contralateral eyes were injected with human IgG as the control. Preretinal nuclei and retinal blood vessels were quantified at peak disease (P17).

RESULTS. EphrinB2 mRNA was constitutively expressed in the developing retina and was unchanged by hyperoxia. In contrast, EphB4 mRNA expression was modulated during normal retinal development and was altered by hyperoxia. Furthermore, p-Eph was detected in developing preretinal tufts, thus implying that Ephrin/Eph signaling system is active in this experimental model. Intravitreal injection of soluble versions of these molecules significantly reduced pathologic neovascularization. The number of preretinal nuclei in hyperoxia-treated mice was reduced by 66% ($P < 0.05$) in EphrinB2-injected eyes, whereas EphB4 treatment yielded a 69% reduction ($P < 0.05$), compared with control injections. Intraretinal vessel development was not altered by the injections.

CONCLUSIONS. These results support the hypothesis that endogenous EphrinB2 and EphB4 are regulators of retinal NV during oxygen-induced retinopathy and may be useful targets for therapeutic intervention.

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The erythropoietin-producing hepatocellular (Eph) receptor family is the largest group of receptor tyrosine kinases known to date. These receptors are further subdivided into groups A and B, depending on whether they preferentially bind to an Eph-interacting (Ephrin) ligand of the A or B class (<http://cbweb.med.harvard.edu/eph-nomenclature/> provided in the public domain by the Harvard Medical School, Boston, MA). However, recent studies indicate that Ephrin ligands can promiscuously bind to Eph receptors of the opposite class, albeit with differing affinities.¹ This emerging characteristic of Ephrin/Eph molecules has added a new tier of complexity on elucidating how the signaling outcomes of these molecules affect cellular behavior at the tissue level.¹

All Ephrin ligands contain an extracellular and transmembrane domain; however, Ephrin ligands of the B class also possess a cytoplasmic tail that the A class lacks.² In general, Ephrin homodimers engage Eph homodimers to regulate such cellular events as cell migration, proliferation, attraction, and repulsion. A unique outcome of Ephrin/Eph interaction is a bidirectional signaling event and is generally referred to as forward or reverse signaling, depending on the direction of signal transduction being studied.^{3,4} Because Ephrins and Ephs of the B class both contain cytoplasmic signaling domains, engaging one another can induce simultaneous phosphorylation of tyrosine residues in these domains, thus initiating signaling cascades within both the EphrinB ligand and EphB-receptor-bearing cells.^{5–8} In cultured endothelial cells (ECs), a typical outcome of forward signaling (EphrinB2 to EphB4) is a decrease in proliferation and migration of EphB4-bearing cells, whereas reverse signaling (EphB4 to EphrinB2) increases the proliferation and migration of EphrinB2-bearing cells.^{9–16}

EphrinB2 ligands and EphB4 receptors have emerged as key regulators of vascular development and are primarily expressed by the ECs of developing arteries and veins, respectively.^{17–20} It has been determined that mouse embryos lacking EphrinB2 or EphB4 exhibit lethal defects in early angiogenic remodeling.^{17–19,21} These mice exhibit malformed capillary beds and poorly differentiated arteries and veins. Recent studies indicate that these molecules are localized to capillaries, along with other Ephrin/Eph molecules, and may also be critical regulators of both neonatal and adult neovascularization (NV).^{9–12,15,20,22,23} For example, Gale et al.²⁰ have demonstrated that EphrinB2 is highly expressed in the adult ovary at sites of physiological NV as well as sites of pathologic angiogenesis within tumors. Similarly, EphB4 localizes to newly budding capillaries and venules of pyogenic granulomas within human gingiva.²² EphrinB2, EphB2, and EphB3 are expressed in the neovascular membranes of a subset of patients with stage 5 retinopathy of prematurity (ROP), indicating that this signaling system may also play a role in ocular neovascular diseases.²⁴

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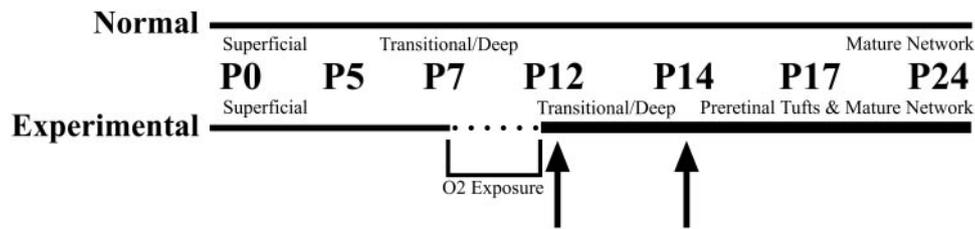


FIGURE 1. Timeline of retinal vessel formation in normal development and oxygen-induced retinopathy in the mouse. This scheme was created based on the protocol developed by Smith et al.³¹ and further characterized by Davies et al.³⁵ Experimental mice placed in 75% oxygen (O_2) on P7 exhibit interrupted development of transitional and deep vascular networks of the central retina. Central superficial vessels also become obliterated during hyperoxia. Mice are returned to room air on P12, which triggers a relative ischemic condition in the retina. The retina then exhibits pathologic growth of vessels in the superficial layer, with preretinal vascular tufts extending through the inner limiting membrane into the vitreous. *Thin line*, normal vessel growth; *dashed line*, interrupted vessel growth; *thick line*, pathologic vessel growth; *arrows*, days of injections.

Recent *in vivo* studies have indicated that the developing retinal vascular system of the mouse does express EphrinB2 and EphB4.²⁵ Newborn mice lack the presence of any retinal blood vessels on their day of birth (P0), but vessels begin migrating outward from the centrally located optic nerve shortly thereafter.²⁶ By P7, vessels cover the entire superficial retina. Retinal arteries and veins are present at this time and are EphrinB2 and EphB4 positive, respectively.²⁵ Many of the retinal capillaries of the superficial vascular bed are also strongly positive for EphB4. At P7, vessels also begin sprouting from the superficial vascular bed to form the deep capillary bed, and by P18 the vascular beds have formed and undergone the remodeling that forms the adult retinal circulation (reviewed in Campochiaro and Hackett²⁶).

In vitro studies performed by our laboratory and others have determined that cultured human retinal ECs express EphrinB2 and EphB4, further suggesting that these molecules may have a role in retinal EC biology.²⁷ *In vitro* stimulation of these and other types of ECs with EphrinB2/Fc or EphB4/Fc can modulate their proliferation and migration.^{15,27} Excessive retinal EC proliferation resulting from pathologic NV is the hallmark of proliferative retinopathies, including ROP, diabetic retinopathy, and age-related macular degeneration.²⁸ The studies just discussed indicate that the Ephrin/Eph signaling mechanism may serve as a therapeutic target in the regulation of retinal NV. In support of this idea, other studies have shown that modulation of various Eph receptors can reduce postnatal angiogenesis in tissues or model systems.^{29,30}

We hypothesize that the Ephrin/Eph signaling mechanism plays a role in the NV process that occurs in oxygen-induced retinopathy and that intravitreal treatment with soluble forms of EphrinB2 or EphB4 can modulate this NV. In this study, we examined the pathologic NV in a model of oxygen-induced retinopathy after intravitreal treatment with soluble forms of EphrinB2 or EphB4.

MATERIALS AND METHODS

Animals

The C57BL/6 mice used in this study were originally obtained from Simonsen Laboratories (Gilroy, CA). The mice were housed and bred in the Oregon Health & Science University animal care facility and treated in accordance with NIH guidelines and the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were provided food and water *ad libitum* and were kept on a 12-hour light-dark schedule. To induce retinopathy, postnatal day (P)7 mice, along with nursing females, were exposed to 75% oxygen for 5 days and then allowed to recover in room air on P12, according

to the protocol of Smith et al.³¹ (Fig. 1) Room air-raised litters were maintained under otherwise identical conditions as the hyperoxia-exposed mice.

RT-PCR Analysis

Retinas were dissected at the selected time points (P7–P24) and four retinas per condition were pooled for RNA extraction. Total RNA was isolated, DNase treated (RNeasy Mini kit; Qiagen, Valencia, CA), and reverse transcribed, to obtain cDNA. Touchdown RT-PCR detection of gene expression was performed as previously described.³² Mouse-specific EphrinB2 ligand primer sets (sense, 5'-TGTCCAGACAAGAGC-CATGA-3', antisense, 5'-TGGTCGTGTGCTGTGGAGAGT-3'; 342-bp amplicon size); and EphB4 receptor primer sets (sense, 5'-ACCTGGCT-GCTCGGAACATC-3', antisense, 5'-GCTGCCGTTGGTCCAAGAGT-3'; 492-bp amplicon size), all from Integrated DNA Technologies Inc. (IDT, Coralville, IA) were used to amplify specific cDNAs. A primer pair for the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was included in each assay as an internal control (sense, 5'-GCATGGCCTTCGGTTCCTA-3', antisense, 5'-CGGCATCGAAGGTGGAAGAGT-3'; IDT; 204-bp pair amplicon size). The PCR products were electrophoresed in 3% agarose gels in Tris-acetate buffer containing ethidium bromide and subsequently photographed under UV light. Densitometry values of RT-PCR bands were obtained from five independent experiments by using image acquisition and analysis software (Labworks; UVP Laboratory Products, Inc., Upland, CA). Although the amplicons detected in our gels matched their predicted base pair size, we sequenced the PCR products and confirmed that the amplicons generated were indeed those of mouse EphrinB2 and EphB4 (data not shown).

Intravitreal Injections

Administration of anesthesia and injections were performed as previously described.³³ Pups were deeply anesthetized by isoflurane inhalation (0.5 L/min in oxygen). Approximately 1.5 μ L of EphrinB2/Fc ($n = 12$ mice) or EphB4/Fc ($n = 13$ mice; 100 ng/ μ L; R&D Systems, Minneapolis, MN) was delivered intravitreally into the right eyes of oxygen-injured mice. Because these chimeric forms of EphrinB2/Fc and EphB4/Fc are monomers dimerized in their active form by the Fc portion of human IgG, 1.5 μ L of human whole IgG (100 ng/ μ L; Sigma-Aldrich, St. Louis, MO; $n = 25$ mice) was delivered into the left eyes of oxygen-injured mice as a control protein injection. Intravitreal injections were performed with a syringe (Hamilton, Reno, NV) connected to an ultrathin pulled borosilicate glass needle (outer diameter, ~ 50 μ m). EphrinB2/Fc, EphB4/Fc, and human IgG proteins were diluted in sterile Dulbecco's PBS (minus Ca^{2+} and Mg^{2+} , pH ~ 7.4 ; Invitrogen-Gibco, Carlsbad, CA) before injection. Control and experimental injections were administered within 5 minutes of each other and were given during the transition from hyperoxia to room air on

P12 and repeated on P14. The mice were allowed to recover until P17 and were killed by CO₂ administration. Both eyes were carefully enucleated from each mouse, placed in 10% neutral buffered formalin overnight, and routinely processed for paraffin embedding. The eyes were then sectioned at 5- μ m intervals, mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA), and stored at room temperature until they were used for immunohistologic analysis. Alternatively, some mice underwent retinal fluorescein angiography, as previously described.^{34,35} The eyes were enucleated, and retinal flatmounts were photographed as previously published.³⁵

Neovascular Nuclei Quantification

To quantitate the retinal NV, tissue sections were stained with hematoxylin and eosin (H&E). Retinal vascular cell nuclei anterior to the inner limiting membrane of the oxygen-injured and room air control retinas were counted at P17 in a masked fashion. Care was taken to avoid counting hyaloid vessel nuclei near the optic disc and lens, which are easily distinguishable from pathologic neovascular tufts extending into the vitreous. The average number of neovascular nuclei per section was calculated for each eye as the mean number counted in 15 sections 40 μ m apart. Twelve to 25 mice per condition were analyzed and represent approximately five different litters of mice. Statistical analysis was based on the median of the averages in the Kruskal-Wallis one-way analysis of variance with the Dunn method of multiple comparison.

Immunohistochemistry

To detect phospho-Eph (p-Eph) receptors, a rabbit pan-anti-p-Eph antibody (1:200) was used, which was generously provided by Catherine Nobes (University of Bristol, UK).³⁶ For the detection of the basement membranes of blood vessels, a rabbit polyclonal anti-mouse type IV collagen antibody (1:400; Collaborative Biomedical Products, Bedford, MA) was used. Before incubating the experimental antibodies with the retinal tissue sections, antigen retrieval was accomplished by digesting with proteinase K for 5 minutes (p-Eph) or 0.1% pepsin for 20 minutes (type-IV collagen) at room temperature. The sections were rinsed with deionized water and then washed with Tris-buffered saline (50 mM Tris, 0.15 M NaCl [pH 7.5]; TBS). Nonspecific binding sites were blocked with 2% normal goat serum (Vector Laboratories, Burlingame, CA), 0.1% BSA, and 0.3% Triton X-100 in TBS for 60 minutes at room temperature. The sections were then incubated with experimental antibodies overnight at 4°C and washed in TBS. Primary antibodies were then detected by incubation with goat anti-rabbit IgG antibodies (1:200; Vector Laboratories), and the antibody-antigen complexes visualized using fast red as the substrate (Biogenex Laboratories, San Ramon, CA).

For quantitation of intraretinal vessels, the type-IV collagen staining was quantitated in the following two categories, as previously described³⁵: superficial vessels, defined as those located between the inner limiting membrane and the ganglion cell layer, and deep vessels, defined as those located in the outer plexiform layer. Vessels were counted in a masked, randomized fashion, with transverse and cross-sectioned vessels being treated equally, while avoiding optic nerve and tangential sections. Two representative sections from each eye were analyzed from either side of the optic nerve with a total of 12 to 25 animals studied per condition. Data gathered in this study were analyzed using a one-way ANOVA with a Tukey multiple comparison procedure.

RESULTS

EphrinB2 and EphB4 mRNA Expression in Normal and Oxygen-Injured Retinas

EphrinB2 and EphB4 have been extensively studied in blood vessel development, but have yet to be studied in the mouse model of oxygen-induced retinopathy. To determine the en-

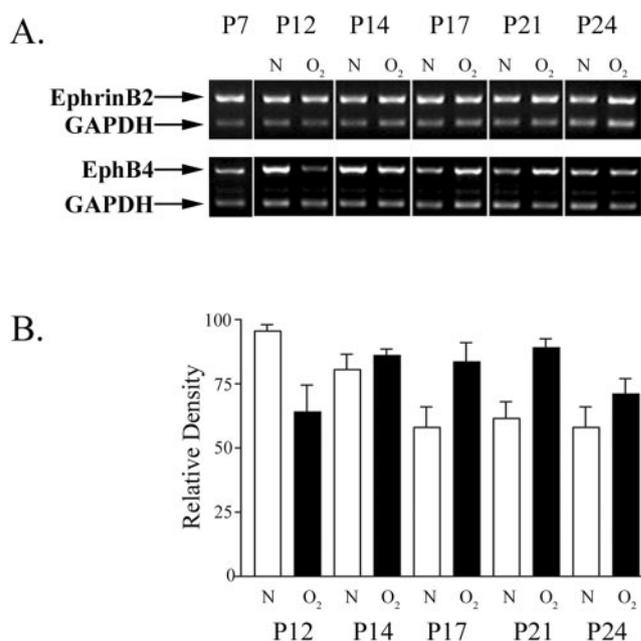


FIGURE 2. RT-PCR analysis of EphrinB2 and EphB4 expression in the retina during normal development and in oxygen-induced retinopathy. Total mRNA was isolated from the retinas at various stages of postnatal development (P7–P24) and analyzed by semiquantitative RT-PCR. (A) EphrinB2 transcript levels remained unchanged in normal developing retinas (N) and hyperoxia-injured retinas (O₂). In contrast, EphB4 transcript levels were modulated during normal development, and 5 days (P7–P12) of O₂ exposure altered this expression pattern. The retinas of four mice per condition were pooled and total RNA isolated. (A) A representative micrograph for one of five experiments conducted. (B) A histogram charting the densitometry values obtained from all five experiments conducted demonstrates the modulation of EphB4 message in normal and hyperoxia-injured retinas.

ogenous expression levels of EphrinB2 and EphB4 mRNA in normal and oxygen-injured retinas, we performed semiquantitative RT-PCR. By this method, we were able to detect the expression of both EphrinB2 and EphB4 in the retinas of P7 mice. We were unable to detect any obvious changes of EphrinB2 mRNA during normal retinal development or during retinal development after oxygen-injury (Fig. 2A). In contrast, EphB4 mRNA expression appeared to be modulated during normal retinal development and was highly detectable between P12 and P14 (Fig. 2). Furthermore, 5 days of hyperoxia resulted in a modest decrease in the levels of EphB4 mRNA (P12) compared with untreated, normal mice on P12. However, on allowing hyperoxia-exposed mice to recover in room air, detection of EphB4 mRNA exhibited a modest increase on P14 and was sustained through P21. By P24, EphB4 mRNA appeared similar between normal, untreated, and O₂-exposed mice.

The Ephrin/Eph Signaling System during Oxygen-Induced Retinopathy

We used a pan antibody, which recognizes all forms of phosphorylated Eph (p-Eph; Ephrin activated) receptors, to determine whether the Ephrin/Eph signaling system was active in the preretinal tufts. Retinal sections from hyperoxia-exposed mice were analyzed at P15 and P17, which represent the timeframe during which peak neovascular disease occurs. Active p-Eph receptors were detected within various blood vessels (i.e., transitional blood vessel; Fig. 3A, arrowhead) of the retina on P15-O₂ exposed. Active p-Eph receptors were also detected within the developing retinal blood vessel tufts (Fig.

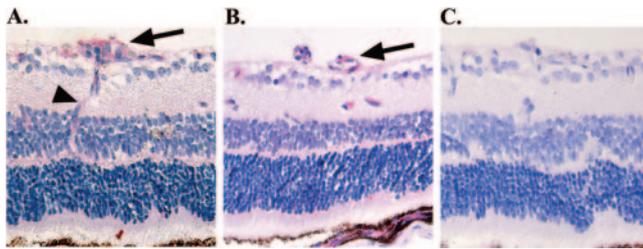


FIGURE 3. The Ephrin/Eph signaling system was shown to be active in the pathologic NV that occurs during oxygen-induced retinopathy. Using a pan-anti-p-Eph antibody, activated Eph receptors were localized to areas of proliferating EC just before breaking through the inner-limiting membrane (A, arrow) and to the transitional vessels (A, arrowhead) on P15. By P17, activated p-Eph receptors were still detectable on the neovascular tufts of oxygen-injured retinas (B, arrow), whereas intraretinal vessel staining appeared to be diminished. (C) A control section incubated with isotype-matched control antibody. Original magnification, $\times 400$.

3A, arrow), thus indicating that the Ephrin/Eph signaling system is engaged during this disease model. p-Eph receptors were also still detected within the preretinal tufts of P17 O_2 -exposed mice (Fig. 3B; arrow). Data obtained in these studies suggest that the Ephrin/Eph signaling system is engaged in this disease model.

EphrinB2/Fc or EphB4/Fc Treatment of Oxygen-Injured Retinas

We next wanted to determine whether we could alter the outcome of neovascular disease through perturbation of the Ephrin/Eph signaling system by treating oxygen-injured retinas in vivo with soluble forms of EphrinB2 or EphB4. The chimeric EphrinB2/Fc and EphB4/Fc proteins used in this study are monomers dimerized in their active form by the Fc portion of human IgG. No further higher-order clustering is necessary for these molecules to be biologically active.⁹ The dimers bind to their respective receptors on the cell surface and induce signaling within the cell that would have occurred normally by cell-cell contact. Our control injections contained human IgG. We exposed mice to high oxygen as described (see Fig. 1) and allowed the mice to recover in room air for 5 days (P17), the day in which peak disease occurs. During room-air recovery, we injected intravitreally 150 ng of either EphrinB2/Fc or EphB4/Fc on P12 and gave a second injection on P14. We next monitored, in a qualitative manner, the pathologic response in the oxygen-injured retinas, through the use of retinal fluorescein angiography (Fig. 4). Unlike normal mouse retinal development, the nature of the oxygen-induced retinopathy model is such that many avascular regions arise within the central retina and appear as dark regions, which are unperfused by fluorescein (Fig. 4A, asterisk). Furthermore, the highest degree of pathology (preretinal tuft formation) is generally observed within the vascular regions that are adjacent to the central avascular regions (Fig. 4A, box). High-power magnification of the boxed region in Figure 4A demonstrated a vast amount of neovascular tufts that formed within the superficial capillary network in the retinas of control injected eyes (Fig. 4B, arrows). Neovascular tuft formation also appeared to be more extensive in control IgG-injected eyes than in the EphB4/Fc (Fig. 4C) or EphrinB2/Fc (Fig. 4D) injected eyes. Indeed, analysis was focused on the avascular-vascular regions (Figs. 4C, 4D, asterisks), because again this is the area in which the highest degree of disease should have occurred. Although qualitative in nature, our observations indicated that the EphB4/Fc and EphrinB2/Fc injections probably had an effect on the pathologic progression of this disease.

We next analyzed NV by immunohistochemistry in the retinas of control and experimentally injected eyes. To add a further level of control to our studies, we also examined the pathologic NV of noninjected eyes. To begin our study, we first compared preretinal tuft formation in the noninjected eyes to the preretinal tufts in the injected eyes. We found that control IgG-injected eyes (Fig. 5B) had slightly reduced preretinal tuft formation compared with noninjected eyes (Fig. 5A). However, on comparing control IgG-injected eyes (Fig. 5B) to experimentally injected eyes, we observed a vast reduction in the presence of preretinal tufts in the EphB4/Fc- (Fig. 5C) and EphrinB2/Fc- (Fig. 5D) injected eyes. To further confirm these observations, neovascular nuclei anterior to the inner limiting membrane were quantified in a masked fashion and the data analyzed (Fig. 6). EphrinB2/Fc injections yielded a 66% reduction over control IgG injections, whereas EphB4 yielded a 69% reduction. Administration of these chimeras did not appear to alter the presence of the already established large vessels (arteries and veins) of the retina (data not shown). The difference in tuft formation between noninjected and control-injected eyes was not statistically significant ($P > 0.05$).

Intraretinal Vessel Development after EphrinB2/Fc or EphB4/Fc Treatment

Because the experimental injections exhibited a profound effect on preretinal tuft formation, we wanted to determine whether the injections also affected the development of the intraretinal vascular beds (superficial and deep) in this disease model. Control and experimental tissue sections generated in the previous study were immunostained for type IV collagen (a blood vessel marker), and we then analyzed the superficial and deep vascular networks. As is demonstrated in the representative micrographs shown in Figure 7, we did not observe a marked change in staining pattern in the superficial and deep vascular beds of mice receiving EphrinB2/Fc (Fig. 7B), EphB4

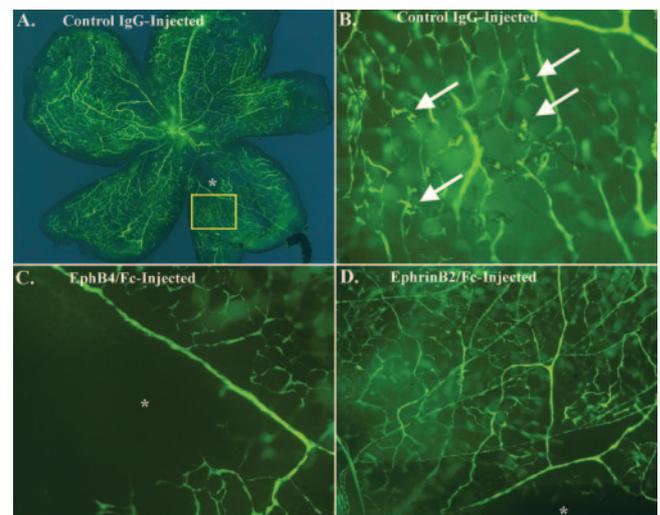


FIGURE 4. Control IgG and EphB4/Fc- or EphrinB2/Fc-injected eyes were analyzed in a qualitative manner using fluorescein angiography of retinal flatmounts. Control IgG-injected retinas (A, yellow box; B, arrows) demonstrated a vast amount of neovascular tufts in their superficial layer located near the avascular-vascular transition zone. (B) A high-power magnification of the avascular-vascular region pictured within the yellow box in (A). In contrast to control injections, EphB4/Fc (C) and EphrinB2/Fc (D) injections exhibited similar affects in the reduction of tuft formation along the avascular-vascular regions. (Asterisk) The avascular, unperfused regions of the retina that are common in this experimental model. Magnifications: (A) $\times 25$; (B, C) $\times 200$; (D) $\times 100$.

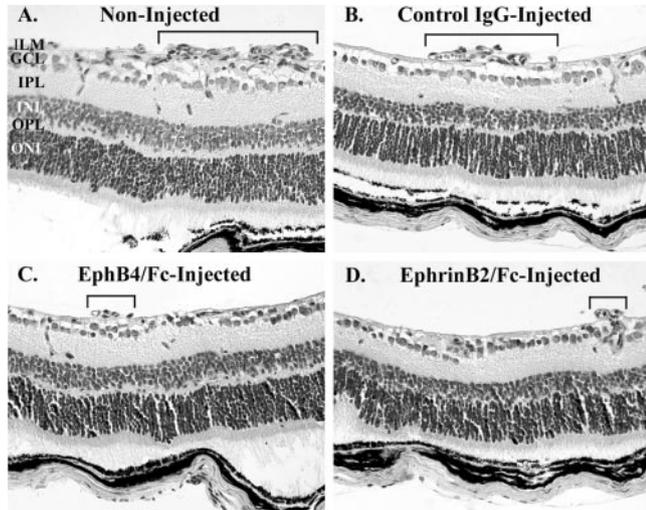


FIGURE 5. Mice were exposed to 75% O₂ from P7 to P12. On P12 the mice were intravitreally injected on return to room air with control IgG, soluble EphrinB2/Fc, or EphB4/Fc and then reinjected on P14. Peak disease was then allowed to develop (P17) before the eyes were enucleated and fixed. All sections are H&E-stained retinas representative of noninjected (A), control IgG- (B), EphB4/Fc- (C), or EphrinB2/Fc- (D) injected eyes. Brackets: neovascular tufts just anterior to the ILM. A slight reduction in neovascular tufts occurred between noninjected and control IgG-injected eyes. EphB4/Fc- and EphrinB2/Fc-injected eyes demonstrated a large reduction in neovascular tufts compared with control IgG-injected eyes. ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Original magnification, $\times 400$.

(Fig. 7D), or their respective controls (Figs. 7A, 7C). This observation was further confirmed on quantitating these vascular beds. Statistical analysis by one-way ANOVA revealed no significant difference between any of the conditions ($P > 0.05$). Neither experimental nor control injections significantly affected the number of vessels in the superficial (Fig. 8A) or deep (Fig. 8B) vascular beds.

DISCUSSION

Expression gradients of various classes of Ephrins and Ephs have been reported in the retina.³⁷⁻⁴⁰ Specifically, EphrinB2 is detected in the neural cells of the retina and plays an important role in retinotectal mapping of axons during visual system development.⁴⁰⁻⁴³ EphB4 is not detected in the neural cells of the retina.^{25,44} However, both EphrinB2 and EphB4 are expressed in the developing retinal vasculature of the mouse, and this expression has been localized to the developing arteries and veins, respectively.²⁵ Herein, we report on the expression of EphrinB2 and EphB4 in mouse retinas during the developmental period from P7 to P24 in both normal and hyperoxia-injured mice. Although we did detect the expression of EphrinB2 within this developmental range, we were unable to detect any modulation of its mRNA. It is important to note that the spoke-patterned arteries and veins of the retina are already developed and in place by P7. Therefore, the EphrinB2 expression data gathered from this developmental period reflects the final developmental stage of the transitional and deep vessels, as well as the final formation of the capillary network. Because our results are reflective of whole retinal expression of EphrinB2, subtle modulation of vascular EC-EphrinB2 expression may occur, but may be masked from detection by background levels of neural EphrinB2 transcript.

Quantification of Pre-Retinal Nuclei

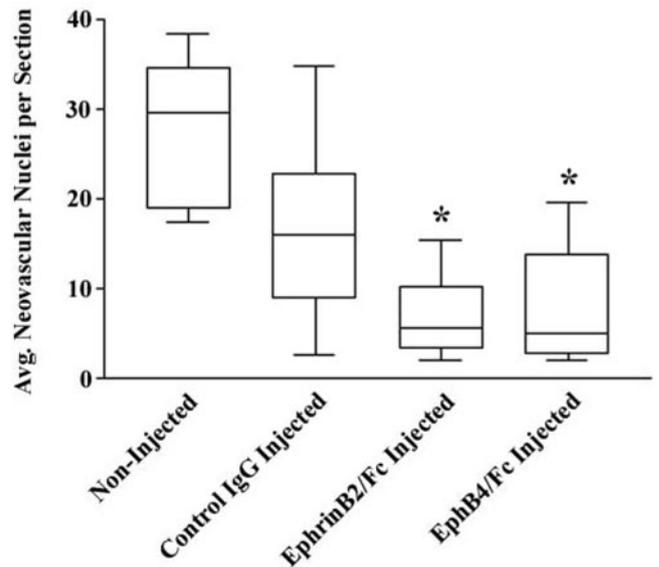


FIGURE 6. Preretinal nuclei were counted in a masked fashion, and data were statistically analyzed. Intravitreal injection of EphrinB2/Fc reduced the number of preretinal nuclei by 66% in comparison to control IgG injections. Similarly, EphB4 injections reduced the number of preretinal nuclei by 69%. * $P < 0.05$ compared with control IgG injections. There was no statistically significant difference between noninjected and control IgG-injected eyes. Statistics are based on the median of the averages, using Kruskal-Wallis one-way ANOVA with the Dunn multiple comparison test.

In contrast to EphrinB2 expression, EphB4 appeared to be modulated during normal retinal vascular development, and this pattern of expression appeared to be altered in hyperoxia-injured mice. Because EphB4 is not detected in the neural cells of the retina,^{25,44} the detection of EphB4 presumably reflects its expression by the smaller developing vessels of the transitional and deep vascular beds. Downregulation of EphB4 tran-

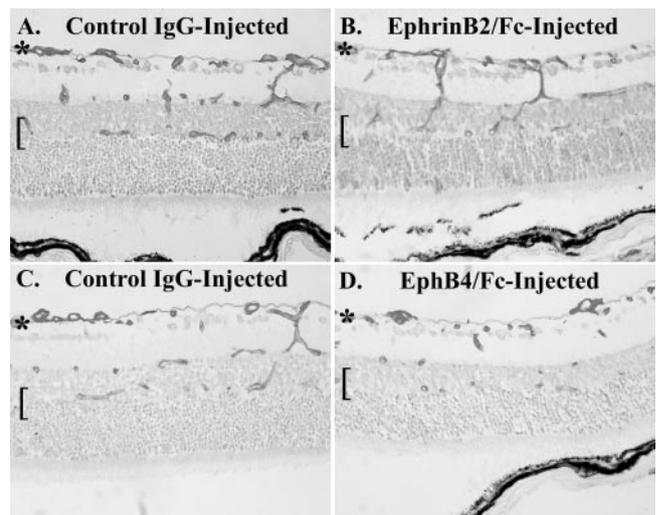


FIGURE 7. Immunodetection of blood vessels in tissue sections (P17) from EphrinB2/Fc- (B) and EphB4/Fc- (D) injected eyes and their respective control injections (A, C). Qualitatively, the amount of blood vessels in the superficial (*) and deep (bracket) layers did not appear to be different between control and experimentally injected eyes (see Fig. 8). Original magnification, $\times 400$.

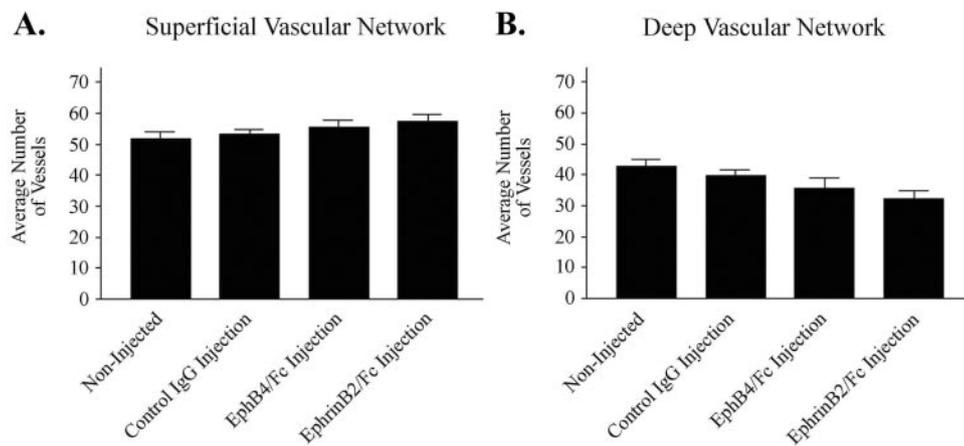


FIGURE 8. Quantification of blood vessels in the superficial (A) and deep (B) vascular beds of the retina on P17. EphrinB2/Fc and EphB4/Fc injections did not significantly alter the amount of blood vessels in either superficial (A) or deep (B) vascular beds, compared with control IgG injections ($P > 0.05$). Blood vessels in superficial and deep regions were counted and analyzed with a one-way ANOVA with a Tukey multiple-comparison test.

script in hyperoxia-injured retinas on P12 compared to normal unexposed retinas of this same developmental period may reflect the halting of vessel growth due to hyperoxia treatment. The increase in expression of EphB4 from P17 to P21 in oxygen-injured retinas presumably reflects the regrowth of vessels during the relative ischemia phase of room air recovery. EphB4 appears to be modulated by oxygen treatment, and the Ephrin/Eph signaling mechanism could play a role in pathologic NV in these retinas.

We next investigated whether Ephrin/Eph signaling is active in the retina of hyperoxia-injured mice. Through the use of an antibody that recognizes the active, phosphorylated forms of Eph receptors,^{36,45} we observed that the proliferating EC of superficial and transitional vessels were positive for active p-Eph receptors on days P15 and P17. This is the time period of peak pathologic NV. The pathologic NV tufts forming during this period were also positive for active p-Eph receptors. These results indicate that Eph receptors are indeed present and activated, presumably via their Ephrin ligands, and suggest that the Ephrin/Eph signaling mechanism play a role in the progression of this disease.

To further investigate this possibility, we next attempted to perturb endogenous Ephrin/Eph signaling by administering soluble EphrinB2/Fc and EphB4/Fc. We took advantage of the inherent promiscuity that Eph receptors show for the same and different class of Ephrin ligands.¹ Retinal fluorescein angiography studies qualitatively indicated reduced retinal tuft formation in hyperoxia-injured retinas as a result of intravitreal injection of EphrinB2/Fc or EphB4/Fc chimeras. Quantitative analysis of preretinal nuclei in H&E-stained tissue sections indicated that intravitreal injection of soluble EphrinB2/Fc or EphB4/Fc resulted in a 66% to 69% reduction in pathologic preretinal tuft formation. The control IgG-injected eyes tended to have less preretinal tuft formation than the noninjected control eyes, but this difference was not statistically significant. We attribute this phenomenon to the release of antiangiogenic factors by the intraocular puncture wound, as has been previously described by Stitt et al.⁴⁶ The exact mechanism by which soluble EphrinB2/Fc or EphB4/Fc reduce preretinal NV remains unknown.

VEGF, the primary initiator of angiogenesis in the retina,⁴⁷⁻⁴⁹ is detected in the mouse retina as early as 1 day after birth⁵⁰ and regulates EC sprouting in this tissue.¹³ VEGF induces the proliferation, sprouting and migration of cultured ECs during angiogenesis, and inclusion of EphrinB2/Fc suppresses these responses.¹⁵ This result parallels our EphrinB2/Fc injection data and follows the general outcome of forward signaling. The reduction in proliferation and migration has been attributed to the inhibition of both VEGF-induced ERK1/2 phosphorylation and the suppression of Ras activity.

Furthermore, Sturz et al.,⁵¹ have provided direct evidence implicating EphrinB2/Fc in the inhibition of EC migration by directly activating the kinase activity of EphB4. Other *in vitro* studies have shown that EphrinB2/Fc can suppress EC spreading in culture due to inhibition of focal contacts, suggesting that EphrinB2 signaling affects EC-EC interactions via cytoskeletal regulation.¹⁰ It is possible that intravitreally injecting EphrinB2/Fc allows it to bind ECs as they penetrate the inner limiting membrane of the retina and reduces their proliferation and migration.

The observation that EphB4/Fc injections reduced angiogenesis in this model is contrary to the general outcome of reverse signaling, because stimulation of endogenous EphrinB2 with soluble EphB4/Fc typically induces the migration and proliferation of purified EC.^{9-16,27} However, similar to our results, a recent *in vivo* study by He et al. (*IOVS* 2004;45:ARVO E-Abstract 2230) demonstrated an inhibition of choroidal NV by soluble EphB4/Fc in a model of laser-induced NV. Likewise, other studies have demonstrated that dominant-negative forms of soluble EphB4 have the ability to inhibit tumor growth and angiogenesis.⁵² It remains to be determined whether the results observed in our studies are due to direct activation of Ephrin ligands by the soluble EphB4/Fc receptors or the effect occurs by EphB4/Fc occupying endogenous Ephrin ligands to prevent them from engaging endogenous EphB4.

A growing body of literature suggests that the outcome of forward and reverse signaling may be dependent on several factors and that data obtained in cell culture systems may not necessarily carry over into *in vivo* scenarios. Several studies have revealed that EphrinB2 and EphB4 can exhibit both proangiogenic and anti-angiogenic properties depending on experimental conditions. Clues as to why these discrepancies in data exist may come from recent studies conducted on human umbilical vascular endothelial cells (HUVECs). Maekawa et al.¹¹ demonstrated that EphrinB2/Fc induces the migration of HUVECs in a dose-dependent manner, whereas Kim et al.¹⁵ report that EphrinB2/Fc inhibits VEGF-induced HUVEC proliferation and migration. The significant difference between the the results of Kim et al.¹⁵ and Maekawa et al.¹¹ are that the former group stimulated their HUVECs with VEGF in conjunction with EphrinB2/Fc, whereas the latter did not. It has been suggested that growth factor-activated ECs, as have been observed during angiogenesis and vessel organization *in vivo*, are particularly responsive to EphrinB2 and EphB4⁵¹ and more accurately represent an *in vivo* scenario.

In contrast to the neovascular tufts, intraretinal vessel formation was unaltered by the soluble chimeras. One possibility is that the chimeric proteins were unable to penetrate the inner-limiting membrane of the retina efficiently due to their large molecular masses (EphrinB2/Fc ~65 kDa; EphB4 ~110

kDa),⁵³ thus sparing intraretinal ECs from encountering the soluble chimeras. However, increased permeability of the diseased tissue argues against this option. Another possible explanation is that cell-cell and cell-matrix interactions, which are known to support EC stabilization and proliferation,⁵⁴ may protect the ECs from the effects of soluble EphrinB2/Fc or EphB4/Fc within the properly formed vessels but not in the tufts.

The data presented in this study demonstrate that EphrinB2 and EphB4 are key regulators of hyperoxia-induced NV of the mouse retina. These observations suggest that these molecules may serve as critical therapeutic targets in treating related diseases (i.e., ROP or diabetic retinopathy). Further studies are necessary to localize the target cells of the soluble chimeras and mechanistically characterize the molecular, cellular, and biological outcomes of these interactions. Ultimately, a better understanding of the mechanistic role that these molecules play during retinal NV should prove useful in better understanding other forms of postnatal angiogenesis.

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