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Altered Vascular Expression of EphrinB2 and EphB4 in a Model of Oxygen-Induced Retinopathy

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Abstract

EphrinB2 ligands and EphB4 receptors are expressed on endothelial cells (EC) of arteries and veins respectively, and are essential for vascular development. To understand how these molecules regulate retinal neovascularization (NV), we evaluated their expression in a model of oxygen-induced retinopathy (OIR). EphrinB2 and EphB4 were expressed on arterial and venous trunks respectively, and on a subset of deep capillary vessels. EphB4 expression was reduced following hyperoxia, while ephrinB2 expression remained unaltered. In addition, a subset of EphB4 positive veins regressed in a caspase-3 dependent manner during hyperoxia. Arteriovenous malformations were also observed with loss of arterial-venous boundaries. Finally, both ephrinB2 and EphB4 were expressed on a subset of neovascular tufts following hyperoxia. These data confirm the contribution of ECs from both venous and arterial origins to the development retinal NV.

Keywords

Angiogenesis; EphrinB2; EphB4; Neovascularization; Arteriovenous Malformation

INTRODUCTION

The Eph receptor and ephrin ligand signaling system has been implicated in the regulation of such cellular events as cell migration, proliferation, attraction, repulsion and apoptosis during neuronal and vascular development (for reviews, see (Davy and Soriano, 2005; Kuijper et al., 2007; Lackmann and Boyd, 2008). Eph receptors and ephrin ligands of the B class are transmembrane proteins which are recognized as one of the largest families of receptor tyrosine kinases (RTKs) (Pasquale, 2008). The ephrinB2 ligand and EphB4 receptor are specifically thought to be key regulators in vasculogenesis and angiogenesis and are expressed on arteries and veins respectively (Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999; Gale et al., 2001; Lackmann and Boyd, 2008). These molecules play a major role in artery-vein demarcation which is critical for the proper balance between these major afferent-efferent vessels and subsequent morphology of their associated capillary plexus. Deletion of either gene leads to embryonic lethality due to vascular defects (Gerety et al., 1999). However, it is thought that the loss of EphB4 forward signaling and not the

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ephrinB2 reverse signaling is essential for early embryonic vascular development (Cowan et al., 2004).

Pathologic neovascularization (NV) of the eye can lead to blindness in several ocular disease processes such as retinopathy of prematurity, diabetic retinopathy and age-related macular degeneration (Saint-Geniez and D'Amore, 2004). The mouse model of oxygen-induced retinopathy (OIR) allows for the study of this pathologic process (Smith et al., 1994). The OIR model is characterized by two distinct phases. In the first phase hyperoxia results in retinal vascular regression and inhibition of the deep vascular plexus development (Smith et al., 1994; Davies et al., 2003). The second phase occurs when, following cessation of supplemental oxygen, relative retinal hypoxia results in the up-regulation of vascular endothelial growth factor (VEGF) with subsequent development of pathologic preretinal NV (Pierce et al., 1995).

Ephrin ligands and their Eph receptors (Umeda et al., 2004; Zamora et al., 2005; Chen et al., 2006) have been implicated in ischemic retinopathies. Direct interference of either ephrin ligands or Eph receptors can disrupt pathologic angiogenesis (Cheng et al., 2002; He et al., 2005; Zamora et al., 2005). Previous work from our group and a recent study by Ehlken and colleagues has shown that intravitreal injection of soluble ephrinB2-Fc, soluble EphB4-Fc, or soluble monomeric EphB4 inhibits retinal NV in the mouse model of OIR (Zamora et al., 2005; Ehlken et al., 2009). However, the exact target cells of the inhibitory soluble molecules remains to be elucidated. In order to identify possible sites of interaction following treatment with soluble ephrinB2 or EphB4, the present study investigates the expression patterns of ephrinB2 and its receptor EphB4 during physiological and pathological angiogenesis in the OIR model.

RESULTS

Expression of EphrinB2 and EphB4 in the Developing Mouse Retinal Vasculature

Arteries can be differentiated from veins within the retina based on both morphological characteristics and by their expression of ephrinB2 and EphB4 respectively (Wang et al., 1998; Adams et al., 1999; Claxton and Fruttiger, 2005; Uemura et al., 2006). Previous studies have demonstrated that the vascular sprouts originating from the optic nerve head during retinal vascular development (postnatal day 2, P2) are of venous origin based on the presence of EphB4 expression and the absence of ephrinB2 expression (Uemura et al., 2006). After the first week of retinal vasculature development, ephrinB2 expression was observed around the major arterial trunks with extension along multiple branch points off the arterial trunks on P8, P10 and P12 (Fig. 1A, C, E). Non-vascular ephrinB2 expression was also appreciated on retinal axons (Fig. 1). In addition to being identifiable by ephrinB2 expression and morphological characteristics, retinal arteries can be identified by α -SMA expression from vascular smooth muscle cells which circumferentially surround arteries and arterioles (Benjamin et al., 1998; Fruttiger, 2002; Stalmans et al., 2002). Expression of α -SMA along the arteriole trunk in the developing retinal vasculature was similar to the ephrinB2 expression (Supp. Fig. 1A, B). However, in contrast to ephrinB2, it did not extend far beyond primary branch points at P8 and P12.

It is around P8 when angiogenic vessels from the superficial vascular plexus begin to migrate toward the posterior retina to form the deep vessel network (Dorrell and Friedlander, 2006). In addition to expression along the major arterioles of the superficial vasculature (Fig. 1B), ephrinB2 expression was observed in a subset (~10–15%) of diving vessels (Fig. 1B, Z-cross section, arrows). Rotation of 3-dimensional reconstructions revealed that expression of ephrinB2 along the descending vessels always occurred directly off an ephrinB2⁺ superficial artery. However, ephrinB2 expression along the sprouting

vessel was limited to the stock cells and not apparent in the filipodia-positive tip cells (data not shown). As the deep vascular network continues to develop, ephrinB2 expression in the superficial arteries persisted at P10 and P12 (Fig. 1C, E). A small subset of vessels between the superficial and deep vascular plexuses continued to express ephrinB2 on P10 and P12 (Fig. 1D, F, arrows), while many of these deep plexus vessels remained devoid of ephrinB2 expression (Fig. 1D, F, arrowheads). As expected, EphB4 expression was seen along veins extending from the optic nerve head in the superficial vascular network on P8, P10 and P12, with limited expression in primary branches (Fig. 2A, C, E). Interestingly, at P8 and P10, EphB4 expression was also observed in limited number of diving vessels in the deep plexus (Fig. 2B, D, arrows). The diving EphB4 vessels originated directly off a venous trunk (Fig. 2D) or a primary venous branch (Fig. 2B). By P12 an occasional vessel extending all the way from the superficial to the deep vascular network was positive for EphB4 expression (Fig. 2F, arrows).

Effects of Hyperoxia on EphrinB2 and EphB4 Expression

Exposure of neonatal mice to 75% oxygen for a period of 5 days results in a central retinal vaso-obliteration observed at P12 with subsequent over-proliferation of ECs cells at P17 (Smith et al., 1994). Following 24 hours (P8) and 5 days (P12) of hyperoxia exposure, ephrinB2 continued to be expressed in the central and peripheral retina along major arteries (Fig. 3A). However, ephrinB2 expression was reduced along branching capillaries, failing to extend beyond secondary branch points (Fig. 3A, arrows). This is in contrast to the expression pattern in the peripheral retina of an age matched room air time point, in which primary, secondary, tertiary and quaternary branching was observed (Fig. 1). As with the room air matched time points, α -SMA expression was observed along the major arteriole trunks in the hyperoxia-exposed retina (Supp. Fig. 2A, 2B).

EphB4 expression was diminished in the central retina at P8 following 24 hours of hyperoxia (Fig. 3B), while some major vessels appeared to be regressing (Fig. 3B, arrowheads). At this same time point, EphB4 expression was absent in peripheral retina (Fig. 3B, asterisk). Following 5 days of hyperoxia exposure EphB4 expression was markedly reduced or absent in both the central and peripheral retina on P12 (Fig. 3B). Despite the reduction in EphB4 expression in the veins, they did not become arterialized as evidenced by the lack of α -SMA labeling on P8 and P12 (Supp. Fig. 2A, 2B). In order to determine if the thinning major veins observed at P8 were regressing via apoptosis, retinas were analyzed following 22hrs of hyperoxia exposure. Qualitative analysis of retinas immunostained for isolectin, EphB4 (anti- β -galactosidase), and activated caspase-3 revealed apoptotic cells within the EphB4 positive vein, while the EphB4 negative artery remained free of caspase-3 positive cells (Fig. 3C). Note the regressing vein in Fig. 3C is approximately the same size as the adjacent artery in the isolectin labeled image, in contrast to the typical morphology on P8 (Fig. 1A). Higher magnification of the EphB4 positive vein demonstrated multiple caspase-3 positive cells within the vessel wall (Fig. 3C, arrows; Supp. Fig. 3), and an occasional apoptotic cell outside the vasculature (Fig. 3C, arrowhead). As expected, central retinal capillaries surrounding the major arteries and veins were also positive for activated caspase-3, consistent with the vaso-obliteration of capillaries observed in the OIR model (Fig. 3C, asterisk).

As a result of our identification of regressing veins on P8, we quantified the number of major vessels in the superficial vascular plexus on P12, which revealed a significant decrease in the total number of veins on P12 following 5 days of hyperoxia compared to P12 room air control retinas (Fig. 3D). In contrast, there was no significant change in the number of arteries after hyperoxia-exposure.

EphrinB2 and EphB4 Expression During Relative Hypoxia

Following 5 days of hyperoxia-exposure neonatal mice were returned to room air on P12 resulting in relative hypoxia in the under-vascularized retina (Dorrell and Friedlander, 2006). This relative hypoxia leads to up-regulation of VEGF resulting in an over proliferation of ECs and secondary neovascular tuft formation at the transition zone between vascular and avascular retina on P17. On both P13 and P15 ephrinB2 expression was still prominent along arterial trunks and in some branches that appeared to anastomose directly with a vein (Fig. 4A, arrows) forming an arteriovenous shunt. These arteriovenous malformations (AVM) were observed in all retinas examined at P15 (n=12). In the majority of the retinas (85%) a “double” shunt was observed with an artery from both sides joining in with the same vein (Supp. Fig.4). At P15 ephrinB2 expression extended from the artery to the vein of the AVM shunt (Fig. 4A, arrowhead). Furthermore, the AVMs persisted at P17, with both the artery and vein were exhibiting α -SMA expression (Supp. Fig. 2C). In regards to ephrinB2 expression in the deep vascular plexus, there are relatively few diving vessels on P13 and of those observed, ephrinB2 expression was not appreciated (Fig. 4B, arrowhead). However, by P15 ephrinB2 expression was found on several vessels spanning the two vascular networks of the retina (Fig. 4B, arrows).

Interestingly, EphB4 expression on P13 was nearly absent except for trace staining located at the optic nerve head (Fig. 4C, arrow) even though morphologic characteristics distinctly showed veins present. While at P15 EphB4 expression had returned along the major venules, there was also low level expression on an occasional artery (as determined by morphology and presence of capillary free zone) at the arteriovenous shunts (Fig. 4C, arrowhead). Unlike ephrinB2, EphB4 expression was not appreciated in the diving vessels at either of the time points (P13, P15) examined along the major venules (Fig. 4D) and rarely in the peripheral regions of the retina (Data not shown).

EphrinB2 and EphB4 Expression in P17 Hyperoxia-Exposed Retinas

On P17, following hyperoxia-exposure, prominent neovascular tufts are observed throughout the retina localized primarily along the transition zone between vascular and avascular retina. Retinal whole mount analysis revealed that some neovascular tufts express ephrinB2 (Fig. 5A, arrows) while others do not (Fig. 5A, arrowheads). In a similar manner to ephrinB2 localization, EphB4 expression was observed in a subset of the neovascular tufts (Fig. 5B, arrows), but not all (Fig. 5B, arrowheads). Expression within the neovascular tufts (arrows) was confirmed in cryo-sections, with some intra-retinal vessels (arrowheads) also staining positive for ephrinB2 or EphB4 (Fig. 5C, D). To determine if ephrinB2 and EphB4 are present on the same distinct neovascular tufts we used an antibody to stain for EphB4 in tissue sections from the ephrinB2^{taulacZ/+} reporter mice, which were labeled with anti- β -galactosidase (to visualize ephrinB2 expression). In approximately half of the neovascular tufts, co-expression of both ephrinB2 and EphB4 was appreciated (Fig. 5E, arrow). In addition, there were subsets of tufts that expressed EphB4 alone (Fig. 5F, arrow), or tufts that expressed only ephrinB2 (Fig. 5G, arrow). Furthermore, some neovascular tufts were observed that were devoid of ephrinB2 or EphB4 expression (Fig. 5H, arrowhead).

While staining for both ephrinB2 and EphB4 in whole mounts and cryo-sections we observed expression outside of the vasculature. In some instances this staining pattern appeared to have Müller cell morphology, therefore we double labeled cryo-sections for β -galactosidase (ephrinB2 or EphB4) and GFAP, a marker for activated Müller cells. We did not observe any co-localization in retinas double labeled for EphB4 and GFAP (Fig. 6A). However, ephrinB2 did co-localize with GFAP (Fig. 6B, arrows) along the Müller cell body. Furthermore, these ephrinB2⁺ Müller cells envelop some intra-retinal blood vessels within the inner nuclear layer (Fig. 6C, arrows). Wild type littermates were used as negative

controls for β -galactosidase staining for both ephrinB2 and EphB4 expressing mice (data not shown).

DISCUSSION

EphrinB2 and EphB4 expression been previously characterized in the developing arteries and veins of the superficial vascular plexus in the neonatal mouse retina (Saint-Geniez et al., 2003; Uemura et al., 2006). The current study extends the prior findings by establishing that ephrinB2 and EphB4 expression was associated with a subset of capillaries in the developing deep retinal vascular plexus and subsets of pathologic preretinal neovascular tufts. Furthermore, ephrinB2 and EphB4 expression helped delineate arteriovenous shunts that were observed in the OIR model.

Expression of EphrinB2 and EphB4 During Deep Capillary Plexus Formation

The deep capillary plexus of the retina begins to develop between approximately P7 and P8 (Dorrell et al., 2002; Davies et al., 2003) by an angiogenic process (Fruttiger, 2007). Our results have revealed that the deep retinal vascular plexus consists of subsets of both ephrinB2⁺ or EphB4⁺ vessels, as well as a large number of ephrinB2⁻/EphB4⁻ capillaries. Expression of ephrinB2 has been previously noted to occur at sites of secondary angiogenesis, including normal physiologic and tumor angiogenesis (Gale et al., 2001; Shin et al., 2001). The ephrinB2 labeled tumor vessels arose from previously existing ephrinB2⁺ arterioles, supporting the notion that ephrinB2⁺ vessels are characteristic of secondary capillary networks. Similar to our results, EphB4 has also been previously noted to be expressed by angiogenic capillaries, while modulation of EphB4 altered vascular patterning, confirming a functional role beyond venous phenotype marker (Erber et al., 2006; Taylor et al., 2007). It has been noted that symmetric distribution of ephrinB2⁺ and EphB4⁺ vessels are essential for normal vascular development (Visconti et al., 2002). This suggests both vessel populations are required for proper formation of the deep retinal plexus, likely providing focal-adhesion and migration clues required for the developing network of vessels (Fuller et al., 2003). However, based on their known repulsive properties it would be unlikely that ephrinB2⁺ and EphB4⁺ vessels would directly connect to each other (Marston et al., 2003; Zimmer et al., 2003). As mentioned above, we observed a large number of ephrinB2⁻/EphB4⁻ deep retinal vessels which would allow intercalation between ephrinB2⁺ and EphB4⁺ vessels permitting formation of a capillary plexus. In support of our data, Oike and colleagues proposed a model for capillary network formation that is regulated by ephrinB2 and EphB4 interactions, in which an ephrinB2⁻ capillary is required to bind with an EphB4⁺ capillary (Oike et al., 2002). The proposed model was subsequently supported experimentally by lacZ staining experiments in which an ephrinB2⁻/EphB4⁻ EC population was found situated between the ephrinB2⁺ and EphB4⁺ ECs (Fuller et al., 2003).

Altered Expression of EphB4 During Hyperoxia Phase

The genetic pre-determination of arterial-venous identity is under the complex control of several gene pathways (Gridley, 2007; Lin et al., 2007), while vessel specification needs to be maintained by local micro-environmental cues and can be altered by changes in blood flow, physiological requirements, and pathologic conditions (Korff et al., 2006; Aitsebaomo et al., 2008; Rocha and Adams, 2009). After hyperoxia exposure ephrinB2 expression is maintained in the developing arteries and arterioles, consistent with a prior study in which ephrinB2 expression in developing retinal arteries was not affected by exposure to 60% hyperoxia (Claxton and Fruttiger, 2005). In contrast, EphB4 expression was present in the central veins but attenuated in the peripheral veins after 24 hours of hyperoxia and completely absent in veins after additional hyperoxia exposure. Similarly, EphB4 mRNA

has also been previously noted to be reduced in the OIR model during hyperoxia (Zamora et al., 2005).

EphrinB2 expression is dependent upon VEGF induction and peak expression has been noted on P6 during normal retinal vascular development, a period of VEGF expression and normal physiologic angiogenesis in the retina (You et al., 2005; Hong et al., 2006; Salvucci et al., 2009). Thus ephrinB2 is associated with arteries and small arterioles when the retina is exposed to hyperoxia on P7, and despite the down-regulation of VEGF during the phase of hyperoxia (Pierce et al., 1995), expression of ephrinB2 is maintained, suggesting alternative regulation. In addition to VEGF, endothelial ephrinB2 expression can also be regulated by contact with smooth muscle cells or pericytes, with expression at points of contact (Korff et al., 2006; Salvucci et al., 2009). As shown by the SMA expression pattern, the relationship between ephrinB2⁺ arterioles and mural cells is maintained during the hyperoxia phase. Although the initial onset of ephrinB2 expression is independent of mechanical forces, shear stress or wall tension could also induce and maintain gene expression of ephrinB2 as a result of arterial blood flow (Othman-Hassan et al., 2001; Obi et al., 2009).

Recently *COUP-TFII* has been identified as a regulator of venous identity, down-regulating the Notch signaling pathway and expression of the VEGF co-receptor, neuropilin 1 (NP-1), thereby inhibiting arterial fate (You et al., 2005; Lin et al., 2007). Although we observed down-regulation of EphB4 expression during the hyperoxia exposure, mRNA expression of *COUP-TFII* is unchanged (unpublished observations). Similar to our results EphB4 expression has also been shown to be lost in a model of vein graft adaptation, without the vessel acquiring an arterial phenotype (Kudo et al., 2007). In regards to alternative regulation of EphB4 expression, erythropoietin (Epo) is capable of inducing a venous phenotype in EC cultures by up-regulating EphB4 expression (Muller-Ehmsen et al., 2006). During the hyperoxia phase in which EphB4 expression is reduced, local Epo levels are also significantly suppressed (Chen et al., 2008), suggesting that the reduced Epo levels observed in the OIR model could potentially result in suppression of EphB4 expression. PECAM is another molecule with the potential to modulate EphB4 expression in the retina (Dimaio et al., 2008). Functional studies will be required to further evaluate the role of these potential regulators of EphB4 expression in the retina.

Retinal EphB4 expression permitted us to appreciate that a subset of veins were more sensitive to hyperoxia exposure as compared to arteries, regressing after 24 hrs of hyperoxia via apoptosis. One molecule with the potential to contribute to vein and capillary survival is Angiopoietin-1 (Ang-1). Ang-1 has been identified as an essential EC survival factor, playing a key role in protecting retinal vessels from apoptosis (Hoffmann et al., 2005; Childs et al., 2008). Additionally, modulation of Ang-1 was observed to specifically alter the number of major blood vessels in both retinal and early vascular development models (Suri et al., 1996; Uemura et al., 2002). In humans, the venous vasculature appears to more sensitive to defects in the Ang-1/Tie-2 pathway than arterial vessels with the development of venous malformations (Gaengel et al., 2009). In regards to the mouse OIR model, the exposure of mice at P7 to hyperoxia has been shown to significantly suppress the retinal expression of Ang-1, increasing vascular susceptibility to EC apoptosis and subsequent vessel regression (Hoffmann et al., 2005). Interestingly, in addition to promoting a venous phenotype, the Epo signaling pathway is also a major positive regulator of Ang-1 expression, but as previously noted, Epo levels are significantly reduced during the hyperoxia phase, potentially making retinal EC at risk for apoptosis from both reduced Epo and Ang-1 levels (Kertesz et al., 2004; Chen et al., 2008). Finally, perhaps the retinal veins are at higher risk for apoptotic-induced regression as compared to arteries secondary to reduced intrinsic numbers of smooth muscle cells and associated EC survival factors (Transforming Growth Factor- β , Platelet-derived Growth Factor) (Shih et al., 2003; Gaengel

et al., 2009). There is also data to suggest that arterial ECs are intrinsically resistant to apoptosis as compared to capillaries and veins by expression of inhibitory Smads (I-smad) (Kiyono and Shibuya, 2006).

EphrinB2 and EphB4 Expression During Relative Hypoxia Phase

We initially sought to compare and contrast the deep vascular plexus expression of ephrinB2 and EphB4 after hyperoxia-induced injury to normal development. On P13 the superficial arteries expressed ephrinB2 while the deep vascular plexus starts to recover (Davies et al., 2003). Although initially deep plexus vessels did not express ephrinB2 or EphB4, there was a recovery of ephrinB2⁺ vessels in the deep network by P15. In contrast, EphB4 was expressed by superficial veins but continued to be absent in the deep plexus vessels on P15. VEGF expression is rapidly increased in the OIR model on days P12.5 to P17 (Pierce et al., 1995) and we have previously demonstrated significant EC cell proliferation in the deep network on P14 (Davies et al., 2006). VEGF also regulates ephrinB2 expression which correlates with the recovery of ephrinB2 in the deep network by P15. Altered signaling pathways, as a result of the hyperoxia exposure, likely accounts for the attenuated EphB4 expression (Hong et al., 2006; Lin et al., 2007). As previously noted, Epo is a molecule that can influence EphB4 expression and is suppressed during the hyperoxia phase (Muller-Ehmsen et al., 2006; Chen et al., 2008). In contrast to VEGF, Epo up-regulation is delayed until P15 during the relative hypoxia phase and could be one factor for EphB4 expression being limited to superficial vessels at this time point.

The finding of arteriovenous malformations (AVMs) during vascular recovery has been previously noted in the OIR model (Lobov et al., 2007) but the altered expression patterns of ephrinB2/EphB4 and loss of artery-vein boundaries has not been previously appreciated in the retina. However, AVMs with altered arterial and venous boundaries has been previously described in mouse studies of early vascular development (Urness et al., 2000; Sorensen et al., 2003). In humans with venous malformations, expression of ephrinB2 has been observed in malformed veins with reduced expression in accompanying arteries (Diehl et al., 2005). In addition, mice in which Delta-like 4 ligand is over-expressed, AVMs also develop with arterIALIZATION of veins with associated ectopic ephrinB2 expression, similar to the AVMs noted in our study (Carlson et al., 2005; Trindade et al., 2008). Altered blood flow force and shear stress are also known to regulate ephrinB2 and EphB4 expression and artery-vein differentiation (le Noble et al., 2004). It remains to be determined if the development of AVM's in the oxygen-injured retina is related to altered ephrin/Eph expression or changes in hemodynamic forces. Direct experimental manipulation of ephrin/Eph expression (e.g., gain of function/loss of function studies) will be required to further delineate their involvement in the etiology of AVM's.

Expression of EphrinB2 and EphB4 in Neovascularization Phase

We found that ephrinB2 and/or EphB4 were expressed in a subset of NV tufts confirming their involvement in retinal NV. Expression of ephrinB2 and EphB4 have been previously observed at sites of NV in models of cutaneous wound healing and tumor angiogenesis, as well as human pathological specimens of inflammatory and ocular angiogenesis (Yuan et al., 2000; Gale et al., 2001; Shin et al., 2001; Umeda et al., 2004). Co-expression of ephrinB2 and EphB4 was also noted in some neovascular tufts, which is consistent with previous observations by Fuller and colleagues, implying a role for these molecules beyond artery/vein demarcation (Fuller et al., 2003). A biologic role for the ephrinB2/EphB4 pathway in retinal NV has recently been confirmed by immunolocalization of phosphorylated ephrinB in neovascular tufts (Salvucci et al., 2009). The promotion of angiogenesis by ephrinB2 and EphB4 is thought to occur through signaling effectors that modulate cell adhesion, migration of ECs, and interactions with pericytes (Foo et al., 2006;

Pasquale, 2008; Salvucci et al., 2009). Activation of EphB4 can also enhance endothelial progenitor cell adhesion and targeting to sites of angiogenesis and this cell population has been localized to neovascular tufts in a mouse model of retinal angiogenesis (Grant et al., 2002; Foubert et al., 2007). Our findings of ephrinB2 and EphB4 expression suggest that pre-retinal neovascular tufts are a viable target for anti-angiogenesis therapies that modulate ephrinB2/EphB4 interactions. Additional studies examining ephrinB2 and EphB4 expression after modulation are warranted because of the complex interactions of these multi-functional ligands and receptors with other cell populations (Pasquale, 2008).

Expression of EphrinB2 in Activated Müller Cells

Retinal Müller cells are known to be activated in the OIR model as evidenced by increased GFAP expression (Smith et al., 1994). Activated Müller cells are often located at the border between the vascular and avascular ischemic retina, an area of neuronal apoptosis and microglia/macrophage activation (Davies et al., 2003; Davies et al., 2006). The expression of ephrinB2 in activated Müller cells suggests function beyond just angiogenic in the ischemic retina. Expression of ephrinB1 and ephrinB2 ligands is up-regulated in activated Müller cells and astrocytes in glaucomatous optic neuropathy and in a model of spinal cord transection supporting the theme of increased ephrin expression at sites of neuronal injury (Bundesen et al., 2003; Schmidt et al., 2007).

In summary, our data demonstrates that ephrinB2 and EphB4 expression are altered during hyperoxia-induced injury to the retina with subsequent localization to neovascular tufts during the phase of relative hypoxic recovery. These data offer an important foundation for additional mechanistic investigations into the role of ephrinB2 and EphB4 in retinal NV. For example, EphB4 gain of function studies, during the hyperoxia phase, are warranted in order to determine the effect upon vein regression and subsequent development of AVMs. In addition, the response of ephrinB2⁺ and/or EphB4⁺ neovascular tufts to a specific agonist/antagonist intervention can be monitored to confirm on-target response.

EXPERIMENTAL PROCEDURES

Animals

Breeding pairs of ephrinB2^{taulacZ/+} and EphB4^{taulacZ/+} reporter mice, on a C57BL/6 background, were purchased from The Jackson Laboratory (Bar Harbor, Maine), provided food and water ad libitum, and kept on a 12 hour light-dark cycle. Mice were housed and bred in the Oregon Health & Science University animal care facilities and treated in compliance with the NIH guidelines and the guidelines outlined in the ARVO statement for “The Use of Animals in Ophthalmic and Vision Research”. Utilizing the mouse model of OIR established by Smith and colleagues (Smith et al., 1994), both ephrinB2^{taulacZ/+} and EphB4^{taulacZ/+} neonates, along with nursing females, were exposed to 75% oxygen for five days beginning on P7 and then allowed to recover in room air on P12. Room air age matched control litters of both ephrinB2^{taulacZ/+} and EphB4^{taulacZ/+} mice were maintained in identical conditions as the hyperoxia-exposed animals. Hyperoxia-exposed and room air control pups were sacrificed by CO₂ euthanasia or cervical dislocation on P8, P10, P12, P13, P15, and P17. For cryo-sections, eyes were carefully enucleated from each mouse and placed in 2% paraformaldehyde (PFA) for 1 hr, and snap frozen in OCT. These eyes were sectioned at 10-μm intervals, mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA) and stored at -80°C until used for immunohistologic analysis. For whole mounts, the retina and lens were dissected and fixed in 4% PFA for 30 minutes at 4°C. After fixation, the lens was removed and the retina fixed an additional 15 minutes in 100% methanol. Immunohistologic analysis was performed immediately as described below.

Immunohistochemistry

For whole mount analysis, retinas were incubated overnight at 4°C, with a rabbit anti-mouse β -galactosidase antibody (Immunology Consultants Laboratory, Inc., Newberg, OR) and rabbit anti-human activated caspase-3 (Cell Signaling Technology, Danvers, MA). Retinas were then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), followed by incubation with fluorescein avidin-D (Vector). Alternatively, some retinas were incubated with a goat anti-rabbit IgG Alexa Fluor® 488 or 647 (Molecular Probes, Eugene, OR) labeled secondary antibody. Additionally, a subset of retinas were incubated with a anti- α SMA-Cy3 (Sigma-Aldrich, MO) antibody. All retinas were simultaneously incubated with isolectin GS-IB₄ Alexa Fluor® 594 or 647 conjugate (Molecular Probes) to label endothelial cells. Retinas were incised radially, the vitreous removed, flat-mounted with *SlowFade*® antifade (Molecular Probes) and visualized via fluorescence or laser scanning confocal microscopy.

Retinal cryo-sections and paraffin embedded sections were immunolabeled with the following antibodies either alone or in combination: a rabbit anti-mouse β -galactosidase antibody (Immunology Consultants Laboratory, Inc., Newberg, OR); a cow anti-GFAP (DAKO, Carpinteria, CA) a marker for activated Müller cells; a rabbit anti-EphB4 antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA); and GS-isolectin (Molecular Probes) and counterstained with DAPI ($n = 6-8$ eyes; 4-8 sections per eye).

Retinal whole mounts ($n=4-8$ retinas per time point) and cross sections ($n= 6-8$ eyes; 4-8 sections per eye) were visualized by light and fluorescence microscopy and photographed with a Leica DC500 digital camera (Leica Microsystems, Bensheim, Germany). Confocal microscopy was used to assess the diving vessels on P8, P10 and P12 retinal whole mounts (Olympus, Fluoview FV1000). Olympus Fluoview software was used to generate 3D reconstructions from confocal z-stacks, which were rotated 90-degrees to view all vessels traversing the retina.

Artery and Vein Quantification

To quantify the major vessels extending out of the optic nerve on P12, retinal whole mounts were immunolabeled with isolectin as described above. As has been described previously, arteries and veins can be identified based on morphological criteria (Wang et al., 1998; Adams et al., 1999; Claxton and Fruttiger, 2005; Uemura et al., 2006). Specifically, arteries tend to be smaller in diameter than veins, and can be identified by the capillary-free zones which veins lack. The total number of arteries and veins were then counted in a masked fashion from the digital images (P12 room air control, $n = 9$; P12O₂, $n = 13$). Results are expressed as the mean $\pm$ standard error of the mean. Statistical significance was determined using the Student *t* test.

Supplementary Material

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Figure 1.

EphrinB2 is expressed in a small subset of diving retinal vessels. Staining for isolectin (red) and ephrinB2 (green), by immunolabeling for anti- β -galactosidase, in LacZ reporter mice in P8 (**A, B**), P10 (**C, D**), and P12 (**E, F**) retinal whole mounts under normoxic conditions. Extending out from the optic nerve head (ON) ephrinB2 expression is observed on arteries (a) but not veins (v). This expression is present on major arterial trunks as well as two and three branch points off the major trunk at all time points evaluated (**A, C, E**: Mag. 200X). From 3-dimensional renderings it was observed that ephrinB2 expression is present on a subset (arrows), but not all (arrowheads), of the diving vessels penetrating into the deep neuroretina (**B, D, F**: Z-cross sections are 90° rotation around the x-axis of enface 3D-image). Mag. 400×

Figure 2.

EphB4 is expressed in a small subset of diving retinal vessels directly under major veins. Staining for isolectin (red) and EphB4 (green), by immunolabeling for anti- β -galactosidase, in LacZ reporter mice in P8 (**A, B**), P10 (**C, D**), and P12 (**E, F**) retinal whole mounts under normoxic conditions. Extending out from the optic nerve head (ON) EphB4 expression is observed on veins (v) but not arteries (a). This expression is restricted to major venous trunks (**A, C, E**: Mag. 200 $\times$). Additionally, EphB4 expression is seen on descending vessels directly underneath major vein trunks (**B, D, F**, arrows; Z-cross sections are 90 $^{\circ}$ rotation around the x-axis of enface 3D-image). As observed before, numerous vessels traversing the neuro-retina are EphB4 negative (**B, D, F**, arrowheads: Mag. 400 $\times$).

Figure 3.

Hyperoxia suppresses vein-specific gene expression in retinal vasculature. Mouse retinas were evaluated following 24 hours (P8) or 5 days (P12) of hyperoxia exposure. **A:** At both time points, ephrinB2 expression remains intense in arterial trunks in both the central and peripheral retina. Although, there is less branching off of the major arteries in the peripheral retina (A, arrows) compared to room air controls. **B:** At P8, EphB4 expression is present on veins (arrows) in the central retina at the optic nerve, but fades as the vein extends to the peripheral retina. Additionally, one vessel appears to be a vein based on EphB4 expression (arrowhead) although it has lost the morphologically characteristic thickness of a typical vein coming out of the optic nerve. Furthermore, at P12 EphB4 expression is absent from both the central and peripheral retina. **C:** Activated caspase-3 staining was observed within capillary plexus (asterisk) in addition to EphB4 positive veins (v), while the EphB4 negative artery (a) was negative for caspase-3 staining following hyperoxia exposure. Higher magnification (600 $\times$) demonstrates multiple caspase-3 positive cells located within the EphB4 positive vein (arrows) in addition to a non-vascular staining (arrowhead). **D:** Quantification of veins (v) and arteries (a) at P12 following hyperoxia exposure reveals a significant decrease in the total number of veins, but not arteries, compared to room air age matched controls. (H= hyaloid vessel) Mag. 200 $\times$ (except where indicated).

Figure 4.

A: Expression patterning of ephrinB2 during the relative hypoxic phase of OIR; note the arteriovenous shunt formation (arrows), and the apparent expression of ephrinB2 on what morphologically appears to be a vein (inset: arrowheads). **B:** At P13 the initial diving vessels appear to be ephrinB2 negative (3D projection of Z-cross sections), while a subset of diving tortuous vessels at P15 express ephrinB2 (arrows), with some remaining ephrinB2 negative (arrowhead). **C:** EphB4 expression remains minimal at P13 and localized to the central retina (arrow), but has returned to the venous trunks (v) by P15. EphB4 expression is also observed on an artery (arrowhead) near the arteriovenous shunt (yellow box) at P15. **D:** At P13 no EphB4 expression is observed in the peripheral retina. However, at P15 EphB4 expression is observed along what appears to be an artery (arrowheads, higher magnification of yellow box in C). At either time point there is no expression along diving vessels (3D projection of Z-cross sections). Additionally, there appears to be some extra vascular staining within the retina (asterisk). Mag. 100× **A** and **C**; 400× **B** and **D**.

Figure 5.

EphrinB2 and EphB4 expression at P17, the peak of retinal NV. On P17 following hyperoxia exposure, prominent neovascular tufts are observed throughout the retina located at the transition zone between the vascular and avascular retina. In retinal whole mounts stained for lectin (red) and β -galactosidase (green), some tufts express ephrinB2 (A, arrow), while others do not (A, arrowhead). Similarly, EphB4 expression is observed in only a subset of neovascular tufts (B, arrow), but not all (B, arrowhead). Expression of ephrinB2 and EphB4 within neovascular tufts (arrows) was confirmed in retinal cross-sections (C & D, respectively). Some intra-retinal vessels were also positive for ephrinB2 and EphB4 (C, D, arrowheads). Retinal cross-sections from ephrinB2^{taulacZ/+} mice were immunolabeled with β -galactosidase (green), lectin (red) and an antibody for EphB4 (cyan) and counterstained with DAPI (blue) at the peak of NV (E–H). Subsets of neovascular tufts were observed that had both ephrinB2 and EphB4 expression (E, arrow); that had EphB4 expression, but no ephrinB2 expression (F, arrows); that had ephrinB2 expression, but no EphB4 expression (G, arrows); or that were devoid of both ephrinB2 and EphB4 expression (H, arrowhead). Mag. 400 $\times$ A–D, 600 $\times$ E–H

Figure 6.

Extra-vascular expression of ephrinB2 and EphB4. In some instances the ephrinB2 staining pattern in tissue sections appeared to have Müller cell morphology. To confirm this observation, EphB4 and ephrinB2 expression was double labeled with GFAP, a marker for activated Müller cells. **A:** Representative micrographs from retinal cross sections labeled for EphB4 expression which did not co-localize with GFAP. **B:** Staining for ephrinB2 expression revealed co-localization with GFAP (arrows) along the Müller cell processes. **C:** Furthermore, ephrinB2 positive Müller cells (green, a, c, d; arrows) surrounded intra-retinal blood vessels (red, b-d; arrows) along the inner nuclear layer. A high resolution image of the yellow box in (c) is shown in (d). Mag. 400× A, B; 600× C (scale bar ~30 microns)