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### Review

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# Retinopathy of prematurity: understanding ischemic retinal vasculopathies at an extreme of life

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**Retinopathy of prematurity (ROP) is a major complication of preterm birth. It encompasses a spectrum of pathologies that affect vision, from mild disease that resolves spontaneously to severe disease that causes retinal detachment and subsequent blindness. The pathologies are characterized by an arrest in normal retinal vascular development associated with microvascular degeneration. The resulting ischemia and retinal hypoxia lead to excessive abnormal compensatory blood vessel growth. However, this neovascularization can lead to fibrous scar formation and culminate in retinal detachment. Present therapeutic modalities to limit the adverse consequences of aberrant neovascularization are invasive and/or tissue-destructive. In this Review, we discuss current concepts on retinal microvascular degeneration, neovascularization, and available treatments, as well as present future perspectives toward more profound elucidation of the pathogenesis of ROP.**

Retinopathy of prematurity (ROP) is the major ocular disorder of the neonate (1, 2) and the dominant cause of severe visual impairment in childhood in North America and Europe. ROP is associated with significant sequelae, the most serious being retinal detachment, which results in blindness. However, even milder forms of ROP increase the incidence of pathologies that negatively impact visual acuity, for example, ametropias, refractive errors that reduce visual acuity; strabismus, a condition in which the eyes are not properly aligned, preventing proper binocular vision and adversely affecting depth perception; and disorders of color discrimination (3–6). ROP proceeds following an initial phase of degeneration of the retinal microvasculature (vasoobliteration) (7, 8) (Figure 1) that is associated with cessation of progression of vascular growth toward the retinal periphery. In the subsequent phase of the disease, the ensuing retinal ischemia predisposes to abnormal compensatory neovascularization (9, 10). Of the various factors that have been associated with the development of ROP, low birth weight, low gestational age, supplemental oxygen therapy, and its associated relative hyperoxia dominate.

The development of the human retinal vasculature commences at approximately the 16th week of gestation and concludes at term (i.e., the 40th week of gestation) (11). Hence, when an infant is born prematurely, its retinal blood supply is incomplete and highly vulnerable to decay. This immaturity in vascular development predisposes the retina to complications. Major advances have been made over the past 30 years in identifying mechanisms implicated in the genesis of ROP. Comprehension of mechanisms underlying this disorder has, in turn, enhanced understanding of the pathogenesis of ischemic retinal vasculopathies in the adult, for example, diabetic retinopathy (a complication of diabetes mellitus) and neovascular forms of age-related macular degeneration (the major cause of visual impairment in adults over 50 years of age).

The oxygen-induced retinopathy (OIR) model of ischemic retinopathy (12, 13), which adequately reproduces the vasoobliterative and neovascularization phases of ROP and accurately assesses treatment outcome (14), has been a valuable tool to researchers studying ischemic retinopathies, providing substantial insight into these conditions. The OIR model has largely been utilized in rodents because development of the retinal vasculature in these animals occurs mainly after birth, allowing retinal angiogenesis to be studied under both physiologic and pathologic conditions. Understanding both the mechanisms of normal retinal vascular development and the pathophysiological processes leading to primary vascular loss is the key to developing new therapeutic approaches to prevent the sight-threatening neovascularization associated with ROP and ischemic retinal retinopathies in the adult.

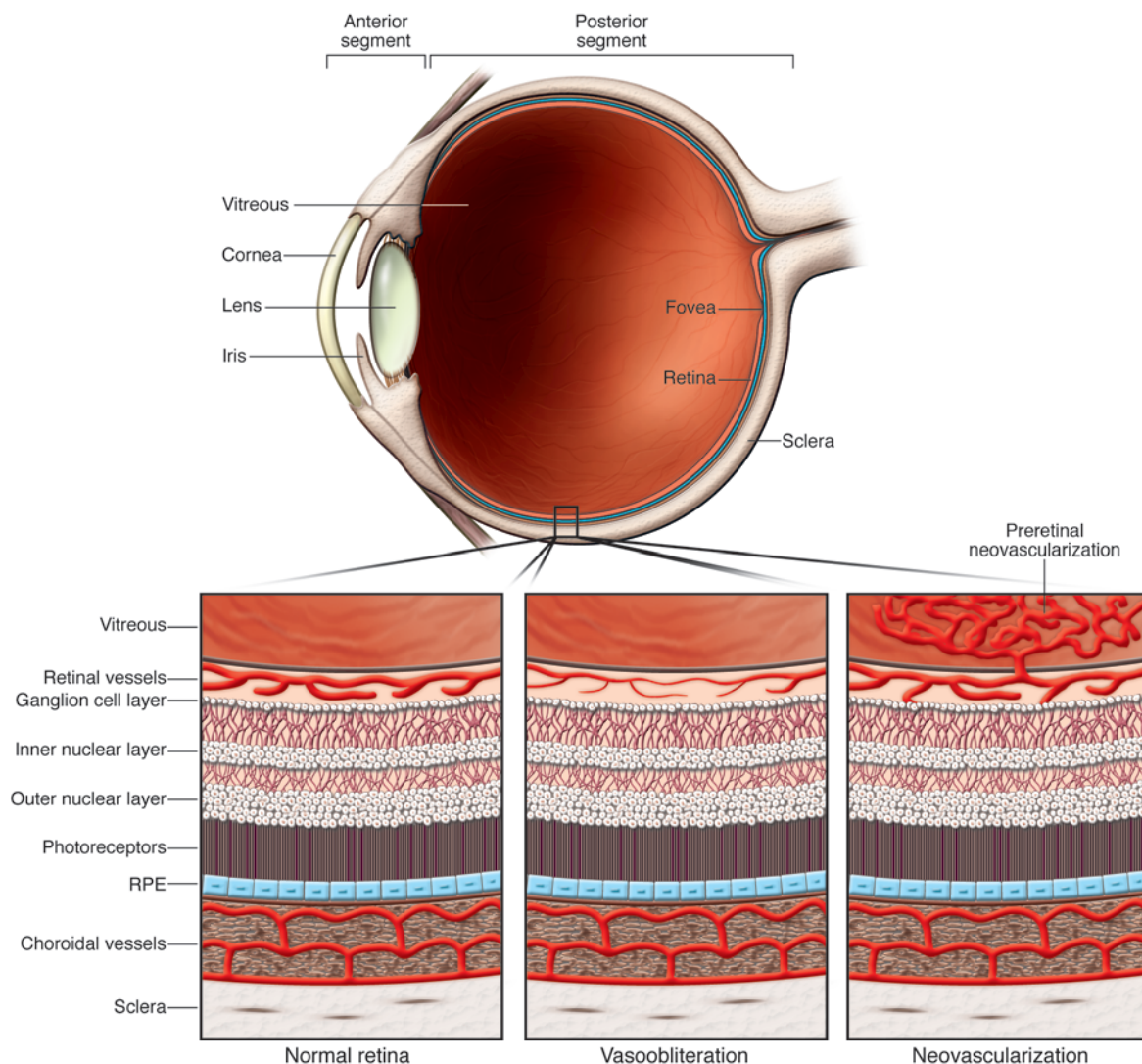
In this Review, we address various aspects of ROP pathogenesis, including mechanisms involved in the control of ocular circulation, vasoobliteration, neovascularization, and therapeutic approaches, and outline future perspectives. Although the field of ROP is dynamically expanding into novel areas of research, here we explain key concepts and concentrate on the most widely accepted theories.

## Regulation of ocular blood flow

Relative excess oxygen supply to the premature infant is one of the most important factors involved in the genesis of ROP (15, 16). Accordingly, limiting hemoglobin saturation with oxygen by reducing oxygen supplementation has been repeatedly shown to be effective in diminishing the rate of ROP (17–19). To understand the effects of oxygen in the genesis of ROP, it is first important to consider how blood supply to the inner retina is governed. The mechanisms that modulate ocular circulation (as elsewhere in the body) are regulated by a complex interplay of systemic factors (including circulating hormones and autonomic innervation) that affect total cardiac output and factors that regulate flow locally (i.e., variations in perfusion pressure,

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**Figure 1**

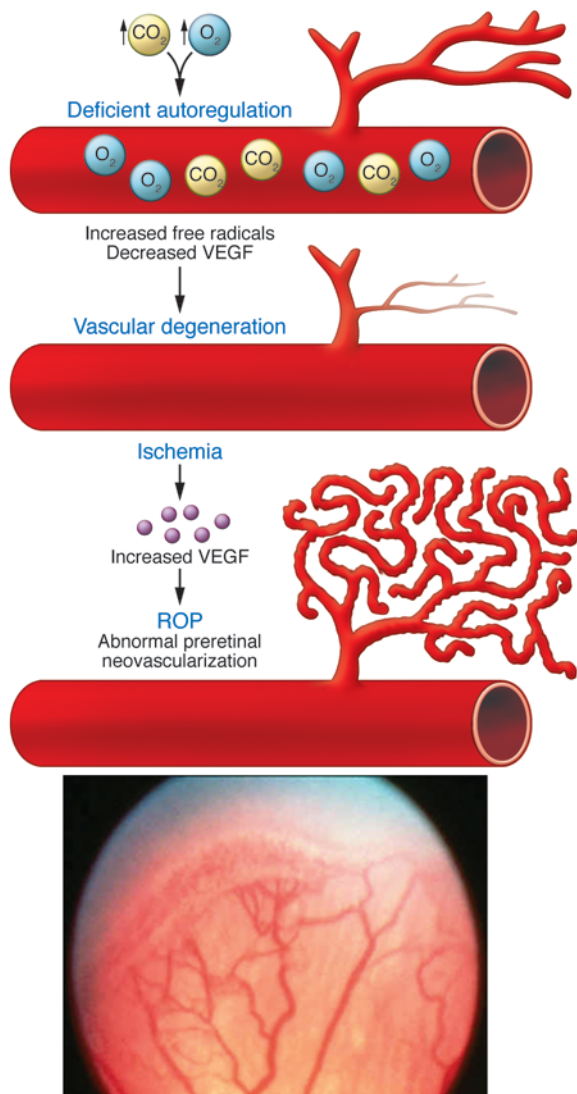
Overview of the pathogenesis of ROP. Schematic depiction of the neural retina and the vascular beds that perfuse it. Retinal vessels (which form preretinal vascular tufts in ROP) are present adjacent to the retinal ganglion cell layer next to the vitreous body. As ROP progresses, there is an initial phase of vascular degeneration (vasoobliteration), followed by a secondary phase of compensatory (but pathologic) angiogenesis toward the vitreous of the retina (preretinal neovascularization). The choroidal vascular plexus, which supplies the outer retina and is affected in age-related macular degeneration, is present behind the photoreceptors at the back of the eye. RPE, retinal pigment epithelium.

pH, partial arterial pressure of oxygen [ $\text{PaO}_2$ ], and partial arterial pressure of carbon dioxide [ $\text{PaCO}_2$ ]). These factors act in concert to ensure adequate blood supply to meet tissue demand. The retina is predominantly influenced by local factors, as it lacks autonomic vascular innervation (20–22).

The capacity to keep blood flow constant over a range of perfusion pressures and oxygen tensions and to adjust blood flow to meet metabolic demand is termed autoregulation (23). In the adult, retinal blood flow (RBF) is maintained constant over a wide range of perfusion pressures, whereas in the newborn, RBF is autoregulated over a much narrower range (24–29); moreover, preterm infants who suffer complications of prematurity exhibit total absence of autoregulation of neural blood flow (30–32). Similarly, blood flow to the choroid (ChBF) — the vascular layer that is the major supplier of oxygen to the outer retina and is incompletely vascularized in the

immature newborn — is also autoregulated in the adult over a wide range of perfusion pressures, while in the newborn, ChBF autoregulation is essentially absent (25–27, 33–36). Autoregulation of ocular blood flow also responds to changes in blood oxygen tension.

In hyperoxia, such as might occur during oxygen supplementation protocols (mechanical ventilation) administered to premature infants to overcome respiratory insufficiency, the retinal vasculature constricts comparably in the newborn and adult (9, 25, 34, 35, 37, 38). However, compared with those in the adult, vessels of the choroid in the newborn do not constrict in hyperoxia. Consequently, during an acute rise in oxygen tension or perfusion pressure, both the RBF and ChBF of the newborn cannot be maintained constant, resulting in an exaggerated delivery of potentially toxic oxygen to the retina (Figure 2) (25, 27, 38–41). This relative inability of the neonate to control oxygen delivery to the retina is largely due



**Figure 2**

Deficient autoregulation in the premature retina. Deficient autoregulation of ocular blood flow in the newborn fails to limit retinal oxygenation during hyperoxia. This results in excess delivery of oxygen to tissues. High carbon dioxide tension increases ocular blood flow and further curtails its autoregulation. Increased retinal oxygenation augments free radical generation and suppresses VEGF expression in the newborn. This leads to arrest in vascular development and microvascular degeneration. The ensuing ischemia triggers the aberrant neovascularization seen in ROP.

low intrauterine to higher extrauterine oxygen tension, results in exposure of immature tissues to a relative excess of oxygen. This provokes endothelial cell death and thus obliteration of retinal vessels, because infants born prematurely are prone to oxidant injury. The neonate retina (and the adult retina) is rich in mitochondria and maintains a high rate of oxidative metabolism readily overwhelmed in this abnormal developmental context (44). The incomplete reduction of oxygen, largely by complex III of oxidative phosphorylation, leads to the generation of ROS (45). In addition, the retina contains numerous molecular photosensitizers that generate free radicals upon excitation (46–48). In contrast to adults, there is more free iron in the neural tissue of newborns to catalyze oxidizing reactions (49, 50). Finally, this ability of the retina to generate excessive ROS is not counterbalanced by antioxidants, which are deficient in the immature subject relative to the adult, since the low oxygen-exposed fetus has modest needs for antioxidants in utero (51, 52).

Necessary intermediates of constitutive biochemical reactions also generate ROS. Endothelial cell enzymes and transporters, including xanthine oxidase, NADPH oxidase, COX, NOS, and to a lesser extent those involved in lipoxygenase pathways, contribute to the generation of free radicals. Interestingly, COX and NOS activities are high in the neonatal period and contribute to peroxidation in ocular tissues, especially in premature subjects with reduced antioxidant defenses and subjected to exaggerated oxygen tension secondary to mechanical ventilation. During oxidative stress, the COX pathway is an important producer of free radicals, amplified by ROS due to a positive feedback loop (53). Increased COX activity produces more prostaglandins (mostly PGD<sub>2</sub> and PGE<sub>2</sub>), themselves upregulating eNOS and NO production to further increase ocular blood flow and oxidant stress to the immature retina. Furthermore, ROS can react with NO to generate highly reactive nitrogen species (RNS), including peroxynitrite, nitrogen dioxide, and dinitrogen trioxide (54). The deleterious effects of these RNSs on cell function are generally referred to as nitrative stress (55) and result in retinal microvascular degeneration (56, 57). In line with these findings, endothelial NOS was shown to contribute to retinal vascular degeneration in a mouse model of ROP and increase the ensuing pathologic neovascularization (58). Similarly, inducible NOS (which is upregulated in hypoxia) inhibits physiologic retinal revascularization subsequent to vasoobliteration and encourages preretinal neovascularization (59).

Lipid peroxidation of cell membranes secondary to inadequate oxygen tension is pivotal to the pathogenesis of ROP and, together with nitrative stress, among the most toxic consequences of increased ROS. Polyunsaturated fatty acids (PUFAs) of membrane phospholipids are common targets for peroxidation, resulting in loss of cell membrane function and structural integrity (60, 61). For reasons that remain ill defined, retinal endothelial cells are

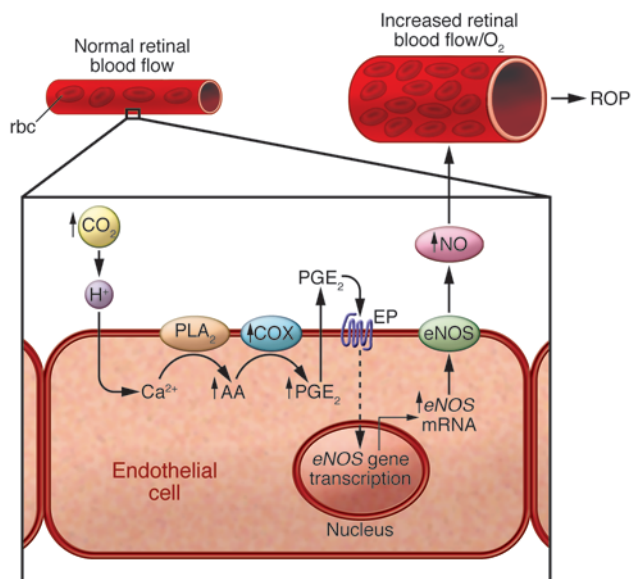
to high perinatal levels of prostaglandins (such as PGD<sub>2</sub> and PGE<sub>2</sub>) and NO that profoundly influence vasomotor tone and override the autoregulatory response (27, 38–40, 42) (Figure 3).

Another factor significantly contributing to the disruption of regulation of RBF and ChBF in infants born prematurely is the rise in carbon dioxide tension (hypercapnia) in conservatively mechanically ventilated and lung-damaged premature infants. Both acute and sustained hypercapnia markedly increase RBF and ChBF in infants born prematurely. The acute rise in ocular blood flow in response to hypercapnia is largely PGE<sub>2</sub> dependent. During sustained hypercapnia, this increase in PGE<sub>2</sub> induces expression of eNOS, which releases NO and in turn mediates the delayed carbon dioxide-induced rise in ocular hemodynamics (Figures 2 and 3) and further curtails ocular blood flow autoregulation (43). This marked induction in ocular hyperemia is clinically relevant, since hypercapnia is associated with ROP in humans and experimental animals (17).

**Vasoobliteration**

*Oxygen-dependent factors: oxidative and nitrative stress and their downstream effects.* The exaggerated retinal oxygenation secondary to oxygen supplementation, along with the sudden transition from



**Figure 3**

The effects of hypercapnia on RBF. Sustained hypercapnia evokes a marked increase in RBF. This effect is sequentially mediated by calcium entry into endothelial cells, inducing increased PGE<sub>2</sub> production and in turn eNOS-derived NO formation.

particularly vulnerable to peroxidation-induced injury, whereas pericytes, smooth muscle cells, and perivascular astrocytes are relatively resistant (62–64). The retina is highly susceptible to lipid peroxidation, being composed of lipids with elevated levels of PUFAs such as docosahexaenoic acid (DHA), *cis*-arachidonic acid (AA), and choline phosphoglyceride.

Prostanoids are synthesized from AA by the sequential action of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and COX triggered by oxidant stress and peroxidation (27, 39, 65, 66). The accumulation of peroxides eventually favors thromboxane (TXA<sub>2</sub>) production over that of prostaglandins (39, 53). In contrast to TXA<sub>2</sub> synthase, prostaglandin synthases function best under reducing conditions (which are required by the cofactors glutathione and NADH). TXA<sub>2</sub> is a potent vasoconstrictor as well as a cytotoxic agent in microvessels (67). Consistent with these events having a role in ROP, inhibitors of COX (indomethacin) and TXA<sub>2</sub> synthase (CGS-12970) selectively curtail oxygen-induced retinal vasoobliteration in mice (67). In contrast to prostaglandins produced by COX, isoprostanes are formed non-enzymatically *in situ* from the peroxidation of AA and then released by phospholipases; they exceed the production of prostaglandins under oxidizing conditions (27, 53, 65, 66). Isoprostanes may contribute to microvascular injury in ROP, as they are indirectly cytotoxic, since they trigger the production of TXA<sub>2</sub> (68–70) (Figure 4).

Nitrative stress results in *cis*- to *trans*-isomerization of AA (TAA), and this was recently shown to contribute to retinal vascular degeneration in a mouse model of ROP (71). This reaction yields four stable TAA monoisomers (72) (two of them being endogenous and not found in diet; ref. 73). Circulating levels of plasma TAA (74) are increased in oxygen-induced microvascular degeneration (75) and are known to be associated with induction of nitrative stress (76, 77); more specifically, TAA formation is abrogated in mice treated with NOS inhibitors and in mice deficient

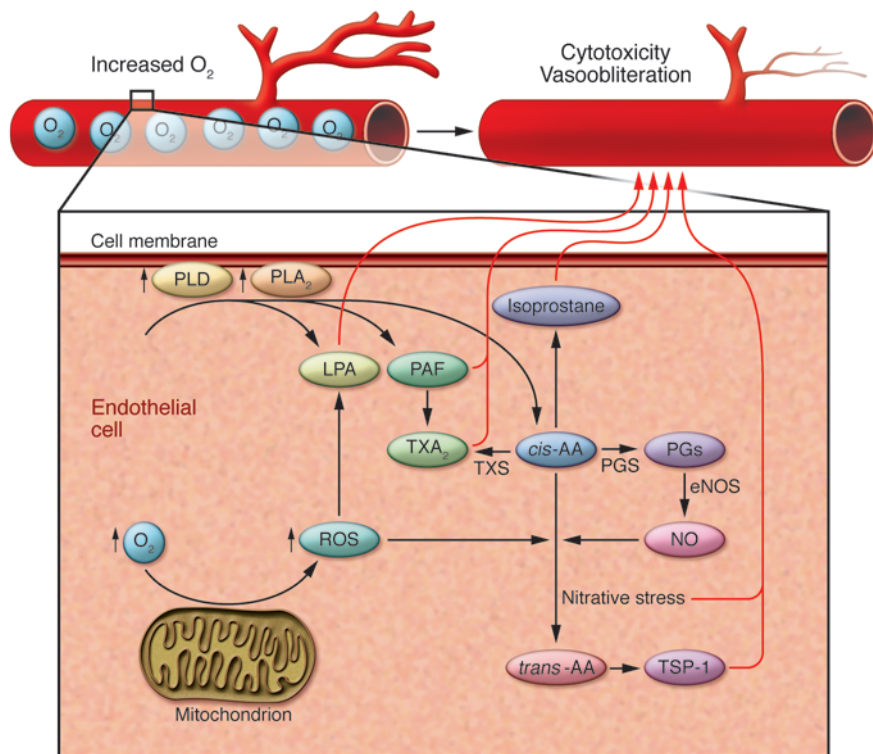
in eNOS (75) (Figure 4). TAA also exerts endothelial cytotoxicity by inducing formation of the antiangiogenic and proapoptotic thrombospondin-1 (TSP-1) (75). Hence, *trans*-fats are not only generated during industrial processing of lipids but also formed *in vivo* and contribute to the microvascular degeneration observed in ischemic retinopathies.

Other lipids generated during peroxidation are important proinflammatory mediators; these include lysophosphatidic acid (LPA) and platelet-activated factor (PAF). Choline phosphoglycerides are the precursors of PAF. Constitutive levels of PAF are maintained by the *de novo* pathway to ensure normal housekeeping cellular function. Otherwise, PAF is abundantly generated under oxidative stress by the remodeling pathway and calcium-dependent activation of cytosolic PLA<sub>2</sub>. In ROP, AAs are cleaved off from membrane phospholipids (by cPLA<sub>2</sub>) to yield lysophospholipids, which in turn can be acetylated and converted to PAF (78). Both vasomotor and cytotoxic effects of PAF in the premature infant are mediated to a large extent by TXA<sub>2</sub> (79, 80). Thus, PAF, which is generated concomitantly with TXA<sub>2</sub> during oxidant stress and amplifies formation of TXA<sub>2</sub>, contributes to retinal vasoobliteration in ischemic retinopathies (Figure 4). Likewise, LPA is also released from lysophosphatidylcholine by the action of lysophospholipase D (81) and can partake in retinal inflammation leading to microvascular cytotoxicity (82).

*Oxygen-dependent factors: suppression of oxygen-regulated growth factors.* The underlying function of a healthy vascular bed is the proper delivery of oxygen and nutrients to the tissue in order to guarantee adequate levels of energy production. This fundamental task ensures cellular respiration and consequently systemic survival. Organisms have therefore developed a series of effective metabolically driven, oxygen-sensing mechanisms to reinstate local capillary supply when perfusion is disrupted (83–85). In a mature organism, tissue oxygenation is tightly regulated to ensure sufficient supply without excess (described above). Conversely, the immature retina of neonates poorly regulates oxygen delivery to its tissue, leading to excess oxygenation when ventilated at high oxygen tension in oxygen supplementation protocols.

Beyond direct oxidative damage to the endothelium, excess retinal oxygenation plays an important role in the pathogenesis of ROP by suppressing key oxygen-regulated growth and survival factors. This occurs at the transcriptional level via the oxygen-sensing transcription factor HIF (86), which is composed of two distinct  $\alpha$ - and  $\beta$ -subunits that must heterodimerize in order to be active (87). *HIF1A* is constitutively transcribed but has an ephemeral half-life of only 5 minutes under normoxic conditions (88). The swift turnover is catalyzed by proline hydroxylation of its oxygen-dependent degradation domain by HIF-1 $\alpha$  prolyl hydroxylases (PHDs). The ultimate result is polyubiquitination and targeting for proteasomal degradation. Conversely, in hypoxic conditions, proline hydroxylation hardly occurs and HIF-1 $\alpha$  is stabilized. It is then free to translocate to the nucleus, where it dimerizes with HIF-1 $\beta$  and initiates transcription of target genes that have a hypoxia-response element (HRE) (89).

HIF-1 $\alpha$  is important for both the vasoobliterative phase of ROP (where the production of key growth factors is suppressed) and the neovascularization phase (where retinal hypoxia provokes the excess production of angiogenic factors). HIF drives the transcription of a cluster of genes (presently over 60 have been described) that encode proteins that promote the adjustment to a hypoxic state (90, 91) and help to reinstate local tissue perfusion. These genes include



**Figure 4**

The effects of oxidant stress on premature retinal vasculature. The premature retina is relatively deficient in antioxidants. Consequently, oxidant stress is more likely to induce peroxidation and nitration that is cytotoxic to retinal microvasculature. Downstream mediators of peroxidation, notably the phospholipids PAF and LPA, the non-enzymatically derived prostanoids, isoprostanes, and nitration products (of particular relevance here are the *trans*-AAs) are all cytotoxic to retinovascular endothelium, causing vasoobliteration. PGS, PG synthase; TXS, thromboxane synthase.

those that encode enzymes involved in energy production, such as glucose transporter-1 (GLUT-1) and the key glycolytic enzyme aldolase A (92). Moreover, HIF promotes production of angiogenic factors such as VEGF (93) and erythropoietin (Epo) (94) that will participate in establishing retinal vascular networks. Once the tissue is revascularized, the hypoxic stimulus is alleviated and HIF degraded. When oxygen levels are exaggerated, as occurs during the early phase of ROP, HIF is preemptively suppressed and its target genes not transcribed. In this context, oxygen-regulated growth factors such as VEGF and Epo are suppressed; VEGF suppression contributes to the arrest in vascular development and compromised endothelial survival (95) seen in the hyperoxic phase of OIR (96, 97). The requirement for VEGF in normal physiological retinal vascular growth is well established. As the neuronal retina differentiates and metabolic activity increases, vascular growth is initiated in response to waves of tissue hypoxia, largely via the production of VEGF by retinal ganglion cells (83) and astrocytes (98). For its part, Epo is well described as a renal-derived hormone produced in response to hypoxia or anemia to stimulate erythrocyte production. Like VEGF, Epo levels drop substantially during hyperoxia (99). Its role in the pathogenesis of ROP has been elegantly addressed in the work of Chen et al. (99), in which this cytoprotective agent was shown to preserve (via activation of pro-survival NF-κB) the retinal vascular bed and protect retinal neurons against apoptosis when administered in early phases of retinopathy. Interestingly, systemic delivery of Epo can also stimulate mobilization of CD34<sup>+</sup> endothelial progenitor cells and colony-stimulating factor 1 receptor<sup>+</sup> (Csf-1R<sup>+</sup>) microglia to the retina. Given that both of these cell populations are important in the repair of injured retinal vasculature, it is likely that in addition to promoting cell survival via activation of its receptor, Epo can also promote retinal vascular repair by recruiting bone marrow-derived proangiogenic cells to the retina.

*Oxygen-independent factors.* In light of the major role of high levels of oxygen and their resulting effects in the genesis of ROP, oxygenation protocols limiting hemoglobin saturation with oxygen have been adapted to the clinic and shown to reduce the incidence of ROP (18, 19, 100). However, these approaches have not eliminated ROP in the industrialized world, suggesting a role for oxygen-independent factors in the development of ROP. Of all these contributors, premature birth remains the greatest risk for ROP (101), which suggests that perhaps certain factors present in utero required for normal fetal development may be lacking in infants born prematurely. This hypothesis was explored in detail in the seminal works of Lois Smith on IGF-1, a polypeptide protein hormone whose fetal plasma levels rise with gestational age and considerably increase during the third trimester of pregnancy but are considerably lower in premature infants born early during the last trimester (102–105). Evidence implicating a paucity of IGF-1 in ROP was demonstrated in IGF-1-deficient mice, in which retinal vessel growth was retarded, akin to patterns noted in premature babies with ROP (102). Similarly, in human subjects, low IGF-1 serum levels directly correlate with the severity of ROP and interestingly may also account for abnormal brain development (106).

In neonatal mice with decreased IGF-1, it was observed that VEGF levels remain elevated in OIR, yet these animals are resistant to hypoxia-induced retinopathy (105). These findings point to a mechanism of action whereby IGF-1 would not directly modulate VEGF levels. In fact, IGF-1 potentiates the maximal VEGF-induced activation of Akt in endothelial cells and therefore modulates vessel survival (104), an essential event in preventing the first phase of ROP. IGF-1 also seems to be permissive for VEGF-induced activation of p44/42 MAPK, which is essential for endothelial cell proliferation and thus the neovascularization observed in the second phase of ROP (105). In this regard, IGF-1 acts as a permissive factor for VEGF-dependent endothelial growth and survival.



The action of IGF-1 is dependent upon the concentration of IGF-binding proteins (IGFBPs), which prolong its circulating half-life and thus augment tissue delivery (107). Once in the tissue, IGFBPs can either potentiate IGF-1 signaling by releasing it in the proximity of its receptors or conversely hinder signaling by sequestering it. In infants born prematurely at 30–35 weeks gestation, levels of the most abundant IGFBP, IGFBP3, were found to be markedly diminished in infants with ROP as compared with those without (103). And in mice subjected to OIR, augmentation of IGFBP3 levels increased vessel survival (in an IGF-1-independent manner) and consequently reduced the severity of the retinal vasculopathy (103).

In line with the obligate presence of adequate levels of IGF-1 (and/or IGFBP3) for normal retinal vascular development, clinical trials are being undertaken to supplement IGF-1 and IGFBP3 to in utero levels in infants born prematurely (108). Moreover, based on the observations presented above, the question of whether poor weight gain is a contributing risk factor for ROP in infants born prematurely has been raised. In this context, it was recently found that inadequate weight gain during the first few weeks of postnatal life is associated with more severe ROP (109). An algorithm based on weight gain and IGF-1 levels was developed to predict the risk of ROP and termed WINROP. Using serial weight and IGF-1 measurements in a cohort of 50 premature babies, WINROP predicted all infants who later developed ROP by a mean age of 10 weeks (109). Furthermore, measurements of weight gain (and exclusion of IGF-1) was sufficient to demonstrate in a cohort of 351 patients that insufficient weight gain could predict all infants that required treatment and 75% of babies who did not develop ROP (110). These findings emphasize the importance of adequate metabolic supply to the developing fetus and its role in proper retinal vascular development.

### Neovascular proliferation

In an attempt to reinstate adequate levels of oxygen and nutrients to the vasoobliterated retina, the hypoxic/ischemic tissue orchestrates a vasoreparative growth program triggered by the now metabolically deficient situation in the retina. In contrast to normal developmental growth, this neovascularization is excessive, disorganized, and misdirected toward the vitreous, which is devoid of vessels under physiological conditions (Figure 1). This destructive secondary angiogenesis is driven by an exaggeration of many of the same hypoxia-driven stimuli that coordinate physiological vessel growth in the embryonic retina. Therefore, valuable insight into the molecular mechanisms governing the pathogenesis of proliferative ROP can be gained through a better understanding of retinal vascular development.

*Physiological retinal vascular development.* Mammalian retinas of various species, including humans and mice, form vascular networks with highly reproducible growth patterns, suggesting a tightly regulated and carefully guided growth process that is the sum of responses to pro- and antiangiogenic factors (111). Retinal vascular growth must be restricted to the retina, as misdirected vessels to the adjacent vitreous body would obstruct incoming light. Several mechanisms are in place to ensure this directed growth, such as formation of junctions with the astrocytic bed (112) and graded production of VEGF by neuroglia (113) and retinal ganglion cells (83) on the retinal surface.

The superficial retinal vascular plexus (which is affected in ROP) forms by angiogenesis (Figure 1) (114–117). As indicated above, regions of the tissue that are poorly supplied with oxy-

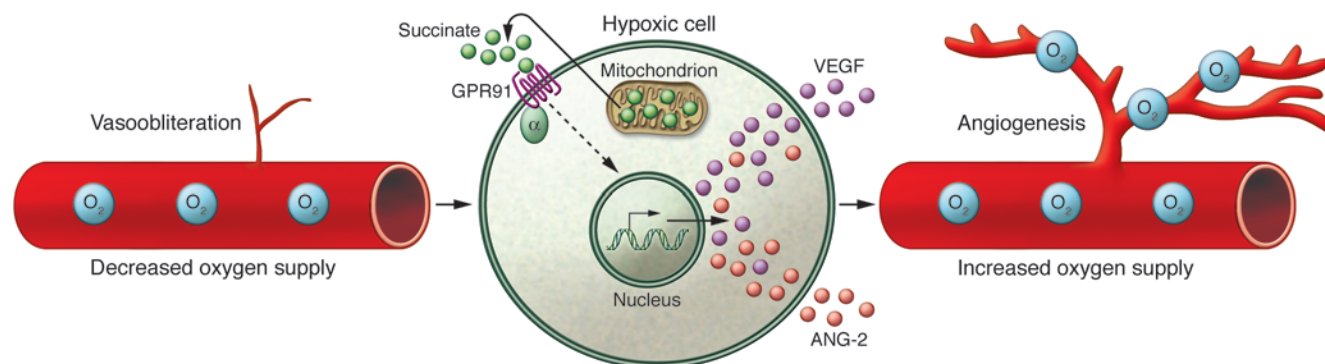
gen and nutrients prompt formation of neovessel sprouts from the walls of preexisting blood vessels in response to oxygen tension-sensitive growth factors such as VEGF and Epo (95, 99) and other oxygen-independent factors such as angiopoietins/Tie2 (118) and IGF-1 (105). Once the new vessel forms, its growth is steered by specialized endothelial tip cells (119) that probe and sense chemoattractive and repulsive environmental cues at the vascular front. Tip cells respond to VEGF by forming motile filopodia enriched in VEGFR2 and rich in guidance receptors such as neuropilin-1, Unc5b, and Eph (120–122) that respond to directional cues (119). Stalk cells (the endothelial cells that follow the tip cell) responding to VEGF with proliferation, follow to form the nascent vessel with a lumen (119, 123). The final maturation of vessels requires pruning of excess vasculature (124) and recruitment of mural cells (pericytes in medium-sized vessels and smooth muscle cells in large vessels) (125).

*Pathological neovascularization.* Throughout the proliferative phase of ROP, the proangiogenic response originates from neurons (83) and supporting astrocytes (98). These cell populations are severely metabolically deprived following the vasoobliteration during the first phase of ischemic retinopathy; therefore, in an attempt to re-equilibrate their metabolic supply, they produce exaggerated amounts (far above normal developmental levels) of oxygen-regulated angiogenic factors such as VEGF and Epo (reviewed in refs. 96, 97, 126). As a consequence, the ensuing regrowth becomes overstated, deregulated, and misguided toward the vitreous and lens. This profuse growth can culminate with the formation of a fibrous scar and contractile band that exert torsion and can ultimately sever the retina from the retinal pigment epithelium (i.e., it can cause retinal detachment).

*Metabolite signaling.* The role of a vascular network is to ensure the essential delivery of nutrients and oxygen. Therefore, it is imperative for a cell during hypoxic/ischemic episodes to respond to an energy imbalance and signal to reinstate vascular supply. Perhaps the most efficient manner in which this can occur is by harnessing the intermediates of its own energy metabolism. In this regard, a new paradigm of signaling via energy metabolites in response to compromised energy status has recently been proposed as an important contributor to both physiologic and pathologic vascularization in the retina (83, 127–129). The best-described examples of this evolutionarily preserved primitive system involve signaling by succinate and adenosine and are described below.

Succinate is a dicarboxylate generated as a metabolite of the Krebs cycle during cellular respiration, while adenosine is a purine nucleoside produced during ATP metabolism. Beyond their well-established roles in energy production, these molecules bind and activate G protein-coupled receptors, implying additional physiological roles. Consistent with mediating compensatory angiogenesis, both intermediates are upregulated under hypoxic conditions. When oxygenation is adequate, the Krebs cycle produces energy, and hence ATP levels are high and adenosine is low. Conversely, in low-oxygen conditions, Krebs cycle intermediates, such as succinate, accumulate, owing to feedback inhibition of succinate (and  $\alpha$ -ketoglutarate) dehydrogenase by nonoxidized flavin and nicotinamide nucleotides and by ROS (130, 131). Consequently, ATP is low and adenosine is high. In turn, succinate and adenosine act as proangiogenic mediators to reinstate adequate blood supply and oxygen delivery (83, 127, 128, 132). The robust vascular response provoked by these metabolites is suggestive of a direct role in linking energy demand to capillary growth. Importantly,





**Figure 5**

The proangiogenic effects of metabolite signaling in ROP. During hypoxia, as seen in ROP/OIR following hyperoxia-induced microvascular degeneration, succinate accumulates. Through its actions on its receptor, GPR91, which is present on retinal ganglion cells (RGCs), succinate induces production of a pleiotropy of proangiogenic factors, which trigger neovascular formation. This new vessel growth attempts to restore oxygen supply to the hypoxic retina; however, in ROP this compensatory vascular expansion is exaggerated and penetrates the vitreous. ANG-2, angiopoietin 2.

succinate signaling is thought to be a master regulator of both development and pathological retinal angiogenesis (83); this signaling pathway is operational before HIF stabilization and is thus an antecedent sensor of hypoxic stress.

The contribution of both succinate and adenosine to the proliferative phase of ROP has been established by inhibiting their cognate receptors in the OIR mouse model. Treatment with either siRNA or lentiviral vectors carrying shRNAs targeting the succinate receptor GPR91 effectively attenuates the proliferative phase of ROP in an OIR model (83). Similarly, selective antagonists of the  $A_{2B}$  adenosine receptor profoundly reduce preretinal neovascularization (on the retinal-vitreous interface) (Figure 1) (129). It is noteworthy that both succinate and adenosine derive their angiogenic potential from cells other than the endothelium; monocytes express adenosine receptors (133), while neurons (retinal ganglion cells) express both adenosine and succinate receptors (83, 134). Deficiency in monocytes or retinal ganglion cells (either by destruction or genetic ablation) interferes profoundly with retinal vascular development in mice (83, 135). The findings provide insight into a signaling paradigm in which cells in the vicinity of hypoxic zones provoke an angiogenic response to restore metabolic equilibrium, thus linking metabolism to vascular supply (Figure 5). Given the growing number of metabolites being identified as ligands for previously orphaned G protein-coupled receptors, these findings open the door for exploration of a novel family of mediators of pathological neovascularization in ROP.

**Lipid signaling and vasoproliferation.** The heightened level of inflammation associated with ischemic retinopathies (136–138) suggests that blocking enzymes that directly contribute to the inflammatory state may be an interesting strategy to curb both the vasoobliteration and neovascularization phases of ROP. The response of a tissue to inflammatory stimuli is largely orchestrated by mediators such as lipids, nucleotides, and peptides. Inflammatory mediators can lead to cytotoxicity or cytoproliferation, depending on the agent, the concentration, and the condition. The role of cytotoxic phospholipids has been addressed above (see *Oxygen-dependent factors: oxidative and nitrate stress and their downstream effects*).

Among the most prominent mediators of inflammation are the eicosanoids derived from essential  $\omega$ -3 and  $\omega$ -6 long-chain PUFAs (LCPUFAs) (139); both  $\omega$ -3 and  $\omega$ -6 PUFAs are highly expressed in

the retina. Simply put, the proinflammatory 2-series prostaglandins and leukotrienes are derived from the  $\omega$ -6 AA. In contrast, the antiinflammatory neuroprotectins and D-series resolvins originate from the  $\omega$ -3 DHA and the E-series resolvins and 3-series prostaglandins from eicosapentaenoic acid (EPA). There are two principal families of enzymes that convert LCPUFAs to biologically active entities: the COXs and the lipoxygenases. The molecular basis for the health benefits of  $\omega$ -3 PUFAs is thought to occur primarily through the direct integration of EPA and DHA at the sn2 position of membrane phospholipids, which thus unseats  $\omega$ -6 PUFAs such as linoleic acid (18:2 $\omega$ 6) and AA. The biological impact of altering lipid intake is thought to occur primarily through modification of membrane microdomain composition and specific receptors.

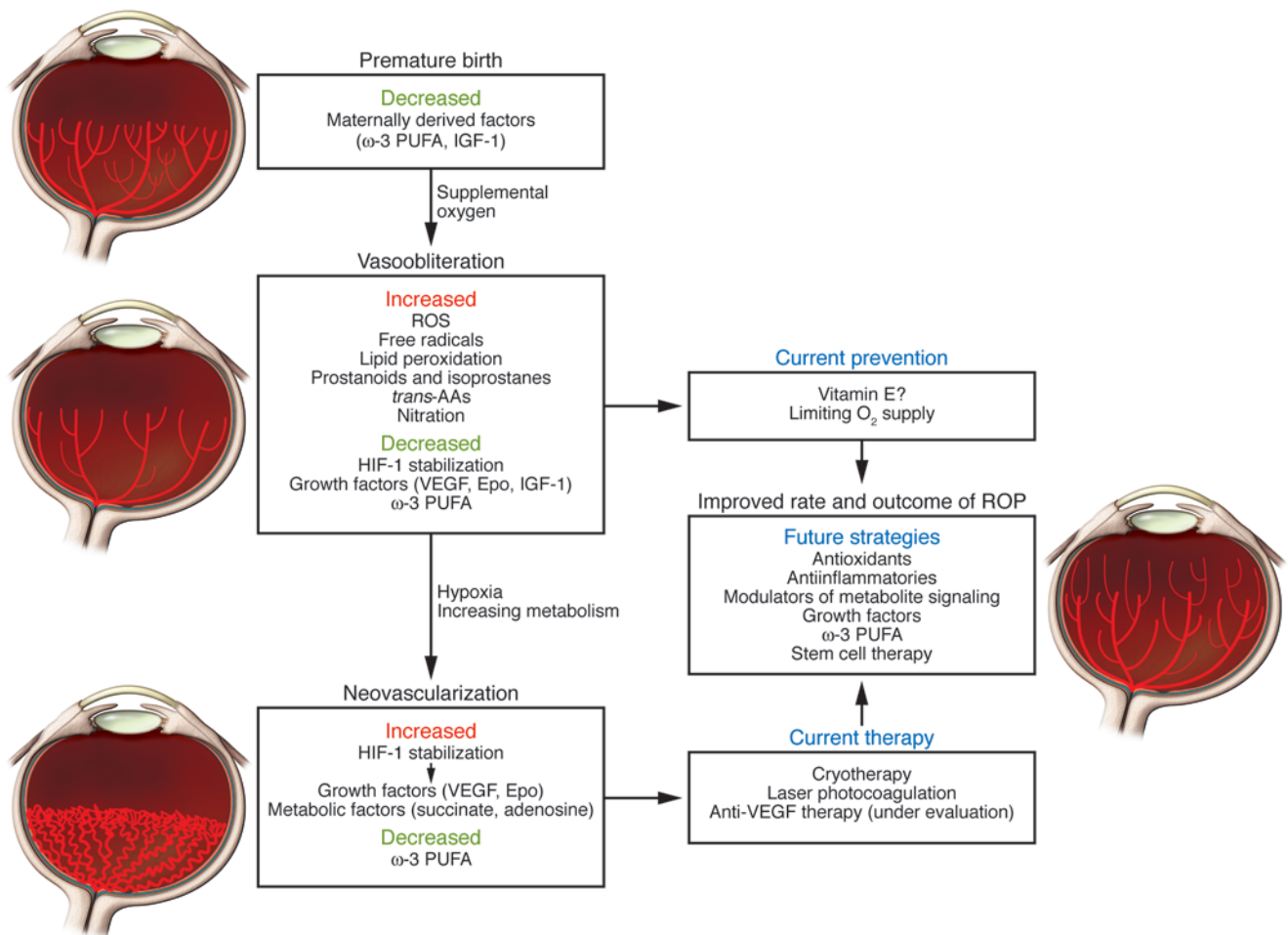
A successful strategy to alter the inflammatory status of the retina stems directly from influencing dietary lipid intake. Studies using the mouse OIR model demonstrate that diets enriched in  $\omega$ -3 LCPUFAs (mimicking Japanese diets) effectively increase total retinal content of  $\omega$ -3 fatty acids and translate into decreased avascular zones and protection against neovascularization when compared with diets rich in  $\omega$ -6 LCPUFAs (mimicking Western diets) (136). This beneficial effect is in part explained by an augmented retinal content of the antiinflammatory neuroprotectins and resolvins derived from  $\omega$ -3 PUFAs, which suppress production of excessive cytotoxic concentrations of TNF- $\alpha$  from microglial cells.

Further evidence of the involvement of cytoprotective inflammatory lipid mediators in ROP comes from work on COX (138). In both patients with symptomatic diabetes and in a mouse OIR model, the inducible form of COX, COX-2, shows altered expression patterns when compared with that in healthy individuals and is localized primarily in the nerve fiber layer (adjacent the retinal vasculature) (140). It also has been shown that COX-2 participates in neovascularization via the generation of PGE<sub>2</sub> and its activation of PGE receptor 3 (EP3) and to a lesser extent EP2 (140). Future studies will be required to address the relevance of the lipoxygenase pathways and their metabolites in ROP.

### Prevention and therapy of ROP

**Prevention.** Preventive measures against ROP have so far been modestly successful, largely due to an incomplete understanding of pathogenesis and limited efficacy of therapeutic modalities. To



**Figure 6**

Summary illustration of the current concepts in ROP. When premature birth occurs, the retinal vasculature, which normally develops until birth, is immature. In addition, the premature infant is deficient in several maternally derived factors (such as  $\omega$ -3 PUFAs and IGF-1, which are transferred during the third trimester) that are essential for healthy blood vessel development, thus further compromising the prognosis. Moreover, the premature patient is mechanically ventilated to overcome pulmonary insufficiencies, and as a consequence, the supplemental oxygen given during mechanical ventilation contributes to retinal vascular obliteration due to oxidant stress and suppression of oxygen-regulated proangiogenic factors such as VEGF and Epo. Following the initial phase of vascular dropout, a second phase of compensatory and destructive neovascularization results and is driven by hypoxia-induced angiogenic factors. Current therapeutic interventions rely on invasive procedures such as laser photocoagulation, whereby affected areas of the retina are cauterized. A number of future treatments, including anti-VEGF therapy and the use of antioxidants, are currently being evaluated.

date, the most effective means to diminish the rates of ROP largely involve restricting tissue oxygenation by maintaining lower levels of hemoglobin saturation with oxygen in infants born prematurely (17, 18, 100). Other preventive strategies rigorously studied include the early use of antioxidants, notably vitamin E. Vitamin E is a naturally occurring potent, free radical scavenger that decreases lipid peroxidation and helps maintain membrane integrity (141). Infants born prematurely have only 10% of adult levels of retinal vitamin E (51), which provides a rationale for using vitamin E in prevention of ROP. Four clinical trials involving premature infants (<1,500 g birth weight) demonstrated that vitamin E suppressed the development of severe ROP. However, subsequent trials could not reproduce earlier results (142–144), and hence the efficacy of vitamin E remains at best modest (145).

**Therapy.** Current therapeutic approaches tackle late-appearing neovascularization. Laser photocoagulation (where an area of the

retina affected by ROP is cauterized) has, for the last decade, been the mainstay of therapy in ROP (Figure 6). Laser photocoagulation was implemented in the clinic following recommendations made as a result of the cryotherapy trial for advancing forms of ROP at high risk of progressive intravitreal neovascularization (146). Early intervention in ROP has led to improved visual outcome (147), although undesired long-term outcome of laser therapy on visual acuity and fields cannot be disregarded (148).

A promising future strategy to counter ROP is to use anti-VEGF therapy. To date, 9 reports (including 6 case reports, 2 retrospective studies, and 1 prospective study) document results of therapy with a VEGF-specific neutralizing antibody (bevacizumab) in ROP, and these have been reviewed in ref. 149. Timing (stage/zone of ROP), dose (0.4–12.5 mg intravitreal), and frequency of administration of bevacizumab, as well as cotreatment with photocoagulation, varied tremendously among reports. Nonetheless, generally favorable



outcome (controlled progressive neovascularization) was noted in 6 of the 9 small reports. However, this leaves a number of issues to be addressed in upcoming randomized trials, including: (a) adjunct photocoagulation therapy with anti-VEGF treatment; (b) impact on retinal ganglion cell integrity, since these neurons express the receptor for VEGF and VEGF can act as a cytoprotective factor for retinal ganglion cells; (c) timing of anti-VEGF therapy; (d) systemic effects of larger doses (1.25 mg) of bevacizumab, notably on cerebral vasculature; (e) long-term impact on visual acuity and visual fields; (f) cost-benefit versus currently available treatment.

### Future perspectives

One of the most destructive manifestations of ROP is preretinal neovascularization (Figure 1). Approaches to prevent vasobliteration or accelerate normal vascular repair following initial microvascular degeneration would ideally curb the development of late-appearing harmful neovascularization (Figure 6). As we continue to decipher the underlying cellular mechanisms governing proliferative retinopathies such as ROP, fostering rapid normal retinal revascularization may open new therapeutic avenues. Insight into the complex cellular interdependent network of retinal neurons, microglia, astroglia, and vessels is being unraveled from the perspective of their respective energy requirements. The metabolic demands of these cell populations link them to vascular supply. In this context, numerous energy metabolites are now being identified as ligands for previously orphaned G protein-coupled receptors. Harnessing components of cellular energy metabolism and metabolite signaling may help modulate normal

and pathologic neovascularization (83). Along similar lines of thought, a more profound understanding of the complex interplay of inflammatory mediators is needed. Novel vascular repair strategies may also emerge from advances in regenerative medicine using stem cells, including the findings that hematopoietic stem cells home into areas of vascular damage and that engineered stem cells injected into the eye hasten vascular repair (150). Future safe and nondestructive therapeutic strategies combined with preventive approaches need to be tailored to the unique developmental requirements of the premature infant.

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