The Effect of Oxygen on Vasoformative Cell Division

Evidence That 'Physiological Hypoxia' Is the Stimulus for Normal Retinal Vasculogenesis

Tailoi Chan-Ling, Bronwyn Gock, and Jonathan Stone

Purpose. To assess the role of oxygen in normal retinal vasculogenesis.

Methods. A new preparation for studying cytogenesis in retinal wholemounts was developed. Nuclei of dividing cells were labeled with a monoclonal antibody to bromodeoxyuridine (BrdU), and vascular cells were covisualized with *Griffonia simplicifolia* lectin. The topography and time course of vasoformative cell division and vessel formation were determined in the kitten retina during normal development and under experimental hyperoxia and hypoxia.

Results. During normal development, vasoformative cell division was maximal at the leading edge of the forming vessels. Normal vessel formation was initially proliferative, and cell division was high. However, after vessel formation occurred, which presumably relieved tissue hypoxia, the mitogenic process was markedly reduced, and many excess capillary segments underwent retraction. The rate of vasoformative cell division and vessel formation increased when the inner layers of the retina were made avascular after exposure to hyperoxia, and it decreased when there was an increase in inspired oxygen.

Conclusions. The authors have shown that between 17% and 45% oxygen, the extent of vasoformative cell division is inversely proportional to the level of oxygen in the inspired gas mixture. They have further shown that dividing vascular cells have a peak density in a region proximal to the edge of the forming vasculature. The density is maximal between P7 and P8, a time when formation of photoreceptor outer segment begins, only a few days before the onset of retinal function. These results led the authors to conclude that the stimulus for normal vasculogenesis is a transient but physiological level of hypoxia induced by the increasing activity of retinal neurons. Invest Ophthalmol Vis Sci. 1995;36:1201-1214.

During normal development of the retina, vessels spread from the optic disk to the margin of the retina in a process called vasculogenesis, involving the transformation of precursor cells into capillary networks from which adult vessels subsequently differentiate. The second process of angiogenesis entails budding of capillary-sized vessels from existing vasculature and is characteristic of the formation of the inner plexus of vasculature at the area centralis, the outer retinal plexus, and the radial peripapillary capillaries.

The role of oxygen in controlling the formation of retinal vessels has been recognized since the work of Michaelson and Ashton. Weiter et al proposed that the maturation of the photoreceptors and the concomitant increased oxygen consumption make it impossible for the choroid to provide adequate full-thickness retinal oxygenation. The resultant progressive change in the inner retinal metabolism thus provides an orderly, controlled stimulus for the subsequent progression of retinal vascularization from the optic disk to the peripheral retina. More recently, Rosen et al have shown that the proliferation of endothelial cells in vitro varies inversely with oxygen tension. On the basis of these observations, we have suggested that the normal formation of retinal vessels depends on a transient period of hypoxia in the inner layers of the retina. We referred to this level of hypoxia as "physiological" because it occurs during normal...
development and is well tolerated by the tissue. This level of hypoxia is sufficient to stimulate the growth of retinal vessels yet insufficient to induce the pathologic sequelae of more intense hypoxia, such as the degeneration of astrocytes and the failure of the blood–retinal barrier. As a result, the retinal vascular plexus has an intact blood–retinal barrier as soon as the vessels become patent. This suggestion is a development of Ashton’s view that “many of the pathologic responses of the retinal vessels are not separate morbidity processes, but exaggerations of normal behavior.”

To test the hypothesis that the development of the retinal vasculature is stimulated by metabolic (oxygen) demand, we examined the rate of vasculogenic cell division in normal development as vessels spread into previously avascular regions of the retina and subsequently mature. The pattern of cell division was related to the pattern of retinal maturation established in previous studies, to the differentiation of arteries and veins, and to the process of capillary retraction during maturation of the initially exuberant capillaryplexuses. Further, we observed the effects of experimentally generating hypoxia and hyperoxia in the inner layers of the retina. A thorough topographic study of vasculogenesis was made possible by the development of a new approach for investigations of cytogenesis in retinal wholenumount preparations. Nuclei of dividing cells are labeled with a monoclonal antibody to 5-bromo-2’-deoxyuridine (BrdU), and vascular cells are covisualized with Griffonia simplifolia isolectin.

Finally, we noted that the effects of oxygen are specific to vasoprotective processes; neurogenesis appears unaffected by the variations in tissue oxygen levels used. The results suggest that tissue oxygen levels control vasculogenic cell division and vessel formation through upregulation in the production of an angiogenic growth factor, and they confirm the concept of “physiological hypoxia” as the normal stimulus for retinal vessel formation.

METHODS

Vasculogenesis and Angiogenesis During Normal Development

Retinas were obtained from two adult domestic cats and from kittens aged postnatal day (P) 4, 5, 7, 8, 12, 28, and 35. The retinas were processed for BrdU and G. simplifolia isolectin immunohistochemistry as described below.

Vasculogenesis During Experimental Hyperoxia and Hypoxia

To determine the relationship between inspired oxygen level and retinal vasculogenesis, newborn kittens within 24 to 48 hours of birth were raised for 4 days at oxygen levels of 17%, 21%, 30%, 45%, and 70%. Using oxygen microelectrodes, Alder and Cringle, Linsenmeier and Yancey, and Yu et al have shown that oxygen tension at the inner retina is directly related to levels of inspired oxygen. The relationship between vitreal oxygen tension and inspired oxygen is also directly related, though it is nonlinear with a central relatively flat region between 50% and 80% inspired oxygen. Retinas from the kittens then were processed for BrdU and lectin immunohistochemistry. To generate intense retinal hypoxia, we used the kitten model of retinopathy of prematurity (ROP). Two newborn kittens were raised in high (70% to 80%) oxygen for 4 days. This period of hyperoxia induces the retinal vessels to degenerate so that, on return to room air, the inner retina is avascular and is presumed to be intensely hypoxic. The retinas were examined after periods of 12 and 25 days in room air.

Vasculogenesis During Supplemental Oxygen Therapy

One newborn kitten was exposed to 70% to 80% oxygen for 4 days and then placed in atmospheres containing 45% oxygen for 4 days, 60% oxygen for 4 days, and 70% oxygen for 4 days. This regimen of supplemental oxygen therapy (derived empirically; see the next article in this issue, pages 1215–1230), we assume, protects the retina from hypoxic damage until it is substantially revascularized.

Preparation and Monitoring of Inspired Gas Mixtures

Kittens were placed in Isolette infant incubators (Narco Medical, Warminster, PA) within 24 to 48 hours of birth. Oxygen levels within the incubator were monitored with polarographic oxygen sensor (Series 90, Commonwealth Industrial Gases, Sydney, Australia). Atmospheres containing 17% to 75% oxygen were delivered at a rate of 4 to 5 L/minute with a low-flow ultrablender (Bird Products, Tag Medical, Victoria, Australia). The mother was exercised regularly and was placed for periods of time in room air. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Retinas for Immunohistochemistry

The protocol described is a modification of procedures reported by various researchers, including Gratzer, Watanabe and Raff, and Del Rio and Sariano, and is fully described in Chan-Ling. The animals were injected with BrdU (Sigma, St. Louis, MO) (0.1 mg/g body weight, intraperitoneally) dissolved in Dulbecco’s modified Eagle’s medium. The animals were anesthetized 1 hour later using an intramuscular...
The retinas were postfixed in cold (-20°C) 70% ethyl alcohol, the anterior segment, crystalline lens, and vitreous were followed by a brief perfusion with 0.5% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The anterior segment, crystalline lens, and vitreous were removed, and the eyecup was filled with cold (−20°C) 70% ethanol. The retina was then dissected free from the choroid and sclera while floating in the ethanol. Radial incisions were made to permit flattening of the retina, which was then fixed by immersion for an additional 2 to 3 minutes in 0.5% paraformaldehyde. The retinas were postfixed in cold (−20°C) 70% ethanol for 15 to 20 minutes and washed for 30 minutes in PBS containing 1% Triton X-100.

**Combined Lectin and BrdU Immunohistochemistry**

To covisualize retinal vessels and dividing cells, retinas were first incubated with biotinylated *G. simplicifolia* (Bandeiraea) isolectin B4 (0.2 μg in 5 ml of sterile PBS) (Sigma) for 4 hours at room temperature. This lectin has a high affinity for terminal α-D-galactose residues and labels vessels and vascular precursor cells. Retinas were washed three times, each time for 10 minutes, in PBS containing 1% Triton X-100, and then incubated in Streptavidin conjugated to fluorescein isothiocyanate (FITC) for 4 hours at room temperature. By this time, both the retinas and washes were gently agitated with an orbital shaker. The retinas were washed in PBS and 1% Triton (as above) and placed in 2 M HCl at 37°C for 1 hour. Each retina was then washed in 0.1 M sodium borate for 30 minutes, washed in PBS containing 1% Triton X-100, and incubated with a monoclonal antibody to BrdU (ascites diluted 1:20 with PBS containing 1% bovine serum albumin) (gift of D. Mason) for 24 to 48 hours at 4°C. The retinas were then washed in PBS and 1% Triton X-100 and incubated in anti-mouse immunoglobulin conjugated to Texas Red (Amersham, Australia) (diluted 1:50 with PBS containing 1% bovine serum albumin) for 2 hours. After a final wash in PBS, the retinas were mounted in PBS containing 33% glycerol.

**Mapping of Retinas**

The use of BrdU and *G. simplicifolia* lectin immunohistochemistry in retinal wholemounts provides a display of mitotic cells associated with the vascular tissue of the retina, from which the vascular tree could be seen intact and mapped without reconstruction. It was also possible to assess the depth of labeled nuclei within the retina, relying on landmarks of the layers (vessels, axon bundles, neuron size, and density) and the focal movements of the microscope. In assessing the distribution of mitotic cells, we made the simplifying assumption that the proportion of the mitotic cycle spent in S-phase did not vary significantly between the various conditions examined and, therefore, that the density of labeled cells provides a direct measure of the density of mitotic cells. All counts were performed without knowledge of the study conditions of the particular retina being counted.

The outline of the retina was obtained with a 1-mm grid incorporated into the ×10 eyepiece of a Vanox Fluorescent microscope (Olympus Optical, Tokyo, Japan) and a ×10 objective, with a 1.25 magnification factor incorporated. The density of BrdU-labeled nuclei (located in the plane of focus of developing vessels) was sampled in 1-mm steps, and at each sampling point the number of labeled cells in an area of 0.16 mm² was counted using a ×20 objective (numbers of labeled nuclei within this area was then multiplied by 6.25 to arrive at cells/mm²). Because the mitotic divisions that generate the neurons and Müller cells of the retina occur in two external layers of the retina, mitotic cells in the inner retinal layers were presumed to be related to the genesis of vasculature. Only dividing cells that were obviously related to the forming vasculature labeled with *G. simplicifolia* isolectin were counted. Beyond the leading edge of the forming vessels, only dividing cells in the same plane of focus as the forming vessels were counted.

**RESULTS**

**Identification of Dividing Vascular Cells**

The fluorescence elicited from BrdU-labeled nuclei appeared to fill the nuclear sac (Fig. 1A), creating an ovoid shape with a clear outline. Labeled nuclei were observed in all the cellular layers of the retina. Many were within cells also labeled by *G. simplicifolia* lectin and formed part of the wall of vessels at the inner surface of the retina (Figs. 1A, 1B, 1D). The nuclei of vascular endothelial cells in arteries tended to be elongated with their long axes along the direction of blood flow (Fig. 1E), whereas the nuclei of endothelial cells in veins were less elongated (Fig. 1F). Labeled nuclei were also numerous among differentiating vessels near the edge of the inner vasculature (Fig. 1G). In some large vessels, such as the artery shown in Figure 1H, some labeled nuclei were oriented around the circumference of the vessel.

Some labeled nuclei were not associated with vessels. Many of these nuclei (such as those to the right of Fig. 1B and to the upper right of Fig. 1D) were observed in the inner layers of the retina, peripheral to the formed vessels, and were presumed to be the nuclei of precursor cells of the vasculature. Many labeled nuclei were observed in the inner and outer nuclear layers and were presumed to be the nuclei of

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dividing cells in the neural retina; these nuclei were most abundant in peripheral regions of the retina, consistent with the restriction of most neurogenesis in the postnatal cat retina to the retinal margin.19–22

**Vasculogenesis and Angiogenesis During Normal Development**

Retinal vessels develop in two sequential processes.2 The first process begins early in gestation with the migration of precursor "spindle" cells from the optic disk over the inner surface of the retina. Beginning at embryonic day 48, precursor cells transform into an exuberant capillary plexus, from which the adult vessels differentiate. The first vessels form at the optic disk, from which vessels spread toward the edge of the retina, completing their coverage of the retina approximately 5 weeks after birth. As the vessels spread, successive stages in the formation of these vessels can be traced in a single retina by scanning from the margin of the retina to the optic disk. At P7 and P8, for example, the inner vessels have formed over the posterior 50% to 60% of the retina. Near the optic disk, relatively large vessels have begun to differentiate (artery in Fig. 1E, vein in Fig. 1F), with mitotic nuclei in their walls. Several millimeters from the disk, the edge of the spreading vessels is apparent (Figs. 1B, 1C), formed by exuberant capillaries, among which labeled nuclei are numerous. Our earlier work2 has

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933191/)
FIGURE 2. (A, B) A region at the edge of the vasculature forming in the retina at P7.5. (A) The first-formed vessels labeled with *Griffonia simplicifolia* lectin are exuberant and coarse, and dividing cells, their nuclei labeled with BrdU, are numerous among them. (C, D) Neater the optic disk, earlier formed vessels are beginning to transform into finer capillaries (C) or are coalescing to form coarser vessels (D). (E, F) During maturation of arteries, retraction of capillary connections occurs to form a capillary-free zone (F). Capillaries to the artery disappear, and connections to its branch appear to be thinning and collapsing (arrow). (G) A vein (v) and an artery (a) are shown in a region close to the optic disk. The artery is flanked by a relatively large capillary-free space, has only a single capillary-size branch, and has a low mitotic density within its walls and in adjacent capillary plexus. The vein, in contrast, shows multiple connections with adjacent capillaries and higher numbers of dividing vascular cells. Scale bars represent 50 μm.

shown that the region just peripheral to the patent vasculature contains numerous lectin-positive vascular precursor cells. These cells are less brightly labeled by the *G. simplicifolia* lectin and show the same topography of invasion as the patent vasculature. BrdU-labeled nuclei were also evident among these cells (Fig. 1D).

In normal development, vessel growth is sharply regionalized. At the edge of the developing vasculature, new capillaries are formed rapidly (Fig. 2A). Capillary formation is initially excessive, with the first-formed capillaries profuse and coarse in caliber (Figs. 2A, 2B) and with dividing nuclei numerous among them. Remodeling of exuberant plexus of capillaries to its adult parameters begins promptly (Fig. 2C), and the processes of retraction and selection are apparent in vessels only a few millimeters (toward the optic disk) from the exuberant capillaries. Remodeling comprises two distinct processes: Some capillaries coalesce into larger vessels (Fig. 2D) and others, particularly those in proximity to arteries (Figs. 2E, 2F arrow) undergo retraction. As a result of their collapse, the vascular tree becomes less exuberant, and capillary-free zones are formed along the flanks of arteries (Figs. 2E to 2G). Where a vein is close to an artery, capillaries are more prominent on the side remote
from the artery (Fig. 2G). Division of angiogenic cells follows this pattern; it is suppressed near arteries and more profuse on the side of a vein remote from an artery (Fig. 2G).

It is a striking feature that vessel formation and degeneration occur in such proximity. We argue in the Discussion that these regional variations in growth are caused by variations in tissue oxygen tension.

The distribution of mitotic cells between the optic disk and the margin of the retina was quantified in normally developing retinas from kittens 4, 5, 7, 8, 12, 28, and 35 days of age. For each age, we plotted the density of mitotic vascular cells or precursors in the inner layers of the retina as a function of distance from the optic disk (Fig. 3). Three observations were striking. First, at each age from P5 to P28, a peak in the density of mitotic nuclei could be identified. The peak consistently occurred immediately central to the edge of the forming vasculature and spread with that edge. Second, the peak density of mitotic nuclei among the ages studied increased with age, reaching a maximum (810 to 1030/mm²) at P7 to P8, and then decreased with age, disappearing by P35. Third, the density of mitotic cells between the edge of the vasculature and the optic disk was approximately 110/mm² at P5 and decreased to low levels (<15/mm²) by P35. In two normal adult retinas examined, no mitotic nuclei were detected in the inner layers of the retina (data not shown).

The dimensions of the retinas presented in Figures 3 and 4 warrant comment because they appear at variance with the steady growth of the retina reported in earlier studies (e.g., Chan–Ling et al²). For example, the P12 retina seems smaller than some younger retinas. This discrepancy is presumably the result of variations in shrinkage induced by the ethanol fixation step of the BrdU protocol. In this step, the retina is exposed to 70% ethanol while free floating so that it is likely to shrink substantially. Despite this complicating factor, the proportion of each retina covered by vasculature increases steadily with age, confirming earlier studies.

The distribution of vasculogenic cells is represented schematically in Figure 4. In the normal retinas shown (P5, Fig. 4A; P7, Fig. 4B; P12, Fig. 4C), dividing vascular cells were present throughout the retina but were concentrated immediately central to the edge of the patent vasculature (indicated by the unbroken line). The maximum mitotic activity associated with the forming vasculature was found at P7. At P7, in a region just peripheral to the edge of the forming vasculature, mitotic activity was also significant.

In the second process of normal angiogenesis, capillary-sized vessels bud from vessels formed by the first process, without the involvement of precursor cells.² The first buds grow from vessels of the inner

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**FIGURE 3.** Density of mitotic nuclei in vascular endothelial or precursor cells during normal postnatal development. Data from the inferior temporal axis of each retina are presented. In each graph, zero on the abscissa represents the center of the optic disk, the open arrow represents the edge of the vasculature, and the closed arrow represents the edge of the retina.
FIGURE 4. Maps of the distribution of BrdU-labeled nuclei in vasculogenic cells (spindle-shaped precursor cells and vascular endothelial cells) in three normally developing retinas at P5 (A), P7 (B), and P12 (C). (D) The distribution of labeled nuclei in the retina of a kitten raised in high oxygen for 4 days beginning within 48 hours of birth, then raised in room air for 12 days (therefore, P17 at sacrifice). All regions where there are no data points represent regions of the retina with a mitotic density of 0 to 15 cells/mm². These were predominantly in the peripheral retina.

layer that skirt the area centralis and extend into the area centralis (Figs. 5A, 5B). During such growth, few labeled nuclei were found peripheral to the forming vessels (Figs. 5A, 5B). In accord with the suggestion that these capillaries do not form by transformation of precursor cells, we found no evidence of a population of dividing cells preceding vessel formation. Instead, labeled nuclei were embedded close to the tips of growing capillaries. The newly formed capillaries are less exuberant than those formed by the first process. The density of labeled nuclei within the newly formed capillaries was much lower (<75/mm²) than that for capillaries formed by the transformation of mesenchymal precursor cells (compare Fig. 1B with
FIGURE 5. New vessel formation at the area centralis and at the outer vascular plexus occurs by a budding process. (A, B) Mitotic cells are only seen close to the tips of vessel buds at the margin of the area centralis, in a P8 (A) and a P5 retina (B). (C, D) Mitotic cells seen close to the tips of new vessel growth in the outer vascular plexus of a P8 retina. Note the clear absence of mitotic vascular precursor cells in the region preceding the growing vessel bud. This contrasts dramatically with the pattern of vascular mitosis seen in the process of vascular transformation associated with the formation of the inner vasculature. Scale bars represent 100 μm in A and B and 25 μm in C and D.
Oxygen Controls the Rates of Retinal Vasculogenesis

Figure 6. Density of mitotic nuclei in vessel-related cells in kittens raised in various levels of oxygen for 4 days after birth. Data are presented along the inferior temporal axis of each retina (from the disk to the temporal margin of the retina, below the area centralis). The zero on the abscissa represents the center of the optic disk, the last data point represents the edge of the retina, and the arrowheads represents the edge of the vasculature.

... sor cells thus appear to respond to hypoxia in a similar manner as endothelial cells. A third source of vascular budding appears to be from vessel remnants that have persisted after vessel ablation (Figs. 7C, 7D).

After 25 days in room air, the vasculature extended further toward the edge of the retina. The peak density of mitotic cells was approximately 1500/mm², less than after 12 days in air but still markedly higher than that observed in normal development (810 to 1030/mm² at P7 to P8), and 200/mm² at P28. Figure 8C shows the distribution of mitotic nuclei in the retina of a kitten subjected to hyperoxia (70% to 80% for the first 4 days of life) and then brought through the regimen of "supplemental oxygen therapy" described in the next article in this issue (pages 1215-1230). The density of mitotic nuclei after supplemental oxygen therapy for 12 days (4 days in 45% oxygen, 4 days in 60% oxygen, 4 days in 70% oxygen) was similar to the density seen at comparable ages during normal development (Fig. 3, P12 and P28). Correspondingly, the vasculature of this retina showed a vascular mitotic density markedly lower than that observed during the hypoxia of room air reintroduction and was at a level resembling that seen in normal development (Figs. 7E, 7F).

Discussion

Oxygen Tension Controls Vascular Mitosis and Vessel Growth

The concept that retinal tissue controls the formation of retinal vessels was first proposed by Michaelson, who later provided the insight that this control is related to retinal metabolism and that this may be mediated through a vasoformative growth factor. Ashton, in his interpretation of the pathogenesis of ROP, suggested that oxygen levels within the retina are a key determinant of both the normal and pathologic formation of retinal vessels. In a reexamination of retinal angiogenesis, we made the additional observations that the timing and topography of formation of the outer vascular plexus are coincident with the onset and location of photoreceptor formation and activity. Moreover, where the thickness of the nerve fiber layer prevents adequate oxygenation of the peripapillary region, a third vascular plexus is formed. Tissue hypoxia is also important in the pathogenesis of ROP, causing proliferative vessel growth and degeneration of astrocytes in the second stage of feline ROP. Because astrocytes are responsible for induction of the blood-retinal barrier, their degeneration results in the formation of new vessels showing gross leakage of horseradish peroxidase.

We have shown that the rate at which vessels spread across the retina varies inversely with oxygen level, confirming Phelps. The importance of this observation for the clinical management of oxygen-induced vasculopathies is discussed in the next article in this issue (pages 1215-1230). We have shown further that the dividing vascular cells show a characteristic topography, their density peaking in a region proximal to the edge of the forming vasculature. The density is maximal between P7 and P8, a time when...
The effect of hypoxia on the density of retinal vascular growth as demonstrated for the same field of view with *Grijonia simplicifolia* isoelectin (A) and anti-BrdU immunohistochemistry (B). Capillaries forming at the edge of the spreading vasculature are proliferative (A) in a kitten raised for 4 days in 70% to 80% oxygen (to obliterate the developing vessels) and for 12 days in room air (to induce vasoproliferation). Mitotic cells associated with the proliferative vasculature have a very high density (B). (C, D) During the vasoproliferation, the vessels that form from vascular remnants (C) also show high densities of dividing nuclei (D). (E, F) For comparison, the newly formed capillaries in a retina raised for 4 days in high oxygen (to obliterate the developing vessels) and for 12 days with supplemental oxygen therapy are less proliferative (E), and the density of labeled nuclei associated with them is much lower (F). Scale bars represent 50 μm.

The formation of photoreceptor outer segments is beginning and only a few days before the onset of retinal function. This period of maximal vasculogenic cell division coincides with the age at which the outer vascular plexus starts to form in the kitten retina. We have shown, too, that the rate of vasculogenic cell division is greater near veins than arteries, suggesting that the higher oxygen tension in arterial blood suppresses vasculogenesis. In addition, the extent of vasculogenic cell division was inversely proportional to the level of oxygen in the inspired gas mixture over a wide range (17% to 45%).

Our experiments did not address the question of the mechanism by which oxygen controls vessel formation and regression in the developing retina. A striking feature of this control is that it is extremely localized, with vessel growth and regression occurring in proximity. Such local control could be mediated, as proposed by Michaelson by vasoformative factors secreted by the retina in response to local conditions. Chen and Chen, Glaser et al, and D'Amore et al demonstrated that extracts of retina from several mammalian species are potent stimulators of angiogenesis. Further, this vasoproliferative effect of retinal extracts is increased several-fold if the retina is harvested when in the hypoxic stage of ROP. We have...
suggested that two antagonistic growth factors modulate retinal vessel formation and degeneration in the developing retina. A candidate for the vasformative factor produced by the retina emerged when Baird et al showed the presence of fibroblast growth factor (FGF) in the developing retina, a factor whose many actions includes the induction of mitosis in vascular endothelial cells. However, FGF lacks the specificity of the more recently described vascular endothelial growth factor (VEGF) for endothelial cells. Also known as vascular permeability factor, VEGF has been described in the developing brain, expressed in relation to developing vessels. Furthermore, VEGF—not FGF—is induced by hypoxia, a factor thought critical for normal development of retinal vessels. In situ analysis of tumor specimens with active vessel growth showed that the production of VEGF is specifically induced in a subset of glioma cells and that there is a clustering of capillaries alongside VEGF-producing cells. In a preliminary report, Plouët et al showed that VEGF can also stimulate the proliferation of endothelial cells in a dose-dependent manner.

Vascular endothelial growth factor is thus a strong candidate for the growth factor responsible for translating physiological hypoxia found during normal development into vasculogenic division. Its presence in the retina remains to be described. In contrast, transforming growth factor-β inhibits the stimulatory effect of basic FGF on the proliferation of cultured bovine retinal capillary endothelial cells. The effect is dose dependent; that is, by increasing the concentration of basic FGF, the inhibitory effect of transforming growth factor-β can be overcome, suggesting a dynamic equilibrium between these factors. Such an interaction between two or more competing growth factors is consistent with our idea of physiological hypoxia as the stimulus for retinal angiogenesis during normal development; local tissue oxygen tension may thus regulate the production of these two antagonistic factors in opposite directions. Depending on the relative concentrations of the two factors, vascular endothelial cell proliferation would be stimulated or inhibited. Although we have not provided direct evidence, our observations lead us to suggest that these factors do not require cell contact for their actions because their concentrations were inversely related to the proximity to the sites of release. The localized nature

**FIGURE 8.** Effects of hypoxia and hyperoxia on the density of vasculogenic cell division, shown on transects of the retina passing from the optic disk to the temporal margin, below the area centralis. (A) Retinopathy of prematurity was induced in newborn kittens by 4 days’ exposure to 70% to 80% oxygen, followed by 12 days (A) or 25 days (B) in room air. Recovery in room air results in the density of mitotic vascular cells reaching a peak of (3000 to 3500/mm²) just central to the edge of the developing vasculature. After 25 days in room air, the revascularization of the retina has relieved tissue hypoxia significantly, and peak density, though still elevated, is now 1100/mm². (C) Using supplemental oxygen therapy, we attempted to protect the retina from massive tissue hypoxia during revascularization of the retina in retinopathy of prematurity. After exposure to 70% to 80% oxygen for 4 days, the kitten was raised in 4 days at 45% oxygen, 4 days in 58% oxygen, and 4 days in 70% oxygen. The density of labeled nuclei again reaches a peak just central to the edge of the developing vasculature, but the level is much lower and is within the range observed in normally developing retinas (Fig. 3). In each graph, zero on the abscissa represents the center of the optic disk, the open arrow represents the edge of the vasculature, and the closed arrow represents the edge of the retina.
Processes of Retinal Vascularization

of the stimulus for angiogenesis also may be reflected in the ability of retinal extracts to act as chemotactants for vascular endothelial cells.

The observation that the inhibitory effect of hypoxia, resulting in obliteration of existing retinal vessels, is lost in adulthood suggests that the signal for the degeneration of retinal vessels either is developmentally regulated or the receptor for the factor mediating this effect is no longer expressed in adulthood.

**Mitotic Activity Associated With the Two Processes of Retinal Vascularization**

Our results confirm the distinction drawn in earlier studies between the two processes observed in the formation of the retinal vasculature. In the first process of vasculogenesis, spindle-shaped precursor cells multiply and differentiate to form an exuberant capillaryplexus, from which the adult vascular tree subsequently differentiates. In the second process of angiogenesis, capillaries bud from vessels formed in the first process. Our present results show that the pattern and intensity of mitotic activity differ between the two processes. In the first process, mitotic activity begins in the precursor cells some millimeters peripheral to the edge of the patent vessels, increases rapidly as the precursor cells transform into capillaries, and then falls to zero in the adult. In the second process, division occurs only in the endothelial cells close to the tips of growing vessels.

**Physiological Hypoxia and Regional Variation of Normal Vasculogenesis**

Our results support the hypothesis that retinal vasculogenesis is controlled by hypoxia in the inner layers of the retina. If we assume that arterial oxygen tension is constant throughout the choroidal plexus and that the choroid does not significantly autoregulate, oxygen tension at any particular location of the retina is affected by two major variables. First, as the neurons (in particular, the metabolically active photoreceptors) of the retina mature, their oxygen consumption increases, reducing the oxygen tension at the inner layers of the retina. The topography of formation and maturation of neurons in the cat retina is centered around the area centralis. Second, the formation of retinal vessels brings blood to the inner layers, relieving this hypoxia. Experimental support for this scenario has been provided by the study of oxygen tension during wound healing in rat muscles with the use of antimony-glass oxygen microcathodes. Oxygen tension remained low in the centers of the wounds as long as vascularization of the wounds was incomplete. This oxygen gradient disappeared when the new blood vessels had grown completely across the wound.

In contrast to the topography of neuronal maturation, the formation of the inner retinal vasculature is centered around the optic disk. Our hypothesis predicts that hypoxia should be maximal just peripheral to the edge of the developing blood vessels. In addition, the area centralis-centered topography of the metabolic demands of the retinal neurons leads us to predict that the hypoxia-induced drive for retinal vascularization should be greater in temporal than in nasal retina. Supporting evidence for both these predictions has been provided by a recent report by Garino et al. These researchers showed that vascular formation in the inner and the outer plexus occurred earlier temporally than nasally in monkey retina. They also observed cystic spaces indicative of hypoxic edema in the nerve fiber layer at the front and immediately peripheral to the leading edge of the forming vasculature.

**Vasculogenesis Itself May Add to Local Hypoxia**

The formation of the capillary plexus is initially exuberant, suggesting that this region of the retina is under an intense hypoxic drive. Why should these capillaries be exuberant and require subsequent retraction? The explanation may lie in the high metabolic activity of dividing vascular cells. If proliferating endothelial cells serve as oxygen sinks, then such proliferation would intensify the hypoxia at the edge of the developing vasculature. Conversely, when blood flows through these vessels, their exuberance would allow a high flow rate and would tend to make the inner retina hyperoxic. The hyperoxia would then induce retraction of the excess capillary segments, as observed in the remodeling of the capillary plexus to its relatively sparse adult form. Capillary degeneration is more extensive close to arteries than veins, producing the capillary-free spaces that flank arteries.

This model of retinal vessel formation is based on many sources, including the work of Michaelson, Ashton, Dollery et al., Wei et al., and our own review and analysis. In addition to contributing to our understanding of the biology of vessel formation, a key observation of our study was that vasculogenic cell division in a retina from an animal with ROP could be brought into the normal range by management of the level of oxygen in the inspired gas mixture. This supplemental oxygen therapy presumably mimics physiological hypoxia, allowing the retina to vascularize with minimal pathology. The value of the concept of physiological hypoxia for the management of proliferative vascular diseases of the retina is explored in the next article (see pages 1215–1230).
Oxygen Controls the Rates of Retinal Vasculogenesis

Key Words
bromodeoxyuridine, cytogenesis, vascularization, oxygen, cell division

Acknowledgments
The authors thank Dr. Takashi Watanabe for helpful advice on BrdU immunohistochemistry, Dr. Don Mason for the gift of anti-BrdU, Mr. Clive Jeffery for photographic assistance, Mr. Leonard Tang for technical assistance, and Dr. David Henderson-Smart for the gift of infant incubators.

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