Clinical and Histopathologic Features of Canine Oxygen-Induced Proliferative Retinopathy

D. Scott McLeod, Salvatore A. D’Anna, and Gerard A. Lutty

PURPOSE. In previous studies the morphologic features of the acute vaso-obliterative and vasoproliferative stages of oxygen-induced retinopathy (OIR) were quantified and described in the dog model of retinopathy of prematurity (ROP). In the present study the sequelae of these events were examined using fluorescein angiography and histologic, enzyme, and immunohistochemical techniques.

METHODS. Thirty newborn animals were exposed to 95% to 100% oxygen for 4 days and returned to room air until they were 22 to 45 days of age. Before death some animals were anesthetized, and fluorescein angiography was performed. Retina and vitreous from some animals were processed for adenosine diphosphatase (ADPase) flat-embedding. In other cases, eyes were prepared for full-thickness eyewall sectioning or frozen for histochemical analysis.

RESULTS. Fluorescein angiography, funduscopic examination, and ADPase preparations showed dilated and tortuous retinal vessels, pigmentary changes, incomplete vascularization of peripheral retina, vitreous hemorrhage, and persistence of massive intravitreal neovascularization. Full-thickness eyewall sections showed tractional retinal folds, tented intravitreal vascularized membranes, and vitreous synchysis. Immunohistochemical analysis showed inner retinal astroglia. Enzyme histochemistry showed high alpha glycerophosphate dehydrogenase activity in poorly differentiated neovascular formations and low activity in formations with mature pericytes and endothelial cells.

CONCLUSIONS. End-stage OIR in the neonatal dog shares many features with the chronic human disease. These results provide additional support for the use of this model in experimental studies of ROP. (Invest Ophthalmol Vis Sci. 1998;39:1918-1932)

Initially retinal blood vessel assemblage in the neonatal dog occurs primarily by a process of vasculogenesis, a term referring to de novo formation of vasculature from mesenchymal precursors or angioblasts. During development, the inner Müller cell processes furnish a scaffold for angioblast attachment and organization. Additionally, the network of interconnecting extracellular spaces they create permits unimpeded vascular growth anteriorly. In the dog, astrocyte migration, assessed with glial fibrillary acidic protein (GFAP) immunolabeling, lags behind formation of the primary retinal blood vessels. Sustained breathing of high oxygen produces a progressive constriction of the developing retinal vasculature that eventually results in vaso-obliteration, or the irreversible closure of many capillary channels and significant degeneration of vasoformative cells. The end product of vaso-obliteration in the neonatal dog model of retinopathy of prematurity (ROP) is a 77% reduction in capillary density, a 60% decrease in luminal diameter of remaining viable retinal capillaries, and a loss of as much as 56% of vasoformative cells. In contrast, the choriocapillaris seems to be unaffected morphologically by prolonged breathing of high oxygen.

In the dog, revascularization after hypoxic insult involves a period of marked vasoproliferation that peaks between 3 and 10 days after return to room air. In addition to the marked increase in endothelial cell proliferation, there is proliferation of perivascular cells, presumably astrocytes. Hyperoxic insult, followed by return to room air, not only stimulates a marked increase in proliferation of vasoformative cells and apparent accessory glia, but also results in a loss of extracellular spaces, which are prevalent in the inner retina during the course of normal development. The diminution of spaces in the peripheral avascular retina after return to room air and the occupancy of these spaces by cells not normally found in advance of forming blood vessels is likely to impede vascular growth anteriorly. Marked vasoproliferation in the abnormal confines of this congested perivascular milieu probably contributes to the prolific capillary overgrowth at the anterior edge of reforming vasculature and to the invasion of vessels into the vitreous cavity.

In this study we examined the clinical characteristics, angiographic features, permeability properties, and vascular patterns in proliferative oxygen-induced retinopathy (OIR). We also studied the morphology and structure of retinal vessels and intravitreal neovascular formations and pathologic changes in the vitreous humor. We used enzyme histochemical local-
FIGURE 1. Funduscopy appearance of the posterior pole region (A, C, E) and peripheral retina (B, D, F) of 22-day-old animals showing varying degrees of proliferative oxygen-induced retinopathy. Mild tortuosity of major arteries (long arrows) of the superior arcade in the peripapillary region (A) and brush border appearance of capillaries (short arrow) at the peripheral edge of vascularized retina (B). Extremely tortuous arteries (long arrows) and dilated veins were present in the posterior pole (C) and, in the periphery, severely retarded vessel growth (D) with sharp demarcation (short arrow) between vascularized and avascular retina. Vessels at the border are severely overgrown and appear somewhat elevated. Pigment clumping is shown (arrowheads) in the avascular retina. Intravitreal vascularized membrane (long arrows) overlying the optic disc (E). A vascularized membrane (long arrows) is present just posterior to a ridge of vessels (short arrow) at the border of vascularized retina (F).
FIGURE 2. Red-free photograph (A) and early arterial phase angiogram (B, C) showing isolated intravitreal neovascular formations (arrows) in the posterior pole of a 45-day-old animal. These intravitreal vessels are fed by a tortuous retinal artery.

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zation of menadione-dependent alpha glycerophosphate dehydrogenase (M-a-GPDH) to examine the distribution of angioblasts in nonvascularized retina and the metabolic activity of intravitreal vessels. Finally, we compared the distribution and density of astrocytes in animals with varying degrees of proliferative retinopathy.

METHODS

Five litters of newborn purebred beagles (6 animals/litter), were exposed to 95% to 100% oxygen continuously for 4 days and then abruptly returned to room air. Animals were killed between 22 and 45 days of age by an intraperitoneal overdose of sodium pentobarbital. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In some animals, fluorescein angiography and fundus photography were performed before death. Animals under halothane anesthesia were examined using indirect ophthalmoscopy, and color fundus photographs were taken. Angiograms were taken in halothane-anesthetized dogs by injecting 0.2 ml 10% sodium fluorescein into a femoral arterial catheter, and photographs were taken using a standard fundus camera (Carl Zeiss, Oberkochen, Germany). Adenosine diphosphatase (ADPase) retinal preparations of some animals were made as described previously. Vitreous ADPase preparations from the same eyes were processed identically to retinal tissue. Eyes designated for immunohistochemical and enzyme histochemical analysis were snap-frozen in ommittine carbamoyltransferase compound (Miles, Elkhart, IN) using isopentane cooled with dry ice. Enzyme histochemical localization of M-a-GPDH, a marker for vascular precursors and immature endothelial cells, was performed on 12-μm sections, as previously reported. Immunohistochemical localization of von Willebrand’s factor (vWF) and GFAP were carried out using a streptavidin peroxidase technique on 12-μm sections, as described previously. The vWF antibody (Accurate Chemical, Westbury, NY) was used at a concentration of 0.2 μg/ml, and the GFAP antibody (Dako, Carpentaria, CA) was used at a concentration of 0.07 μg/ml. Primary antibody incubations were conducted at 4°C for 20 hours.

Four 22-day-old animals were anesthetized using halothane and infused with 500 mg/kg horseradish peroxidase (HRP type II; Sigma, St. Louis, MO) in phosphate-buffered saline (PBS), through a femoral artery. After 15 minutes of circulation time, the animals were killed by an intra-arterial overdose of sodium pentobarbital, and retinas were dissected and fixed in one-quarter strength Karnovsky’s paraformaldehyde-glutaraldehyde fixative for 2 hours at room temperature. Horseradish peroxidase was developed using 3,3-diaminobenzidine. Eyes designated for full-thickness eyewall sectioning were slit at the limbus and fixed in one-quarter strength Karnovsky’s paraformaldehyde-glutaraldehyde fixative before the anterior segment was removed. Tissues were dehydrated and embedded in glycol methacrylate (JB-4; Polysciences Inc, Warrington, PA) as recommended by the manufacturer.

Morphometric analysis of ADPase flat-embedded retinas was performed en bloc before sectioning, using digitized darkfield images collected from a photomicroscope (Photomicroscope II; Carl Zeiss) equipped with a charge-coupled device camera (Hamamatsu City, Japan) and a computer (Macintosh Ilci computer; Apple, Cupertino, CA) with NIH image software (version 1.44; National Institutes of Health, Bethesda, MD). Vitreous ADPase preparations were analyzed in 0.1 M cacodylate buffer, using the system described above. Microdensitio-
FIGURE 3. Red-free photograph (A) and fluorescein angiogram (B, C, D) of a 22-day-old animal with an intravitreal vascularized membrane. The membrane (long arrows, A, B), which extends peripherally from the optic disc along the temporal vascular arcade, fills during the early arterial phase of the angiogram (B, C). Dye readily leaks from the membrane and from isolated tufts of neovascularization near the edge of the membrane (short arrows, B, C).

metric analysis was performed on digitized images of GFAP-immunolabeled sections and M-α-GPDH-incubated sections using the plot profile function of the software (NIH). The offset and gain of the CCD controller (Zeiss) were adjusted before data collection to provide images with gray-scale values that were within the 0 to 255 range of the program (black = 255; white = 0). All images were collected under identical conditions. Density plot profiles were generated using rectangular field selections (100 μm wide × 200 μm high) through the inner retina and vitreous cavity for GFAP-stained sections. The peak density of each plot, which coincided with labeling of astrocytes within the nerve fiber layer, was subtracted from the background density of the vitreous. Sections incubated for M-α-GPDH activity were analyzed by generating density plot profiles in a similar manner, except rectangular selections (20 μm wide × 200 μm high) were made through the neovascular formations and vitreous. Again, the peak densities, which in this case coincided with the intravitreal vessels, were subtracted from the background densities of vitreous. The mean density and standard deviation were calculated for each region or structure from a minimum of six density plot profiles, and statistical analysis of the data was performed using Student's t-test.

RESULTS

Funduscopic examinations revealed varying degrees of oxygen-induced pathologic changes that ranged from tortuosity of posterior pole vessels (Figs. 1A, 1C) and capillary overgrowth at the border of vascularized retina (Figs. 1B, 1D) to severe vasoproliferation that resulted in tractional retinal folds and vitreous hemorrhage. In the posterior pole intravitreal neovascularization was often seen extending from the optic nerve head peripherally along the major vascular arcades to the border of vascularized retina. The neovascularization appeared as dense matlike structures that formed opaque membranes in the vitreous (Figs. 1E, 1F). Additionally, isolated neovascular fronds were observed in the posterior pole and near the border of vascularized retina (Fig. 2). In 20% of the animals, retinal folds were associated with intravitreal vascular membranes. These folds extended radially from the optic disc peripherally
FIGURE 4. Fluorescein angiogram (A, B, C) and horseradish peroxidase flatmount preparation (D) from a 28-day-old animal showing intraretinal vessels at the border of vascularized retina. Fluorescein dye readily leaks from these vessels during the early arterial filling phase. Horseradish peroxidase, however, is confined mostly to the lumen of these vessels with minimal extravasation of tracer.

and incorporated the major retinal vessels, which seemed to feed and drain the overlying neovascular network. In some cases only one vascular arcade had a membrane and fold associated with it, whereas in other cases, all three vascular arcades were involved. In two animals, mottled pigmented changes were observed in the pole region and peripheral to the border of vascularized retina (Fig. 1D).

Peripheral changes ranged from mild capillary overgrowth producing a characteristic brush border appearance or finger-like projections of the peripheral vessels (Fig. 1B) to an elevated pile or ridge of overgrown capillaries forming a well-defined line of demarcation at the border of the vascularized retina (Figs. 1D, 1F). This “ridge” in the dog did not appear opaque nor was it the primary area of neovascular growth, as has been described in human ROP. Isolated neovascular fronds or dense confluentplexuses of intravitreal vessels or both were located posterior to the border. Angiography showed rapid filling of isolated neovascular fronds (Fig. 2) and vascularized membranes (Fig. 3) during the arterial filling phase of angiograms. The vessels in these formations readily leaked dye, which obscured any detail of the intravitreal and intraretinal vessels. Dye also leaked from intraretinal capillaries at the anterior edge of vascularized retina (Fig. 4A, 4B, 4C), which masked the vascular border. The areas with more severe capillary overgrowth at the border tended to leak more profusely.

Retinas prepared from animals infused with HRP showed mild leakage of tracer from capillaries at the border of vascularized inner retina (Fig. 4D). Intraretinal vessels in the posterior pole showed no apparent extravasation of tracer. The weak extravascular HRP reaction product did not mask the adjacent retinal capillaries, which appeared dark brown against a pale-tan background (Fig. 4D). The leakage of HRP seemed much less profuse than that of fluorescein. In some areas near the border of vascularized retina, small focal intraretinal hemorrhages were observed near or at the tips of the capillaries. Erythrocytes of these hemorrhages were stained with 3,3'-diaminobenzidine, apparently because of their normally high levels of endogenous peroxidases. Of note, these hemorrhages were usually not visible during funduscopic examination and therefore may have been artifactually produced by the infusion of HRP, which significantly raised the animal's arterial blood pressure (data not shown).
In the dog, revascularization and the proliferative response after hypoxic insult varied considerably, even among littermates of the same gestation, similar birth weight, and identical oxygen exposure (Figs. 5, 6, 7). In some animals, vascular growth was severely retarded, and vessels reformed in a circular pattern equidistant in growth peripherally in all directions from the optic nerve head (Fig. 5A). In these animals the vessels showed extreme and uniform capillary overgrowth. In other animals, however, the response was asymmetrical with vascular arcades exhibiting markedly different pathologic changes and extent of peripheral growth (Fig. 5B). Generally, the most severely retarded arcades showed the more significant capillary overgrowth at the border of vascularized retina. Despite the variability among animals, however, the response between eyes of the same animal were remarkably similar (Fig. 6). When vascularized areas of retina in all right and left eyes were compared in a litter of animals, no significant differences were found (Student's t-test; \( P = 0.9 \)). In eyes in which vitreous was incubated for ADPase activity and analyzed to measure the area of intravitreal neovascularization in relation to retinal vascular area, an inverse correlation was found between the two parameters. A comparison of retinal vascular area and intravitreal neovascular area in 45-day-old littermates (40 days after return to room air after oxygen exposure) is shown in (Fig. 7). Animals that had more vascularized retinas had less intravitreal neovascularization.

Neovascular formations in the vitreous varied in morphology and structure (Fig. 8). Some were isolated polypoid nodules that were highly cellular, contained few canalized lumens, and consisted of poorly differentiated cellular components, especially at their tips (Figs. 8A, 8B). These formations were most often located peripherally, nearest the border of vascularized retina. Other formations seemed to be more mature morphologically and consisted of isolated fronds of delicate vessels with canalized lumen and well-developed endothelial cells and pericytes (Figs. 8C, 8D). These formations were typically located more posteriorly than the polypoid formations. Activity of M-a-GPDH was much higher in poorly differentiated polypoid formations than in the more mature posteriorly located intravitreal vessels (Fig. 9). Often, individual formations became confluent with neighboring formations and coalesced to form an anastomosing vascular membrane (Fig. 10). These membranes, which contained new collagen, were typically seen extending from the optic nerve head along the major arcades in the posterior pole (Fig. 11). In these advanced stages, hemorrhages were common and occasionally were accompanied by acute inflammatory cell infiltrates, vitreous condensation, tractional fibers, and in severe cases, vitreous liquefaction (Fig. 12). In eyes with vitreous degeneration, the membranes were tented, retinal folds were present, and the posterior vitreous was often detached. Vessels overlying the optic nerve head generally represented frank neovascularization and only rarely were associated with a persistent hyaloid system (data not shown).

Immunohistochemical localization of GFAP and vWF showed inner retinal astrogliosis in animals with proliferative retinopathy. The pattern of GFAP immunolabeling was consistent with astrocytes because of its confinement to the nerve fiber layer (Fig. 13). The radial processes of Müller cells that extend through the inner plexiform, inner nuclear, and other retinal layers were rarely labeled. Unlike the close temporal relation of forming vasculature and migrating astrocytes in

**Figure 5.** Adenosine diphosphatase retinal flatmount preparations from three 22-day-old littermates showing the variability in response among animals. Note that in (A) revascularization resulted in a small circular pattern of vessels extending equidistant from the optic nerve head. In (B) and (C), however, the response is asymmetrical with the superior arcade (arrowhead, B) showing more severely retarded vascular growth than the other arcades. Magnification, \( \times 3.5 \).
normal retina, in eyes with proliferative retinopathy, astrocytes had spread throughout the inner retina to the far periphery (Fig. 13D), despite the failure of vessels to form in these regions (Fig. 13B). Even though blood vessel assemblage had not occurred, M-a-GPDH-labeled angioblasts were consistently seen occupying avascular inner retina (Fig. 13F).

**Figure 6.** Comparison of the area of vascularized retina in right eyes (OD; gray bars) and left eyes (OS; striped bars) of five 22-day-old littermates. Even though the response among animals was variable, the response between the eyes of each animal was remarkably similar (maximum 9% difference in vascular area).

**Figure 7.** Comparison of total retinal area (shaded bars), retinal vascular area (Vas area; white bars) and neovascular area (NV area; striped bars), determined by measurements derived from adenosine diphosphatase-incubated retina and vitreous from four 45-day-old littermates.
FIGURE 8. Morphology and structure of isolated tufts of intravitreal neovascularization in the periphery (A, B) and in the posterior pole region (C, D) in an adenosine diphosphatase vitreous preparation (A, C) and in cross sections (B, D) from a 45-day-old animal. (A, B) Immature formations were polypoid nodules that contained numerous poorly differentiated cells with bulbous nuclei (arrowheads) and few canalized lumen (arrows). (C, D) Mature, preretinal neovascular formations consisted of delicate capillary-like vessels (C) with canalized lumen and well-developed endothelial cells (arrowheads; D) and pericytes (arrows; D). These two cell types were often juxtaposed (D) and had 1:1 ratios. (A) Dark-field illumination; magnification, X130; (B) toluidine blue-basic fuchsin; magnification, X350; (C) dark-field illumination; magnification, X130; (D) periodic acid-Schiff stain and hematoxylin; magnification, X1100.

The most severe astrogliosis was seen in regions of retina with the most severe proliferative retinopathy. Perhaps this correlation was best shown in animals that had asymmetrical disease (similar to the one shown in Fig. 5B). Vascular arcades with severely retarded vessel growth peripherally, extreme capillary overgrowth, and intravitreal neovascularization posteriorly had significantly greater GFAP immunoreactivity than arcades in which little or no disease was present and vessel growth had reached the far periphery (Fig. 13). Little or no GFAP staining was localized to intravitreal vascular formations except in astrocytic processes at the base of these structures where connections were made with retinal vessels (Fig. 14). We never observed a deficiency in astrocytes at locations where vessels breached the internal limiting membrane and erupted into the vitreous cavity. In fact, we found increased GFAP immunolabelling at these sites compared with areas of the same retina that did not give rise to intravitreal vessels.

DISCUSSION

Proliferative OIR in the neonatal dog has many clinical characteristics in common with human ROP. Dilation and tortuosity of posterior pole vessels, referred to as plus disease in the human, was common in the dog model and was generally an indicator of severe peripheral retinopathy, as has been reported in man. Peripheral changes in the dog ranged from slightly retarded vascular growth and brush border-like peripheral capillaries to severe overgrowth of capillaries producing an elevated ridge of vessels and sharp demarcation at the border of vascularized retina. This feature in dog mimics some aspects of the ridge seen in human ROP. Intravitreal neovascularization occurred posterior to the border of vascularized retina and persisted for relatively long periods (at least 40 days after return to room air). In some cases individual neovascular formations coalesced to form dense membranes with collagenous perivascular matrices that extended along the major vas-
Figure 9. Menadione-dependent alpha glycerophosphate dehydrogenase (Mα-GPDH) in intravitreal neovascular formations in a 28-day-old animal. (A) Cryosection showing an immature intravitreal neovascular formation with a polypoid morphology (arrow) and extremely high Mα-GPDH activity. This lesion is 1.13 mm from the border of vascularized retina. (B) Same section as that shown in (A), except this mature formation (arrow) with delicate capillary-like vessels and weak Mα-GPDH activity was located in the posterior pole region, 5.36 mm from the border of vascularized retina. (C) Higher magnification view of (B). (D) Microdensometric analysis of Mα-GPDH activity in neovascular formations shows that intravitreal vessels with an immature structure have significantly greater Mα-GPDH activity than mature formations (Student's t-test; P < 0.001). Magnification, (A) ×200; (B) ×150; (C) ×530.

cular arcades from the optic nerve head. Retinal folds were present in some eyes with intravitreal vascularized membranes, and their appearance was remarkably similar to retinal folds reported in human cases of cicatricial retrolental fibroplasia. Although optic nerve head neovascularization and posterior vascular tufts are somewhat common in the dog and cat models of OIR, these features are not routinely associated with today's human ROP cases. Nevertheless, there are clinical cases and histologic studies describing these features in human retrolental fibroplasia.

Fluorescein angiography has been used to assess the severity of proliferative disease in premature humans and in animal models of OIR. As has been reported in humans, we observed varying degrees of proliferative retinopathy in the dog, even among littermates of identical gestational age, duration of oxygen exposure, and similar birth weight. Even though the response among animals varied significantly, there was remarkable symmetry of disease between the two eyes of a given dog. Also, the active vasoproliferative stages of the disease persisted for the duration of this study (40 days after return to room air) and in some animals progressed to tractional retinal folds without evidence of regression. Bilateral symmetry, massive neovascularization, and the extended period of active proliferative disease in a relatively large eye may offer some benefits in comparison with other animal models.

The ability to screen dogs with conventional ophthalmoscopy and fluorescein angiography should prove advantageous in future therapeutic studies. Treating severely affected animals and observing the progression of disease clinically for prolonged periods may be beneficial in determining the efficacy of pharmacologic or other types of interventions on massive persistent intravitreal neovascularization that is typically featured in human ROP cases with poor outcome.

There was an inverse correlation between area of vascularized retina and area of intravitreal neovascularization in the canine model of OIR; that is, the more severely retarded the peripheral vascular growth was, the more robust the intravitreal neovascular response. Patz made a similar observation in
human subjects, in that the incidence of retrolental fibroplasia was inversely related to degree of retinal vascularity. More recently, the Cryotherapy for Retinopathy of Prematurity Cooperative Group observed that the worst outcome rates were in eyes with zone 1 disease: The more posterior the zone of ROP, the greater the extent of stage 3+ disease. Whether this inverse correlation is related to amount of vascular growth factor produced in avascular periphery, degree of hypoxia in the retina, the spatial constraints preventing peripheral growth of new vessels, or a combination of these factors has yet to be determined.

Intravitreal neovascularization in the canine model of OIR appeared as isolated tufts, dense anastomosing networks, or both. Ashton et al. also observed two distinct morphologic forms of preretinal neovascularization in the feline model of OIR: long, convoluted loops and glomerular tufts, which seem to be similar to the polypoid formations we observed. In dog, isolated tufts varied in morphology and structure, depending on their location. Peripherally, near the border of vascularized retina, they appeared as polypoid angioblastic masses with few canalized lumen and poorly differentiated cellular components. All cells within these formations expressed high M-α-GPDH activity reminiscent of angioblasts and primordial capillaries in normally developing retina and were weakly labeled with the antibody to vWF. Posteriorly, these formations consisted of delicate canalized capillaries with well-developed endothelial cells and pericytes with ratios of nearly 1:1. Cells within these vessels expressed significantly less M-α-GPDH activity, as do normal mature retinal vessels, whereas endothelial cells expressed increased vWF. Because polypoid angioblastic formations seem to evolve into mature capillarylike networks with well-differentiated cellular components, these and previous results suggest that, as in normal retinal vasculogenesis, pluripotential vasoformative cells contribute to formation of intravitreal vessels. Angioblasts may therefore give rise to endothelial cells and pericytes, and their ultimate fate may be influenced by their position (luminal or abluminal) on the vessel wall, as was suggested by Ashton.

The dense intravitreal vascular networks that were present in the dog seemed to evolve by coalescence of neighboring isolated tufts. In older animals these formations were membranous in that they exhibited a perivascular collagensomes. All cells within these formations expressed high M-α-GPDH activity reminiscent of angioblasts and primordial capillaries in normally developing retina and were weakly labeled with the antibody to vWF. Posteriorly, these formations consisted of delicate canalized capillaries with well-developed endothelial cells and pericytes with ratios of nearly 1:1. Cells within these vessels expressed significantly less M-α-GPDH activity, as do normal mature retinal vessels, whereas endothelial cells expressed increased vWF. Because polypoid angioblastic formations seem to evolve into mature capillarylike networks with well-differentiated cellular components, these and previous results suggest that, as in normal retinal vasculogenesis, pluripotential vasoformative cells contribute to formation of intravitreal vessels. Angioblasts may therefore give rise to endothelial cells and pericytes, and their ultimate fate may be influenced by their position (luminal or abluminal) on the vessel wall, as was suggested by Ashton.

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**Figure 10.** Possible sequence leading to the evolution of interanastomosing neovascular networks in the vitreous from a 22-day-old animal. (A) Single isolated polyplike nodule in the vitreous near the border of vascularized retina. (B) Adjacent nodules with independent feeder vessels apparently growing to confluence. (C) Same as (B) except the number of cells and the size of the formation is greatly increased. (D) Network of vessels with well-differentiated cellular components and perivascular collagen elevated above the retinal surface in the posterior pole region. Magnification, (A, B) ×400; (C) ×300; (D) ×200.
FIGURE 11. Tented vascularized membrane and tractional retinal folds in a 45-day-old animal. (A) Red-free fundus photograph showing an opaue membrane (arrow) extending from the optic nerve head radially along the major vascular arcades. (B) Gross photograph of the same specimen showing the vascularized membrane (long arrow), tractional attachments to the retina (short arrow), and folding of the retina (curved arrow). (C) Full-thickness eyewall section through the region indicated by the long arrow in (A) and (B) shows tenting of the membrane (arrow) and folding of the retina at the borders of the tent. (D) Section through the region indicated by the short arrow in (B) showing a full-thickness retinal fold (curved arrow) and traction with the vascularized membrane (short arrow). Magnification, (C) ×150; (D) ×125.

(component. In some cases vitreous hemorrhage and acute inflammatory cell infiltrates were present in eyes with vascularized membranes (Fig. 12). The combination of increased serum proteins from leaky intravitreal vessels and the presence of acute inflammatory cells from persistent or recurrent hemorrhage probably altered the colloidal structure of the vitreous and resulted in condensation in some areas and liquefaction in others, as occurs in other proliferative retinopathies. It is in these eyes that tractional attachments developed between the contracted vascularized membranes and the retina, producing full-thickness retinal folds.

Accumulating evidence suggests that glial cells play a major role in normal retinal vasculogenesis and oxygen-induced proliferative retinopathy. Our previous studies in the dog have shown an important role for Müller cells in normal vasculogenesis, whereas others have shown that astrocytes may be important mediators of these events in other animal models. Stone et al. have provided evidence that astrocytes are the major source of vascular endothelial growth factor in the normally developing cat and rat retina and that their exclusive transient expression of this growth factor in advance of developing superficial retinal vessels stimulates vasculogenesis in this layer. These investigators have also provided evidence that preretinal neovascularization in cat and rat models is preceded by degeneration of astrocytes and expression of vascular endothelial growth factor by neurons of the ganglion cell layer. In the normally developing dog retina, astrocytes seem to trail formation of primordial capillaries and therefore may not function in the capacity of initiating vasculogenesis. A recent report on developing human retina described limited associations between astrocytes and forming vessels in some regions at early gestational ages (less than 20 weeks) and no clear specific correlations in other areas. Therefore, it seems that developing vessels in the cat and rat conform strictly to astrocyte processes, whereas in the dog and human they are less closely associated.

Astrocyte degeneration has been implicated in the formation of preretinal vessels in some models of OIR. Zhang and
FIGURE 12. Vitreous changes in a 45-day-old animal (same specimen as shown in Fig. 11). (A) Vitreous condensation (v) at the apex of a tented vascularized membrane. (B) Lamellar appearance of condensed vitreous (v) along the surface of the membrane posterior to the region shown in (A). The blood vessels (arrows) are surrounded by a perivascular collagenous matrix (c). (C) Acute inflammatory cells surrounding blood vessels (arrows) in a vascularized membrane in the vicinity of a small vitreous hemorrhage (arrowheads). (D) High-magnification micrograph shows liquefaction of the vitreous in a pocket between a retinal fold and a tented membrane. Stain in all panels, toluidine blue–basic fuchsin. Magnification, (A, B) ×325; (C) ×130; (D) ×620.

Stone22 reported that preretinal vessels form in astrocyte-deficient areas of rat retina after cyclic hyperoxia and suggested that the weak anatomic barrier created by these deficiencies in the glial limitans allows vessels to grow into the vitreous. In the dog, we find that astrocyte degeneration does not occur during or after exposure to hyperoxia27 and, on the contrary, astrocytes proliferate after hyperoxic insult and return to room air producing inner retinal astrogliosis.5 Eyes that had more severe vasoproliferative disease had more severe astrogliosis. This was most clearly shown in retinas in which asymmetrical disease was present. Areas of retina that were more vascularized and had no intravitreal neovascularization had normal astrocyte densities (similar to those in control subjects exposed to room air). In contrast, areas with severely retarded vascular growth and proliferation of vessels into the vitreous had significantly more astrocytes. These cells even occupied the entire avascular inner retina, which in some cases was more than 4 mm beyond the edge of vasculature. Whether astrocyte degeneration occurred before the time points examined in this study may be inconsequential, considering that vessels were still actively growing from retina into vitreous in these animals and that this neovascular growth originated from regions abnormally rich in astrocytes. Our observations in the dog suggest that astrocytes proliferate and invade extracellular spaces of the inner retina before blood vessel assemblage after hyperoxic insult, disrupting the milieu required for normal vasculogenesis.5 Vessels then erupt into the vitreous because peripheral growth is impeded by astrocytes and their processes. As is the case in the dog, the presence of extracellular spaces in the normally developing human avascular inner retina and their absence in peripheral avascular ROP retinas has also been noted.29 Additionally, Hamada et al.29 used GFAP immunolabeling to show astrogliosis in a clinically documented case of ROP. Because angioblasts coexist with astrocytes in avascular periphery of diseased dog retinas, the potential exists for vasculogenesis to proceed anteriorly, but the morphologic conditions favoring blood vessel assemblage (i.e., a network of extracellular spaces) are absent.

In conclusion, the canine model of OIR has many features in common with the cat model of OIR and human ROP. The
FIGURE 13. Astrocyte distribution and density (C, D, glial fibrillary acidic protein [GFAP] immunolabeling) in relation to retinal vascularity (A, B, von Willebrand's factor [vWF] immunolabeling) in a 28-day-old animal with asymmetrical proliferative disease. (A) Ora serrata region of a fully vascularized inferior retina showing primary vasculature (arrow) and secondary capillaries (arrowhead) of the retina. (B) Identical section of the same animal showing the ora serrata region of the superior retina, which has severely retarded vascular growth peripherally (4.6 mm from the ora serrata), capillary overgrowth at the border of vascularized retina, and intravitreal neovascularization posterior to the vascular border. Absence of vWF staining indicates that there are no vessels in this area. (C) Serial section showing immunolabeling of astrocytes (arrows) in the fully vascularized arcade. (D) Same section as in (C) showing intense labeling of astrocytes (arrows) in the avascular inner retina along the severely affected arcade. (E) Microdensitometric analysis of GFAP reaction product in the far periphery of an age-matched air control and in the two regions of the oxygen-treated animal shown in (C) and (D). There was no significant difference in density of the GFAP reaction product in the fully vascularized arcade of the oxygen-treated animal and that in the control animal breathing air ($P = 0.65$). There was, however, significantly greater density of the GFAP reaction product in the nonvascularized arcade compared with that in the vascularized arcade in the oxygen-treated animal ($P < 0.001$). (F) Serial section of the region in (D) showing menadione-dependent alpha glycerophosphate dehydrogenase activity in angioblasts within the avascular inner retina. Magnification, (A, B, C, D, F) X75.

Neovascularization that occurs is exuberant compared with that in rodent models of OIR. $^{30,31}$ The severity may be in part caused by the degree of vaso-obliteration during exposure to hyperoxia, in that dog, $^{4}$ cat, $^{19}$ and human $^{32-34}$ have extensive obliteration of the forming vasculature compared with rodents. In the mouse, for example, vaso-obliteration is limited to the posterior pole. $^{31,35}$ Neovascularization in the dog and human persists for an extended period, whereas the neovascularization in rodents has a much shorter duration. $^{31,36}$ Retinopathy progressed to tractional retinal folds in some animals.
in our study, which was in keeping with observations in the cat by Ashton et al.19 and is often seen in human ROP.37 These observations show that the canine model of oxygen-induced ROP is an appropriate model of human ROP.

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References