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ANIMAL MODELS OF OXYGEN-INDUCED RETINOPATHY

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1. ABSTRACT

Retinopathy of prematurity (ROP) is a neovascularizing disease of the retina affecting premature infants. Much of our current knowledge regarding development of both normal and abnormal blood vessels in the retina has been obtained from animal models of retinopathy. The retina is an excellent organ for studying angiogenesis, since the progress of blood vessel growth can be monitored by angiography or fundoscopy. Also, the entire retinal vasculature can be viewed in flat-mounted retinal preparations. Although these animal models were previously used to study the gross aspects of vasculogenesis and angiogenesis, they are increasingly being used to identify the genes and molecular mechanisms involved in these processes. Knowledge gained from these studies can be applied to non-ocular angiogenic conditions. This paper provides historical perspective on the development and use of animal models of retinal neovascular disease since the 1950's and on the key studies that have led to our current understanding about the pathogenesis these conditions.

2. BACKGROUND

Retinopathy of prematurity (ROP) is a neovascularizing disease of the retina that affects premature infants. In its most severe form it can lead to severe visual impairment and blindness. Although the true incidence of ROP is unclear, recent studies estimate approximately 500 infants are blinded each year by retinopathy, while another 2,300 develop visual impairment from retinal scars (1, 2). Much of our knowledge regarding development of both normal and abnormal retinal vascularization has been obtained by meticulous studies conducted in animal models of retinopathy. It is obvious that our current level of understanding of the pathogenesis of ROP would not have been possible without the use of animal models of disease, but their usefulness is dependent on several factors; the degree of similarity between the model and the human with respect to the insult used to produce ROP, the gestational age of the model, retinal location of both avascularity and neovascularization, the outcome of disease, the cost and ease of reproducing the model, and the correlation between the pathology of the disease in human infants and the animal model.

An ideal model of ROP would require the use of a premature animal with a pattern of retinal vascular development similar to that of the human infant. One of the problems with the use of animal models is the changing nature of ROP in the human population over the past several decades. Cases of ROP seen in the 1980's occurred mainly in infants > 1000g. The advent of modern

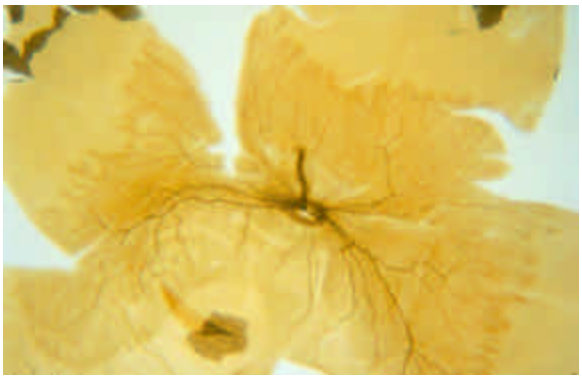

Figure 1. Flat mount of a 21-week gestational age human retina showing extent of peripheral avascular area.

resuscitation techniques has lead to an increased survival of infants < 1000g (3); thus most cases of ROP seen today occur in infants of much lower birth weight than were seen previously. Improved respiratory management and an increased vigilance of oxygen saturations has led to a decreased incidence of ROP in larger infants (4).

The retina is one of the last organ systems to be vascularized in the developing fetus. It has a dual blood supply, with the outer (scleral) portion being supplied with oxygen and nutrients by the adjacent choroid plexus and the inner layers supplied by a superficial and deep plexus of blood vessels lying within the retina itself. Vascularization is initiated by a process of vasculogenesis early in gestation in the posterior region of the retina, and it proceeds in a wave-like fashion towards the periphery by a process including both vasculogenesis and angiogenesis; completing vascularization of the retina by 36 weeks of gestation in the nasal quadrant and slightly later in the temporal quadrant (5). *In utero*, the process of retinal vascularization is believed to occur in response to physiological hypoxia; the highest pO_2 in the human fetus is 35 mm Hg. After birth, retinal vascularization in the premature infant proceeds under conditions of relative hyperoxia (55-80 mm Hg). The pathogenesis of ROP is hypothesized to occur in two phases. The first phase includes an arrest or retardation of existing retinal blood vessel growth by either noxious stimuli or the relative hyperoxia of the ex-uterine environment, potentially complicated by oxygen therapy. Retinal hypoxia occurs secondary to the increased metabolic requirements of the developing retina that are unable to be met by the attenuated or injured vasculature. This is followed by the second phase of compensatory, but often unregulated, neovascularization. It is important to note that fully one-third of all infants who develop ROP do so while still on oxygen therapy. Clearly, the retinal hypoxia that is believed to initiate neovascularization cannot be entirely explained by cessation of therapeutic oxygen. (Figure 1).

The retina then enters a quiescent period with the formation of a mesenchymal ridge between the avascular and vascular regions of the retina. This structure is pathognomonic of ROP in the human infant. In a large number of infants the ridge gradually disappears with subsequent growth of normal blood vessels towards the periphery of the retina. However, in some infants, for reasons that remain unclear, abnormal blood vessels develop from the ridge-like structure, and break through the inner limiting membrane causing vitreous hemorrhage, fibrosis, vitreous contraction and ultimately retinal detachment.

ROP is almost exclusively a disease of premature infants, although it has been reported in term infants. There was virtually an epidemic of blindness from ROP in the 1940's. An association between oxygen and ROP was first reported in 1951 (6). Subsequent studies by several investigators confirmed the relationship between the use of oxygen and ROP and led to the practice of using less than 40% FiO_2 for premature infants (7-9). The 1950's saw a decrease in the number of cases of ROP, but an increase in the cases of hypoxemia-related cerebral palsy and death. Subsequent improvement in resuscitation techniques and ventilation of premature infants led to increased survival of preterm infants and a resurgence of ROP.

3. ANIMAL MODELS OF ROP

3.1. Kitten

The kitten model was one of the first to be used for the study of retinal vascularization. It was developed in the 1950's and has been used subsequently by several investigators. The retinal vasculature in the kitten, as in other mammals, develops from spindle-shaped mesenchymal cells in the region of the optic disc early in gestation (10-12). These cells proliferate and migrate across the surface of the retina to form a capillary meshwork. The level of retinal vascular development in the newborn kitten is very similar to that in an infant of 28 weeks gestation (13). It has been hypothesized that physiological hypoxia, created by the increased metabolic demands of the fetal retina, is the major stimulus for the growth of the retinal blood vessels by vasculogenesis and angiogenesis *in utero;* retinal hypoxia leads to the release of vascular endothelial growth factor (VEGF) providing the stimulus for the growth of retinal blood vessels (14-16). Much of our knowledge of the response of the immature retinal vessels to oxygen as well as our current hypothesis of the pathogenesis of ROP has been obtained by meticulous studies done in the kitten model. Exposure of a newborn kitten to an oxygen-rich environment leads to constriction of larger retinal vessels and closure and atrophy of retinal capillaries. The oxygen requirements of the maturing neural retina are initially met by diffusion from the choroid plexus (17). However, return of the animal to room air leads to inadequate oxygen delivery within the retina. This local hypoxia, in turn, causes release of vascular endothelial growth factor (VEGF) as well as other angiogenic factors from the hypoxic cells in the inner layers of the retina (16). Elevation of these factors induces abnormal intraretinal and intravitreal neovascularization and arteriovenous shunting as well as intraretinal and vitreous hemorrhages.

3.1.1. Vasoconstriction of retinal vessels

The phenomenon of oxygen-induced vasoconstriction has been studied extensively in the kitten

retina. In other tissues several homeostatic mechanisms come into play in response to hyperoxia (18). Vasoconstriction of blood vessels, an autoregulatory response to hyperoxia, is a protective phenomenon that defends against oxygen damage by balancing tissue oxygen supply and demand. The immature retinal vessels are exquisitely sensitive to hyperoxia. This sensitivity gradually decreases with advancing maturity of the retinal vessels, which acquire mature characteristics in the kitten retina by 21 days of age. The severity of the response is proportional to the concentration and duration of oxygen exposure (19, 20). These studies in kittens provided the impetus for clinical studies by Kinsey *et al*, who subsequently confirmed an increased severity of disease with an increased duration of oxygen exposure in premature infants (8, 21). Exposure of kittens to less than 35% oxygen has been shown to be free of ill effects. Exposure to more severe hyperoxia leads to immediate and delayed effects on the retinal vessels; kittens placed in 80% oxygen developed constriction of the main blood vessels and capillaries after 3 - 5 minutes followed by reopening after 10 minutes. Prolonged exposure up to 8 hours led to complete closure. Subsequent removal to room air was associated with a reopening of the vessels within 5 minutes. However, exposure for 36 hours resulted in irreversible closure (19, 22). Similar results were reported by Flower *et al* in a study of blood velocity in the retinal arterioles of kittens maintained in hyperoxia (23). Vasoconstriction does not always lead to permanent closure and atrophy. Ashton *et al* showed that intermittent administration of a high concentration of oxygen does not produce this effect (24). The threshold for permanent closure in the kitten has been extrapolated from several experiments to correspond to an inspired oxygen mixture of 40% (19).

Several studies have been conducted in the kitten to elucidate the mechanism of oxygen induced vasoconstriction. The branching pattern of mammalian retinal vessels consists of dichotomous branching at an acute angle as well as smaller daughter vessels branching at right angles from the major arterial trunk (25, 26). Side arm branches contain a smooth muscle sphincter (27). Constriction of both the arteriole and the side arm vessel sphincter in response to hypoxia affects red cell and plasma flow such that the side arms are perfused mainly with plasma. Studies by Lemington *et al* and Flower *et al* have shown that reduction in the flow of plasma into the side arms leads to widening of the plasmatic zone with narrowing of the central column of red blood cells in the major retinal vessel (23, 28, 29). This phenomenon gives an appearance of apparent vasoconstriction by direct ophthalmoscopy. However, extrapolation of these results to the intact eye should be done with caution since these studies were conducted without controlling for changes in intraocular pressure. Also, Bulpitt *et al* were unable to reproduce the above observation of plasma skimming in the adult pig or Rhesus monkey (30).

A decrease in flow into the side arm branches results in the flow of red blood cells from the major retinal vessels into preferential channels that function as arterio-venous shunts. These channels have been shown to exist in the kitten retina and are the last to close in response to hyperoxia (29, 31).

3.1.2. Permanent Vessel Closure

Unlike vasoconstriction, permanent closure of vessels is a phenomenon unique to the developing retina. Normal vascular development consists both of formation of new blood vessels and removal of excessive capillary growth by a process of endothelial cell retraction or migration. This process of molding of the retinal vasculature is exaggerated by hyperoxia thus resulting in capillary closure (32, 33). Retractions of endothelial cells in response to extreme hyperoxia have been demonstrated in both kitten and human retina (34, 35). Although it is unclear if the process of retraction is entirely due to a toxic effect of oxygen on endothelial cells, detailed experiments involving antioxidant therapies argue against this (36). Another possibility is sluggish microcirculatory flow, but a lack of injury to the underlying perivascular neurons in the damaged capillaries suggests this to be unlikely also (37). Other suggested mechanisms for vascular retraction include intravascular thrombus formation (19, 38), adherence of capillary walls and platelet aggregation (39), reduced levels of survival/anti-apoptosis factors, such as VEGF or increased levels of pro-apoptosis factors like pigment epithelium-derived factor.

3.1.3. Retinal hypoxia/ischemia and vasoproliferation

In the early 1950's hypoxia or too rapid withdrawal of oxygen was suggested to be responsible for the increase in incidence of ROP (40-42). Some reports suggested that a return of infants with ROP to oxygen was associated with a decrease in severity of ROP (41). This apparent paradox was subsequently explained by studies done in the kitten and other models. Permanent closure of the retinal vessels in the oxygen exposed kitten retina is followed by retinal ischemia and hypoxia upon removal of the animals to room air. Removal of the kittens to a hypoxic environment (< 21% oxygen) was followed by a more severe retinopathy than those removed to room air (43). Retinal hypoxia was hypothesized to lead to the release of an angiogenic factor from the cat retina (44). Subsequent studies in cell culture as well as in several animal models have identified VEGF to be a key growth factor responsible for increased angiogenesis in response to hypoxia. Thus, return to oxygen after onset of ROP has the potential to reverse these events.

Vasoproliferation is produced in response to vaso-attenuation caused by either hyperoxia or other causes such as retinal embolization. Electron microscopic studies have confirmed the earlier observations by Ashton *et al* of the vasoproliferative response, which consists of intraretinal and intravitreal neovascularization, and arteriovenous shunting (45, 46). Ashton *et al*, in electron microscope studies, have shown the proliferative lesions to consist of endothelial buds emerging through the internal limiting membrane into the vitreous (47), but the location and pattern of the proliferating vessels in kittens is distinctly different from that seen in infants. Proliferating endothelial cells show mitochondrial swelling with loss of cristae and a vacuolated cytoplasm (48). Ingrowths of

Muller fibers from the inner layers of the retina and growth of collagen fibers from the mesenchymal cells occurs in the vitreous (47). During the proliferative phase, the kitten shows anterior segment abnormalities such as iris vascular engorgement and pupillary rigidity similar to that seen in the human infant.

3.1.4. Supplemental Oxygen

The role of supplemental oxygen for treatment of retinopathy was first studied in the kitten model of oxygen-induced retinopathy (OIR). Recovery of hyperoxia-exposed kittens in a variably hypoxic and hyperoxic environment was associated with less severe retinopathy than room air recovered animals (43). However, gradual withdrawal from high oxygen to room air has not been shown to reduce retinopathy in the kitten models (43). The results of these studies formed the basis of the supplemental therapeutic oxygen protocol for retinopathy of prematurity (STOP-ROP) multi-center clinical trial (49). The STOP-ROP trial tested the hypothesis that administration of supplemental O_2 to infants with prethreshold ROP to maintain their O_2 saturations at 96-99% versus 88-94% would lead to a decrease in the proportion of infants with at least one eye at prethreshold who converted to threshold ROP. Infants whose O_2 saturations were 88-94% in room air were excluded from the study. Although results showed a trend towards a decrease in conversion in supplemented infants (48.5% versus 40.9 %), the results were not statistically significant. A statistically significant improvement (46% versus 32%) was, however, noted in a small group of infants with no plus disease. One of the surprising findings of the study was that supplemented infants tended to have exacerbation of chronic lung disease (8.5% versus 13.2%), although there were no ill effects on the eye.

3.1.5. Retinal Detachment

One of the drawbacks of the kitten model is that it apparently does not develop retinal detachment as is seen in infants with severe ROP. Although the retinal vessel complement and architecture are never completely normal, the neovascularization gradually regresses over a period of many months. Previous studies had suggested that this is likely due to the lack of mesenchymal cells in the kitten retina in contrast to the situation in the human (20, 50, 51). Subsequent work by Halasz et al and others showed the presence of these cells in kitten retina (12, 52, 53). Another possibility is that, since mesenchymal cells are present in isolated foci, they are unable to produce sufficient traction for detachment. Ashton has proposed that anatomic factors, such as the mechanical organization of the vitreous or the small size of the hemorrhages in the experimentally produced lesion, may explain the absence of retinal detachment in kittens.

Hyperoxia-induced vasoconstriction does not occur in kittens with experimentally detached retinas (54), thus arguing with the theory of the direct effect of oxygen on the endothelial cells; one possibility is the lack of diffusion of vasogenic factors from neighboring sources. However, recovery of these animals in room air is associated with an outward proliferation of vessels into the subretinal space and an inward proliferation of vessels forming tufts into the vitreous.

3.1.6. Prevention and Treatment of ROP

Some investigators have suggested that endothelial cell damage by oxygen is due to the action of oxygen-derived free radicals. The interest in using the antioxidant Vitamin E for treatment of ROP was based on the premise that scavenging of free radicals would result in decreased cellular damage (55). Bougle *et al* showed that pretreatment of kittens with Vitamin E prevented a decrease in retinal superoxide dismutase activity in response to hyperoxia (56). Taki *et al*, in studies in kittens exposed to oxygen, showed an increase in lipid peroxide levels in exposed animals as well as an increase in platelet aggregation in these animals. These effects were reduced by Vitamin E administration during and after the exposure (39). Of the several studies conducted to study the effect of Vitamin E supplementation in infants, only one study demonstrated a statistically significant beneficial effect of therapy (57, 58). Raju *et al* conducted a meta analysis of 6 randomized trials of Vitamin E prophylaxis designed to reduce ROP. Although there was an important 52% reduction of severe or Stage III ROP in the treated group in comparison to the control group, there was no reduction in the incidence of ROP (59).

3.2. Rabbit

Retinal vessels in the rabbit are limited to a small region of the retina which contains medullated nerve fibers and extends horizontally on each side of the optic disk, termed a merangiotic pattern (60). The rabbit's immature retinal vessels are permanently closed by elevated ambient oxygen, as are the kitten's (19). One of the unique features of this model is the damage to retinal neurons that is caused by hyperoxia (61). Retinal detachment does not occur in this model, although the growth of new retinal vessels after regression is not completely normal. Prior to the use of the rabbit model there was a notion that retinal edema may be responsible for vessel closure in response to hyperoxia (10). However, since retinal vessels in the rabbit grow on the surface of the retina, it is unlikely that retinal edema can impinge on these vessels; thus contradicting the importance of retinal edema in causing permanent closure.

In vitro studies have been conducted after removal of the intact growing vascular complex from the rabbit eye and placing it in culture medium (50, 62). It is difficult to extrapolate the meaning of these studies to the pathogenesis of ROP or the relevance of the oxygen exposures to physiologic levels *in situ*, but exposure of immature capillaries to high oxygen for 4 – 6 hours resulted in shrinkage and retraction of the capillaries. Removal of the cultures to room air after varying periods in 100% oxygen resulted in regrowth of the capillaries. However, prolonged exposure to oxygen for 48 hours resulted in complete degeneration of the cultured cells and no regrowth when exposed to room air (62). Electron microscopy studies on degenerating rabbit endothelial cells reveal an initial increase in size and number of lysosomes and the formation of autophagocytic vacuoles, followed by enlargement of the Golgi apparatus and focal degeneration of the cytoplasmic matrix and widespread disruption of cell membranes (63, 64).

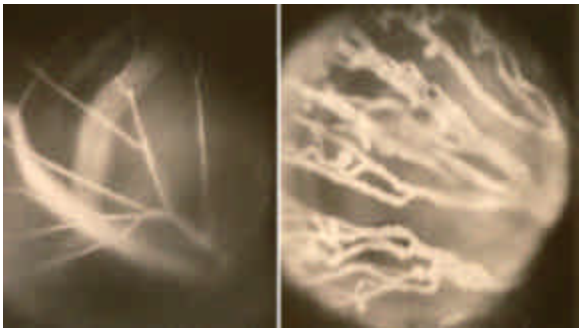

Figure 2. Fluorescein angiograms depicting "hyaloidopathy" in the rat model of ROP. The left panel shows the eye of a 14-day old room air-raised rat with normal hyaloid regression. The right panel shows tortuosity and non-regression of the hyaloid in the eye of an age-matched rat after 14 days of hyperoxic treatment.

3.3. Puppy

The development of the retinal vasculature in the beagle puppy has been described in detail by Flower *et al* (65). The authors demonstrated several similarities between canine and human retinal vascular development. In comparison to the kitten retina the puppy retina is much more vascularized at birth and the rate of postnatal vascularization is much faster (65).

McCleod *et al* studied the pattern of "vaso-obliteration" in the canine model of OIR. Capillary constriction began after 1 hour of extreme hyperoxia and peaked by 24 hours. However, arteries and veins continued to close after 3 days of oxygen breathing. The pattern and severity of the reaction of the developing blood vessels to hyperoxia is similar to that in kittens (66). In a separate study these investigators exposed newborn pups to 95 – 100 % oxygen for 4 days and removed them to room air until 22 – 45 days of age. Retinal folds and intravitreal vascularized membranes were seen in this model. These investigators have suggested that the beagle is a superior model for studying the human disease as it is the only model in which a high percentage of animals develops retinal scarring and detachment (65, 67), although these problems develop in only a small percentage of infants with ROP. Moreover, it is important to note that retinal detachments and hereditary retinal folds have been known to occur spontaneously in dogs (68-70).

Flower *et al*, with their work in the beagle puppy, also challenged the earlier notion that oxygen induced vaso-constriction was an essential element of oxygen-induced damage to developing retinal vessels. They created a more severe retinopathy with a cicatricial fold in the beagle puppy by reversal of the oxygen induced vaso-constriction by aspirin (inhibitor of prostaglandin synthetase) or by exposing the animals to an oxygen/carbon dioxide mixture (69, 71). These results could not be reproduced in the kitten model (71). Recently, the puppy has served as the experimental venue for testing the angiostatic potential of VEGF receptor antagonists (72).

3.4. Rat

Because genetically stable dams are inexpensive and average litter sizes can be as large as 15 depending upon strain, the rat is a more economical model for the study of retinal angiogenesis than kittens or puppies. It differs from other experimental animals in that there is little retinal vascular development until, or shortly before, birth. The retinal vessels of the rat develop initially from mesenchymal precursor cells, forming first a superficial network, which subsequently gives rise to a deeper capillary layer. The superficial network, which develops into arteries, begins developing soon after birth and is complete by day 11 of life while the deep capillary plexus, which develops into veins, begins growing by day 9 and is complete by day 15. Regression of the hyaloid vessels begins about day 4 and is nearly complete by day 12 shortly after the deep plexus forms (47). The hyaloid persists under conditions of high oxygen, as retinal vessel growth is retarded (73, 74). (Figure 2).

While the response of the developing retinal vessels has varied with differing oxygen protocols, it is clear that human-like patterns of vaso-attenuation and subsequent neovascularization can be produced in the rat. Appropriate exposure protocols lead to retarded growth of both superficial and deep vessels, resulting in a peripheral avascular zone. Removal from oxygen exposure to room air causes neovascular growth at the boundary between vascular and avascular areas in the retinal mid-periphery. The abnormal new vessels arise just posterior to the advancing edge of vessel growth and derive from post-capillary venules near the peripheral extent of the major veins. The initial pre-retinal tufts can combine into larger sheets of endothelium. When tufts arising from adjacent veins join, the result is a ridge of pre-retinal vessel growth that is highly reminiscent of the human pathology. Mature retinal vessels of the rat are not affected by hyperoxia. The rat retina is reported to detach under some experimental protocols in a small percentage of cases (75). (Figure 3).

Early attempts by Patz, Ashton, and Gole to develop a rat model of ROP or OIR were inconsistent (22, 76, 77). However, Penn *et al* developed a successful protocol for inducing consistent retinopathy in the newborn rat (78). They noted that protocols requiring exposure of newborn rats to a constant level of extreme hyperoxia, commonly used in previous studies, created substantial vaso-attenuation but did not consistently produce abnormal vasoproliferation upon removal to room air. A systematically varied protocol using alternating twenty-four hour exposure cycles of 80% and 40% oxygen for the first 14 days followed by removal to room air resulted in less vaso-attenuation, but led to neovascularization in 62% of treated animals (79). These studies raised the possibility of neovascularization being caused by other factors besides retinal avascularity and clearly placed in doubt the widely-held notion that "more oxygen means more ROP." In order to mimic the clinical setting in the neonatal intensive care unit more accurately, Penn altered the exposure paradigm to vary between 50% and 10% oxygen. Arterial blood gases measured during the exposure were more reflective of a sick premature infant

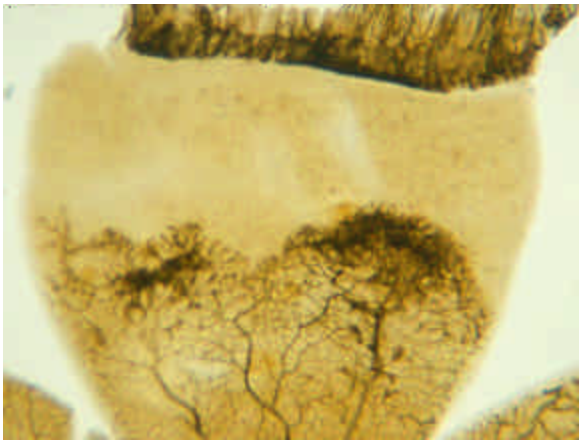

Figure 3. Retinal neovascularization in the rat ROP model occurs, as it does in infants, at the advancing edge of new vessel growth on the boundary between centrally vascular retina and peripherally avascular retina.

than in previous studies. The 50%/10% oxygen cycle resulted in a greater retardation of retinal blood vessel development than the 80%/40% cycle and led to a higher incidence and severity of neovascularization. This indicates that neither the overall amount of oxygen the subject receives nor the range of variation is as important for the development of retinopathy as avoidance of hypoxia during exposure (79). In a subsequent series of experiments this group tested several different exposure paradigms to create a more clinically relevant model; a minimal threshold for developing proliferative disease was determined to be a difference in oxygen supplementation of 20% within a cycle. Retinal avascularity was shown to increase linearly with increasing change in supplemental oxygen (80). (Figure 4).

Because the rat yields a human-like pattern of retinal neovascularization, investigators have often used methods to quantify or stage the pathology which are similar to those used in the clinical setting. This includes measuring the extent of neovascular growth by counting the retinal clock hours containing pathological tufts. This system is much less laborious than alternative methods that involve counting endothelial cell nuclei in transverse retinal sections and it has a clinical corollary, but the precision of the method is not up to general laboratory standards. Still, Zhang *et al* confirmed the validity of the clock hour system in the rat by comparing it to the cell counting method and showing a highly significant positive correlation (81), and clock hour estimation remains in wide use.

The rat model has been used by several investigators to study the molecular basis of ocular angiogenesis and it is widely used in pre-clinical efficacy trials to study the effectiveness of anti-angiogenic agents. Dorey *et al*, in a modified version of the neonatal rat model, showed VEGF mRNA, as measured by *in-situ* hybridization, to be increased in the inner nuclear layer especially around the Mueller cells at a time preceding neovascular growth (82). Robbins et al extended these studies to describe the spatial and temporal distributions of VEGF protein and its receptors in a rat ROP model (83, 84). Shafiee et al used peptides derived from thrombospondin-1, a tumor suppressor glycoprotein, to inhibit angiogenesis in a rat model of ROP. Treated animals had significantly decreased neovascularization (85) and a heparin-binding region of the protein was concluded to be primarily responsible. Anecortave acetate, an angiostatic steroid with little glucocorticoid or mineralocorticoid activity, has been shown to inhibit ROP in the rat model (86). Plasminogen activator inhibitor (PAI-1) mRNA was found to be increased in treated animals indicating a mechanism of action for this class of compounds.

Berkowitz et al were the first group to address a critical question that remained to be unequivocally answered: namely, is retinal hypoxia present at the time and location that neovascularization is initiated? Using a novel magnetic resonance imaging technique to measure retinal oxygenation, the investigators provided compelling evidence of compromised oxygen delivery prior to neovascularization in rats treated in 50%/10% exposures (87). Later, Berkowitz used this system to measure the effects of supplemental oxygen on preretinal neovascularization in the newborn rat model of ROP. Animals were recovered during a 6-day period after the50%/10% exposure in either room air (controls) or 28% oxygen. Animals recovered in 28% oxygen had a decrease in incidence of neovascularization along with a decrease in panretinal oxygenation (88).

The rat is perhaps the best characterized of all retinopathy models. It has served as the testing ground for numerous therapies, including antioxidants, antiangiogenic agents and anti-inflammatory drugs. Rat retinopathy models have served to test numerous drug delivery routes, including intraperitoneal, intramuscular and oral systemic routes, encasement of compounds in liposomes or as chimeric proteins, as well as intraocular and topical local delivery. Substantial pharmacokinetics data have been generated to support these experimental systems in the rat. In addition, the rat model was the first to support real time fluorescein angiogram images to track progression (73, 74, 89), the first to be assessed by computer-assisted image analysis (90) and the first to be used for direct comparisons of vessel staining techniques to optimize these for the understanding of ROP pathogenesis (91).

3.5. Mouse

Within the past decade, the mouse model of OIR has grown in use, and it is now the most common model for studying retinal angiogenesis. Much of our current knowledge of the molecular mechanisms underlying ocular angiogenesis derive from this model. The developing retinal circulation of the mouse is very similar to that of the rat. The vascular maturity of the retinal vessels at birth is equivalent to that of a 25-week fetus (92). The superficial vascular network begins to develop shortly before birth and the deeper network develops at day 9.

In early experiments described by Gyllensten *et al*, 100% oxygen was administered to newborn mice for 1-3 weeks. In 33% of the animals there were hemorrhages into

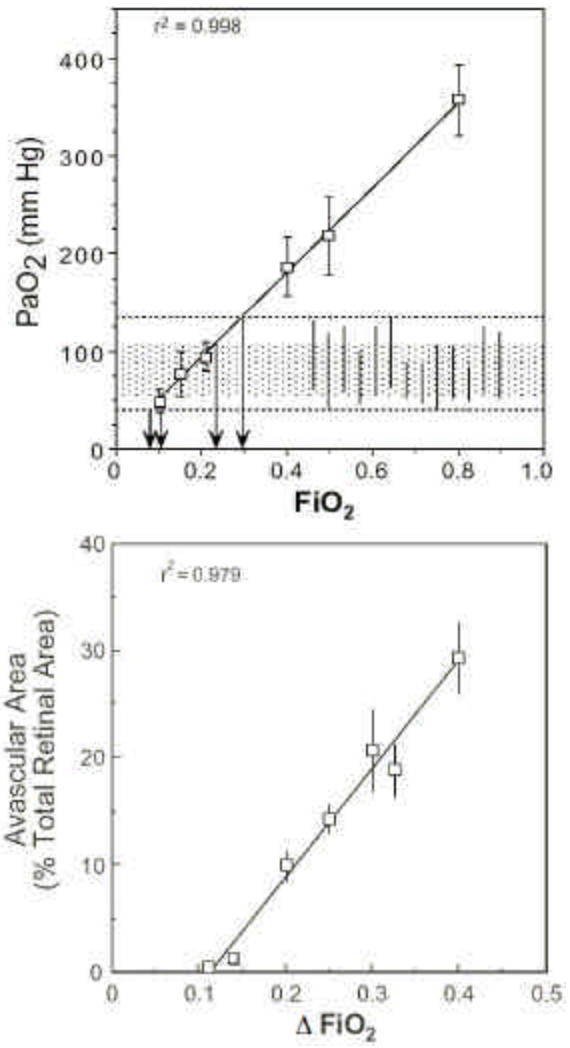

Figure 4. Top panel - The relationship between Fio_2 and Pao_2 was defined in newborn rats exposed to extended periods of various Fio_2. The relationship is linear (r^2 = 0.998). The average range of Pao_2 variation in a group of infants in whom threshold retinopathy of prematurity developed is superimposed upon the plot (doted horizontal lines). The 13 vertical lines on the right side of the graph illustrate the individual Pao_2 ranges of 13 infants in whom "threshold ROP" developed. The x-axis has no bearing on the position of these lines. The Fio_2 at which the limits of the two estimated infant Pao_2 ranges cross the relationship curve (arrows) may represent clinically relevant ranges of variable oxygen exposure for animal experiments. Bottom Panel - The correlation between ?Fio_2 and avascular retinal area immediately after oxygen exposure is direct and linear (r^2 = 0.979). Retinal avascularity on removal to room air is hypothesized to contribute to retinal hypoxia, which leads to subsequent abnormal vasoproliferation. Reproduced with permission from Investigative Ophthalmology and Visual Science.

the vitreous body and anterior chamber as well as retinal folding and fibrovascular proliferation in the vitreous (92).

In subsequent studies the experimental model consisted of exposing newborn mice to 100% oxygen for five days followed by a recovery period in room air. Vasoproliferation of the retinal vessels occurred in these animals. Exposure of animals at 5-10 days of age resulted in a less severe retinopathy. Proliferative lesions in newborn mice were present mainly in the region around the optic disk, while lesions were localized to the peripheral region in older animals. Gerschman *et al* confirmed the above observations (93). Subsequent studies in mice reproduced the observation in the kitten model that gradual withdrawal from oxygen did not decrease the severity of retinopathy (13, 94). Newborn mice exposed to hypoxia alone do not show vasoproliferative changes (95).

In addition to changes in the retinal vasculature, studies in the mouse model have also demonstrated a change in vasculature of the developing lens, iris and hyaloid system. Studies done by Patz *et al* (96) noted a marked proliferation of the hyaloid vessels and neovascularization of the iris in newborn mice exposed to 80% oxygen for varying time periods followed by removal to room air. Later studies by Ashton et al involving exposure of the newborn mice to 100% oxygen for 5 days followed by removal to room air was associated with dense vasoproliferation of the hyaloid system. An abnormal persistence and vasoproliferation of the tunica vasculosa lentis was seen in newborn mice exposed to 70% oxygen for 6 days followed by a return to room air for 8 days (97).

Many of the earlier studies conducted in the mouse model used different experimental designs that produced an inconsistent neovascular response. Smith *et al* further refined the timing and length of exposure of the animals to produce a more consistent, reproducible and quantifiable OIR model. They also described a fluorescein-dextran infusion method to assess the vascular pattern. Mice at day of life 7 (P7) were exposed to 75% oxygen for 5 days and then removed to room air. Mice were euthanized and enucleated at P17 to P21. Fluorescein-labeled dextran infusion was used to delineate the entire vascular pattern as well as neovascular tufts. (Figure 5). Mice treated in this way show a vascular pattern opposite that displayed by infants with vasoproliferative stages of ROP. The posterior capillaries are absent at P21 and the neovascular growth occurs in a star-shaped pattern at the junction of the vascularized and avascular retina (98). In this case, the retinal periphery is vascularized, whereas in infants and other animal models the peripheral retina is avascular. While the late onset of oxygen exposure, the extreme hyperoxemia produced by 75% oxygen breathing, and the unique pattern of neovascularization may limit relevance of this model to ROP, the prevalence of pathology is high and it is robust. This mouse model of OIR does not develop retinal detachment. This is possibly due to the fact that the lens occupies approximately 40% of the volume of the rodent eye, thus making retinal detachment more difficult. The fluorescein-dextran infusion method (98), in contrast to the India-ink perfusion method (43), was shown to be more rapid with lower background fluorescence and the delineation of the entire retinal vasculature was enhanced. The rat and puppy

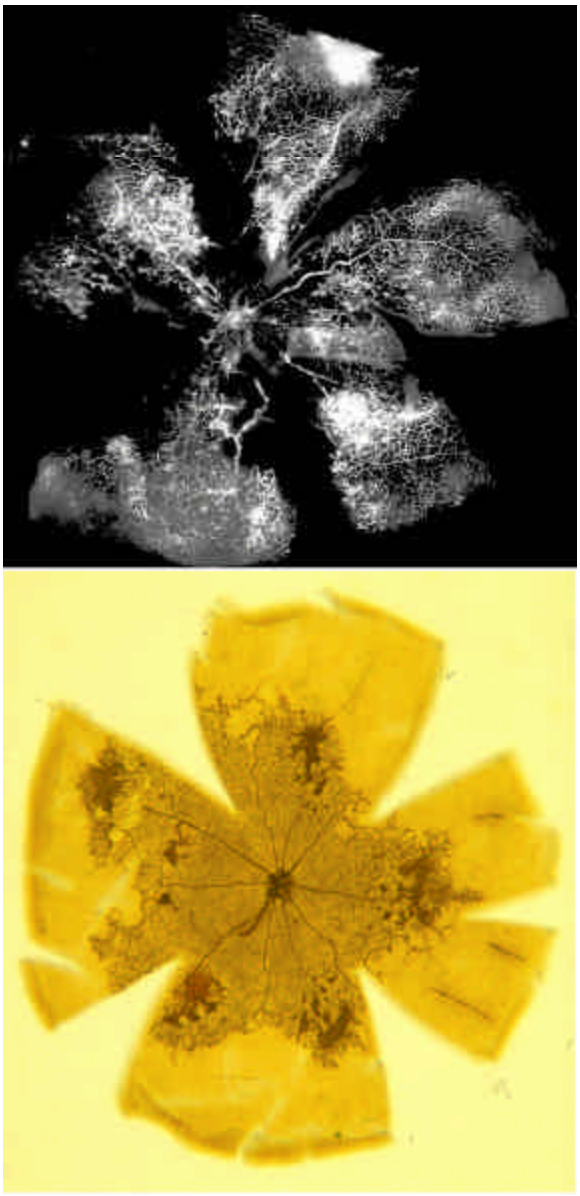

Figure 5. Comparison of patterns between mouse and rat retinal neovascularization. The mouse retina (top panel) shows central neovascularization while peripheral neovascularization is seen in the rat retina (bottom panel). Mouse retinal flat mount courtesy of Dr. Maria B. Grant, University of Florida, Gainesville, FL, U.S.A.

models have relied more heavily upon histochemical stains, such as adenosine diphosphatase (ADPase). This and similar enzymes constitute surface markers of vessels and they can be used to lable mesenchymal precursor cells and non-patent vessels. Thus, ADPase staining is not dependent upon the presence of physiologically patent lumens, which pre-retinal vascular growths often lack. This feature of the pathology limits the usefulness of perfusants or infusants (91).

The mouse model as described by Smith *et al* has been used extensively by others in the study of angiogenesis (98). It is inexpensive, as well as easily reproducible, and the advent of transgenic mouse technology has facilitated molecular investigations and made it a valuable tool for dissecting the details of the angiogenic cascade.

4. MOLECULAR MECHANISMS OF ANGIOGENESIS

Angiogenesis is a complex process that involves disruption of the vascular basement membrane and extracellular matrix by proteolytic enzymes, migration towards angiogenic stimuli, lumen formation, cell division at the base of the sprout and anastomoses of adjacent loops followed by cell proliferation and migration. Formation of the early vascular network is followed by several maturation steps involving attraction of smooth muscle cells and pericytes around the endothelium, as well as vascular remodeling involving changes in lumen diameter and vessel wall thickness to accommodate the needs of the local tissue.

The development of new blood vessels depends on the balance between angiogenic and angiostatic factors.

4.1. Angiogenic factors

Several angiogenic factors such as fibroblast growth factor, transforming growth factors α and β, hepatocyte growth factor, tumor necrosis factor α and interleukin-8 are involved in angiogenesis. However, vascular endothelial growth factor (VEGF) appears to be the major driving force behind ocular blood vessel growth.

4.1.1. Vascular endothelial growth factor

In the 1950's both Michaelson (99) and Ashton (19) proposed that an angiogenic factor was released from the retina. However, it was not until the 1980's that VEGF was identified as the key molecule responsible for angiogenesis (100, 101). VEGF exists in at least 5 different isoforms (102). It acts by binding to tyrosine kinase cell surface receptors Flt-1 and Flk-1; neuropilin-1, a neuronal cell receptor that mediates neuronal guidance, has been also identified as a receptor for VEGF (103). Knockout mice with disruption of even one allele of the VEGF gene have impaired blood vessel formation, and targeted disruption of the Flk-1 gene results in defects in differentiation of endothelial cells (104, 105). VEGF gene expression is hypoxia-inducible (106). This increase in expression is due to an increase in gene transcription as well as mRNA stability. The mechanism of hypoxia inducible expression has been shown to be mediated via hypoxia inducible factor-1alpha (HIF-1alpha), a transcription factor that transactivates several hypoxia inducible genes (107). VEGF levels are increased in the vitreous of patients with diabetic retinopathy and other neovascular disorders of the eye (108, 109), as well as in a primate model of iris neovascularization (110). Increased expression of VEGF mRNA is seen anterior to the developing blood vessels on P7 in the normal retina as well as after 6-12 hours of relative hypoxia in the mouse OIR model. *In situ* hybridization studies have localized the site of production of VEGF during the process of neovascularization to the

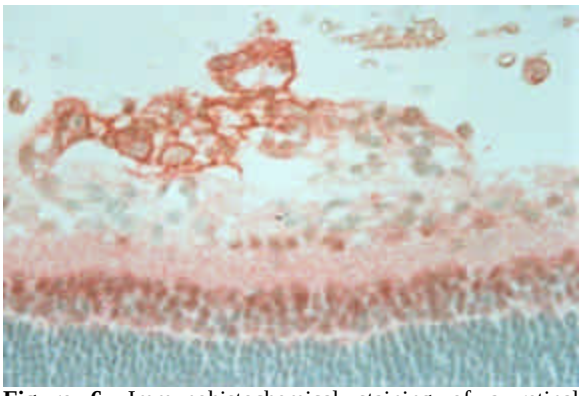

Figure 6. Immunohistochemical staining of a retinal transverse section showing elevation of VEGF protein in the inner nuclear layer of the retina and associated with neovascularization in the rat.

inner nuclear layer of the retina (111). VEGF expression was shown to be down-regulated during the hyperoxic phase, attenuation of the vessels was inhibited by administration of exogenous VEGF (112). Inhibition of VEGF in the mouse model by use of antisense oligonuleotides, receptor binding chimeric proteins and monoclonal antibodies has been shown to decrease neovascularization (113-115). Transgenic mice created by overexpression of VEGF in the photoreceptor layer develop intraretinal and subretinal neovascularization (116). (Figure 6).

4.2. Angiogenesis

Binding of VEGF to the receptor is followed by degradation of the endothelial cell basement membrane by proteases such as metalloproteinases. These degrade collagen and other extracellular matrix components, disrupting the basement membrane barrier, thus enabling endothelial cells to migrate from the vessels and proliferate (117). Vascular cell adhesion molecules such as integrins alphaV-beta3 and alphaV-beta5 help in endothelial cell migration by mediating binding of the endothelial cell to the extracellular matrix (118). Inhibition of either matrix hydrolysis or integrin/ligand interaction can inhibit normal and pathological retinal blood vessel formation (118). Migration and proliferation of cells is followed by formation of tubes with patent lumens. E-selectin, a glycoprotein that mediates endothelial cell-cell contacts, is important for lumen formation (119). The later steps involve remodeling of vessels and recruitment of mesenchymal cells and differentiation to pericytes. Platelet-derived growth factor (PDGF) plays a role in pericyte recruitment (120). PDGF-B deficient mice have a lack of pericytes in their blood vessels and microaneurysms.

TIE receptors, TIE1 and TIE2, are tyrosine kinases expressed by endothelial cells, and their ligands, angiopoietins, play a critical role in embryonic angiogenesis. TIE1 is required for structural integrity of endothelial cells and mice deficient in TIE1 die at birth (121). Mice deficient in TIE2 have defects in the endothelial lining of the heart (121). Angiopoietin-1, a ligand for TIE2, induces tyrosine phosphorylation of TIE2 in endothelial cells and induces sprouting of capillaries and survival of endothelial cells (122). Disruption of the Ang1 gene is lethal to embryos and leads to a lack of periendothelial supporting cells (123). Angiopoietin-2 is a natural antagonist of Ang1 that blocks Ang1 activation of TIE2 and angiogenesis (124), thus promoting endothelial cell differentiation and stasis.

4.3. Inhibitors of angiogenesis

Several inhibitors of tumor angiogenesis have been recently identified (125). Some of these are naturally occurring molecules such as angiostatin, endostatin, platelet factor-4, thrombospondin-1, alpha-interferon, troponin I, and tissue inhibitors of metalloproteinases. In addition, several synthetic inhibitors have been designed with a view to interrupting the mechanism of new vessel formation at various steps along the pathway; by inhibition of VEGF or its signaling, inhibition of matrix proteolysis, or inhibition of integrin/matrix interaction. Many of these agents have shown promise in early, non-clinical trials using the models described herein.

Several genes implicated in retinal angiogenesis have been studied in the mouse model as described by Smith *et al* (98). Although basic fibroblast growth factor (bFGF, FGF-2) was previously implicated in ocular neovascularization, FGF-2-deficient mice developed the same degree of neovascularization as control animals in the OIR model (127). Il-8 levels are increased in the vitreous fluid of patients with proliferative diabetic retinopathy. NF-kappaB and a rat homolog of IL-8 are increased in endothelial cells and glial cells in OIR (128). Administration of an alphaV integrin antagonist peptide reduced retinal neovascularization (129). Retinal neovascularization was shown to be inhibited in transgenic mice expressing a growth hormone antagonist gene and the degree of inhibition was shown to be proportional to serum levels of growth hormone as well as insulin-like growth factor-1 (IGF-I). Inhibition was reversed by administration of exogenous IGF-I, thus suggesting a potential therapeutic option for diseases with intraocular neovascularization (130). Transgenic mice deficient for the endothelial nitric oxide synthase (eNOS) gene have decreased hyperoxia-induced attenuation in comparison with normal controls, thus suggesting a possible therapeutic role for inhibitors of eNOS activity (131). Angiopoietin-2 mRNA expression has been shown to be increased in the inner nuclear layer and ganglion cell layer in the mouse model (132). Angiotensin II has also been shown to play a possible role in hyperoxia/normoxia-induced neovascularization in the mouse OIR model (133). Administration of perindopril, an angiotensin-converting enzyme inhibitor, to hypoxic mice led to a significant decrease in the number of endothelial cells in the retinas of treated versus untreated animals (133). Immunohistochemistry and *in situ* hybridization studies in the mouse model showed HIF-1 alpha protein and VEGF mRNA was increased in the hypoxic inner retina but not in the outer retina 2 hours after removal of the mice from the hyperoxic environment. HIF-1 levels decreased to baseline by 24 hours, while VEGF levels remained elevated for several days (127).

Increased understanding of endothelial cell physiology and tumor angiogenesis has led to the identification of several inhibitors of angiogenesis to block tumor growth and inhibit retinal and choroidal neovascularization, seen in ischemic retinopathies and age related macular degeneration (134, 135). This novel approach, which is currently being tested in several clinical trials, holds promise as an effective therapeutic strategy for these conditions (136, 137).

5. CONCLUSIONS AND PERSPECTIVES

The retina is an excellent organ in which to study angiogenesis, since progress can be followed non-invasively by angiography or fundoscopy. Moreover, after sacrifice of experimental animals, the entire vasculature can be closely scrutinized in flat mount preparations. The retina's ready accessibility has led to its widespread use to improve our fundamental understanding of vascular biology; to study basic growth and development of blood vessels, their exquisite sensitivity to oxygen tension, and the cellular and molecular basis of unregulated growth, all of which are then applied to both ocular and non-ocular contexts. More specifically, the majority of our current understanding of ROP pathogenesis, the role of oxygen as well as the molecular mechanisms underlying vasculogenesis and angiogenesis, has been obtained from these animal models. However, none of the models is perfect, as the animals are not premature and they are healthy in comparison to the premature infant with severe lung disease. The kitten model develops intraretinal and intravitreal neovascularization, but the pattern of this pathology is not human-like. The mouse model suffers from the same drawback, but it is currently the most popular choice because of the advent of transgenic technology. The rat model has been characterized extensively and is the one that most closely mimics the human disease, but rat genotypes are difficult to manipulate. Most of the models have combined extreme oxygen exposures and intrinsically healthy animals, yielding systemic (and presumably retinal) oxygen tensions that are not relevant to the clinical setting. Some models have addressed this by providing variable oxygen exposures within ranges that produce more clinically representative arterial partial pressures. Even these attempts are limited by the temporal aspects of the oxygen variation, which are much less random than those associated with variable respiratory function in premature infants. Our models must be continually refined, so that as new molecular techniques are applied to them, the results will be pertinent to the human pathology in the ever-changing NICU setting. For example, the use of cDNA microarray technology, investigating retinal tissue from animal models of retinopathy will likely yield new information about the various genes involved in the angiogenesis pathway. However, the experimental conditions must be such that the gene expression is influenced by relevant conditions. Using this and similar technologies, future studies will likely focus on the development of antiangiogenic drugs that work selectively on very specific aspects of abnormal retinal vessel growth that are not involved in normal blood vessel growth. This is critical to the treatment of ROP, where both processes occur simultaneously and within close proximity.

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