Quantification of Oxygen-Induced Retinopathy in the Mouse

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Purpose. To describe a quantifiable model of vascular proliferation in oxygen-induced retinopathy (OIR) in the mouse.

Methods. Neonate Quackenbush mice were subjected to high-ambient oxygen (~95%) for the first 5 days after birth, effecting a total inhibition of retinal vascular growth. Animals then were returned to room air, and the rates of subsequent vascular development in the plane of the retina and estimates of retinal capillary density were measured from flatmounts of ink-perfused eyes. Observations were confirmed with fluorescein isothiocyanate–lectin labeling of the peripheral vasculature. Abnormal growth of vascular sprouts into the vitreous was recorded from cross-sections. Observations in OIR were compared against those of age-matched control animals.

Results. The slower rate of retinal revascularization in OIR mice was quantified and compared against the normal rate. Lectin-binding studies confirmed the reliability of ink preparations. The number of vitreous sprouts in OIR peaked 8 to 10 days after animals were returned to room air (13 to 15 postnatal days). Sprouts then regressed, to disappear by postnatal day 20. In all respects, bar a slightly lower peripheral capillary density, the normal retinal vascular pattern was achieved in OIR within 15 days of exposure to room air (as opposed to the 10 days required in control mice).

Conclusions. The protocol described for quantifying retinal proliferation in mouse OIR is reproduced readily, and the data recorded here will allow the effectiveness of subsequent treatments that may affect retinal vascular growth to be evaluated better. Invest Ophthalmol Vis Sci. 1997;38:1168-1174.

Oxygen-induced retinopathy (OIR) has been studied in experimental animals for more than 40 years, primarily to gain better understanding of the pathogenesis of human retinopathy of prematurity (ROP). Retinal vascular proliferation is the defining event in acute ROP, and several protocols exist to induce the proliferation in experimental models. However, there has been relatively little description of OIR in quantitative terms. Semiquantitative approaches have been described in the kitten, in which the extent of the retinal proliferation, as indicated by vascular spread in retinal flatmounts, was mapped or scored by eye. These approaches, also applied to beagle pups, illustrate differential vascular spread, but often have lacked "quantitative rigor."

The density of retinal vessels in OIR and the area of vascularized retina, as opposed to retinal spread (the distance from the optic disc to the most peripheral retinal vessels), are further measures of proliferation. Computerized digital image analysis of retinal vascular density and area, and other graphical representations, have been published on rat OIR. Attempts to follow changes over time with fluorescein angiography, although a potentially powerful approach, have not proved as successful. Although retinal vascularity is important, progress to retinal detachment in ROP is associated with development of vascular sprouts into the vitreous, which is otherwise avascular in the mature eye. Numeric estimates of vitreous sprout (or tuft) formation in histologic cross-sections of mouse OIR, as indicated by the presence of vascular nuclei, have been published recently.

We describe further measures of retinal neovascu-
lar growth in the mouse model against which pharmacologic treatments or other interventions can be better compared. The mouse model, which is reproduced easily, was chosen because of low cost, short gestation, and larger litter sizes.

MATERIALS AND METHODS

Induction of Retinopathy

Outbred Quackenbush mice were used, obtained from the Central Animal Breeding House (University of Queensland). Ethics clearance for the project was obtained (NHS/0/1/94), under NH&MRC guidelines on Animal Experimentation (these guidelines conform with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research). Experimental litters of newborn mice, not more than 12 hours old, were placed into an oxygen incubator (a clear perspex chamber, 25 × 15 × 20 cm high) with their mothers. Humidified medical grade oxygen (100%) was piped into the chamber at a flow rate of 0.5 to 0.75 l/min; this ensured exposure to an oxygen concentration of 90% to 95%, as monitored by a Tele-dine O₂ analyzer (TED 100; Fisher & Paykel, Queensland, Australia). This comparatively high concentration of oxygen is necessary to achieve vasoproliferation in the mouse model of OIR.²

The neonate pups were left in the chamber for 5 days and then returned to room air. The chambers were opened to room air for a maximum of 6 to 8 minutes per day as a result of maternal rotation and a once-daily cleaning regimen. Surrogate mothers were rotated with natural mothers every 12 hours to prevent maternal pulmonary oxygen toxicity. Mothers subjected to this treatment showed few obvious short- or long-term ill effects and generally lived to produce many experimental litters. Control litters were maintained in room air, with the same natural—surrogate mother cycle. The animals were kept at 22 ± 1.5°C ambient temperature and exposed to a 12-hour light-dark cycle. Generic rodent pellets and water were available ad libitum to the mothers, whether in or out of the chamber. A column of soda lime was placed in the chamber to prevent accumulation of carbon dioxide. Litters were gained from mothers that arrived pregnant and from matings in our laboratory (care was taken to ensure that litter parentage varied).

Sampling Regimen

Pups were killed on days 0 (birth), 3, 6, 10, 15, and 20 for measurements of retinal vascular growth. For experimental animals, day 6 indicates sacrifice within 1 day of return from oxygen to room air. Before enucleation of the eyes, deep anesthesia was achieved with diethyl ether inhalation, to a point at which breathing ceased. Under these conditions, heart contractions usually were maintained. Body weight was recorded. After a thoracotomy, the right atrium was cut to ensure outflow. Blood washout was achieved by left ventricular puncture with a 25-gauge × 19-mm Vein Infusion Set (Baxter, New South Wales, Australia), using a hand-held syringe. The washout medium contained heparin (CSL, Queensland, Australia), a vascular relaxant (papaverine hydrochloride; Aldrich Chemical, Milwaukee, WI), and a colloid (polyvinyl pyrrolidone; Sigma, St. Louis, MO) in 0.9% saline.¹⁴ Pressure on the aorta distal to the diaphragm ensured perfusion was limited to the upper body.

Once outflow was clear of blood, animals were perfused with 1 to 2 ml of 80% India ink (Rotring, Hamburg, Germany) in saline perfusant. Successful perfusion was indicated by marked blackening of the eyes, snout, and ears. Eyes were enucleated and placed in 10% phosphate-buffered formaldehyde solution for a minimum of 15 minutes, before production of flatmounts. The tissue was not dehydrated or cleared. For analysis of vitreous sprouts, whole fixed eyes were embedded in paraffin and sectioned anteriorly and posteriorly, through or very close to, the optic disc. Sections were stained with hematoxylin and eosin.

Retinal Flatmounts

Neonate eyes in day 0 and day 20 are approximately 1.5 and 3 mm in diameter, respectively, and dissection was performed with the mice under stereo microscopy. A midline incision into the cornea was extended to the limbus and then circumferentially. The lens was removed, and the choroid and sclera peeled away, and the remaining hyaloid vessels removed. Retinal flatmounts were obtained after three or four radial cuts into the cup of remaining retinal tissue, mounted, and photographed immediately at ×40 (Zeiss [Oberkochen, Germany] Axioskop) using Kodak film (Tech Pan 100; Eastman Kodak, Rochester, NY). Measurements were taken from developed photomicrographs and, if necessary, photographic montages were produced. One hundred forty-four flatmounts were analyzed, 24 per sample day, obtained from equal numbers of control and experimental animals.

The extent of maximum retinal vascular spread (Fig. 1) was taken from a straight line through the optic disc that linked the most distal portions of the peripheral vasculature. Estimates of capillary density were obtained by counting capillary intersections with a series of marked squares on a transparent overlay (Fig. 2). Capillary densities in central, mid, and peripheral regions of the vasculature were obtained from a total of eight equidistant axes per flatmount, using the line of maximum retinal spread as the first of the axes. Sampling boxes bisected by a major artery or vein were discounted and counts taken from regions...
FIGURE 1. Retinal flatmounts from neonatal mice. Photomicrographs of ink-perfused retinal vasculature. (A) Day 10, normal development and (B) day 10, oxygen-treated at the same magnification. The optic disc (arrow) and retinal periphery (asterisk) are indicated. Bar = 0.25 mm.

Neovascular Responses at the Inner-Limiting Membrane

Abnormal, vascular accumulations confined to the inner-limiting membrane were distinguished from those that had penetrated into the vitreous (termed vitreous sprouts). Remnants of the hyaloid vessels cannot always be separated readily from vitreous new vessels, and as a result, only those vascular elements in the vitreous that were observed to have definite anatomic continuity with retinal vessels were counted as vitreous sprouts. Qualitative observations of the microvasculature, including the deep retinal vessels, also were recorded.

RESULTS

Retinal Vascular Spread

The obvious difference in vascular development between OIR mice and control animals is illustrated in the composite photomicrograph (Fig. 1); maximum vascular spread in the retina is plotted against days of development (Fig. 4A). Exposure to high oxygen prevented retinal vascular development. On return of OIR animals to room air, the rate of retinal vascular growth approached that for control animals, and maximum retinal spread was achieved within 15 days (approximately 5 days longer than observed in control animals). However, the rates of vascular growth, although linear in each case, are significantly different, with slower growth in oxygen-treated animals. The

FIGURE 2. Retinal ink-perfused flatmount from neonatal mice. Estimations of capillary density. Day 10, normal development. Numbers of microvessel intersections with the squares illustrated provided numeric data on capillary density graphed in Figure 6. Retinal periphery is indicated (asterisk). Bar = 0.25 mm.
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rate of normal vascular growth (Fig. 4) is consistent with previous accounts.2,16

The possibility exists that the delay in vascular development reflects a lack of growth generally and has little to do with oxygen exposure per se. However, as indicated (Fig. 5), body weight in OIR was never greater than 20% under control animals, and growth is delayed maximally by 2 to 3 days. The marked difference in retinal vascular growth between OIR and control animals remains significantly different if the OIR curve in Figure 4 is shifted to the left by 2 to 3 days (i.e., if commensurate account is made for the slower growth in oxygen-treated animals).

In terms of technical problems, obtaining flatmounts from animals from days 0 to 6 required some practice because of the small eye size. However, as shown, good, reproducible results are obtainable using stereo microscopy.

Capillary Densities in Retinal Flatmounts

Observations are graphed (Fig. 6). In OIR, central zone capillary density increases with time and in the pattern observed in control animals, but is delayed by the 5 days of oxygen exposure. In the midzone, capillary density in OIR is reduced markedly by day 6, but recovers to normal levels by day 20. Again, in the peripheral zone, capillary density is lower in OIR at day 6 and increases with time. However, the rate is slow and normal densities still are not achieved by day 20.

Fluorescein Isothiocyanate-Lectin Observation of Peripheral Capillaries

The lectin labeling (Fig. 3), as expected, indicated the presence of small patent, and nonpatent, vascular pathways that were not delineated by India Ink. In qualitative terms, the complete peripheral vasculature

![Figure 3. Double-labeled retinal flatmount. Ink (A) and fluorescein isothiocyanate-lectin (B). Day 10, control animal. The limit of the peripheral vasculature is indicated (P). Arrows indicate vascular elements delineated by fluorescein isothiocyanate-lectin that are not obvious in the ink preparation. Bar = 25 μm.](image)

![Figure 4. Diameter of maximum vascularized retina (mm) versus days of development in the mouse. (A) Control (normal development) versus oxygen-treated. Mean values (±1 SD) between days >0 and <20 are significantly different (P < 0.001) between the two groups. (B) As in A, but with estimated gradients for linear periods of growth. The regression curves are accompanied by 95% confidence limits, and the rates of growth are significantly different (P < 0.01) between the two groups. Vc = control, Vo = oxygen-treated, D = days.](image)
DAYS OF DEVELOPMENT

**FIGURE 5.** Body weight (g) versus days of development in control (normal development) and oxygen-treated mice. Mean differences (±1 standard deviation) between day 6 and day 10 animals are significantly different ($P < 0.001$).

was better detailed by the lectin label. However, maximum vascular spread, as indicated with the use of ink, was not underestimated to any significant degree (Fig. 3) (i.e., the most peripheral vessels invariably contained ink, although sections of the proximal network did not). The microvascular network at the growing edge in OIR is primitive, with vessels of widely varying diameters and numerous interconnections, as reported previously.  

**Cross-Sectional Analysis of Vascular Abnormalities**

Vitreous sprouts, as seen in cross-section, were not common until day 10 in OIR. Between day 10 and day 17, sprouts, as identified in histologic cross-section, were found in 30% to 65% of eyes, with never more than two sprouts per section (the sections were 3 to 4 μm thick). Six nonserial sections were analyzed per eye. The sprouts were confined to the midregion of the vascularized retina, and their development was preceded by obvious neovascular activity just deep to the inner-limiting membrane. A total of 36 eyes (OIR) were sampled and 190 sections analyzed. By day 20, little evidence of sprouts could be found; that is, abnormal vascular growth in OIR animals obviously is established before day 10 and peaks between days 13 and 17, but essentially normalizes, as far as can be determined with light microscopy, once the normal mature vascular spread is achieved by day 20 (Fig. 4). Vitreous sprouts were never observed in control eyes.

Potential problems with incorrect identification of hyaloid remnants as vitreous sprouts already have been alluded to; any vitreous vessels adjacent to the inner-limiting membrane, but without obvious connections to retinal vessels, consequently were ignored in the count. This may have caused the number of vitreous spouts to be underestimated. The situation is

**FIGURE 6.** Capillary density in central, mid, and peripheral zones of the vascular retina, normalized to day 20 control animals. Control (normal development) versus oxygen-treated mice. Significant differences ($P < 0.05$) in mean estimates are indicated (asterisk). Bars = ±1 SEM.
further complicated should remnants of hyaloid vessels maintain connections with peripheral retinal vessels, as reported. Nevertheless, the data clearly record differences between OIR and normal mice, facilitating quantitative comparisons between the two.

**Qualitative Observations**

Vitreous hemorrhages increased as the number of sprouts reached a maximum, in accordance with previous observations. Pathologically leaky vitreous vessels are implicated strongly in ROP. However, confirmation that the hemorrhages reported in our current article are not artifacts of saline perfusion would require analysis of nonperfused tissue.

Development of the deep retinal vascular network (which arises from down-growth of the superficial layer) is delayed in OIR but becomes apparent within approximately 8 days of return to room air. By day 20, no obvious differences remain between OIR and control animals. The morphology of the deep layer was not investigated further, and all quantification of retinal vessels in flatmounts is of the superficial layer. The major radial arteries and veins (as seen in flatmounts) also are affected by oxygen exposure, are comparatively dilated, and exhibit abnormal branching patterns (the latter observations, however, were not a consistent feature of OIR).

**DISCUSSION**

**Experimental Considerations**

Obtaining retinal flatmounts from neonatal animals requires practice because of the small eye size, particularly in animals younger than 1 week old. However, as shown here and in the early literature, reproducible results are not difficult to obtain. Moreover, most continued interest with the model is likely to involve day 10+ animals in which neovascularization is well established and in which the eyes are approximately twice the size they are at birth and, thus, are processed more easily. The measure of retinal spread is easily obtained manually but could be readily quantified with basic image-analysis software. Accurate measures of vessel density within the vascularized retina are not as easily obtained with image analysis for a number of reasons. Superficial and deep retinal beds exist in close proximity to each other and this is a prominent difficulty here. Regarding the experimental regimen in more general terms and presuming that Smith et al. are correct in their contention that mice that are left out of oxygen until day 7 provide a better model of ROP, it may prove useful to change the experimental regimen we used accordingly. The regimen of Smith et al. reduces the occurrence of hyaloid vessels, which largely regress by day 7 under normal conditions.

Use of India Ink to denote the spread of the retinal vasculature, although reliant on proper perfusion and vessel patency (neither of which can be ensured in all vessels), is adequate for the OIR model described, as the use of the lectin label in the current study clearly indicated. Moreover, ink flatmounts are comparatively cheap to produce, and fading problems, which occur with use of the fluorescent labels, are avoided. Capillary density will tend to be underestimated in ink preparations, as the use of lectin indicated. However, lectin labels endothelial basement membranes and therefore highlights vascular elements regardless of vessel patency. ADPase histochemistry has been used successfully to indicate retinal vessels in flatmounts and is another method that could be used in the mouse model.

**Retinal Neovascularization**

The observation that retinal vascular growth in neonatal mice can be inhibited totally by exposure to high oxygen partial pressure (reviewed in other literature) is verified. Unlike previous accounts, however, the subsequent regrowth of retinal vessels in mouse OIR has now been measured accurately. Normal rates of retinal vessel growth (as determined by the spread of the superficial layer) are approximated as soon as oxygen-treated animals are returned to room air. This ensures that the mature vascular spread is achieved in the OIR within 15 days of oxygen (day 20 postpartum). Although there is a contrary view, the procedure we describe for inducing OIR produces reliable and consistent results, as Figure 4A indicates.

Abnormal retinal neovascularization in OIR is indicated primarily by the presence of vitreous spouts and the dilated, immature vessels at the growing edge of the vascular retina. Generation of the sprouts peaks within 8 to 10 days after oxygen treatment has ceased. These sprouts then regress and disappear by day 20. Capillary density within the superficial retinal layer is reduced initially in OIR, but vessels grow to approach normal density by day 20 in all but the most peripheral vasculature. Similarly, development of the deep retinal capillary layer is delayed initially but becomes established by day 20. The large radial vessels in OIR also appear normal by day 20. That is, all of the evidence indicates that a normal retinal vasculature is largely achieved in OIR mice by day 20 (i.e., within 15 days of the cessation of oxygen treatment). Confirmation that the mature capillary bed in OIR functions as normal requires investigation of microvessel permeability.

**Applications of the Oxygen-Induced Retinopathy Mouse Model**

Regarding animal models of ROP, much speculative attention and, to a lesser extent, empirical investigation have been focused on the mechanisms that drive
the proliferation of retinal and vitreous vessels (which originate from the retinal bed). Retinal neovascularization in the mouse model of OIR has now been measured accurately. The current study augments the study of Smith et al.\textsuperscript{5} in which quantification was limited to cross-sectional analysis of neovascular responses. As a result, effects that angiogenic inhibitors-modulators may have can now be better measured in quantitative terms.

Although the model has general application to the study of angiogenesis and inhibition of vessel growth, it remains directly important for our understanding of ROP. In ROP, it is not the complete cessation of retinal neovascularization that is a primary therapeutic aim, but a reduction in the growth of the pathologically leaky new vessels, the most harmful of which occurs within the vitreous. Leakage from these vitreous neovascular sprouts is implicated strongly in the fibrovascular response that, in turn, is linked causally to the traction, and subsequent detachment, of the retina. Any treatment in OIR that could specifically ensure normal rates of growth of the superficial and deep retinal layers while reducing or preventing vitreous sprout formation may well have important consequences for the treatment of ROP.

**Key Words**

mouse, neovascularization, oxygen-induced retinopathy, quantitative model, retinal vasculature

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**References**