Oxygen-Induced Retinopathy in the Rat: Relationship of Retinal Nonperfusion to Subsequent Neovascularization

John S. Penn, Barbara L. Tolman, and Mark M. Henry

Purpose. To confirm a relationship between oxygen-induced retinal vasoattenuation and subsequent abnormal neovascularization in the newborn rat.

Methods. Beginning at birth, some litters of Sprague-Dawley rats were exposed to 80% constant oxygen while others received oxygen varying between 40% and 80% in a cyclic fashion. The frequency of the change in inspired oxygen (FiO₂) was either 6, 12, 24, or 48 hours. The exposure periods lasted for 14 days, at which time some rats from each exposure group were sacrificed and assessed for retinal vasoattenuation with injection of fluorescein-labeled dextran. The remaining rats from each group were transferred at day 14 from the hyperoxic atmosphere to room air for an additional 4 days. These animals were then killed and assessed for retinal neovascularization by staining for vascular ADPase activity.

Results. Of all rats raised in variable oxygen, 62% exhibited abnormal retinal neovascularization after 4 days in room air. Only 18% of the rats exposed to constant oxygen responded with abnormal neovascularization. Among the four groups of variable oxygen-exposed rats, there was a direct correlation (R² = 0.96) between degree of retinal avascularity upon removal from oxygen and the propensity for subsequent abnormal neovascularization. Constant oxygen-exposed rats did not exhibit this relationship. This exposure produced the greatest retinal avascularity upon removal from oxygen but the lowest incidence of abnormal neovascularization after 4 days in room air.

Conclusions. Retinal avascularity may not be the single overriding stimulus for neovascularization in oxygen-induced retinopathy. Other hypotheses merit consideration, including the possibility that variable oxygen leads directly to vascular endothelial cell mitosis, a common retinal manifestation of ischemia—reperfusion. Invest Ophthalmol Vis Sci. 1994; 35:3429-3435.

Retinopathy of prematurity (ROP) is a condition of growing concern in the United States. The increased incidence of the disease is closely correlated to the increased survival of very low birthweight infants. It can be inferred from past estimates that approximately 3,500 neonates suffered lasting visual deficit because of ROP in 1993. Retinopathy of prematurity is one of a group of retinopathies whose pathologic course incorporates abnormal growth of retinal blood vessels. These diseases, collectively called proliferative retinopathies, include sickle cell retinopathy, diabetic retinopathy, branch vein occlusion retinopathy, and retinopathy of prematurity. They all have in common a degree of retinal nonperfusion prior to the abnormal vascular growth. This has led to the suggestion that ischemia-induced retinal hypoxia, an assumed consequence of nonperfusion, plays a causal role. However, there is no proven correlation between the degree of nonperfusion and the propensity or severity of abnormal proliferation in these pathologies, and therefore, a causal relationship remains hypothetical.

Examination of the pathophysiology of ROP reveals a two-staged process: first, a period of retinal vasoattenuation that occurs during the infant's oxygen therapy, then an abnormal proliferation of retinal vessels that is promoted by removal to room air. It is hypothesized that this proliferation is the result of retinal hypoxia—the expected consequence of re-
duced diffusion of oxygen from the choroid in room air, combined with a compromised complement of retinal vessels.

We have developed two protocols for oxygen-induced retinopathy in the newborn rat. One protocol relies on an exposure to a constant level of oxygen. This exposure consistently produces the first stage of vasoattenuation, but seldom the subsequent abnormal proliferation upon removal of the rats to room air. The second protocol uses a systematically varied oxygen exposure that consistently produces both vasoattenuation and severe vasoproliferation. These two protocols offer a well-controlled means of studying the relationship between the degree of retinal nonperfusion upon removal to room air and the propensity for subsequent neovascularization. We hypothesized that rats raised in constant oxygen (infrequent, mild neovascularization) will exhibit less vasoattenuation immediately after exposure than will rats raised in variable oxygen (consistent, severe neovascularization). Further, it was our assumption that by increasing the frequency of the changes in oxygen level during the variable oxygen exposure, we would increase the inherent insult and, similarly, the pathology. Our intent was to establish a relationship between vasoattenuation and subsequent neovascularization in groups of animals that suffered varying incidences or severities of retinopathy.

MATERIALS AND METHODS

Immediately after birth, litters of Sprague-Dawley rats were placed with their mothers in one of several hyperoxic environments. Some litters were initially exposed to 40% oxygen for 6, 12, 24, or 48 hours, after which the oxygen level was rapidly increased to 80%, where it remained for the same number of hours. Variation in oxygen level between 40% and 80% was continued in this stepwise fashion during the first 14 days of the rats' lives. This yielded four separate variable oxygen paradigms, each consisting of a different cycle period (Fig. 1). Other litters were maintained in constant 80% oxygen for the same duration. Rats were either killed on day 14 and assessed for retinal capillary nonperfusion or were removed to room air for an additional 4 days before being killed for assessment of abnormal neovascularization. All investigations conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Areas of capillary nonperfusion were measured by an adaptation of the method of D'Amato and Smith. Briefly, rats were deeply anesthetized with 40 mg/kg sodium pentobarbital and 50 µl of phosphate-buffered saline containing 12.5 mg of fluorescein isothiocyanate-dextran (145,000 average molecular weight, Sigma, St. Louis, MO) was injected into the left ventricle. The rats were killed and enucleated 80 seconds after injection, and the retinas were dissected fresh and flat mounted on microscope slides for examination at ×20 magnification with an inverted fluorescence microscope (Olympus IMT-2, Olympus Optical, Tokyo, Japan).

Apparent avascular areas in the central retina (overlying the vessel origin) and in the peripheral retina (adjacent to the ora) were measured with the aid of an image analysis apparatus described elsewhere. Both the degree of retinal avascularity and the capillary density in vascular areas were measured in all experimental groups. The latter measurements were made in digitized binary images of randomly selected regions of the retinal midperiphery measuring 1 mm². Three regions from each retina were analyzed for capillary density.

Abnormal neovascularization was assessed in flat-mounted retinas that had been stained for ADPase activity by a previously described adaptation of a method developed by Flower and coworkers. Briefly, retinas were incubated in lead nitrate, magnesium chloride, and adenosine diphosphate, followed by washing and staining with ammonium sulfide. ADPase activity is associated with lead sulfide precipitate, which is limited to vascular endothelial cells and their precursors. After staining, retinas were flat mounted...
TABLE I. Effect of Variable and Constant Oxygen Exposures on Retinal Avascularity and Neovascularization

<table>
<thead>
<tr>
<th>Oxygen Exposure</th>
<th>40%/80%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
<td>6-Hour Cycle</td>
<td>12-Hour Cycle</td>
</tr>
<tr>
<td>Avascular area* (% total area)</td>
<td>6.7% ± 3.0 (6)</td>
<td>21.2% ± 5.3 (7)</td>
</tr>
<tr>
<td>Incidence of neovascularization†</td>
<td>34.6% (8/26)</td>
<td>71.4% (15/21)</td>
</tr>
<tr>
<td>Severity of neovascularization‡ (clock hours/retina)</td>
<td>3.7 ± 4.6</td>
<td>4.7 ± 3.1</td>
</tr>
</tbody>
</table>

* Assessed immediately after 14 days in oxygen exposure.
† Assessed after 4 days postexposure in room air.
‡ Average number of clock hours of neovascularization was measured only in retinas showing some degree of disease.
Significantly different from 12-hour cycle; †P < 0.002, ‡P < 0.05.
Significantly different from 12- and 24-hour cycles; †P < 0.002, ‡P < 0.05.

and assessed for the presence or absence of preretinal vascular proliferation at high magnification. Trained observers were able to determine the presence of vascular tissue that had penetrated the inner limiting membrane by using the microscope’s plane of focus. In retinas displaying any degree of disease, severity was measured by superimposing a clock face on the retinal surface and counting the number of clock hours in which abnormal vessel growth was found. Areas between the separated quadrants of the flattened retinas were omitted from the assessment. A few select retinas were removed from slides after assessment and were processed and embedded for histologic sectioning.

Differences in the degree of retinal avascularity (in % total retinal area) were submitted to an arcsine transformation and analysis of variance. Differences in incidence of neovascularization (in % of unilateral or bilateral occurrence in the sample) were analyzed by the chi-squared test. Sample sizes for all treatment groups and assessments are found in Table 1.

RESULTS

Vasoattenuation

Figure 2 illustrates the difference in the extent of oxygen-induced vasoattenuation in two groups of rats exposed to variable oxygen and in rats exposed to constant oxygen. Panel A displays the fluorescein-perfused retinal vessels of a room air-raised rat at day 14. The retina is fully vascularized, with major vessels and capillary arbor extending to the ora serrata. Panel B shows the fluorescein-perfused retinal vessels of a 14-day-old rat exposed to variable oxygen on a 6-hour cycle. The retinal area without vessels was 6.7% of the total area. Panel C shows the retina of a 14-day-old rat exposed to variable oxygen on a 48-hour cycle. The avascular area was 26.1% of the total in this group of animals. The two intermediate cycle frequencies resulted in degrees of vasoattenuation that fell between the two extreme cycles, although values from the 24- and 48-hour cycles were not significantly different.

The observed pattern of nonperfusion in rats exposed to constant oxygen was opposite from that expected (Fig. 2D). This group was known to produce little subsequent retinal neovascularization, but it displayed more vasoattenuation upon removal to room air than any group of rats raised in variable oxygen. This overall difference in retinal vascularity between constant and variable oxygen-exposed rats was the combined result of three characteristics. First, rats from the constant exposure had a larger avascular area in the central retina than even the most affected variable oxygen group (5.51 ± 1.59 mm² for the constant exposure versus 3.84 ± 1.96 mm² for the 48-hour cycle; P < 0.03). This corresponds to 15.1% and 10.4% of the total retinal areas, respectively. Second, rats from the constant exposure had a larger avascular area in the peripheral retina (10.52 ± 3.27 mm² versus 5.74 ± 2.65 mm² for the 48-hour cycle; P < 0.001). This corresponds to 28.8% and 15.7% of the total retinal area, respectively. Third, the constant oxygen-exposed rats had less capillary density where there were capillaries in the midperiphery (0.27 mm² of vessel/mm² of retina versus 0.41 for the 48-hour cycle; P < 0.005).

Vasoproliferation

Table 1 displays the incidence of abnormal neovascularization in the groups of oxygen-exposed rats. This
FIGURE 2. Differences in the extent of oxygen-induced vasoattenuation are illustrated in flat-mounted retinas of 14-day-old rats from four separate treatment groups. Retinal vessels are filled with fluorescein-labeled dextran. (A) Room air-raised; (B) near-average result of 6-hour variable oxygen cycle; (C) near-average result of 48-hour variable oxygen cycle; (D) constant oxygen.

FIGURE 3. Differences in the degree of abnormal neovascularization (white arrows) are illustrated with representative ADPase stained retinas from four treatment groups. Animals were sacrificed after a 4-day postexposure period in room air. (A) Room air-raised (black arrow, remnant of the hyaloid vasculature); (B) near-average result of 6-hour variable oxygen cycle; (C) near-average result of 48-hour variable oxygen cycle; (D) constant oxygen.

Assessment was made 4 days after removal to room air. In addition to areas of persistent capillary nonperfusion and mild vessel tortuosity, preretinal vessel growth was found in 62% of all variable oxygen rats examined. Significant differences were found in the incidences of neovascularization between groups with the exception of the 12- and 24-hour cycles (chi-squared G statistic for all variable exposure groups = 15.5, $P < 0.002$). Figure 3 shows representative retinas from 18-day-old rats raised in several of the treatment conditions. Panel A exhibits the retina of an animal raised in room air. Panels B and C illustrate the condition resulting from the 6-hour and 48-hour cycles plus 4 days in room air. After postexposure room air maintenance, some rats raised in constant oxygen had persistent areas of peripheral capillary nonperfusion (Fig. 3D, 9 o'clock), infrequent mild vessel tortuosity, and occasional regions of abnormal capillary architecture (not shown), but in only 18% of these rats was there any tissue observed penetrating the inner limiting membrane.

The severity of abnormal neovascularization was estimated only in those retinas that displayed some degree of preretinal growth. The average numbers of clock hours containing abnormal vessel growth in these rats are listed in Table 1. The most severe cases are represented by Figure 4. At 4 days after exposure, retinas of variable oxygen-exposed rats often developed vascular ridges immediately posterior to the advancing front of vessel formation. These ridges included large caliber shunts (Figs. 4A, 4B, arrows). Peripheral to the shunt, the retina was often thickened as evidenced by the increased distance between the ganglion cell layer and the inner limiting membrane. This region included a dense accumulation of cell nuclei and a collection of small caliber lumenized capillaries. There was also mild dysplasia in these retinas as evidenced by the outer nuclear layer architecture. This was a frequent result of oxygen exposure during retinal development. In many respects, the abnormal vessel growth appeared to be similar to that in pathologic samples from infants with stage III ROP.

The relationship between vasoattenuation immediately after removal from oxygen and subsequent vasoproliferation in room air was plotted and fitted with a linear regression curve (Fig. 5). The correlation coefficient for this relationship in the variable oxygen groups was 0.96. The slope describing these data is significantly different from zero ($P < 0.005$). The constant oxygen group was not considered in the regression analysis, but its position on the graph is denoted.

The only group of rats in which any degree of abnormal proliferation of vessels was observed immediately upon removal from oxygen was the 48-hour cycle group. In cross-sections, this mildly abnormal...
FIGURE 4. A severe form of neovascularization is demonstrated in two views of an area of the midperipheral retina of a rat exposed to the 24-hour variable oxygen cycle. At the border between vascular and avascular retina, ADPase staining reveals a ridge of retinal and preretinal neovascular growth. (Top) Preretal vessels appear out of focus in the plane of superficial retinal vessels in the top panel. There is a large caliber shunt (white arrow in top panel, black arrow in bottom panel) underlying the preretinal vessels. (Bottom) A transverse section shows the retina thickened just peripheral to the shunt where a clump of unidentified cells has accumulated within the inner retina. The inner limiting membrane is intact in this location. C = More central; P = more peripheral.

growth was evidenced by clumps of cell nuclei in the plane of the superficial vessels (Fig. 6). These clumps included cells with spindle-shaped nuclei and others with the morphologic characteristics of endothelial cells.

DISCUSSION

It has long been hypothesized that retinal vasof ormation occurs as the direct or indirect result of low tissue PO₂. The relatively hypoxic environment in utero creates a local retinal PO₂ that is conducive to normal vasof ormation. In the context of ROP, the acutely hypoxic environment of oxygen therapy may prompt a high choroidal PaO₂ that translates to a high tissue PO₂ in the inner retina. This might arrest the retinal vasof ormation process, exerting a greater effect on the deep capillary net closer to the source of oxygen diffusion. Removal from therapy to room air would greatly reduce the level of oxygen diffusion from the choroid. In combination with the extent of retinal avascularity, this could cause a low retinal PO₂ and a stimulus for new vessel growth. Unfortunately, the new growth is often abnormal, perhaps because the mechanisms that usually regulate this process are no longer functional. The proliferation of new vessels may be stimulated by the release of an as yet unidentified growth factor, or it may be the direct response of vascular tissue to local PO₂.

Given this hypothetical series of events, we should not be surprised that rats housed in variable oxygen formed more retinal vessels because they received considerably less oxygen over the course of the exposure period than constant 80% exposure rats. It is what transpired after removal to room air that is surprising. Rats raised in the four variable oxygen paradigms showed a significant correlation between vasoatenuation and vasoproliferation, whereas rats exposed to constant oxygen did not. Studies in kittens have shown that ambient hypoxia during recovery from oxygen-induced retinal vascular injury leads to more severe retinopathy than recovery in normoxia, supporting the idea that retinal hypoxia is correlated to propensity for neovascularization. If this is the case, then variable oxygen-raised rats should have been less likely to develop proliferative retinopathy than their con-

FIGURE 5. Regression analysis resulted in a linear relationship between the degree of retinal avascularity upon removal from oxygen and the incidence of later neovascularization for variable oxygen-exposed rats. The axis is the incidence of abnormal vessel growth in each group; the axis is the average area of retinal nonperfusion for each group. The correlation coefficient for the four points is 0.96. The relationship of avascularity and neovascularization in constant oxygen-exposed rats does not fall on the regression line.
FIGURE 6. The 48-hour variable oxygen exposure is the only treatment in which abnormal neovascularization occurred during the exposure period. All other treatments required a period of room air before this condition was observed. The abnormal growth in the 48-hour cycle consisted of clumps of apparent endothelial cells that formed at vascular-avascular interface in the plane of superficial vessels (arrows in top panel). This abnormal growth, albeit relatively mild, was easily distinguished from the pattern of vascular cells in normal vasoformation.

stant oxygen counterparts. Clearly, the end result of the constant exposure contradicts the theory that retinal nonperfusion is the single overriding cause of neovascularization. Further, the failure of the constant exposure to lead to consistent abnormal vessel growth calls into question the assumption that retinal nonperfusion leads to tissue hypoxia. However, it should be realized that intrinsically healthy rats exposed to sustained FiO2 of 80% have PaO2 levels far above those found in premature infants and that, therefore, the vascular damage sustained by rats in the constant exposure may be clinically irrelevant.

To measure the extent of oxygen-induced vasoattenuation, the true physiologic patency of retinal vessels was determined by a relatively new application of a technique long used for assessment of microvasculature— injection of fluorescein-labeled high molecular weight dextrans. In this way, we hoped to avoid the common artifacts associated with more conventional perfusion techniques. If we assume that fluorescein-perfused vessels actually represent those (and only those) that are patent to blood-borne oxygen, the discrepancy presented by the constant exposure is difficult to explain. Three suppositions arise.

**Damaged Endothelium**

Perhaps the retinal endothelial cells of constant oxygen-exposed rats are not viable enough to proliferate, even though the stimulus for angiogenesis is present. This explanation is weakened by the relatively normal vasoformation that does occur in these retinas upon removal to room air. However, normal formation of superficial retinal vessels proceeds by differentiation of vascular precursors, whereas abnormal proliferation requires budding and mitosis of endothelial cells. If the constant high oxygen severely injured the existing vascular endothelium, the former process might continue in the absence of the latter.

**Lack of Angiogenic Stimulus**

The manner in which neovascular tissue proliferates by growth of discrete tufts suggests that a relatively focal release of angiogenic factor(s) is the stimulus, rather than simply low retinal PO2. It may be that the considerable retinal nonperfusion of rats in constant oxygen does cause hypoxia upon removal to room air and that this hypoxia does exert its effect on the cells that produce such factors. But these cells may be too damaged to produce them after constant high-level oxygen, or, if they are produced, the factors may be defective and unable to provoke a proliferative response from the surrounding vasculature. Again, differentiation might proceed under a different set of regulatory mechanisms.

**Ischemia—Reperfusion**

It has been shown that brief occlusion of the central retinal vessels in adult rats followed by reperfusion resulted in a severe proliferative response. Specifically, mitotic figures were observed in vascular endothelial cells of retinas treated in this way. It is possible that our variable oxygen protocol mimics repeated ischemia—reperfusion insults that lead directly to endothelial cell proliferation.

Our initial assumption was that the 6-hour cycle would result in the greatest degree of disease because we thought that the rapid transition between 40% and 80% constituted the primary tissue insult and that the more times it occurred (28 times for the 6-hour cycle versus 3 times in the 48-hour cycle), the greater would be the damage. Perhaps the change occurred so often in the 6-hour group that the retina was never able to adjust to its environment. In contrast, 48 hours between changes in oxygen level may have allowed the retina time to adapt to its environment by altering its
degree of physiologic vessel patency or by other, as yet unknown, means. Under these circumstances, the change in oxygen level might constitute a more severe insult.

The 48-hour rats are the only group ever to display abnormal proliferation during oxygen exposure in our experiments. This is a significant finding because the vasoproliferative phase is often seen during oxygen therapy in neonates, but animals have always required a room air postexposure period to mimic this phase. It appears that the relative hypoxia at 40% was stimulus enough, and the 48 hours was time enough, for the retina to initiate its abnormal proliferative response before the severely hyperoxic 80% phase arrested it. Neonatologists should be alerted to the potential danger of prolonged periods of relative hypoxia during the oxygen therapy of their charges. In the context of ROP, the emphasis is more often on episodes of hypoxia.19

The least equivocal means of testing the correlation of retinal nonperfusion to ischemia-induced hypoxia is to measure directly retinal PO2 immediately upon removal to room air. This is a necessary first step before any conclusions can be drawn regarding the exact stimulus for subsequent neovascularization. Further studies using oxygen microelectrodes designed for this purpose are planned.

Key words

oxygen, rat, neovascularization, retinopathy of prematurity, ischemia

Acknowledgments

The authors thank K. Conaway and B. Burnett for their contributions to the analysis of retinal avascular area and J. Bunch for processing the manuscript.

References