Photoreceptor Dystrophy in the RCS Rat: Roles of Oxygen, Debris, and bFGF

Krisztina Valter, Juliani Maslim, Felicity Bowers, and Jonathan Stone

PURPOSE. To examine the roles of oxygen, basic fibroblast growth factor (bFGF), and photoreceptor debris in the photoreceptor dystrophy of the Royal College of Surgeons (RCS) rat.

METHODS. Pups were exposed during the critical period of their development (postnatal day [P] 16–24) and for some days thereafter to hypoxia and hyperoxia. The effects of these exposures on photoreceptor death, debris accumulation in the subretinal space, and the expression of bFGF protein and mRNA by surviving cells were studied.

RESULTS. During the critical period hyperoxia slowed photoreceptor death in a dose-related fashion and decreased bFGF protein levels, whereas hypoxia accelerated death and increased bFGF levels. At the edges of the retina, where photoreceptors survive longest in normoxia, hypoxia had little effect on either photoreceptor death or bFGF protein levels. Oxygen-induced modulation of rates of death could not be related to the accumulation of debris in the subretinal space. After P27, the relationship between oxygen and photoreceptor death changed markedly, hypoxia no longer delaying and hypoxia no longer accelerating death.

CONCLUSIONS. The death of RCS rat photoreceptors in the period P16 to P27 is precipitated by hypoxia that may result from the accumulation of photoreceptor debris in the subretinal space. This debris, the result of the phagocytotic failure of the retinal pigment epithelium in this strain, lies in the normal pathway of oxygen diffusing to the photoreceptors from the choriocapillaris. During this period the retina responds to hypoxia by increasing expression of a potentially protective protein (bFGF), but hypoxia-induced damage overwhelms any protection provided by this or other mechanisms. Later stages of the dystrophy may not be hypoxia-induced. (Invest Ophthalmol Vis Sci. 1998;39:2427–2442)

The photoreceptors of the Royal College of Surgeons (RCS) rat are apparently normal in their function and structure, yet undergo almost complete dystrophy. Evidence of the normality of the photoreceptors came from transplantation and chimera experiments that demonstrated that RCS rat photoreceptors survive and function where apposed to retinal pigment epithelial cells of non-RCS rat origin but die where apposed to retinal pigment epithelial cells of RCS rat origin. Their death follows an accumulation of opsins-labeled debris in the subretinal space first detected by Dowling and Sidman. This accumulation occurs because the retinal pigment epithelium (RPE) fails in its normal function of phagocytizing the discarded tips of the outer segments.

Despite this understanding of RCS rat photoreceptor dystrophy and knowledge of the pattern of its inheritance (autosomal recessive), the genetic defect and the specific cause of photoreceptor death have not been identified. Although the primary defect is believed to lie in the RPE, abnormalities preceding the onset of photoreceptor dystrophy have been described in the carbohydrate chemistry and neurochemistry of the RCS rat retina. Interest in this strain of rat has persisted, nevertheless, because it has proved possible to slow the dystrophy with growth factors, laser lesions, and hyperoxia.

The present article develops previous evidence that hypoxia delays the RCS rat photoreceptor dystrophy, adding evidence that hypoxia accelerates the dystrophy, and that the dystrophy is not caused directly by debris that accumulates in the subretinal space or by a failure of expression of the trophic factor basic fibroblast growth factor (bFGF). Rather the degeneration is caused by hypoxia and persists despite an upregulation of bFGF. Preliminary data were reported previously in Valter et al.

METHODS

All experimental procedures were designed to conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the National Health and Medical Research Council of Australia.

Oxygen Exposure

Animals were placed in standard cages inside a box made of clear plastic, built to function as an oxygen chamber. The level of oxygen in the box was set and maintained by a computer-controlled feedback device (OXYCYCLER; Reming Bioinstruments). RCS rat pups were exposed to 10%, 21%, 50%, or 70%
oxygen from postnatal day (P) 16. In the descriptions of our experiments below, "hypoxia" refers to 10% oxygen and, except where the use of 50% oxygen is specifically indicated, "hyperoxia" refers to 70% oxygen.

Animals in hypoxia or hyperoxia appeared normal in activity, feeding behavior, and grooming. Hypoxia slowed the weight gain of pups, without increasing mortality or morbidity.

Preparation of Eyes for Labeling
Animals were euthanatized with an overdose of sodium pentobarbital (60 mg/kg IP, Nembutal; Abbott, North Chicago, IL). The temporal side of each eye was marked with a subconjunctival injection of India ink. The eyes were enucleated perimortem and immersion-fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at pH 7.4 for 1 to 3 hours. After immersion for cryoprotection in 15% sucrose solution for 30 minutes, eyes were embedded in ornithine carbamoyltransferase (Tissue-Tek OCT compound; Saeura Finechnical Co, Japan) embedding medium, were snap-frozen in liquid nitrogen, and were cryosectioned at 20 μm. Sections were mounted on slides that had been coated with both gelatin and poly-L-lysine.

General Conditions for Immunocytochemical Analysis, Lectin Histochemical Analysis, and Labeling
Unless otherwise stated, reactions were elicited at room temperature on sections fixed to slides, sections were washed by covering the slide with 0.1 M PBS for two successive periods of 5 minutes, sections were incubated by covering them with the respective solution, and sections were coverslipped with a 1:1 mixture of PBS and gelatin-glycerol.

Labeling and Lectin Histochemical Analysis
To detect dying (apoptotic) cells in situ, we used the TdT-dUTP terminal nick-end labeling (TUNEL) technique, according to our own protocol, using Cy-3 (Jackson ImmunoResearch Laboratories, West Grove, PA) as the fluorophore. To label, blood vessels and microglia sections were washed and then incubated overnight at 4°C in fluorescein isothiocyanate-conjugated goat anti-mouse (Fab specific) IgG diluted 1:100 in 0.1 M PBS; and then washed and coverslipped.

Immunochemical Analysis
Detection of bFGF. To detect bFGF protein in situ we used monoclonal anti-bovine bFGF, type I (Upstate Biotechnology, Lake Placid, NY), according to the method reported by Xiao et al. For successful localization it proved essential to limit the fixation time to less than 6 hours; usually we used fixation times of 1 to 3 hours. Sections were washed for 20 minutes and incubated in 0.3% Triton X-100 for 15 minutes; incubated in 10% normal goat serum for 30 minutes; incubated for 1 hour at room temperature in anti-bovine bFGF diluted 1:200 in 0.1 M PBS containing 1% normal goat serum and 0.3% Triton X-100; washed and then incubated for 1 hour in biotin-conjugated goat anti-mouse IgG (Fab specific Sigma) diluted 1:100 in 0.1 M PBS; and then incubated for 1 hour in Cy3 diluted 1:500 or 1:1000 in PBS and washed. To provide a second labeling for Müller cells, sections were incubated overnight at 4°C in a mouse monoclonal antibody to vimentin (Zymed Laboratories, San Francisco, CA) diluted 1:100 in 0.1 M PBS; washed and then incubated for 1 hour in fluorescein isothiocyanate-conjugated goat anti-mouse (Fab specific) IgG diluted 1:100 in 0.1 M PBS; and then washed and coverslipped.

Detection of Opsin. To label photoreceptor outer segment and debris we used an antibody to rod opsin designated rho4D2 (gift from Robert S. Molday, University of British Columbia, Canada). Rho4D2 is an N-terminal-specific anti-bovine monoclonal antibody. Sections were immersed in 70% ethanol for 20 minutes; washed in double distilled water followed by PBS and then incubated in 0.1% Triton X-100 for 10 minutes followed by 1% BSA-PBS for 10 minutes; incubated in rho4D2 diluted 1:100 in 0.1 M PBS for 1 to 2 hours in 37°C over a water bath; washed and then incubated in fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 1 hour at room temperature; and then washed and coverslipped.

DNA Labeling
For general staining, sections were incubated in the DNA-specific dye SYTO 12 (diluted 1:1000 in PBS; Molecular Probes, Eugene, OR) for 20 to 40 seconds.

bFGF In Situ Hybridization
Preparation of cRNA Probes. Probes were prepared from a 477-bp cDNA strand corresponding to nucleotides 533 through 1009 of a rat ovarian bFGF (or FGF-2) cDNA. This cDNA (from the Whittier Institute, La Jolla, CA) incorporates the complete bFGF coding sequence and a 75-nucleotide 3'-flanking sequence. The strand was cloned into pBluescript SK+ (Stratagene) vector. The plasmid was linearized with BamHI (antisense) or XhoI (sense) to obtain DNA templates. Labeled (digoxigenin-UTP) antisense and sense probes were transcribed from the cDNA using T7 and T3 RNA polymerases (DIG RNA Transcription Kit, Boehringer-Mannheim, Mannheim, Germany), respectively.

General Procedures. Tissue preparation was the same as for immunocytochemical analysis, making sure that the slides were kept RNase free. Unless otherwise stated, reactions were at room temperature, sections were washed by covering the slides with 0.01 M PBS for two successive periods of 5 minutes, sections were incubated by covering them with the respective solution, and sections were coverslipped with a 1:1 mixture of PBS and gelatin-glycerol.

Specific Procedures. Sections were brought to room temperature and dried in a vacuum for at least 1 hour, were immersed in 4% paraformaldehyde for 7 minutes, and then were washed. Sections were then treated for 30 minutes with 5 μg/ml proteinase K (Boehringer-Mannheim), diluted in 0.01 M PBS, and washed. Sections were next immersed in 4% paraformaldehyde for 5 minutes and washed and, to block positive charges, were treated with acetic anhydride in 0.1 M triethanolamin (2.5 μl/ml) for 10 minutes and washed twice for 2 minutes. They were immersed then in 2X SSC for 5 minutes.

Each probe was diluted to 0.05 μg/ml in a prehybridization solution (50% formamide, 1X Denhardt's solution, 2X SSC, 10% dextran sulfate, 10 μl/ml tRNA, 10 μl/ml torula yeast). To hybridize, 100 μl diluted probe was spread over the sections and covered by plastic coverslips. The hybridization proceeded for 16 hours at 50°C in a moist chamber. Slides were rinsed in 2X SSC to remove coverslips and hybridization solution.

To reduce nonspecific binding, sections were treated in a series of stringency washes at 50°C with gentle agitation, starting with 2X SSC for 20 minutes followed by 2X SSC 50%
formamide for 30 minutes, 1X SSC 25% formamide for 30 minutes, and, finally, 0.1X SSC for 45 minutes. Sections were then incubated in Tris-NaCl buffer (pH 7.5) for 5 minutes and then in a blocking solution (1% Blocking Reagent in Tris-NaCl buffer) for 60 minutes. Sections were next incubated in 1:2000 anti-digoxigenin-alkaline phosphatase antibody (Boehringer-Mannheim), washed in Tris-NaCl at pH 7.5 for 5 minutes, and then washed for 10 minutes in a buffer solution (1 M Tris-HCl, 2 M NaCl, 1 M MgCl₂ diluted in H₂O at pH 9.5). Sections were then incubated in the same buffer containing 1.5% 5'-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride (NBT/BCIP, Boehringer-Mannheim) for 70 to 90 minutes in a dark, humid chamber. The sections were observed periodically, and the staining reaction was stopped at the required level by washing in distilled water. To prevent light-induced fading, the sections were fixed in 4% paraformaldehyde for 20 minutes and then washed in water and coverslipped.

**Analysis of Sections**

The density of TUNEL-positive profiles was quantified by scanning along retinal sections from one margin to the other, in successive 800-μm steps. The number of TUNEL-positive profiles present in the full width of the outer nuclear layer (ONL) along each 800-μm length was recorded, and counts were kept in sequence. From these counts we estimated average density per field and the variation between fields. We could also compare densities in different retinal regions (nasal, temporal, central, peripheral). The significance of differences in counts was assessed with Student’s t-test.

To quantify differences in bFGF protein levels, the intensity of specific fluorescence-labeling retinas was measured on confocal microscope images with an image analyzer program (NIH Image, National Institutes of Health, Bethesda, MD). Care was taken to prepare the sections to be compared in the same staining runs, to obtain the confocal images in the same session, and to set and then maintain the photomultiplier settings of the confocal microscope so that they were standard throughout the sessions.

**RESULTS**

**Hypoxic Acceleration and Hyperoxic Deceleration of Photoreceptor Death**

**Qualitative Observations.** Evidence of the acceleration of the RCS rat photoreceptor dystrophy caused by hypoxia during the period P16 to P27 is shown in Figure 1. Each panel shows a representative region of the mid-peripheral retina of an RCS rat labeled for fragmenting DNA (the TUNEL technique, red) and for blood vessels and microglia (with the G. simplicifolia lectin, green). The inner and outer boundaries of the ONL are marked with asterisks.

The effects of hyperoxia and hypoxia at P20 are shown in Figures 1A, 1B, and 1C. Figure 1B is from a P20 RCS rat raised in normoxia and shows dying cells in the ONL, at the beginning of the dystrophy characteristic of this strain. Figure 1A suggests that hyperoxia since P16 has reduced TUNEL, indicating a delay of photoreceptor death (see Fig. 2). Figure 1C shows that hypoxia since P16 has increased TUNEL, suggesting an acceleration of photoreceptor death.

The effects of hyperoxia and hypoxia at P24 are shown in Figures 1D, 1E, and 1F. Figure 1E is from an animal raised in normoxia; comparison with Figure 1B shows the increase in TUNEL caused by the dystrophy characteristic of this strain. Comparison of Figure 1E with Figure 1D shows that hyperoxia since P16 has reduced TUNEL. Comparison of Figure 1E with Figure 1F shows that hypoxia since P16 has increased TUNEL.

The effects of hyperoxia and hypoxia at P27 are shown in Figures 1G, 1H, and 1I. Figure 1H is from an animal raised in normoxia; when Figure 1H is compared with Figures 1B and 1E one sees the increase in TUNEL characteristic of the RCS rat photoreceptor dystrophy. By this age there is some evidence of microglial invasion of the ONL, seen as the irregularly labeled shapes in the ONL. Comparison of Figure 1H with Figure 1G shows that hyperoxia since P16 has reduced TUNEL, suggesting that hyperoxia continued to delay cell death. Comparison of Figure 1H with Figure 1I does not show a clear difference in the labeling of the ONL.

If the hypoxic retinas from P20 to P27 (Figs. 1A, 1D, 1G) are examined, TUNEL increased with age despite the hyperoxia; hyperoxia thus delayed the RCS rat photoreceptor dystrophy but did not stop it. When the hypoxic retinas from P20 to P27 are compared (Figs. 1C, 1F, 1I), one sees that TUNEL of the ONL increases up to P24 and that there was a further small increase from P24 to P27. A new feature was prominent at P27, that is, TUNEL in the inner nuclear layer (INL) (Fig. 1I). The labeling of the INL was diffuse; it did not appear to be concentrated in the nuclei and may represent fragmented DNA dispersing from the ONL, perhaps taken up by cells of the INL.

Overall, the thickness of the ONL decreased with age (as seen from the top to the bottom rows in Fig. 1) and with the level of oxygen (seen from left to right in Fig. 1). In Figure 1G, however, the ONL is slightly thinner than in Figure 1H. This may result from individual variability in rates of degeneration between animals.

**Quantitative Observations.** Figure 2 shows some of the trends from Figure 1 in quantitative form. These graphs conflate counts for four experiments.

The bars in the front row of the histogram shown in Figure 2 represent data for three levels of oxygen-rearing of RCS pups during the period P16 to P20, thus for 4 days. The density of labeled cells was greatest in the retina raised in hypoxia and least in the retina raised in hyperoxia. The difference between the hyperoxic and normoxic retinas was not statistically significant (P = 0.61), but the differences between normoxic and hypoxic retinas and between the hyperoxic and hypoxic retinas were highly significant (P < 2 × 10⁻¹¹).

The bars in the second row of Figure 2 show data for four levels of oxygen-rearing during the period P16 to P24, thus for 8 days. The data show the same inverse relationship between oxygen levels and TUNEL seen for P20. In this case, all differences were statistically significant, the least significant difference being between the 50% hyperoxic and normoxic samples (P = 0.043). Comparison between P20 and P24 (i.e., between the first and second rows of data) shows that TUNEL increased with age at all oxygen levels tested.

The bars in the third row of Figure 2 show data for four levels of oxygen-rearing during the period P16 to P27, thus for 11 days. TUNEL of the ONL was higher than at P24 for all four oxygen levels tested. The delaying effect of hyperoxia on cell death was prominent, but by this age the hypoxic retina had no more TUNEL in the ONL than the normoxic retina. By P30 (back row of histogram bars), the retina reared in hypoxia...
Figure 1. Effect of oxygen levels on photoreceptor death in the Royal College of Surgeons (RCS) rat. Pups were raised in normoxia to postnatal day 16, and then in hyperoxia, normoxia, or hypoxia. Sections were labeled with the Tdt-dUTP terminal nick-end labeling (TUNEL) technique for fragmenting DNA (red) and with a lectin (Griffonia simplicifolia), which shows vessels, microglia, and (less reliably) inner segments. The borders of the outer nuclear layer (ONL) are marked by asterisks. (A, B, C) At P20, hyperoxia (70% oxygen, A) appeared to decrease TUNEL below normoxic levels (B). Hypoxia (C) increased TUNEL. (D, E, F) At P24, hyperoxia (D) decreased TUNEL of the ONL below normoxic levels (E), whereas hypoxia (F) increased labeling.
Hypoxia Accelerates the RCS Dystrophy

Hypoxia Accelerates the RCS Dystrophy

Figure 2. Quantification of experiments illustrated in Figure 1. Each bar shows the mean number of TdT-dUTP terminal nick-end-labeled cells in the outer nuclear layer (ONL) per 800-μm length of sections. At P20 (front row) and at P24 (second row), the density of dying cells in the ONL varied inversely with oxygen exposure from P16. All differences were statistically significant (P < 0.05, Student's t-test) except that between 21% and 70% oxygen at P20 and that between 10% and 21% oxygen at P27 (third row). At P30 (back row), 10% oxygen since P16 was associated with lower levels of cell death in the ONL. All scales, 50 μm.

Since P16 had significantly less TUNEL than the normoxic retina. This apparent reversal of the effect of hypoxia on cell death was observed in the two experiments pursued to this age.

The data shown in Figure 2 were also analyzed in five regional segments: nasal periphery, nasal mid-periphery, central, temporal mid-periphery, and temporal periphery. The effects shown in Figure 2 were observed in all five subregions (data not shown). Except close to the edges of the retina (see below), the effects of hyperoxia and hypoxia were detected throughout the retina.

Relation between ONL Thickness and the Effect of Hypoxia

Figure 2 shows evidence that hypoxia from P16 accelerated cell death in the ONL at P20 and P24 but that after P24 the toxic effect of hypoxia faded and was reversed. Figure 3 shows an initial attempt to understand this fading and reversal. Figures 3A and 3B show the fragmenting DNA of photoreceptors undergoing apoptotic death (left panels) and the DNA of other nuclei (right panels) in two P30 retinas, one raised in normoxia (Fig. 3A) and the other in hypoxia (Fig. 3B) since P16. The ONL is thinner in Figure 3B than in Figure 3A, the result (presumably) of the higher levels of photoreceptor death in the period P16 to P24. However, the density of TUNEL-stained cells is lower in Figure 3B than in Figure 3A; this relationship is shown quantitatively in Figure 2 (back row). When we first noticed the low level of death in the P30 hypoxic material we surmised that depletion of the ONL had left too few photoreceptors for the toxic effect of hypoxia to be apparent. It is clear from Figure 3B, however, that only a minority of the ONL cells still present are undergoing apoptosis; there remain many nuclei that are candidates for DNA fragmentation, but they are not affected. We suggest in the Discussion section that the excessive photoreceptor death caused by prolonged hypoxia itself limits the hypoxia of the ONL and limits photoreceptor death due to hypoxia.

Oxygen Modulation of Photoreceptor Death

Protein Distribution. Fibroblast growth factor (in its basic form, bFGF) applied exogenously to RCS rat photoreceptors delays their death, and bFGF is expressed by the developing RCS rat retina. Xiao et al. have recently reported that bFGF is upregulated around laser lesions, in photoreceptors delays their death. bFGF is expressed by the developing RCS rat retina.
FIGURE 3. Effect of hypoxia from P16 to P30 on Royal College of Surgeons (RCS) rat retinas. Sections were labeled with the TdT-dUTP terminal nick-end labeling (TUNEL) technique for fragmenting DNA (left panels) and with the DNA-specific SYTO 12 dye (right panels). (A) In a P30 retina raised in normoxia, TUNEL of the outer nuclear layer (ONL) is extensive. (B) In a P30 retina raised in hypoxia since P16, the ONL is thinner, and the density of TUNEL is lower. All scales, 50 μm.

...tors whose death is delayed by such lesions.11 We therefore tested the possibility that hyperoxia delays the death of RCS rat photoreceptors by upregulating the retinal expression of bFGF and, conversely, that hypoxia accelerates their death by downregulating its expression. Figures 4 and 5 explore this possibility and suggest a different conclusion.

Figure 4 shows part of a hyperoxia/normoxia/hypoxia experiment similar to that shown in Figure 1. In this experiment the retinas were also labeled for bFGF protein. Figures 4A and 4C show that bFGF-labeling is lower in the retina of a P20 pup raised in hypoxia from P16 to P20 than in the retina of a pup raised in normoxia. Figures 4B and 4D show that the hyperoxia caused a reduction of TUNEL in the ONL, confirming what's shown in Figures 1 and 2 and the findings of Maslim et al.12 For two P24 retinas, bFGF protein is less strongly expressed in the retina of a pup raised in hyperoxia since P16 (Fig. 4F) than in the retina of a pup raised in hypoxia for that period (Fig. 4G). In adjacent sections, Figures 4F and 4H show the higher level of TUNEL in the hypoxic retina. These images suggest a direct rather than an inverse relationship between TUNEL and bFGF-labeling.

Protein Distribution Quantified. Figure 5 shows a more quantitative analysis of the effects of oxygen on bFGF protein expression. Regardless of oxygen levels, protein levels were initially highest in the layer of Müller cell bodies and lowest in the ONL. Hypoxia from P16 to P20 (Fig. 5A) changed bFGF levels little from those found in a normoxic retina, but hyperoxia during the same period reduced bFGF expression below normoxic levels in all sublayers of the retina measured. In the retinas made hyperoxic and hypoxic from P16 to P24 (Fig. 5B), bFGF levels were lower in the retina exposed to hyperoxia than in the retina exposed to hypoxia. By P27, with cell death still accelerating in the hyperoxic retinas and steady at a high rate in the hypoxic retinas (Fig. 2), bFGF expression varied little between hyperoxic and hypoxic retinas (Fig. 5C).

mRNA Distribution. Sections hybridized with the probe for bFGF mRNA were examined from P20 RCS rats (hypoxic, normoxic, hyperoxic), from P24 rats (hypoxic and hyperoxic), and from P27 rats (hypoxic and hyperoxic). Only one clear variation in mRNA expression was observed. At P27, there was a marked upregulation of expression in the ONL in the hypoxic retina (compare Figs. 6A, 6B). The bFGF expression in the hypoxic ONL was patchy, concentrating around a few cells (Figs. 6C, 6D, 6E), in some cases extending radially across the ONL (Figs. 6D, 6E).

In summary, bFGF expression (protein or mRNA) was not inversely correlated with the level of photoreceptor death, as might be expected if modulations in the level of cell death were being achieved by regulation of bFGF levels. On the contrary, our material suggested a direct correlation between bFGF expression and photoreceptor death.
FIGURE 4. Effect of oxygen levels on basic fibroblast growth factor (bFGF) protein levels in Royal College of Surgeons rat retinas. Sections were labeled with two antibodies, one against bFGF protein (red), the other against vimentin (green). (A, B, C, D) Effect of hyperoxia (70% oxygen) at P20. Hyperoxia since P16 reduced bFGF protein expression below the level in a normoxic retina (compare A and C) and also reduced TdT-dUTP terminal nick-end-labeling (TUNEL) (compare B and D). (E, F, G, H) Effect of oxygen at P24. Hyperoxia since P16 resulted in lower bFGF expression than in a retina raised in hypoxia since P16 (compare E and G), and in lower TUNEL of the ONL (compare F and H). All scales, 50 μm.
FIGURE 5. Effect of oxygen levels on basic fibroblast growth factor (bFGF) protein quantified. The intensity of bFGF-labeling was measured from confocal images of sections prepared in the same staining runs and imaged in the same session with photomultiplier tube settings held constant. Measures were made using the NIH Image program (National Institutes of Health, Bethesda, MD), in the inner plexiform layer (IPL), the full width of the inner nuclear layer (INL), over just the row of Müller cell bodies (MC) within the INL, over the inner and outer halves of the ONL (ONLi, ONLo, respectively), and over the inner segments of photoreceptors (IS). Hypoxia, 10% oxygen; normoxia, 21% oxygen; and hyperoxia, 70% oxygen.
FIGURE 6. Effect of hypoxia on levels of basic fibroblast growth factor (bFGF) mRNA. These sections have not been counterstained; the digoxigenin-labeled probe appears red-blue, both as fine-grained labeling and, where intense, as coarser granules. (A, B) At P27 bFGF mRNA levels in the inner layers of retina (ganglion cell, inner plexiform, inner nuclear layer) were not more prominent in the hypoxic material (B) than in the hyperoxic (A). In the outer nuclear layer (ONL), however, the hypoxic (10% oxygen) retina showed more patchy intense labeling than in a retina raised in hyperoxia (A). The ONL labeling in the hypoxic retina occurred in patches (C, D, E) that clustered around a few cells, and in some cases (example in E) appeared to extend radially across the layer. All scales, 50 μm.
FIGURE 7. Edge phenomena in photoreceptor death and basic fibroblast growth factor (bFGF) expression. (A) TdT-dUTP terminal
nick-end labeling (TUNEL) on either side of the optic disc from a P27 Royal College of Surgeons (RCS) rat raised in hypoxia since
P16. Labeling is mostly in the outer nuclear layer (ONL; o), with some labeling in the inner nuclear layer (INL; i). (B) TUNEL in
the ONL on either side of the optic disc from a P20 RCS rat raised in hypoxia since P16. Pairs of arrows on the two sides of the
disc indicate TUNEL-sparse zones. (C) Immunocytochemical labeling for bFGF protein in a section adjacent to (B). (D) TUNEL at
the peripheral margin of a P27 RCS rat retina raised in hypoxia since P16. Two arrows mark a TUNEL-sparse zone at the edge of
the retina. (E) bFGF-labeling in a section adjacent to (D). (G) TUNEL at the edge of the optic disc in a P27 RCS rat retina raised
in hypoxia since P16. The field is several hundred microns away from the disc edge and shows the limit (arrow) of the
TUNEL-sparse region of the INL (i). (H) TUNEL at the peripheral margin of a P27 RCS rat retina raised in hypoxia since P16. Three
arrows mark TUNEL-sparse zones in the ONL and INL. All scales, 50 μm.
Hypoxia Accelerates the RCS Dystrophy

Figure 8. Quantification of the edge effects on TdT-dUTP terminal nick-end labeling (TUNEL) illustrated in Figure 7. (A) Number of TUNEL-positive cells in successive 40-μm lengths of the ONL, averaged over three adjacent sections, from the peripheral edge of retina toward the optic disc in P24 Royal College of Surgeons rats. In a rat raised in normoxia (21% oxygen), the average number over the full length of the sections was 2.7 (lower dotted line). Within 200 μm of the edge, density was consistently less than this average. In the retina from a rat raised in hypoxia (10% oxygen) since P16, the full-length average was 8.1 (upper dotted line), and counts within 600 μm of the edge were consistently less than this average. (B) Comparable data for the edges of the retina at the optic disc. Again, close to the edge of the retina, counts are consistently below the full-length averages. All scales, 50 μm.

ONL Survival at Retinal Edge without bFGF Upregulation

The effects of hypoxia shown in Figures 1, 2, 3, 4, and 5 were apparent throughout almost all of the RCS rat retina. At two sites, however, the rate of photoreceptor death was low during normoxia and was little affected by hypoxia. These two sites were the peripheral margin of the retina and the margin of the retina close to the optic disc.13

At all ages in the period studied, photoreceptor degeneration assessed by TUNEL in the ONL was relatively low at the edges of the retina around the optic disc (Figs. 7A, 7B) and at the peripheral margin of the retina (Figs. 7D, 7H). The TUNEL-sparse region appeared wider in the hyperoxic material (Figs. 7B, 7D) than in the hypoxic material (Figs. 7A, 7H) but within 100 μm of the edge the sparseness of TUNEL was always clear. Furthermore, in the hypoxic P27 retina in which TUNEL had spread to the INL, a similar sparsity of labeling was seen at the edge of the retina (Figs. 7G, 7H) and was more extensive than the TUNEL-sparse region in the ONL. To determine whether this resistance of degeneration was mediated by bFGF, we examined adjacent sections labeled for the protein bFGF. The reduced TUNEL at the edges of the optic disc shown in Figure 7B was not matched by a rise in bFGF in the ONL (‘o’ in Fig. 7C). Similarly, the reduced TUNEL at the peripheral edge of the retina shown in Figure 7D was not related to increased bFGF-labeling in the ONL (‘o’ in Fig. 7E).

Figure 8 shows the low levels of TUNEL at the retinal edges more quantitatively, for RCS rats aged P24. The mean number of TUNEL-positive cells per 40-μm length of the ONL, averaged over six sections, increased from the peripheral mar-
Figure 9. Relation of oxygen levels to opsin expression. Sections are labeled with an antibody to opsin. The margins of the outer nuclear layer are shown by asterisks. Opsin concentrates in the outer segments and in debris in the subretinal space. (A, B) Results from Royal College of Surgeons (RCS) rat retina at postnatal day (P) 20;
gin toward the optic disc (Fig. 8A). In a P24 RCS rat kept in normoxia, the mean density was less than the overall average for the full length of the sections (lower dotted line) in the most peripheral 200 μm of the section. In a P24 RCS rat kept in hypoxia since P16, mean density was less than the average for the full length of the sections (upper dotted line) for more than 600 μm of the section. Figure 8B shows similar data for the two sides of the optic disc; averaged over three adjacent sections. In a P24 RCS rat kept in normoxia, mean density of TUNEL-positive cells was less than the average for the full length of the section (lower dotted line) for 300 μm to 600 μm. In a P24 RCS rat kept in hypoxia since P16, mean density was less than the full length average (upper dotted line) for at least 300 μm.

Relationship of RCS Rat Photoreceptor Dystrophy to Debris in the Subretinal Space

In the RCS rat the debris from photoreceptors accumulates in the subretinal space.\(^2\)\(^2\)\(^2\) This debris lies between the photoreceptors and their source of oxygen, in the choriocapillaris; it may therefore make the outer layers of the RCS rat retina hypoxic, inducing the dystrophy. Alternatively, the debris may be toxic to photoreceptors through some other mechanism. In either case, the rescue effect of hyperoxia might be achieved by reducing the debris (perhaps by improving the phagocytic performance of the RPE), while the exacerbating effect of hypoxia might result from an increase in debris deposit. Figure 9 explores this possibility, comparing the subretinal space and adjacent layers of retinas from RCS rats raised in normoxia, hypoxia, and hyperoxia.

In a P24 RCS rat kept in normoxia, mean density of TUNEL-positive cells was less than the average for the full length of the section (lower dotted line) for at least 300 μm. In a P24 RCS rat kept in hypoxia since P16, mean density was less than the full length average (upper dotted line) for at least 300 μm.

Failure of phagocytosis by the RPE

Debris accumulation in the subretinal space

Reduced oxygen diffusion to photoreceptors

Hypoxia-induced death of photoreceptors

(C, D) results from RCS rat retina at P24; (E, F) results from RCS rat retina at P27. There was no trend for debris accumulation in the subretinal space to be greater where photoreceptor death was greater (i.e., in B, D, F). All scales, 50 μm.

Discussion

The present results confirm and expand our previous findings that hyperoxia delays\(^1\)\(^2\) and hypoxia accelerates\(^1\)\(^3\) the RCS rat photoreceptor dystrophy and provide evidence that the delay and acceleration cannot be explained either by modulations of the expression of the trophic factor bFGF or by variations in the accumulation of debris in the subretinal space. Our previous report\(^1\)\(^3\) that the edges of the RCS rat retina are protected against hypoxic damage is confirmed, and the observation is added that this protection does not involve the upregulation of bFGF.

Precipitating Cause of Photoreceptor Dystrophy in the RCS Rat

The present results, taken with those of earlier studies, suggest that the RCS dystrophy is initiated because a genetic abnormality prevents the normal ingestion of photoreceptor debris by the RPE (Fig. 10), the debris accumulates in the subretinal space and blocks the diffusion of oxygen from the choroid to the photoreceptors, and the resultant hypoxia induces photoreceptor death. Experimental hyperoxia relieves the hypoxia, reducing photoreceptor death, whereas experimental hypoxia worsens the hypoxic state, accelerating photoreceptor death. Because hyperoxia relieves the prime cause of retinal damage, it downregulates the expression of bFGF protein, whereas, conversely, hypoxia reinforces the prime cause of damage and upregulates bFGF. The delaying effect of hyperoxia and the accelerating effect of hypoxia cannot be explained as the result of changes in the accumulation of debris in the subretinal space.

A simplifying feature of this hypothesis is the postulate that the immediate cause of the RCS rat photoreceptor dystrophy is the same as that of the death of photoreceptors during...
normal development (i.e., hypoxia). Of course, this hypothesis does not exclude a contribution to hypoxia in the RCS rat retina by other factors, such as the abnormality of choroidal circulation described by McLaren et al.

**Edge Effects**

The sparing of the edges of the retina from the RCS rat photoreceptor dystrophy was first noted by LaVail and Battelle. In a previous study and here we have presented evidence that this difference persists during hyperoxia and that it is emphasized by hypoxia. Whatever is protecting the edges of the retina from the RCS rat photoreceptor dystrophy also protects it from experimental hypoxia. Furthermore, the protection is not mediated by the protective factor bFGF. One explanation of these observations is that the edges of the retina have a richer supply of oxygen than regions away from the edges. At the optic disc, the extra supply of oxygen may come from the large vessels that pass through the optic nerve head. At the peripheral margin, the additional supply of oxygen may come by diffusion from the choriocapillaris "around the edge" of the retina. We suggest that this additional supply limits photoreceptor death and bFGF expression.

**Limitations of the Hypoxia Hypothesis**

We previously reported that after approximately P22, hyperoxia is ineffective in delaying the RCS rat photoreceptor dystrophy; we note here (Fig. 2) that after P24, hypoxia becomes ineffective in accelerating the dystrophy. We further note that the toxicity of hypoxia fades as the ONL becomes thin. It is possible that this fading results from the upregulation of protective factors such as bFGF, but it is also possible to propose an explanation of the fading in terms of oxygen supply. We suggest that when the ONL is depleted of oxygen, the hypoxia caused by the debris in the subretinal space is relieved because there are fewer photoreceptors using oxygen. Hypoxia then ceases to cause death and the delaying effect of hyperoxia and accelerating effect of hypoxia diminish correspondingly. It thus seems that hypoxia can account only for the initial stages of the RCS rat photoreceptor dystrophy and that the full course of the dystrophy must involve further mechanisms. One very speculative sequence that might account for the full course of the RCS rat photoreceptor dystrophy is as follows.

As the photoreceptors enter their critical period of vulnerability, hypoxia caused by debris accumulation in the subretinal space causes photoreceptor death, as argued above and previously. The reduction of photoreceptor numbers reduces the oxygen demand of the ONL so that, by the late P20s, the ONL is no longer hypoxic. Clearance of photoreceptor debris over the next several weeks reduces that barrier to the flow of oxygen into the ONL, and it becomes hyperoxic. Hyperoxia is toxic through an oxygen-radical mechanism, driving photoreceptor death to completion. We are currently investigating the late (post-P25) stages of the RCS rat photoreceptor dystrophy with these possibilities in mind.

**Opsin in the RCS Rat Retina**

Our present observations confirm previous reports that the development of opsin-labeled protein (presumably rhodopsin) in the RCS rat is initially normal in timing and location and that it becomes abnormal in location once the outer segments of the photoreceptors begin to develop. The abnormalities include retention of opsin in the ONL and inner segments and accumulation of opsin in the subretinal space. In the present observations, the opsin label was used to estimate debris accumulation and to provide evidence (Fig. 9) that the greater death of photoreceptors in retinas kept in hypoxia is not caused by increased debris accumulation in the subretinal space.

**bFGF Regulation in the RCS Rat Retina**

Little is known of the regulation of bFGF in the retina. Our data indicate that extensive changes in protein distribution can occur (Fig. 4) with only limited changes in mRNA levels (Fig. 6). These observations warrant further study, because they raise the possibility that the movement of bFGF protein (rather than a new translation of protein) may be a major element in the deployment of this protein within the retina.

**bFGF in the RCS Retina: Normal or Abnormal?**

Whether measured by immunocytochemical detection of bFGF protein or by polymerase chain reaction detection of bFGF mRNA, the RCS rat retina develops normal levels of bFGF and the protein and message are normal in location. Gao and Hollyfield described an abnormality of expression, specifically high levels of bFGF expression in the ONL, but interpreted this as a response to the dystrophy. One study has reported an abnormally low level of bFGF expression in the RPE of the developing RCS rat eye and a slower development of the choroidal circulation.

The antibody we used, from the technique of Xiao et al., localizes bFGF protein to the cytoplasm and nuclei of Müller cells, to the nuclei of astrocytes, to the cytoplasm of endothelial cells, and to the nuclei and cytoplasm of RPE cells, confirming previous studies of bFGF localization in rat retina. Lower levels usually can be detected in the processes of Müller cells and in the cytoplasm of larger neurons. Under conditions of stress, bFGF protein levels are raised in the inner segments of photoreceptors and in the adult the protein becomes prominent in the cytoplasm of photoreceptors. The implications of these patterns for the function of bFGF remain to be worked out.

The pattern of bFGF mRNA expression observed here is consistent with earlier descriptions of that for the rat, but a comparison of protein and mRNA raises several questions. The mRNA of bFGF appears to concentrate in neurons rather than in macroglia; in our study protein levels varied more obviously with photoreceptor dystrophy than did mRNA levels. Resolution of these issues will require close testing; Cao et al. have, for example, recently reported that bFGF protein can produce an upregulation of bFGF mRNA in Müller cells in vitro. Nevertheless, given the effectiveness of bFGF in delaying photoreceptor death in several circumstances, present results do seem to justify the conclusions that the delay of photoreceptor death by hyperoxia is not achieved by an upregulation of bFGF and that the acceleration of photoreceptor death by hypoxia is not mediated by a downregulation. In our study, by contrast, bFGF levels varied in directions opposite to those that would implicate it as a causal factor in either hyper-
oxic inhibition or hypoxic acceleration. A simple explanation of these relationships is that the stimulus that causes both photoreceptor death and bFGF upregulation in the early stages of the RCS rat photoreceptor dystrophy, is hypoxia.

Environmental and Genetic Factors

The RCS dystrophy is, as we argued above, caused by a sequence of genetic and environmental factors and can be slowed and accelerated by environmental interventions, including growth factors, laser lesions, and oxygen. It is possible that among the large number of human photoreceptor dystrophies now identified, the causes of many include environmental parameters. Given sufficient knowledge, such dystrophies may prove amenable to environmental intervention, in short to therapy.

If, as Mullen and LaVail and Li and Turner have argued, RCS rat photoreceptors are genetically normal, the present observations allow a further comment. As many as half the cases of human photoreceptor dystrophy are “simplex”; they occur without a familial history. This is evident among sufferers in the United Kingdom, Spain, and Japan. There is currently debate as to how many cases of simplex retinitis pigmentosa are in fact genetic, either reemerging recessive or fresh mutations, but it is possible that many cases of retinal dystrophy seen in humans occur in patients with genetically normal retinas. The RCS rat model suggests that dystrophy can result from a perturbation of the diffusion pathway between the choroid and photoreceptors, and it seems possible that damage to this diffusion pathway from nongenetic causes, for example, an inflammation of the RPE that reduces its transport or diffusion properties or a thickened Bruch’s membrane, could induce lethal levels of hypoxia in the ONL. It thus seems possible that photoreceptor dystrophies can be understood as being precipitated by a sequence of genetic and environmental mechanisms or even (in some cases) by environmental insult to a genetically normal retina. Several groups have noted the “incomplete penetrance” of some RP genes, only one of two siblings with the same gene showing the disease phenotype. These investigators have concluded that “some other factor must be involved” that other factor may be nongenetic damage to the interface between photoreceptors and the choriocapillaris.

Acknowledgments

The authors thank Tania Novikova for skilled technical assistance and Guy Cox and Eleanor Kable of the Electron Microscope Unit of the University of Sydney for support and help with confocal microscopy. Diana Oaks provided skilled help in optimizing in situ hybridization procedures.

References


Hypoxia Accelerates the RCS Dystrophy


