Limiting Photoreceptor Death and Deconstruction During Experimental Retinal Detachment: The Value of Oxygen Supplementation

KYLE MERVIN, BSC, KRISZTINA VALTER, MBBS, JULIANI MASLIM, MBBS, PHD, GEOFFREY LEWIS, PHD, STEVEN FISHER, PHD, AND JONATHAN STONE, DSC

PURPOSE: To assess the role of hypoxia in causing the death and deconstruction of photoreceptors in detached retinas and the effectiveness of supplemental oxygen in limiting such damage.

METHODS: Retinal detachment was induced surgically in the right eye of each of 10 cats. The cats were allowed to survive surgery for 3 days. Two were kept for these 3 days in normoxia (room air, 21% oxygen) and eight in hyperoxia (70% oxygen). The retinas were examined for cell death by use of labels for normal and fragmenting DNA, with antibodies and a cone sheath-specific lectin to demonstrate the status of their inner and outer segments, the synaptic structures of the outer plexiform layer, and the distribution of basic fibroblast growth factor (bFGF) and with in situ hybridization to demonstrate bFGF mRNA.

RESULTS: Retinal detachment without oxygen supplementation caused the death of some photoreceptors; the loss of cytochrome oxidase from the inner segments and the collapse of the outer segments of surviving photoreceptors; the loss of synaptophysin profiles from the outer plexiform layer; and the loss of bFGF protein from retinal neurons and neuroglia but not from retinal vessels. Oxygen supplementation (hyperoxia) during detachment mitigated all these changes, reducing photoreceptor death, maintaining the specialized structures of surviving photoreceptors, and stabilizing the bFGF within the retina.

CONCLUSIONS: In experimental retinal detachment, hypoxia caused by the separation of outer retina from its normal source of nutrients is a factor in inducing the death and deconstruction of photoreceptors as well as in the loss of bFGF from the detached retina. Hyperoxia offered to human patients between diagnosis of retinal detachment and surgery may enhance the function of the reattached retina. (Am J Ophthalmol 1999;128:155–164. © 1999 by Elsevier Science Inc. All rights reserved.)

Detachment of the neural retina is a common pathology, which may be induced by trauma or occur apparently spontaneously. Clinical experience has shown that even when reattachment surgery is successful, detachment threatens vision in two distinct ways: a reattached retina rarely regains normal sensitivity or acuity and may also become the focus of proliferative reactions (proliferative vitreoretinopathy and subretinal fibrosis), which can damage previously undisturbed retina.1,2

Both the loss of visual function and the proliferative reactions can be understood to result from damage to the retina while detached, as documented by Fisher and associates3-6 working principally in a feline model. Detachment of a patch of retina causes death in retinal neurons, which is specific to photoreceptors.7 Detachment also induces the surviving photoreceptors to dismantle...
their outer segments and the terminals of their axons in the outer plexiform layer. At the same time, concentrations of mitochondria in photoreceptor inner segments (essential for their normal metabolism) disperse and the Müller glial cells of the retina proliferate and hypertrophy, their processes spreading along both outer and inner surfaces of the retina. Some of these observations, particularly the death of some photoreceptors, have been confirmed in the detached human retina and also in detached rabbit retina.

The concentration of death and degeneration in the photoreceptors of the detached retina can be explained by the pattern of nutrient supply to the retina. The inner layers are supplied by the retinal circulation, which is unaffected by detachment. The outer layers, comprising the photoreceptors, are supplied by diffusion from the choriocapillaris; detachment separates the photoreceptors from their only source of the two molecules essential for their metabolism, oxygen and glucose. Analysis of the metabolism of photoreceptors has shown their metabolic rate to be much higher than that of other neurons; this presumably heights their vulnerability to detachment. Conversely, photoreceptors have been shown to have high capacities for both oxidative metabolism and anaerobic glycolysis, which allow them to rely on oxidative metabolism when hypoglycemic and on anaerobic glycolysis when hypoxic. When starved by detachment of both oxygen and glucose, however, photoreceptors undergo complete or partial degeneration. The present experiments were designed to test whether supplementing the supply of one of the main energy sources of photoreceptors (oxygen) would enhance photoreceptor survival. Oxygen reaches the outer retina specifically and rapidly when inhaled levels are supplemented. Furthermore, oxygen is readily available in most clinical settings, and there is much expertise and experience in its administration in cardiac and respiratory disease. That availability and expertise could enable a ready deployment of oxygen to improve the outcome of reattachment surgery if the value of supplementation for photoreceptor survival could be established.

**METHODS**

**THE STUDY WAS PERFORMED ON ADULT CATS (FELIS DOMESTICUS).** The protocols were approved by the Animal Ethics Committee of the University of Sydney and the Animal Care Council of the University of California Santa Barbara. All procedures conformed to the standards set by the Association for Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgery was performed under surgical anesthesia and sterile conditions. Anesthesia was induced with a mixture of xylazine, 10 mg per kg, and ketamine 50 mg per kg, supplemented by lesser doses of the same drugs (1 mg/kg, 5 mg/kg, respectively) as needed to maintain anesthesia. Surgery to induce detachment followed published protocols except that the lens and vitreous were left in place. In brief, a glass micropipette was inserted through a 20-gauge hole in the sclera at the region of the pars plana. A solution of 0.25% sodium hyaluronate in balanced salt solution (BSS) was then infused between the neural retina and retinal pigment epithelium. A single detachment was produced in the right eye of each of the 10 cats.

For recovery from surgery animals were placed in a large (75 × 75 × 65 cm) chamber built of Plexiglas, initially on soft bedding. Recovery was monitored closely for several hours. The animals remained in the chamber for 3 days. For two animals the chamber contained room air (21% oxygen); for the other eight the level of oxygen in the chamber was raised to 70% and was maintained at that level for the next 3 days by means of a feedback control device. The animals had food and water ad libitum, and ambient illumination was on a 12-hour/12-hour light/dark cycle, the intensity of the light phase being approximately 50 lux.

Three days after detachment surgery, the animals were sacrificed with an overdose of sodium pentobarbitone (120 mg/kg), and the eyes were enucleated perimortem and immersion fixed for 10 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4. The cornea and lens were then removed, and the eyecup was immersion fixed for 50 minutes in the same fixative. The eyecup was then divided into segments that spanned the point of detachment. The tissue was sectioned on a cryostat or on a Vibratome (Technical Products International, Polysciences, War- rington, Pennsylvania). For cryosections, the segments were washed briefly in PBS and then placed in 15% sucrose until they sank. The pieces were then embedded and cryosectioned at 20 μm. The tissue used for Vibratome sectioning was not dehydrated; after fixation it was rinsed in PBS and embedded in 5% agarose in PBS. Sections 100 μm in thickness were cut with the Vibratome.

To detect dying (apoptotic) cells in situ, we employed the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique to demonstrate the fragmentation of DNA characteristic of apoptosis following our previous protocol for cryosections using a fluorescent marker, usually Cy3. To provide general DNA labeling, the Vibratome sections were incubated for 4 hours in propidium iodide (0.5 μg/ml in PBS).

To label cone sheaths by the method of Blanks and associates the cryosections and Vibratome sections were incubated in biotinylated peanut agglutinin (Vector Laboratories, Burlingame, California). The cryosections were incubated for 1 hour in peanut agglutinin diluted in PBS to a final dilution of 400 μg per ml, followed by incubation for 1 hour in streptavidin-Cy2 or Cy3. The Vibratome sections were incubated overnight in the same solutions.

For the immunocytochemical study of the cryosections, incubation times for the blocking serum (10% normal goat serum) and for the primary and secondary antibodies were...
1 to 2 hours, and the primary antibody was made up in PBS containing Triton X-100 0.3%. For the Vibratome sections, staining times for the blocking serum (normal donkey serum, 1:20), primary antibody, and secondary antibody were 24 hours for each step to allow adequate penetration into the thick sections. Buffer rinses of 1.5 hours were performed between all antibody steps. All antibodies and rinse solutions were made up in PBS containing bovine serum albumin (0.5%) and Triton-X 100 0.1%. On completion of the staining, the sections were mounted in 5% n-propyl gallate in glycerol. The cryosections and Vibratome sections were labeled with antibodies to cytochrome oxidase (Molecular Probes, Eugene, Oregon) at 1 μg per ml; antibodies to rod opsin (gift from Dr R. Molday, University of British Columbia, Vancouver, Canada) at 1:100; antibodies to blue and red-green cone opsins (gift from Dr J. Nathans, Johns Hopkins Medical School, Baltimore, Maryland) at 1:1000; to synaptophysin (Dako, Carpinteria, California) at 1:100; antibodies to glial fibrillary acidic protein (Dako, Carpinteria, California) 1:500; antibodies to Ki-67 (the MIB-1 antibody of Immunotech, Inc, Westbrook, Maine) at 1:100; antibodies to β-tubulin (gift from Dr M. Klymkowsky, University of Colorado, Boulder, Colorado) at 1:1000; and antibodies to basic fibroblast growth factor (bFGF) (Upstate Biotechnology, Lake Placid, New York) 1:200. The secondary antibodies conjugated to Cy2 or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) were diluted 1:200 or 1:1,000.

In situ hybridization was performed with cRNA probes prepared from a 477-bp cDNA strand corresponding to nucleotides 533–1009 of a rat ovarian bFGF cDNA. This cDNA incorporated the complete bFGF coding sequence and a 75 nucleotide-3’ flanking sequence. The strand was cloned into pBluescript SK+ (Stratagene) vector. The detailed procedures have been published.

Observations of the thickness of the outer nuclear layer were made for two animals kept in room air after retinal detachment and three animals kept in hyperoxia. The thinning of the outer nuclear layer observed for animals kept in room air confirmed extensive data reviewed by Fisher and Anderson. The numbers of animals studied with each technique were as follows: TUNEL for two animals kept in room air and six animals kept in hyperoxia; cone opsin labeling for two and three animals, respectively; peanut agglutinin labeling, two and three animals, respectively; synaptophysin labeling, two and six animals, respectively; cytochrome oxidase labeling, two and five animals, respectively; and bFGF labeling, two and five animals, respectively. Images of retinal tissue were digitized by confocal microscopy. When two fluorophores (red and green) were both digitized, the images were obtained sequentially to maximize signal separation. Wherever signal intensities were to be compared, the photomultiplier tube settings were held constant.

Molecule-specific signals from immunolabeled proteins were quantified by use of NIH Image software (the Analysis tool) from the confocal images. Any optimization of images done before quantitation was kept identical between images to be compared.

RESULTS

RETINAL DETACHMENT WITHOUT OXYGEN SUPPLEMENTATION (that is, in normoxia) caused the thinning of the outer nuclear layer (compare Figure 1, A and B), confirming earlier reports. Loss of neurons was apparent only in the outer nuclear layer, which was reduced in thickness by 18%. Further, pyknotic nuclei were common in the detached outer nuclear layer (two at the right of Figure 1B). Labeling by the TUNEL technique (not illustrated) confirmed earlier reports that the death of cells in detached retina was apoptotic and was specific to cells of the outer nuclear layer, that is, to photoreceptors. In all three hyperoxic detachments studied, the thickness of the outer nuclear layer appeared to be fully maintained (compare Figure 1, C and D), and the frequency of pyknotic nuclei in detached retina was reduced (compare Figure 1, D and B). With TUNEL, some DNA fragmentation in the outer nuclear layer of the hyperoxic detached retina was disclosed however, suggesting that hyperoxia slows photoreceptor death but does not to stop it completely.

In the retinas detached in normoxia there was evidence of deconstruction of outer segments (Figure 1B). This deconstruction was greatly reduced by hyperoxia during detachment (compare Figure 1, B and D) in all five retinas examined. The effect of detachment on rod outer segments was also apparent in retinas labeled with an antibody to rod opsin. The normal concentration of opsin in outer segments (Figure 2A) was disrupted by detachment in normoxia (Figure 2B). The outer segments appeared shortened and distorted, and opsin became prominent in rod somas in the outer nuclear layer. Hyperoxia limited these effects, stabilizing the structure of outer segments and limiting the accumulation of opsin in outer nuclear layer somas (Figure 2C). With the use of an antibody to β-tubulin, the effect of detachment on the cilia of photoreceptors could be demonstrated. The normal pattern of fine, highly oriented cilia, each forming the stem of an outer segment, was clear in attached retinas (Figure 2D). In normoxic detached retinas, the cilia were shortened and only a few were long enough to be identified (shown by an arrow in Figure 2E). In hyperoxic detached retinas, the cilia were longer and more numerous (Figure 2F), although less well aligned than in attached retina.

Detachment caused a loss of cytochrome oxidase labeling from inner segments (compare Figure 3, A and B, green signal), which was reduced by hyperoxia during detachment (compare Figure 3, B and C). The same pattern of cytochrome oxidase loss in normoxia and retention in hyperoxia is apparent in Figure 3, D, E, and F and 3, J, K, and L.
FIGURE 1. Effect of oxygen supplementation during retinal detachment on photoreceptor death. All sections are labeled for DNA with propidium iodide. For this comparison, care was taken that obliquity of section did not affect the comparison. (A) Normoxic attached retina. (B) Normoxic detached retina. The arrows indicate disintegrating outer segments. (C) Hyperoxic attached retina. (D) Hyperoxic detached retina.
Detachment also affected the morphologic characteristics of cone outer segments, shown with labels for cone opsins (red in Figure 3, A to I). In attached retinas the cone opsins were largely confined to the outer segments (Figure 3, A, D, and G) but could sometimes be traced along the inner segments to the cone somas (Figure 3D). Where the peanut agglutinin lectin demonstrated the cone sheaths (Figure 3G), the opsin labeling was seen to lie within the sheath. In detached retinas in normoxia, opsin was abnormally prominent in cone somas, opsin-bearing outer segments were absent or misshapen, and the cone sheaths were correspondingly misshapen as in Figure 3, B, E, and H. These observations confirm earlier reports.21,22 In detached retinas kept in hyperoxia, by contrast, the structure of outer segments of many cones was close to normal (Figure 3, C, F, and I). The tendency for opsin to accumulate in cone somas was greatly reduced, and the opsin lay normally within cone sheaths (Figure 3I).

Detachment caused a marked loss of synaptophysin from the outer plexiform layer (Figure 3, J and K, red signal), confirming Lewis and associates,6 but the loss was greatly reduced in animals given oxygen supplementation after
detachment (Figure 3L). The loss of synaptophysin in normoxic detached retina was specific to the outer plexiform layer; the synaptophysin labeling of the inner plexiform layer appeared unaffected by detachment (not illustrated).

Detachment caused marked changes in bFGF protein distribution (Figure 4, A to D). In attached retina at some distance (more than 2 mm) from the point of detachment, bFGF distribution was approximately normal (Figure 4A), the protein concentrating in somas in the inner nuclear layer (i), in the inner segments of photoreceptors (is), and in the walls of blood vessels in the retina and choroid. In attached retina less than 2 mm distant from the point of detachment, bFGF levels were sharply reduced in neurons and glial cells (Figure 4B) but remained high in vessel walls. In detached retina in normoxia, bFGF protein levels were low in the inner layers except in blood vessels (Figure 4C). At the point of detachment, a sharp increase was apparent in bFGF levels in the inner and outer segment layers (Figure 4D). These images suggest that detachment causes bFGF to move from its normal storage sites outward and, where the retina is detached, to accumulate in the inner and outer segments or in the interphotoreceptor matrix.

Hyperoxia during detachment stabilized bFGF in its normal storage sites and in three of five cases examined, it produced a small increase in bFGF protein levels in these normal sites, which is apparent on comparing Figure 4, E and F. This stabilization extended throughout the detached segment of retina. However, hyperoxia did not totally stabilize bFGF distribution. In addition to the increase in bFGF levels in normal sites, already noted, evidence of bFGF movement into the layer of inner segments was detected (arrows in Figure 4F).

The morphologic characteristics of the outward movement of bFGF were unexpected. The bFGF forms in the layer of inner segments in blobs, many associated with a breach in the outer limiting membrane, as shown in Figure 4, G to J, by using an antibody to glial fibrillary acidic protein. At these breaches, GFAP+ processes extended abnormally outward, often in association with a blob of bFGF (Figure 4, G and H). When we added a label that demonstrated cone sheaths (the peanut agglutinin lectin), it was evident that the blobs of bFGF and glial fibrillary acidic protein were located within cone sheaths.

Detachment in normoxia caused a weak upregulation of bFGF mRNA in the detached portion of retina (compare Figure 4, K and L). The upregulation was apparent in all layers in which bFGF mRNA is usually detected but seemed most prominent in the inner segments of photoreceptors. A similar difference was observed for the second control animal. A weak upregulation of bFGF mRNA was also apparent in hyperoxic detached retina (compare Figure 4, M and N) and again seemed most prominent in the inner segments. The overall level of hybridization signal is considerably higher in the hyperoxic than in the normoxic retina in the cases shown in Figure 4, K to N. These tissues were processed through the same runs but not on the same glass slide, and further work is needed before an effect of hyperoxia on bFGF mRNA levels can be inferred.

The upregulation of bFGF in normoxic detached retina contrasts with a loss of bFGF protein, which may involve movement of the protein or some other posttranslational mechanism, as discussed below. The upregulation of bFGF in hyperoxic detached retina correlates directly with an observed upregulation of protein (Figure 3, E and F).

The effect of detachment and hyperoxia on cytochrome oxidase levels in the inner segments could be quantified (Figure 5A). Detachment without hyperoxia reduced cytochrome oxidase labeling of inner segments so that the ratio of cytochrome oxidase labeling in attached retina to that in detached retina was greater than 1 (mean, 2.41; range, 1.33 to 3.78, four separate comparisons in two animals). In animals kept in hyperoxia after detachment (six animals examined), the ratio was closer to 1 (mean, 1.03; range, 0.68 to 1.45).

Similarly (Figure 5B), detachment reduced synaptophysin labeling of photoreceptor synapses in the outer plexiform layer, so the ratio of labeling in attached retina to that in detached retina was greater than 1 (mean, 2.88; range, 1.86 to 4.96, five separate comparisons in two animals). In animals kept in hyperoxia after detachment, the ratio was closer to 1 (mean, 1.078; range, 0.490 to 1.648, seven comparisons in six animals).

**DISCUSSION**

The present results provide evidence that oxygen supplementation during retinal detachment reduces the death of photoreceptors, mitigates sublethal degenerative changes in the survivors, and stabilizes the distribution of...
bFGF protein within the retina. These effects strengthen the hypothesis that the degenerative changes in photoreceptors and redistribution of bFGF that follow detachment result from hypoxia or from a combination of hypoxia and hypoglycemia caused by the separation of the retina from the choriocapillaris. They suggest that in humans the visual function of reattached retina may be improved by providing the patient with oxygen supplementation between diagnosis and surgery.

The biochemistry of adenosine triphosphate (ATP) production by photoreceptors shows that they require ATP in large amounts and have developed excess capacity for ATP production, which gives them the protective ability to switch from principally anaerobic to principally oxidative production of ATP. If, however, both glucose and oxygen are in short supply, as they presumably are in the detached retina, photoreceptors cannot continue to function or in some situations, to survive.11–13

The present evidence that detachment-destabilized bFGF is stored in the retina (Figure 4) is novel and may prove to be an important component of the changes that occur in detached retina. It is known that bFGF is highly protective to the retina and particularly to photoreceptors, whether applied exogenously23,24 or upregulated endogenously by prior damage to the retina25,26 or to the optic nerve.27,28 The movement of bFGF out of the detached retina may increase the vulnerability of retinal neurons to the stresses that follow detachment. Hyperoxia appears to slow the extrusion of bFGF from detached retina without completely stopping the process. The images in Figure 4, F to J, may have caught the process of extrusion at an early stage. The close association of blobs of bFGF with cone inner segments was unexpected, and for the moment its significance is unclear.

The loss of bFGF protein from normoxic detached retina apparent in Figure 4, C and D, contrasts with the upregulation of bFGF mRNA shown in Figure 4L. This suggests that the loss of bFGF protein occurs by some posttranslational mechanism, perhaps simply by separation of bFGF from the sites to which it normally binds and its subsequent movement. The signals controlling such separation and movement are not well understood, however.

The death of photoreceptors in detached retina is believed to be apoptotic, that is, to involve the upregulation of a series of “suicide” genes in the dying cells, which accomplish their death and dismemberment. The present results suggest that the sublethal changes to photoreceptors in detached retina, such as the collapse of their inner and outer segments, are not simply damage collateral to the trauma of detachment but are also programmed responses of the cell. The initial trauma of detachment was not reduced by the oxygen supplementation utilized in the present experiments. Nevertheless, oxygen supplementation reduced all components of sublethal changes to photoreceptors that we have examined, presumably by preventing upregulation of the mechanisms that drive those changes.

The optimistic inference to be drawn from this idea is that the clinical management of detached retina may be improved by measures that control the pathology of detachment, specifically by measures that counter the factors activating suicide and deconstruction mechanisms. The present results

FIGURE 4. Effect of oxygen supplementation on bFGF expression in detached retina. (A to D) Immunolabeling for bFGF protein in normoxic retina: (A) attached retina more than 2 mm distal from the point of detachment; (B) attached retina close to the point of detachment; (C) detached retina; (D) the point of detachment. (E and F) Immunolabeling for bFGF protein in (E) attached and (F) detached hyperoxic retina. (G and H) Immunolabeling for bFGF protein (green) and glial fibrillary acidic protein (red) in hyperoxic detached retina. (I and J) Triply labeled regions of outer retina from a hyperoxic detached region. They are immunolabeled for bFGF (green) and for glial fibrillary acidic protein (red). The third label (blue) is PNA labeling of cone sheaths. (K to N) In situ hybridization for bFGF mRNA in (K and L) attached and detached normoxic retina and in (M and N) attached and detached hyperoxic retina. is, layer of inner segments; olm, outer limiting membrane; os, layer of outer segments.

FIGURE 5. Quantification of the effects of oxygen supplementation on cytochrome oxidase labeling of (A) inner segments and (B) synaptophysin in the outer plexiform layer. The number near each bar shows the number of comparisons of attached and detached retina that were made. The error bars show the ranges of values obtained for the attached to detached signal ratios. The probability that the values obtained from normoxic and hyperoxic tissue represent the same distribution of label was low: (A) \( P < .042 \) on a one-tailed \( t \) test for cytochrome oxidase and (B) \( P < .038 \) on a two-tailed \( t \) test for synaptophysin in the outer plexiform layer.
indicate that hypoxia is such a factor and suggest an intervention, oxygen supplementation between diagnosis and reattachment surgery, that may counter it. Further work may disclose other factors that upregulate self-destructive mechanisms, such as hypoglycemia, in photoreceptors in detached retina and open corresponding opportunities to improve clinical management of the detached retina. The companion article by Lewis and associates29 presents evidence that oxygen supplementation also mitigates the proliferation of retinal glia induced by detachment. Careful trials will be necessary to extend these experimental findings to the clinical situation.

ACKNOWLEDGMENT
The authors wish to thank Peter J. Kappel and Tania Novikova for skilled technical assistance.

REFERENCES