The Efficacy of Delayed Oxygen Therapy in the Treatment of Experimental Retinal Detachment

GEOFFREY P. LEWIS, PHD, KEVIN C. TALAGA, BA, KENNETH A. LINBERG, MA, ROBERT L. AVERY, MD, AND STEVEN K. FISHER, PHD

• PURPOSE: To evaluate the ability of delayed hyperoxia to slow or prevent degenerative and gliotic changes initiated by retinal detachment.
• DESIGN: An experimental study.
• METHODS: Rhegmatogenous detachments were produced in the right eyes of eight cats. After 1 day in room air (21% O_2), four cats were placed in chambers with the O_2 concentration regulated at 70%; the other four were left in room air. At 7 days the retinas were harvested and examined by light and confocal microscopy. Cell specific antibodies, TUNEL and proliferation assays, outer segment length, and photoreceptor counts, were used to assess the condition of the retina. The contralateral unoperated eyes were used as controls.
• RESULTS: Animals maintained in elevated O_2 showed a dramatic preservation of rod and cone outer segments as well as in the organization of the outer plexiform layer. The number of surviving photoreceptors was increased in the hyperoxia-treated animals. Neurite sprouting, a characteristic of detached retina, was rarely observed in the experimental eyes. Proliferation of non-neuronal cells was reduced, but not halted, by hyperoxia. GFAP and vimentin expression was not effected by hyperoxia; these intermediate filament proteins increased in Müller cells similar to that observed in control detachments.
• CONCLUSIONS: Exposure to hyperoxia, delayed by 1 day after the onset of retinal detachment, was highly effective in preserving photoreceptor cells and in reducing proliferation within the retina. It did not, however, reduce the hypertrophy of Müller glia. There were no apparent detrimental effects of exposure to 70% O_2 for 6 days. These results suggest that human patients may benefit from breathing elevated oxygen levels while awaiting reattachment surgery, even if the hyperoxia is delayed relative to the time of detachment. (Am J Ophthalmol 2004;137:1085–1095. © 2004 by Elsevier Inc. All rights reserved.)

RHEGMATOGENOUS RETINAL DETACHMENT IS DEfined as the separation of the neural retina from the underlying epithelial cell layer and vascular supply with a *hole* or tear occurring across the retina between the vitreous and subretinal space. Here we use the simpler term "detachment," because comparisons between this animal model, in which a small full-thickness *break* is produced, animal models in which a larger *break* is produced, and samples from human complex detachments show that we are modeling the cellular effects of a rhegmatogenous detachment (Sethi, CS. ARVO meeting, 2001, Abstract). Detachment can result from trauma as well as diseases of the eye but always causes visual impairment in the detached region. Even though surgical repair of detachment is remarkably successful, patients often describe lingering defects in color perception as well as a decline in visual acuity, and in fact, improvement in visual acuity has been observed to continue for years after surgery.[1,2] This suggests that reattachment alone is not always adequate to initiate complete visual recovery and indicates the need for additional therapeutic intervention.

While the immediate loss of visual capacity after detachment may be attributable to the retina being physically moved to a new focal plane, the more serious loss probably comes from cellular effects starting with the degeneration of photoreceptor outer segments.[3–6] Other events besides outer segment degeneration, especially in rhegmatogenous detachments, may contribute to the imperfect return of vision after reattachment. It has been shown previously that detachment in a feline model initiates a cascade of events in photoreceptors, including significant loss of structural integrity and changes in protein expression[7–10] as well as the actual loss of some cells by apoptosis.[11] Additional cellular events that seem

Biosketch and/or additional material at www.ajo.com.
Accepted for publication Jan 14, 2004.
From the Neuroscience Research Institute (G.P.L., K.C.T., K.A.L., R.L.A., S.K.F.) and Department of Molecular, Cellular, & Developmental Biology (S.K.F.), University of California, Santa Barbara, Santa Barbara, California.
Inquiries to Geoffrey P. Lewis, Neuroscience Research Institute, University of California, Santa Barbara, Santa Barbara CA 93106; fax: 805–893–2005; e-mail: g_lewis@lifesci.ucsb.edu

0002-9394/04/$30.00
doi:10.1016/j.ajo.2004.01.045

TABLE 1. A List of Molecular Probes Used in This Study

Probe	Dilution	Source
Anti-glial fibrillary acidic protein	1:500	DAKO, Carpinteria, CA
Anti-rod opsin	1:500	Dr. R. Molday, Univ. British Columbia, BC
Anti-M cone opsin	1:2000	Dr. J. Nathans, Johns Hopkins Univ., Baltimore, MD
Anti-vimentin	1:500	DAKO, Carpinteria, CA
Anti-calbindin D	1:1000	Sigma, St. Louis, MO
Anti-S cone opsin	1:2000	Dr. J. Nathans, Johns Hopkins Univ., Baltimore, MD
Anti-cytochrome oxidase	1:100	Molecular Probes, Eugene, OR
Anti-synaptophysin	1:100	DAKO, Carpinteria, CA
Anti-neurofilament	1:500	Biomeda, Hayward, CA
Anti-protein kinase C	1:100	Biomol Research Labs, Plymouth Meeting, PA
Biotinylated peanut agglutinin	1:50	Vector labs, Burlingame, CA
Anti-36H	1:100	Dr. S. Hoffman, Med. Univ. of S Carolina, Charleston, SC
Anti-cellular retinaldehyde binding protein	1:400	Dr. J. Saari, Univ. WA, Seattle
Anti-glutamine synthetase	1:600	Dr. P. Linser, Univ. FL, St. Augustine, FL
Anti-carbonic anhydrase II	1:600	Dr. P. Linser, Univ. FL, St. Augustine, FL
Anti-protein kinase C	1:100	Biomol Research Labs, Plymouth Meeting, PA

Those joined by a line segment were used together in double-labeling experiments.

likely to affect visual recovery include neurite outgrowth from second- and third-order neurons,[10,12,13] proliferation of non-neuronal cells,[14,15] and extensive hypertrophy of Müller cells.[16,17] All of these changes are highly predictable in the animal model and can be used as indicators of the response of the retina after therapeutic intervention.[18–20]

The photoreceptors of the retina are among the most metabolically active cell types in the body[21,22] and are dependent on the choroidal vascular supply for 90% of their oxygen[23] and other nutrients. Physical measurements of O_2 gradients and modeling of events after detachment suggest that detachment will produce hypoxia in photoreceptors.[24] Experimentally increasing the environmental oxygen concentration to 70% mitigates the effects of detachment both in rod-dominant feline[18,25] and cone-dominant ground squirrel retinas.[26] While rapid reattachment appears to slow or reverse many of the events initiated by detachment,[20] there will always be practical limits on how rapidly reattachment can occur. Therefore, some type of adjunctive therapy that would delay the degenerative effects would seem desirable. In our previous studies, hyperoxia delivered immediately after creating a detachment dramatically reduced the cellular effects of detachment. In practice, however, it would be rarely possible to begin hyperoxia treatment immediately after the retina detaches. The purpose of this study was to evaluate the effects of hyperoxia when treatment was delayed by 24 hours, thereby more closely modeling what may occur in a clinical setting.

METHODS

• **RETINAL DETACHMENTS:** The retinal detachment procedure used in our feline model is described in detail in Lewis and associates.[19] In this study, the lens and vitreous were removed from the right eyes of eight cats and a 0.25% solution of sodium hyaluronate (Healon; Pharmacia, Kalamzoo, Michigan, USA) in balanced saline solution (Alcon, Ft. Worth, Texas, USA) was infused between the neural retina and the retinal pigment epithelium using a glass micropipette. A large retinal *break* was not produced by the procedure but the *access retinotomy* created by the pipette remained. The detachments were created in the superior retina, thus covering approximately 50% of the retinal surface. After the detachment, all animals were returned to a central animal holding room, where they breathed room air (≈21% O_2) for 24 hours. After this period, four animals were placed for 6 days, in large Plexiglas chambers, where the O_2 concentration was maintained at 70% by a feedback control system ("Oxycycler"; BioSpherix, Ltd., Redfield, New York). As controls, the remaining four animals continued breathing room air for the next 6 days. The total detachment time for both groups was 7 days. The opposite eye of each animal served as a control. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the UCSB Institutional Animal Care and Use Committee.

• **TISSUE PREPARATION:** After the 7-day experimental period, animals were euthanized with sodium pentobarbital (120 mg/kg) and the eyes bisected. One half was prepared for optimum histologic analysis by fixing the retina in 1% paraformaldehyde and 1% glutaraldehyde (both from Electron Microscopy Sciences, Fort Washington, Pennsylvania) in phosphate buffer (PBS; 0.086 mol/l, pH 7.3) overnight at 4 C. The tissue was then fixed in osmium tetroxide (2%) for 1 hour, dehydrated in increasing con-

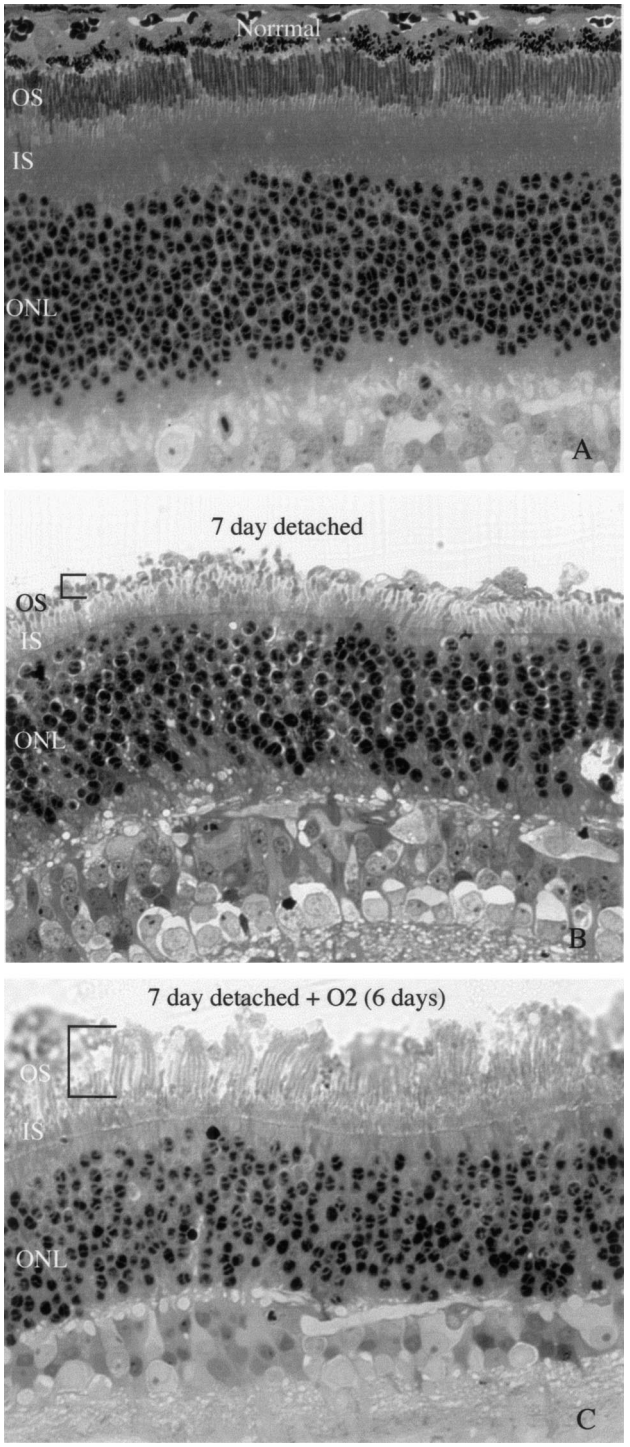

FIGURE 1. Light micrographs showing the outer retina from control (A), 7-day detached (B), and 7-day detached plus 6 days of oxygen (C). After 7 days of detachment in normoxia the outer segments are highly truncated and disorganized (B, brackets), and the ONL appears less densely packed compared with control retina. In hyperoxia-treated animals, the outer segments are longer and more organized (C, brackets), although the ONL is not as densely packed as in control retina. OS, outer segments; IS, inner segments; ONL, outer nuclear layer.

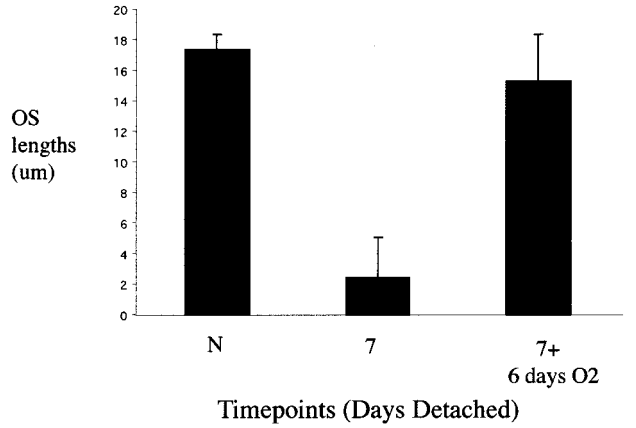

FIGURE 2. Width of the outer segment layer in control retinas (N), retinas detached for 7 days in normoxia (7), and retinas detached for 7 days plus 6 days in hyperoxia (7 plus 6 days O_2).

centrations of ethanol, and embedded in Spurrs resin (Polysciences, Warrington, Pennsylvania). Each retinal location was sectioned at 1-μm thickness and counterstained with toluidine blue and saturated aqueous p-phenylenediamine to enhance the appearance of the outer segments.

The other half of the eye was prepared optimally for immunocytochemical analysis by fixing and storing the retinas in 4% paraformaldehyde in sodium cacodylate buffer (0.1 mol/l; pH 7.4; Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA). Before sectioning, 2-mm^2 pieces of retina were excised, rinsed in PBS and embedded in 5% agarose (Sigma, St Louis, Missouri, USA) in PBS. Sections (100 μm) were cut using a Vibratome (Technical Products International, Polysciences, Warrington, Pennsylvania, USA) and incubated in normal donkey serum (1:20; Jackson ImmunoResearch, West Grove, Pennsylvania, USA) in PBS containing 0.5% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, Pennsylvania, USA), 0.1% Triton X-100 (Boehringer Mannheim, Indianapolis, Indiana, USA) and 0.1% sodium azide (Sigma, St. Louis, Missouri, USA) overnight at 4 C on a rotator (PBS + BSA + triton + azide = PBTA). The next day the blocking serum was removed and primary antibodies added. Six sets of double-label combinations were used as listed in Table 1. All probes were diluted in PBTA. After rotating overnight at 4 C on a rotator, the sections were rinsed in PBTA and incubated in the secondary antibody overnight at 4 C on a rotator. For each double-label combination, donkey anti–mouse and donkey anti–rabbit, conjugated to Cy2 or Cy3 (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) were used. Streptavidin-Cy3 was used with the biotinylated PNA. All secondary reagents were used at a 1:200 dilution. The sections were rinsed, mounted in n-propyl gallate in glycerol, and viewed using a laser scanning confocal microscope (BioRad 1024, Hercules, California, USA).

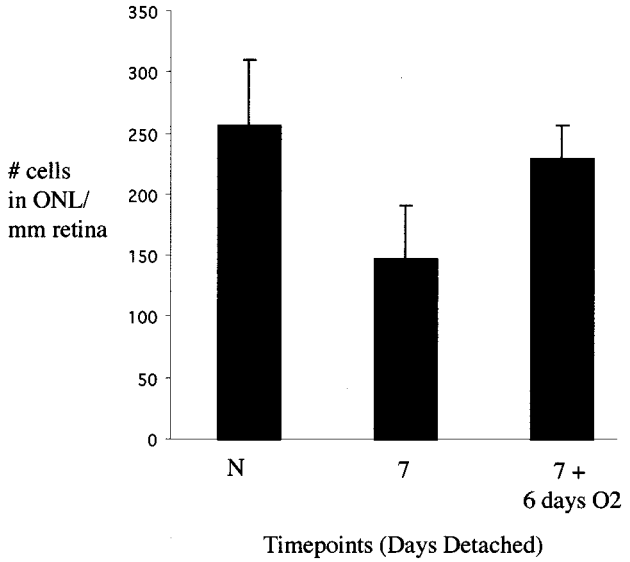

FIGURE 3. The number of photoreceptor nuclei counted/mm of retina in control retinas (N), retinas detached for 7 days in normoxia (7) and retinas detached for 7 days plus 6 days in hyperoxia (7 plus 6 days O_2).

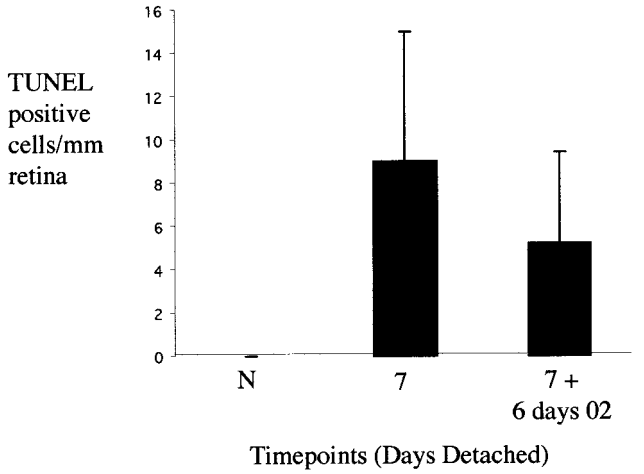

FIGURE 4. The number of TUNEL positive cells counted/mm of retina in control retinas (N), retinas detached for seven days in normoxia (7), and retinas detached for seven days plus six days in hyperoxia (seven plus six days O_2).

To evaluate cellular proliferation, retinal samples were fixed in 4% paraformaldehyde in sodium cacodylate buffer (0.1 mol/l; pH 7.4) overnight at 4 C. The tissue was dehydrated in increasing concentrations of ethanol and embedded in wax (Paraplast X-tra, Fisher Scientific, Pittsburgh, Pensylvania, USA). The tissue was sectioned at 4 μm and placed on capillary gap slides (Fisher Scientific), dewaxed in xylene, rehydrated in graded ethanol, and labeled with the MIB-1 antibody (1:100; DAKO, Carpinteria, California, USA) to the Ki67 protein. At least three areas were sampled from each eye, and three sections within these areas were labeled. Anti-MIB-1-labeled cells were counted, and the section length was measured to give the number of cells labeled/mm of retina.

To assess the occurrence of apoptotic cell death, retinal samples were embedded in agarose as described above and stained using the TdT-dUTP terminal nick-end labeling (TUNEL) method. Briefly, 100 μm thick Vibratome sections were rinsed in PBS, incubated in 70% ethanol for 30 minutes, rinsed in water, incubated in a citrate/triton solution, washed in water, incubated in the TdT buffer for 30 minutes, and finally the TdT reaction solution for 2 hours in a humidified chamber at 37 C. After rinsing in PBS and then BSA with triton, streptavidin conjugated to Cy3 (1:250, Jackson ImmunoResearch) was added for 3 hours. At least three areas were sampled from each eye, and three sections within these areas were probed. TUNEL-labeled cells were counted, and the section length was measured to give the number of cells labeled/mm of retina.

Outer segment measurements and counts of nuclei in the ONL were made on 1 μm-thick resin embedded sections stained with toludine blue and p-phenylenediamine. For sampling, sections were taken from three different regions from three eyes within each condition. The width of the outer segment layer was measured using an optical reticule in three representative areas on each section, whereas counts of photoreceptor nuclei came from one region at the center of each section.

RESULTS

• **LIGHT MICROSCOPY AND MEASUREMENTS:** Hyperoxia had a dramatic effect on the preservation of photoreceptors, and, in particular, their outer segments. In control retinas, the outer segments are highly organized in parallel arrays (Figure 1, A). After 7 days of detachment they become truncated and disorganized (Figure 1, B). In the animals with detachments but exposed to 6 days of hyperoxia, the outer segments appear equally as long as those in the nondetached control retinas (Figure 1, C) and were considerably more organized when compared with retinas without hyperoxia. This observation is reflected in the outer segment length data (Figure 2). Outer segments averaged 17.4 μm long in control retinas and only 2.5 μm after 7 days of detachment in normoxic animals. In the hyperoxic animals, however, the outer segments averaged 15.3 μm in length. Even though there was some variability within retinal regions in these animals, as indicated by the error bars, the mean was not statistically different from that obtained in control retinas.

Also apparent in the light micrographs are morphologic changes in the outer nuclear layer (ONL) after detachment (Figure 1). Normally, the photoreceptor cell bodies are packed closely together (Figure 1, A), however, after detachment in either room air or hyperoxia, there are large

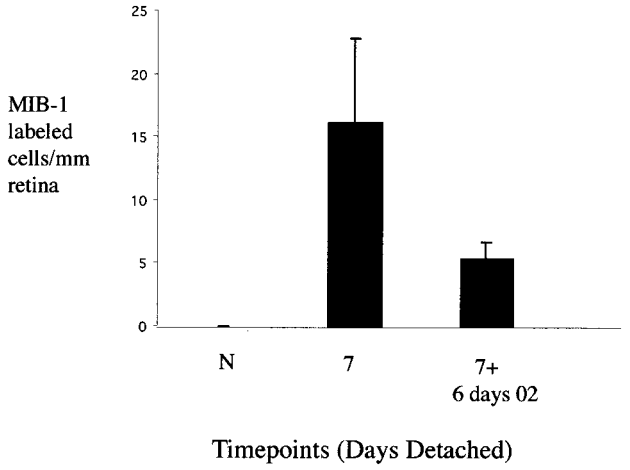

FIGURE 5. The number of MIB-1 labeled cells (that is, proliferating cells) counted/mm of retina in control retinas (N), retinas detached for 7 days in normoxia (7), and retinas detached for 7 days plus 6 days in hyperoxia (7 plus 6 days O_2).

gaps present among the photoreceptor nuclei (Figure 1, A and B). In the hyperoxia-treated retinas we found an average of 230 cells/mm of retina in the ONL compared with 147 cells/mm in the control detachments ($P = .007$) (Figure 3). However, the number of cells in the ONL of the hyperoxia-treated retinas is not considered significantly different from that observed in the nondetached control retinas (230 vs 257). As suggested by the ONL counts, hyperoxia does not completely stop cell death; TUNEL-positive cells occur in both sets of experimental retinas in roughly the same number at 7 days (Figure 4); nine cells/mm retina in control detachments vs 5.2 cells in hyperoxia-treated detachments; $P = .1$). However, 7 days are well past the peak (3 days) of apoptosis in the feline model of detachment,[11] so that the actual effect on apoptotic cell death is not shown in these data but is more accurately reflected in the cell counts. No TUNEL labeling was observed in the contralateral control eyes from normoxia- or hyperoxia-treated animals (data not shown).

• **NON-NEURONAL CELL PROLIFERATION:** After a detachment period of 7 days in normoxia, approximately 16 cells/mm retina were labeled with the MIB-1 antibody (Figure 5). Labeled cells include Müller cells, astrocytes, endothelial cells, pericytes, and microglia.[14,15] In hyperoxia, the number was significantly reduced to 5.4 cells/mm retina ($P = .009$). Additionally, no MIB-1 labeling was observed in the contralateral control eyes from either the hyperoxia- or normoxia-treated animals, indicating that oxygen, given for a period of 6 days, does not stimulate proliferation in the retina.

• **IMMUNOHISTOCHEMISTRY:** As a general result, the antibody labeling patterns were identical in the "normal" contralateral eyes of animals exposed to room air and those exposed to hyperoxia (data not shown).

• **PHOTORECEPTORS:** In the normal retina, anti-M cone opsin labels a subpopulation of outer segments, assumed to be those of M cones (Figure 6, A; red). After a 7-day detachment with the animals in room air, most sampling areas had no outer segments labeled with this antibody (Figure 6, B), although a few greatly truncated outer segments were occasionally observed in retinal folds (Figure 6, C; arrows). In hyperoxia-treated animals, labeled cone outer segments were routinely detected (Figure 6, D, E and F). The labeled cone outer segments usually appeared well organized and almost the same length as those observed in normal control retinas. Anti-S cone opsin, which labels only S cone outer segments, and anti–calbindin D, which labels both cone cell types, showed similar results to those observed with the M cone opsin; that is, these proteins are lost or downregulated after detachment in normoxia[9] and preserved in hyperoxia (data not shown).

Biotinylated PNA and anti-36H were used to examine the status of the interphotoreceptor matrix after detachment. In the normal retina biotinylated PNA labels the matrix sheath surrounding the cone outer and inner segments, whereas anti-36H, a marker for the core protein of a currently uncharacterized matrix proteoglycan (Johnson, PT. ARVO meeting, 2002, Abstract), labels the matrix associated with rods (Figure 6, G; red and green). In the control detachments labeling occurs as a narrow band around the inner segments, indicating a greatly reduced IPM (Figure 6, H).) Additionally, there is an accumulation of punctate labeling in the ONL with the 36H antibody that is not observed in control retinas. In hyperoxia-treated detached retinas, both rod and cone matrix domains appear remarkably well-preserved and there is no detectable anti-36H labeling in the ONL (Figure 6, I).

The anti–rod opsin labeling shows a dramatic preservation of rod photoreceptors by hyperoxia comparable to that observed for cones (Figure 7, A through C; red). In the normal retina, rod opsin is detected in the outer segment region of the cell (Figure 7, A). In the animals that remain in room air, rod opsin-labeling becomes redistributed to the rod cell bodies as the outer segments degenerate (Figure 7,B). In the animals maintained in hyperoxia, this redistribution is infrequently observed, as the rod outer segments maintain their length and overall organization (Figure 7,C).

The synaptic terminals of rods also undergo less remodeling in the hyperoxia-treated animals, as illustrated with anti–synaptophysin labeling (Figure 7 D, E and F; green). In the normal retina, this antibody labels synaptic vesicles in the rod and cone synaptic terminals in the outer plexiform layer (OPL) (Figure 7 D). In the control detachments this labeling can be observed throughout the ONL, leaving the OPL highly disorganized (Figure 7, E; arrows).

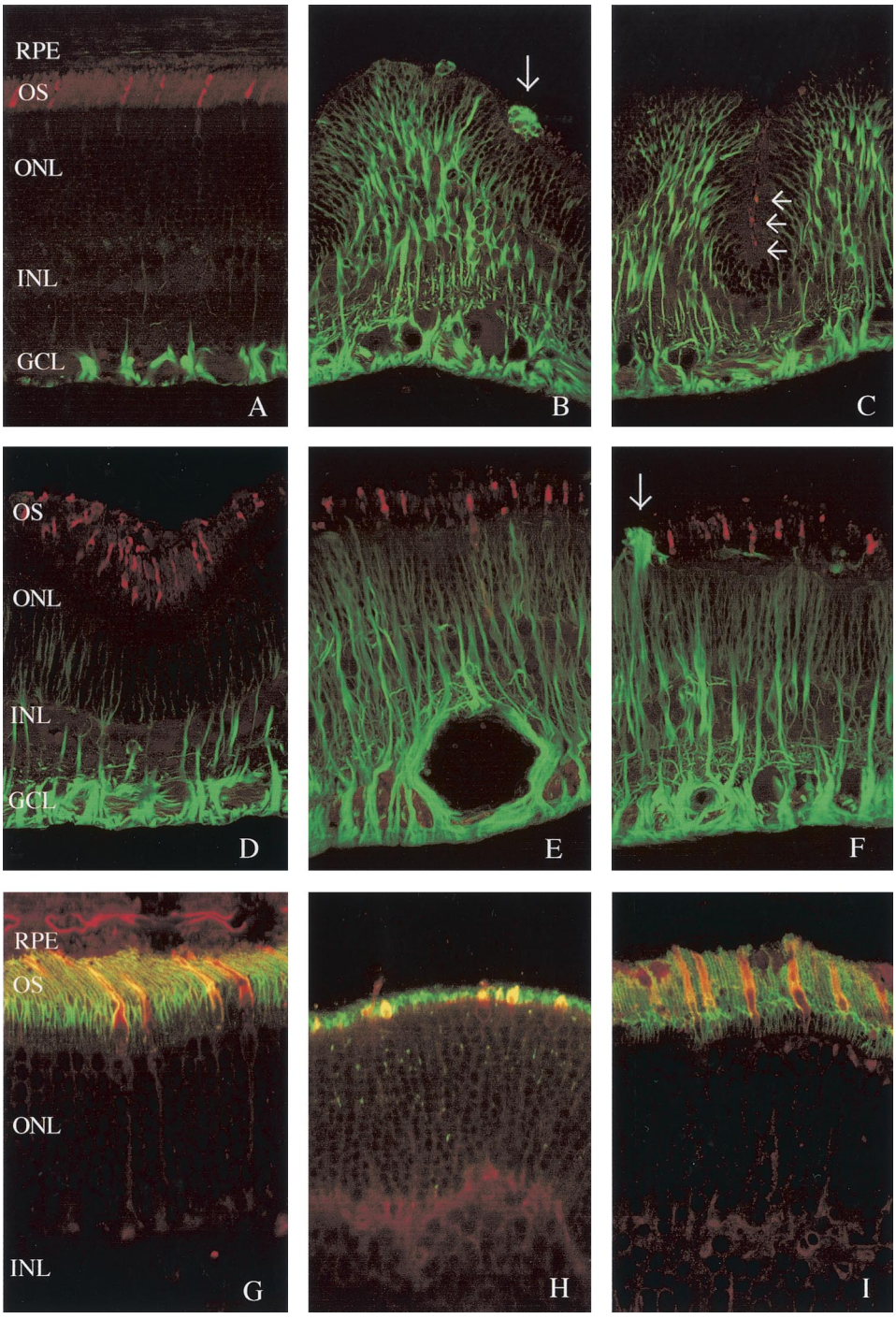

FIGURE 6. Laser scanning confocal microscope images showing changes in antibody labeling of photoreceptor and Müller cell proteins that occur after detachment in normoxia and hyperoxia. In control retina, anti-M cone opsin labels the cone outer segments (OS) of the M cones (A, red). After a 7-day detachment in normoxia, usually no labeling is observed in the cones (B), although occasional highly truncated outer segments can be detected within retinal folds (C, arrows). In hyperoxia-treated detachments, relatively long and organized cone outer segments can always be detected (D, E, F, red). Anti-36H (green) and biotinylated PNA (red) label the matrix associated with the rod (green) and cone (red) photoreceptors (G, H, I). After a 7-day detachment in normoxia, the interphotoreceptor matrix becomes highly degenerated (H) compared with that observed in control retina (G). In detachments maintained in hyperoxia, the matrix is remarkably preserved (I). Anti–vimentin labels only the end foot portion of the Müller cell in control retina (A, green). After a 7-day detachment in normoxia, anti–vimentin labeling increases in the Müller cells throughout the retina (B, C, green) and occasionally into the subretinal space (B, arrow, green). In detachments maintained in hyperoxia (D, E, F, green), anti–vimentin labeling appears, in most cases, very similar to that observed in detachments maintained in normoxia, although slightly less labeling can sometimes be seen (D, green). Müller cell growth can also be observed extending into the subretinal space (F, arrow, green). RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

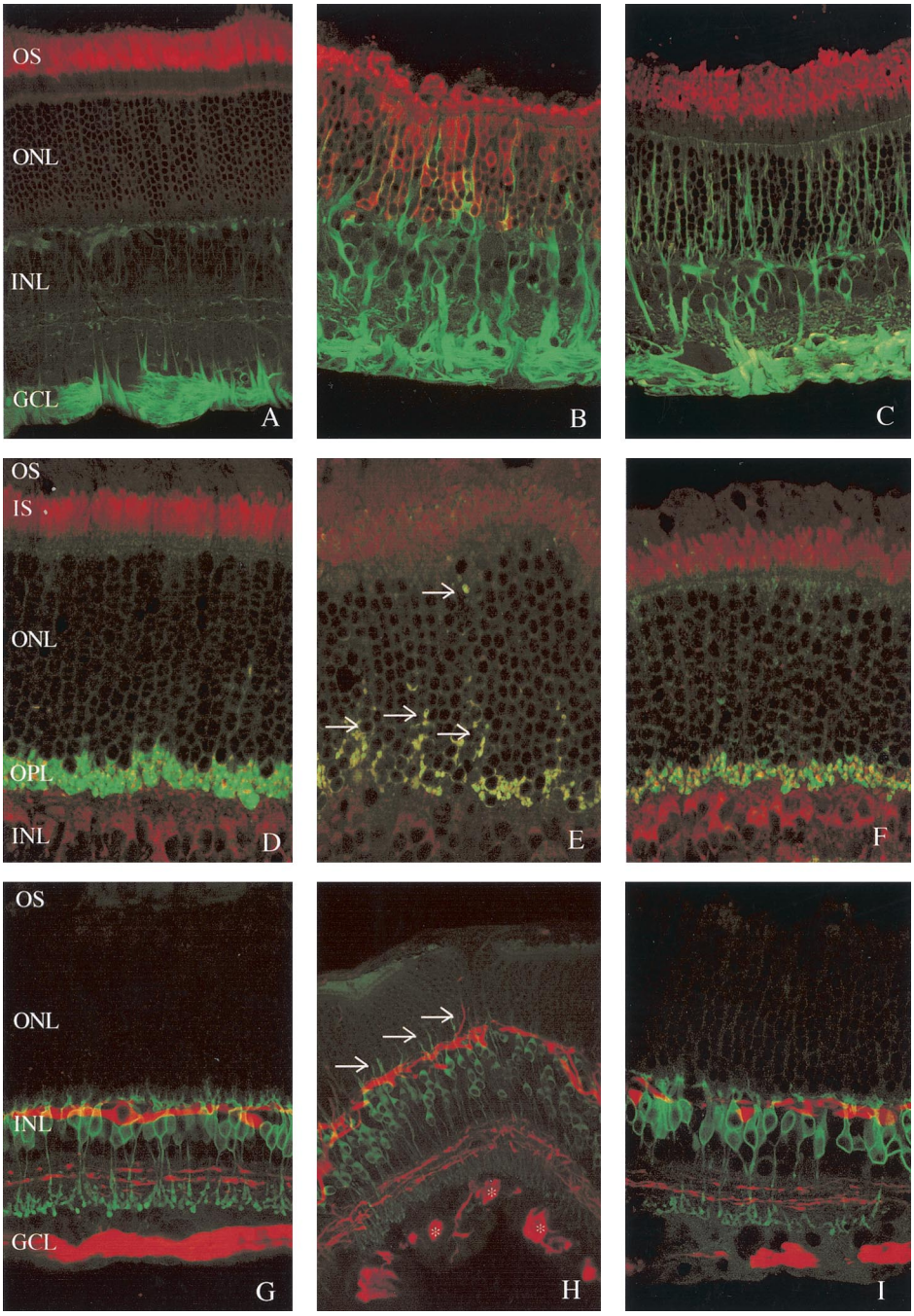

FIGURE 7. Laser scanning confocal images showing the changes in the labeling patterns of various proteins in normal (A, D, G), 7-day detachments maintained in normoxia (B, E, H) and 7-day detachments maintained in hyperoxia (C, F, I). Anti–rod opsin labels rod outer segments (OS) only in control retina (A, red). After a 7-day detachment maintained in normoxia, anti–rod opsin labels the highly truncated OS as well as the opsin that is redistributed to the rod cell bodies in the outer nuclear layer (ONL; B, red). In detachments maintained in hyperoxia, anti–rod opsin labels only the long rod OS (C, red). Anti–glial fibrillary acidic protein (GFAP) labels Müller cell end feet and astrocyte processes in control retina (A, green), and increases in Müller cells after detachment in both normoxia (B, green) and hyperoxia, (C, green). Anti–synaptophysin labels rod and cone synaptic terminals within the OPL in control retina (D, green). After detachment in normoxia, many synaptic terminals that have retracted from the OPL are observed in the ONL (E, arrows, green). In hyperoxia detachments, very little terminal retraction is observed, leaving the OPL more organized (F, green). Anti–cytochrome oxidase labeling of mitochondria in the inner segments (IS) appears relatively intense in control retina (D, red), decreases in normoxia detachments (E, red) but appears closer to control intensities in hyperoxia detachments (F, red). (Labeling in the INL also appears more intense in the hyperoxia treated retinas; F, red.) Anti–protein kinase C labels rod bipolar cells in control retina (G, green). After a 7-day detachment in normoxia, many fine dendritic outgrowths can be observed extending into the ONL (H, green, arrows) and these extensions are rarely observed in the hyperoxia detachments (I, green). Anti–neurofilament protein labels horizontal cells and ganglion cell axons in control retina (G, red). After detachment in normoxia, horizontal cell outgrowths can be seen in the ONL (H, red), and ganglion cell bodies become immunoreactive (H, red, asterisks). Horizontal cell outgrowths and immunoreactive ganglion cells are rare in detachments maintained in hyperoxia (I, red). OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.

Anti–synaptophysin labeling in the ONL has been shown by electron microscopy to correspond to rod terminals that are withdrawn into the ONL.[12] Hyperoxia, however, prevents the withdrawal of the synaptic terminals, resulting in an almost normal-appearing pattern of anti–synaptophysin labeling in the OPL (Figure 7, F).

Anti–cytochrome oxidase, a marker for mitochondria, also shows a dramatic change in photoreceptors after detachment (Figure 7 D, E and F; red). In the normal retina, the most concentrated labeling occurs in the inner segments of the photoreceptors (Figure 7 D). The intensity of this labeling is greatly reduced in the control detachments (Figure 7, E) but appears closer to that in normal retinas in the hyperoxia-treated animals (Figure 7, F). Single, very large mitochondria occur in cone synaptic endings, and these are easily visualized by the anti–cytochrome oxidase labeling (Figure 7 D). When the retina is detached, these intensely labeled structures disappear from the OPL (Figure 7, E). They are, however, present in the retinas of animals kept in hyperoxia (Figure 7F).

Second- and third-order neurons undergo fewer changes in hyperoxia than in normoxia. In the normal retina anti-PKC labels rod bipolar cells (Figure 7, G; green) Anti-neurofilament labels horizontal cells and ganglion cell dendrites and axons but not the cell bodies (Figure 7, G; red). After detachment in normoxia, labeled rod bipolar and horizontal cell, neurites can be identified extending deep into the ONL (Figure 7, H; arrows)) and ganglion cell bodies become intensely labeled with the neurofilament antibody (Figure 7, H; asterisks). In hyperoxia-treated animals, there is little evidence of second-order neuron sprouting and the ganglion cell labeling is limited to dendrites and axons as occurs in the normal retina (Figure 7, I).

• **MÜLLER CELLS:** The intermediate filament proteins GFAP and vimentin are good indicators of the presence of a retinal detachment; these proteins are dramatically upregulated in the Müller cells in detached retina with little to no increase in the adjacent attached retina.[27] In control retina, vimentin (Figure 6, A; green) and GFAP (Figure 7, A; green) are located in the end foot region of the Müller cell (GFAP is also present in the astrocytes within the ganglion cell layer). After 7 days of detachment in room air, both of these proteins noticeably increase their distribution in the Müller cell cytoplasm, now extending to the level of the outer limiting membrane and thus filling nearly the entire cell with intermediate filaments (Figure 6, B) and (Figure 7, B). Also at this time, some Müller cell processes can be observed extending into the subretinal space (Figure 6, B, arrow). The delayed hyperoxia appears to have little effect on the upregulation of these intermediate filament proteins. While in some areas, their expression appears lower than what would be expected at this detachment time (Figure 6 D), for the most part, labeling extends throughout the Müller cells (Figure 6, E) and (Figure7, C) and, in some cases, can be observed in the process of growing into the subretinal space (Figure 6, F; arrow).

While the increased expression of intermediate filament proteins appears to occur unabated when hyperoxia is delayed by a day, it does appear that the observed down-regulation of several cytoplasmic Müller cell proteins that routinely accompanies detachment is affected. In the normal retina labeling with antibodies to glutamine synthetase (GS), cellular retinaldehyde binding protein (CRALBP), and carbonic anhydrase II (CAII) is found throughout the Müller cell cytoplasm, with the heaviest labeling occurring in the end foot region (Figure 8, A, D, and G). In normoxic control detachments the labeling declines to almost undetectable intensities (Figure 8, B, E, and H). Hyperoxia, however, results in labeling intensities closer to those observed in normal retina (Figure 8, C, F, and I).

DISCUSSION

THREE PREVIOUS STUDIES PRODUCED RESULTS STRONGLY suggesting that the simple breathing of 70% oxygen for three days, beginning immediately after the production of a retinal detachment, could have many beneficial effects on the retina.[18,25,26] These results, which include preservation of photoreceptors and a significant diminution of inner retinal effects, including glial proliferation, may well result in superior visual recovery upon reattachment and perhaps lessen the incidence of complications such as proliferative vitreoretinopathy (PVR). Determining the effects on visual outcome is difficult in animal models, although preliminary data using the cone-dominated ground squirrel retina suggests a more rapid recovery of S- and M-cone-specific electroretinogram signals in animals treated with hyperoxia (Jacobs GH, unpublished data, 2003). The issue addressed by this study is when hyperoxia therapy begins, with respect to the starting point of the detachment, and the results show very clearly that photoreceptors are rescued to a significant degree, even when hyperoxia is delayed for 24 hours after the production of a detachment. This rescue includes both maintaining photoreceptor structure and decreases the amount of cell death.

Photoreceptor hypoxia has been proposed as the first step in a cascade of cellular events that occur after detachment.[25] Neuronal remodeling and glial reactivity were hypothesized to be "downstream" from cellular events originating in photoreceptors. The results from this study suggest that some effects on Müller cells may occur independently of the effects on photoreceptors. Müller cell proliferation was significantly reduced by delayed hyperoxia, and the expression levels of soluble proteins appeared to be closer to those in normal retinas, thus correlating

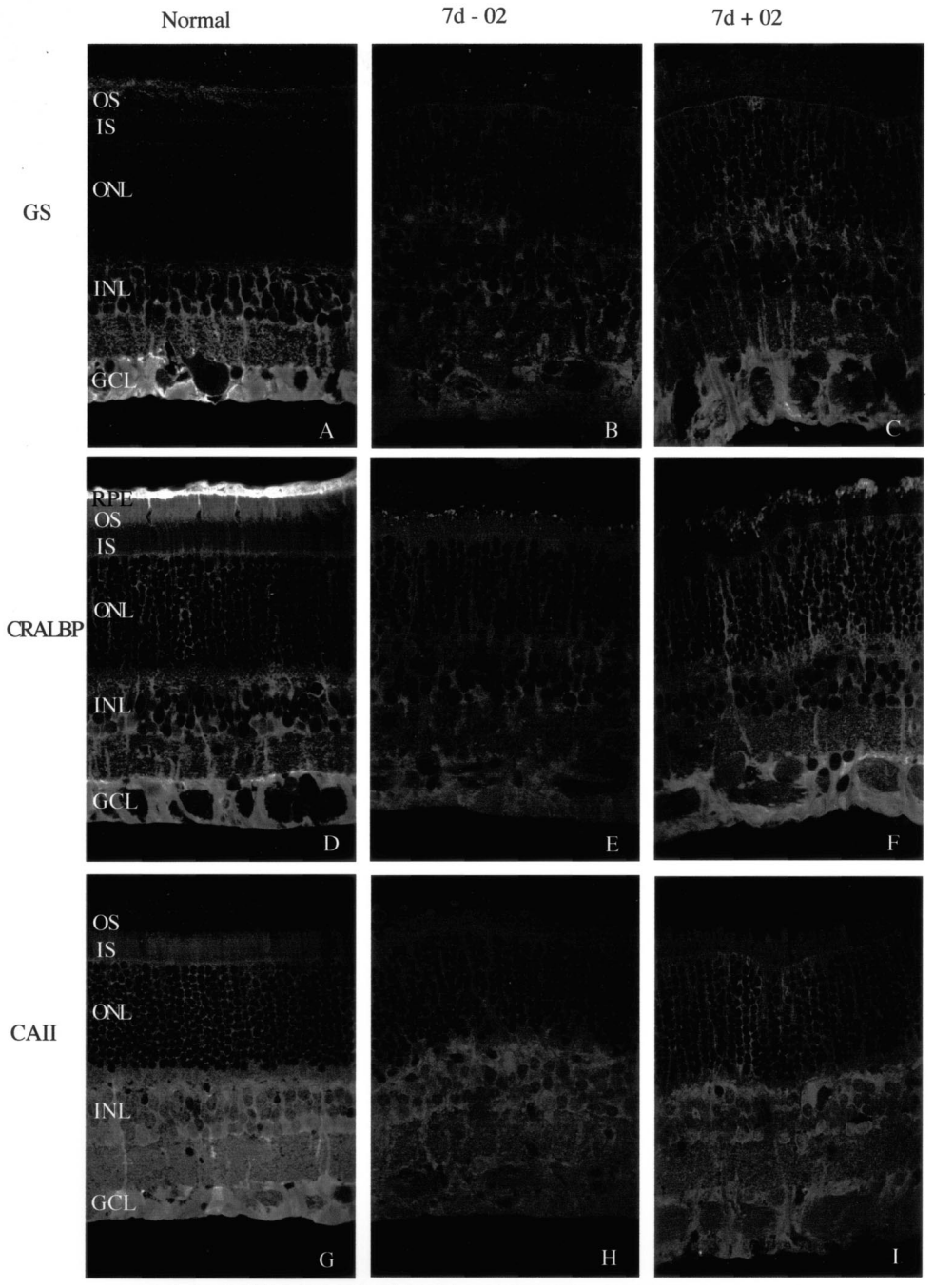

FIGURE 8. Laser scanning confocal images of Müller cell cytoplasmic proteins glutamine synthetase (GS; A, B, C), cellular retinaldehyde binding protein (CRALBP; D, E, F), and carbonic anyhydrase II (CAII; G, H, I) in control (normal, A, D, G), 7-day detachments maintained in normoxia (7d - O2; B, E, H), and 7-day detachments maintained in hyperoxia (7d + O2; C,F,I). All three proteins are present in Müller cells in the normal retina, decrease their expression after detachment in normoxia, and are expressed at more normal intensities in the detachments maintained in hyperoxia. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

with photoreceptor rescue. The increase in intermediate filament proteins was not affected nor apparently was the propensity of Müller cells to grow in the subretinal space, suggesting that these events proceeded even with a significant rescue of photoreceptors. Geller and associates[28] showed that signaling events occur in Müller cells within minutes of detachment, before any photoreceptor effects have been identified. Thus, there is the possibility that the increase in O_2 levels in the retina directly affects Müller cells by interrupting the early signaling events, a possibility

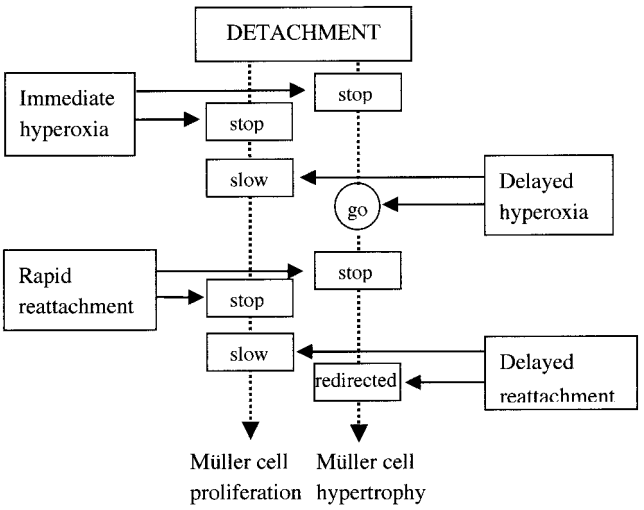

FIGURE 9. Retinal detachment induces pathways that lead to both the proliferation and hypertrophy of Müller cells. Treatments with hyperoxia and reattachment can modify these responses in similar but different ways.

supported by the fact that immediate hyperoxia is more efficacious in preventing proliferation, intermediate filament upregulation, and the subretinal growth of Müller cells than delayed hyperoxia.[18] Interestingly, the fact that only proliferation, and not hypertrophy, is effectively decreased when hyperoxia is delayed by 24 hours also suggests that these two events in Müller cells can occur independently. Hyperoxia at 24 hours postdetachment is apparently more effective at interrupting intracellular events leading to proliferation than to Müller cell hypertrophy.

There are some important similarities between the effects of hyperoxia[18] and those of reattachment[20,29] on Müller cell proliferation and hypertrophy (Figure 9). Immediate hyperoxia (within 1 hour) and immediate reattachment (within 1 day) both stop Müller cell proliferation and hypertrophy. Delayed hyperoxia (24 hours) and delayed reattachment (3 days) slow Müller cell proliferation but not hypertrophy. The hypertrophy continues unabated for 7 days with delayed hyperoxia. With delayed reattachment, the hypertrophy continues, but Müller cell growth is redirected from the subretinal space toward the vitreal cavity.[29] The similarities between results with hyperoxia and reattachment suggest that simple reoxygenation of the retina is a critical mechanism for recovery after reattachment. Because proliferation is slowed by delayed reattachment and hyperoxia, but Müller cell hypertrophy is not, augmenting reattachment with some form of treatment that prevents glial hypertrophy may be more effective in preventing the formation of subretinal and epiretinal membranes than using antiproliferative agents.

The TUNEL results suggest little difference between normoxic and hyperoxic animals at 7 days where there is still a low-level of apoptotic cell death occurring in both. The ONL cell counts suggest, however, that there is a significant effect on photoreceptor survival. This implies that hyperoxia suppressed apoptotic cell death earlier in the detachment period, probably during its peak at day 3.[11] It may not be surprising that hyperoxia does not completely suppress apoptosis. Evidence suggests that reattachment must occur within a day to stop this event.[20] If the retina remains detached for 3 days, we find TUNEL-positive cells and morphologic signs of apoptosis among photoreceptors 28 days later.[29]

Based on the result of anti–synaptophysin labeling, rod synaptic terminal retraction is dramatically reduced in the hyperoxia-treated animals as is neurite sprouting from second-order (rod bipolar and horizontal cell) and third-order (ganglion cell) neurons. Whether these latter events, downstream from photoreceptors, are diminished by the increased survival of photoreceptors or the superior preservation of their synaptic terminals is difficult to determine until we know more about signaling events that stimulate or inhibit neurite outgrowth. Neurite outgrowth in this model, however, is not a product of advanced retinal degeneration (as may be the case in retinitis pigmentosa[30]) but occurs rapidly within the first few days of detachment.[12]

Currently, rapid reattachment produces the best prognosis for a favorable outcome in visual recovery, but even under the best conditions, foveal involvement probably rules out a complete return to predetachment vision in most patients. Thus, adjuncts to reattachment may be significant contributors to visual recovery. Hyperoxia is an inexpensive, practical, and safe therapy that may prove beneficial to human patients in a variety of conditions ranging from detachment to macular translocation surgery or, in the future, techniques such as subretinal gene therapy or photoreceptor transplantation.[31,32]

REFERENCES

1. Burton TC. Recovery of visual acuity after retinal detachment involving the macula. Trans Am Ophthalmol Soc 1982;80:475–497.
2. Kusaka S, Toshino A, Ohashi Y, Sakaue E. Long-term visual recovery after scleral buckling for macula-off retinal detachments. Jpn J Ophthalmol 1998;42:218–222.
3. Machemer R. Experimental retinal detachment in the owl monkey: IV. The reattached retina. Am J Ophthalmol 1968;66:1075–1091.
4. Kroll AJ, Machemer R. Experimental retinal detachment in the owl monkey: V. Electron microscopy of reattached retina. Am J Ophthalmol 1969a;67:117–130.
5. Kroll AJ, Machemer R. Experimental retinal detachment and reattachment in the rhesus monkey. Am J Ophthalmol 1969b;68:58–77.
6. Anderson DH, Stern WH, Fisher SK, Erickson PA, Borgula GA. Retinal detachment in the cat: the pigment epithelial-photoreceptor interface. Invest Ophthalmol Vis Sci 1983;24:906–926.

7. Erickson P, Fisher S, Anderson D, Stern W, Borgula G. Retinal detachment in the cat: the outer nuclear and outer plexiform layers. Invest Ophthalmol Vis Sci 1983;24:927–942.
8. Fisher SK, Anderson DH. Cellular effects of detachment on the neural retina and the retinal pigment epithelium. In: Ryan S J, Wilkinson C P. Retina. Vol 3. Surgical Retina. St. Louis: Mosby, 2001:1961–1986.
9. Rex TS, Fariss RN, Lewis GP, Linberg KA, Sokal I, Fisher SK. A survey of molecular expression by photoreceptors after experimental retinal detachment. Invest Ophthalmol Vis Sci 2002;43:1234–1247.
10. Fisher SK, Lewis GP. Müller cell and neuronal remodeling in retinal detachment and reattachment and their potential consequences for visual recovery. Vis Res 2003;43:887–897.
11. Cook B, Lewis GP, Fisher SK, Adler R. Apoptotic photoreceptor degeneration in experimental retinal detachment. Invest Ophthalmol Vis Sci 1995;36:990–996.
12. Lewis GP, Linberg KA, Fisher SK. Neurite outgrowth from bipolar and horizontal cells following experimental retinal detachment. Invest Ophthalmol Vis Sci 1998;39:424–434.
13. Coblentz FE, Radeke MJ, Lewis GP, Fisher SK. Evidence that ganglion cells react to retinal detachment. Exp Eye Res 2003;76:333–342.
14. Fisher SK, Erickson PA, Lewis GP, Anderson DH. Intraretinal proliferation induced by retinal detachment. Invest Ophthalmol Vis Sci 1991;32:1739–1748.
15. Geller SF, Lewis GP, Anderson DH, Fisher SK. Use of the MIB-1 antibody for detecting proliferating cells in the retina. Invest Ophthalmol Vis Sci 1995;36:737–744.
16. Lewis GP, Guerin CJ, Anderson DH, Matsumoto B, Fisher SK. Rapid changes in the expression of glial cell proteins caused by experimental retinal detachment. Am J Ophthalmol 1994;118:368–376.
17. Lewis GP, Matsumoto B, Fisher SK. Changes in the organization of cytoskeletal proteins during retinal degeneration induced by retinal detachment. Invest Ophthalmol Vis Sci 1995;36:2404–2416.
18. Lewis GP, Mervin K, Valter K, et al. Limiting the proliferation and reactivity of retinal Müller cells during detachment: the value of oxygen supplementation. Am J Ophthalmol 1999a;128:165–172.
19. Lewis GP, Linberg KA, Geller SF, Guerin CJ, Fisher SK. Effects of the neurotrophin BDNF in an experimental model of retinal detachment. Invest Ophthalmol Vis Sci 1999b;40:1530–1544.
20. Lewis GP, Sethi CS, Charteris DG, Leitner WP, Linberg KA, Fisher SK. The ability of rapid retinal reattachment to stop or reverse the cellular and molecular events initiated by detachment. Invest Ophthalmol Vis Sci 2002;43:2412–2420.
21. Anderson B, Saltzman HA. Retinal oxygen utilization measured by hyperbaric blackout. Arch Ophthalmol 1964;72:792–795.
22. Ames A. III. Energy requirements of CNS cells as related to their function and to their vulnerability to ischemia: a commentary based on studies on retina. Can J Physiol Pharmacol 1992;70:S158–S164.
23. Wangsa-Wirawan ND, Linsenmeir RA. Retinal Oxygen. Fundamental and clinical aspects. Arch Ophthalmol 2003;121:547–557.
24. Linsenmeier RA, Padnick-Silver L. Metabolic dependence of photoreceptors on the choroid in normal and detached retina. Invest Ophthalmol Vis Sci 2000;41:3117–3123.
25. Mervin K, Valter K, Maslim J, Lewis GP, Fisher SK, Stone J. Limiting the death and deconstruction during retinal detachment: the value of oxygen supplementation. Am J Ophthalmol 1999;128:155–164.
26. Sakai T, Lewis GP, Linberg KA, Fisher SK. The ability of hyperoxia to limit the effects of experimental retinal detachment in cone dominated retina. Invest Ophthalmol Vis Sci 2001;42:3264–3273.
27. Lewis GP, Erickson PA, Guerin CJ, Anderson DH, Fisher SK. Changes in the expression of specific Müller cell proteins during long term retinal detachment. Exp Eye Res 1989;49:93–111.
28. Geller SF, Lewis GP, Fisher SK. FGFR1, signaling, and AP-1 expression following retinal detachment: reactive Müller and RPE cells. Invest Ophthalmol Vis Sci 2001;42:1363–1369.
29. Lewis GP, Sethi CS, Linberg KA, Charteris DG, Fisher SK. Experimental retinal reattachment: A new perspective. Mol Neurobiol 2003;28:159–175.
30. Fariss RN, Li ZY, Milam AH. Abnormalities in rod photoreceptors, amacrine cells, and horizontal cells in humans with retinitis pigmentosa. Am J Ophthalmol 2000;129:215–223.
31. Del Cerro M, Lazar ES, Diloreto D. The first decade of continuous progress in retinal transplantation. Micros Res Tech 1997;36:130–141.
32. de Juan E, Loewenstein A, Bressler NM, Alexander J. Translocation of the retina for management of subfoveal choroidal neovascularisation II: a preliminary report in humans. Am J Ophthalmol 1998;125:635–646.