The Ability of Hyperoxia to Limit the Effects of Experimental Detachment in Cone-Dominated Retina

Tsutomu Sakai,[1,2] *Geoffrey P. Lewis,* [1] *Kenneth A. Linberg,*[1] *and Steven K. Fisher*[1,3]

Purpose. To determine the ability of oxygen supplementation to ameliorate the effects of retinal detachment in a cone-dominated retina.

Methods. Retinal detachments were created in the right eyes of ground squirrels and the animals immediately placed in normoxic (room air) or hyperoxic (70% oxygen) conditions for 3 days. The retinas were sampled from different regions and investigated morphologically or immunocytochemically by light or confocal microscopy. Agarose embedded sections were immunostained with antibody probes to cytochrome oxidase, synaptophysin, medium-to-long wavelength–sensitive (M/L) cone opsin, rod opsin, excitatory amino acid transporter 1 (EAAT1), glutamate synthetase (GS), cellular retinaldehyde-binding protein (CRALBP), and peanut agglutinin (PNA) lectin. Retinal wholemounts were labeled with PNA and antibodies to short (S)-wavelength–sensitive cone opsin and rod opsin. Cell death was examined using a TUNEL assay on agarose sections or using toluidine blue staining on semithin sections.

Results. The percentage of dying cells relative to the total nuclei in the photoreceptor layer was significantly reduced, and the total number of nuclei was greater in hyperoxic animals. Triple labeling using TUNEL, anti-M/L cone opsin and anti-rod opsin showed that hyperoxia had a remarkable effect both on the reduction of cone cell death and the maintenance of the overall structure of cone photoreceptors. Analysis of the retinal wholemounts demonstrated the preservation of PNA, S-cone, and rod opsin antibody labeling in the detachments maintained in hyperoxic conditions. Although the disruption of cytochrome oxidase and synaptophysin was seen in normoxic animals, there was minimal disruption in hyperoxic animals. Labeling with anti-EAAT1, anti-GS, and anti-CRALBP was increased in the Müller cells of normoxic animals with detachments, but was decreased in the hyperoxic animals.

Conclusions. Hyperoxia prevents the degeneration of both rods and cones in retinas heavily dominated by cones and mitigates the effect of detachment on Müller cell reactivity. The current results suggest that the rescue of cones is not secondary to that of rods. (*Invest Ophthalmol Vis Sci.* 2001;42: 3264–3273)

From the [1]Neuroscience Research Institute and the [3]Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara; and the [2]Department of Ophthalmology, Jikei University School of Medicine, Tokyo, Japan.

Supported by National Eye Institute Grant EY-00888 and a grant from Santa Barbara Cottage Hospital, Santa Barbara, California.

Submitted for publication July 3, 2001; revised August 4, 2001; accepted September 5, 2001.

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Corresponding author: Steven K. Fisher, Neuroscience Research Institute, Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, Santa Barbara, CA 93106-5060.
fisher@lifesci.ucsb.edu

Recent studies of experimental retinal detachment have led to possible mechanisms that underlie the genesis of the retinopathy induced by detachment.[1] The investigators hypothesized that detachment of the neural retina creates both hypoxic and hypoglycemic conditions in the photoreceptor layer, because it increases the distance between that layer and the choroidal circulation. To test one of these hypotheses, supplemental oxygen, which may increase oxygen flow from the choroid to the photoreceptors, was provided to cats for 3 days after experimental detachment.[1,2] The results showed hyperoxia to mitigate various cellular effects of the detachment. However, the feline retina is heavily dominated by rod photoreceptors,[3] and the most serious effects of retinal detachment in human patients occur when the cone-dominant macula is involved. If the macula is detached, functional improvement in vision remains disappointing after successful reattachment.[4–6] The damage to cones during an episode of detachment may cause visual impairment, including acquired color vision loss, even though anatomic recovery is regarded as successful. Therefore, it is important to determine whether hyperoxia has a protective effect on the cone photoreceptors during detachment, specifically when these cells are in the majority. In addition, it has not yet been determined whether the efficacy of hyperoxia is related to the height of detachment. Recent modeling analyses predict that hyperoxia can protect photoreceptor oxidative metabolism in small and low detachments.[7,8] The effect of the detachment height, however, has not been evaluated experimentally.

Müller cells play various roles in maintaining the retinal environment. It has been suggested that they interact with photoreceptors and RPE cells in both glutamate cycling and the visual cycle.[9–14] However, specific interactions between Müller cells and cone photoreceptors in vivo remain mostly unknown. Indeed, there are striking differences in the reactivity of Müller cells in the cone-dominated ground squirrel and rod-dominated cat retinas.[15] Müller cells in the ground squirrel do not proliferate, upregulate glial fibrillary acidic protein (GFAP) expression, or lose their expression of various soluble proteins, as they do in the cat retina in response to detachment.

In the present study, hyperoxia had a protective effect on cone degeneration after retinal detachment in the cone-dominated California ground squirrel (*Spermophilus beecheyi*). This effect was dramatic, even in a region of high detachment. Moreover, we provide evidence that hyperoxia mitigates the active responses of Müller cells during detachment in this model, even though these responses are very different from those in rod-dominated retinas.

Materials and 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 Animal Resource Center of the University of California, Santa Barbara.

Retinal Detachments

Retinal detachments were created in the right eyes of ground squirrels (n = 6 normoxia; n = 5 hyperoxia), as described previously.[15,16]

Briefly, ground squirrels were anesthetized with an injection of a mixture of ketamine hydrochloride (18 mg/ml) and xylazine hydrochloride (1.2 mg/ml). A scleral incision was made 2 mm from the limbus at the 10 o'clock position. Using a fundus contact lens, a glass micropipette (100-μm approximate tip diameter) attached to a micromanipulator was inserted through the scleral incision and advanced while its progress was viewed with an operating microscope. A solution of 0.25% sodium hyaluronate (Healon; Pharmacia, Piscataway, NJ) in balanced salt solution (Alcon, Fort Worth, TX) was injected slowly through the pipette to maintain a constant detachment height. By this procedure, a single medium-sized detachment was produced in the inferior retina. The scleral and conjunctival incision sites were closed with 8-0 nylon suture. The height of the detachment was noted at this time, through the surgical microscope, and at the time of the animal's death by observation through a dissecting microscope.

Oxygen Exposure

Animals were placed in standard cages which in turn were put 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, Redfield, NY). Ground squirrels were exposed to 70% oxygen immediately after the surgery and remained in the chamber for 3 days. The animals were provided food and water as usual, and ambient illumination was kept on a 12-hour/12-hour light–dark cycle. In the descriptions of our experiments, "hyperoxia" refers to 70% oxygen, as described previously.[1,2,17]

Tissue Preparation

Three days after detachment surgery, the animals were killed with an overdose of sodium pentobarbitone (120 ng/kg), and the eyes were enucleated and immersion fixed for 10 minutes in 4% paraformaldehyde in sodium cacodylate buffer (0.1 N; pH 7.4). The cornea and lens were then removed and the eyecup was cut in half. One half of the tissue was stored in the fixative solution. From this sample, small areas of retina were excised and embedded in low-melting-point agarose for immunocytochemical analysis by confocal microscopy. The other half was immersion fixed in 1% glutaraldehyde and 1% paraformaldehyde in sodium phosphate buffer (0.086 M; pH 7.3) overnight at 4°C for high-resolution transmitted light or ultrastructural analysis. This tissue was then fixed in phosphate-buffered osmium tetroxide (2%) for 1 hour and embedded in Spurr resin.

Sampling Area

Previous immunocytochemical labeling experiments have provided maps of the density distribution of cones and rods in the ground squirrel retina.[18] The density for the cones reaches a plateau 2 mm inferior to the elongated optic nerve head, defining a horizontally oriented visual streak (VS). Rod density is lowest in the VS, where they comprise less than 5% of the local photoreceptor population, and increases conspicuously in the ventral retina, where the rods achieve 30% of the population.

In this series, to examine the effect of detachment height as well as areas with differences in rod population densities, three different regions within the detached retinas were chosen as sample areas. Figure 1 shows examples of these sampled areas in a photograph of a detached retina. Sampled areas A and C were approximately 1 to 2 mm from the superior and inferior detachment edges, respectively, and area B was from the center of the detachment. The length of detached retina averaged 6.95 $\pm$ 1.05 mm (range, 5.60–8.34). The sampled area and the size of the detachment were measured on computer (Image Tool software; University of Texas Health Science Center, San Antonio, TX). Similar sampling areas were used for the retinas embedded in resin.

Immunocytochemistry for Confocal Microscopy

For confocal analysis, 100-μm-thick agarose-embedded sections were cut on a microtome (Vibratome; Technical Products International,

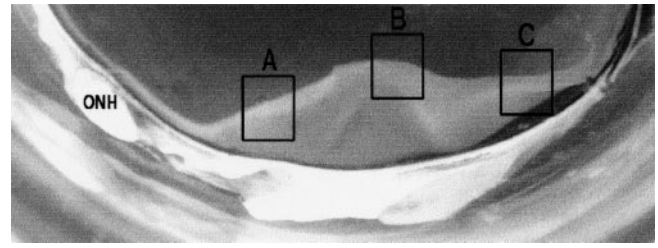

FIGURE 1. A photograph of a retinal detachment with the three areas sampled for histology and immunohistochemistry indicated by the *rectangles*. The center of area A was approximately 1 to 2 mm from the superior edge of the detachment, area B was at the center of the detachment, and area C was approximately 1 to 2 mm from the inferior edge of the detachment. ONH, optic nerve head. Magnification, $\times$8.

Polysciences, Warrington, PA) and blocked overnight in normal donkey serum (1:20; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 4°C. The next day, the sections were incubated in primary antibodies overnight at 4°C on a rotator. The sections were then rinsed in 0.1 M phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA), 0.1% Triton X-100 (Roche Molecular Biochemicals, Indianapolis, IN), and 0.1% sodium azide (Sigma, St. Louis, MO), together referred to as PBTA, and incubated in donkey anti-mouse or anti-rabbit IgG conjugated to the fluorochrome Cy2, Cy3, or Cy5 (Jackson ImmunoResearch Laboratories, Inc.) overnight at 4°C on a rotator (PBTA comprising PBS, BSA, Triton, and azide). When needed for labeling of DNA, the sections were incubated for 4 hours in propidium iodide (PI; 0.5 mg/ml in PBS). Finally, the sections were mounted in 5% *n*-propyl gallate in glycerol, and viewed on a laser scanning confocal microscope (model 1024; Bio-Rad, Hercules, CA). All antibody solutions were made in PBTA.

The primary antibodies used in this study were a mouse monoclonal antibody to rod opsin (Rho4D2, provided by Robert Molday, University of British Columbia, Vancouver; 1:50), two rabbit polyclonal antisera to short wavelength-sensitive (S) and medium/long wavelength-sensitive (M/L) cone opsins (JH455 and JH492 provided by Jeremy Nathans, Johns Hopkins Medical School, Baltimore, MD; both 1:1000), a mouse monoclonal antibody to cytochrome oxidase (1 μg/ml; Molecular Probes, Eugene, OR), a rabbit polyclonal antibody to synaptophysin (1:100; Dako, Carpinteria, CA), a rabbit polyclonal antiserum to cellular retinaldehyde binding protein (CRALBP, 1:400; provided by John Saari, University of Washington, Seattle, WA), a rabbit polyclonal antibody to glutamine synthetase (GS, 1:600; provided by Paul Linser, University of Florida, St. Augustine, FL), and a goat polyclonal antibody to excitatory amino acid transporter 1 (EAAT1, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA). In addition, staining with biotinylated peanut agglutinin (PNA) lectin (400 μg/ml; Vector Laboratories, Burlingame, CA) was used on some sections.

Retinal Wholemount Immunocytochemistry

Retinal wholemount immunocytochemistry was performed according to the following protocol. Retinas were removed and rinsed three times in PBS. They were incubated with blocking solution for 6 hours and incubated in biotinylated PNA, anti-rod opsin and -S opsin (all at 1:10,000) for 3 days. After the incubation, they were washed in PBTA and then incubated in a mixture of streptavidin-Cy3 and anti-mouse and -rabbit IgG conjugated to the fluorochromes Cy5 and Cy2 respectively. The retinal wholemounts were rinsed in PBTA and mounted with the photoreceptor side up.

TUNEL Assay

To detect dying (apoptotic) cells, we performed terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick-end labeling (TUNEL), as described previously.[17,19] The agarose-embedded sections were used for this series. After the sections were rinsed three

times in PBS, they were immersed in 70% alcohol for 30 minutes, followed by a wash in double-distilled water. They were then incubated in 1% citrate and 1% Triton in PBS at 4°C for 4 minutes and, after washing, were placed in terminal deoxynucleotidyl transferase (TdT) buffer (Roche Molecular Biochemicals) at room temperature for 30 minutes, followed by reaction with TdT enzyme and 2 μM biotinylated deoxyuridine triphosphate (dUTP; Roche Molecular Biochemicals) at 37°C for 120 minutes. The sections were then washed in SSC (150 mM sodium chloride and 15 mM sodium citrate, pH 7.4) for 15 minutes. After a rinse in PBS, avidin-Cy2 or -Cy3 (1:200) was added for 3 hours, at which time the sections were examined with a confocal microscope. For double or triple labeling, the sections were incubated with primary antibodies overnight after the TUNEL procedure and, after rinsing, were reacted with appropriate fluorescent secondary antibody.

TUNEL-positive cells, which were regarded as dying cells, were counted in four areas of each section from three different eyes. The percentage of dying photoreceptor cells was expressed as the number of TUNEL-positive cells divided by the total number of nuclei in the outer nuclear layer (ONL). The nuclear counts were represented by the number of cells per millimeter of retinal length.

Light Microscopic Analysis

For light microscopy, three different eyes were embedded in resin, divided into four regions extending from superior to inferior retina, sectioned at 1 μm, and stained with toluidine blue. The cells with dark nuclei were counted as representative of dying photoreceptors. In this study, the identity of the tissue was masked from the observer.

Statistical Analysis

All data are presented as means ± SD. The cell counts for the study of photoreceptor cell death were analyzed by one-way ANOVA and Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Morphology

The photoreceptors of normoxic attached retinas differed morphologically in each of the three sampling areas (Figs. 2A, 2D, 2G). The differences in photoreceptor density, thickness of ONL and inner nuclear layer (INL), and size of inner segments (IS) were consistent with the results reported previously in this species.[20] At 3 days after detachment in room air, we found many small, dark nuclei in the ONL and a remarkable decrease in the total nuclei in the ONL (Figs. 2B, 2E, 2H). In area B, the remaining nuclei were mostly small and dark (Fig. 2E). Disruption of inner and outer segments (OS) on all surviving photoreceptors was observed in all areas, with the most severe damage always occurring in area B. A morphologic description of the effects of detachment in this species is provided in Linberg et al.[21]

Figures 2C, 2F, and 2I illustrate the morphology of retinas treated with oxygen supplementation during the detachment. Although their morphology was not normal, they showed dramatic changes by comparison to the normoxic detachments (Figs. 2B, 2E, 2H). In all areas, the percentage of small, dark nuclei was less, there was a greater number of nuclei remaining in the ONL, and IS morphology was less disrupted than in the untreated eyes.

Quantitation of the Effects of Hyperoxia

Figure 3 reflects how hyperoxia effects the relative percentage of dying cells and the actual number of nuclei in the ONL, as obtained from cell counts in semithin resin sections. In normoxic detached retinas, the percentage of dying cells was highest in area B. There was no significant difference between areas A and C. The percentage of dying photoreceptors in the oxygen-treated eyes was significantly decreased compared with that in untreated eyes ($P < 0.01$). In contrast, the number of nuclei in the ONL was significantly greater in the hyperoxic detached retinas ($P < 0.01$), where the effects were dramatic in all areas.

Figure 4 shows the effects of hyperoxia on the percentage of TUNEL-positive cells and the number of nuclei in the ONL on the agarose-embedded sections. In normoxic detached retinas, the percentage of TUNEL-positive cells in the ONL was greatly increased in all sampling areas. As before, the increase in area B was particularly striking. In hyperoxic detached retinas, we found a significant reduction of the percentage of cells in the ONL that were TUNEL-positive in all areas ($P < 0.01$) and the number of nuclei in the ONL was significantly greater (areas A and C: $P < 0.01$; area B: $P < 0.05$).

Immunocytochemistry

The patterns of antibody labeling were compared between normoxic attached, normoxic detached, and hyperoxic detached retinas from sample area B.

Anti-Cytochrome Oxidase and Anti-Synaptophysin. In normal retina, immunocytochemical labeling of the outer retina with the antibody to cytochrome oxidase occurred mainly in the IS of photoreceptor cells (Fig. 5A). At 3 days after detachment, the IS were disrupted and their labeling was faint (Fig. 5B). In contrast, the oxygen-treated eyes showed less of a reduction of labeling with this antibody (Fig. 5C), even though the IS morphology was far from normal.

Anti-synaptophysin labeled the synaptic terminals of rods and cones in the outer plexiform layer (OPL; Fig. 5A). In normoxic detached retinas, this labeling was greatly decreased, both in the intensity and the extent of labeling within individual terminals and in the number of synaptic terminals within the OPL (Fig. 5B). Labeling also occurred in abnormal locations within the photoreceptor cells. In detached retinas treated with oxygen, the collapse of anti-synaptophysin labeling in the OPL was greatly reduced and the number of synaptic terminals increased (Fig. 5C).

The Effects of Detachment Height: Triple-Labeling Studies. To further examine the correlation between detachment height, TUNEL and the expression patterns of various proteins, we performed triple-labeling experiments with TUNEL, anti-cytochrome oxidase, and anti-synaptophysin (Figs. 5D–G) as well as TUNEL, anti-rod, and anti-M/L cone opsins (Fig. 6). In these studies the transition zone from attached to detached retinas (Figs. 5D, 5E, 6A, 5B) was also included and comparisons made between normoxic and hyperoxic conditions. In both experiments the transition to a higher detachment showed a more intense TUNEL signal (blue in Fig. 5; red in Fig. 6) and an attenuation of staining by the other antibodies in the photoreceptor layer. A comparison of normoxic (Figs. 5D, 6A) and hyperoxic (Figs. 5E, 6B) retina showed the same trend but an overall attenuation of the response. The same effects are shown at higher magnification in Figures 5F and 5G and 6C and 6D. One striking observation we made throughout was that cells double labeled with TUNEL and the antibodies to rod opsin were rarely observed, although the rods showed other characteristic signs of photoreceptor degeneration.[15]

Retinal Wholemount Study. In the low-magnification images of sectioned retina it was difficult to obtain an overview of the effect of detachment and hyperoxia on the photoreceptor cells. Therefore, we prepared wholemounts of samples from control (Fig. 7A) and 3-day detached retinas in either normoxia (Fig. 7B) or hyperoxia (Fig. 7C). All cone OS were labeled with the lectin PNA (red), the S cones with the antibody to S-cone opsin (green; yellow signals colocalization with PNA), and the

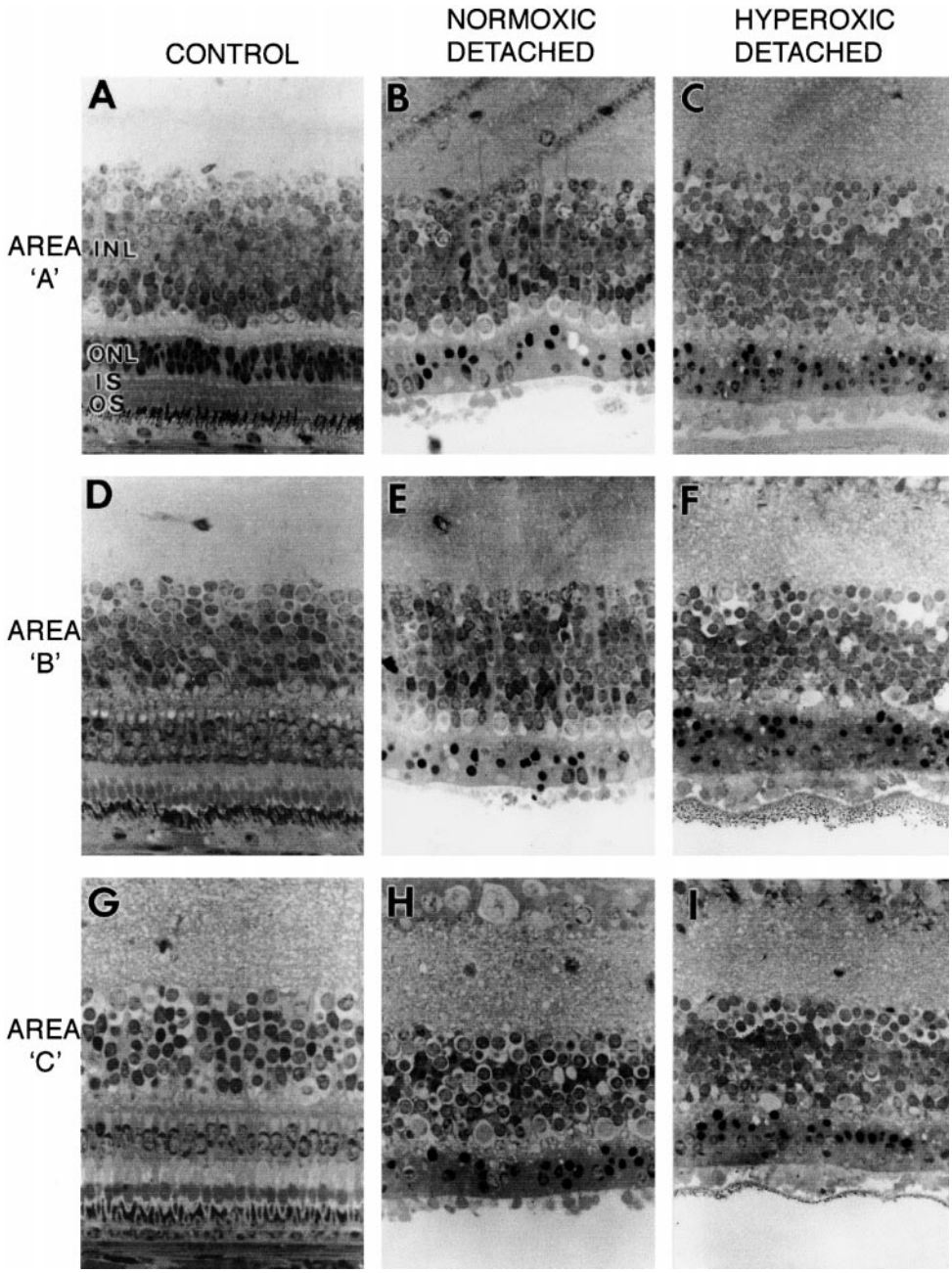

FIGURE 2. Light micrographs selected from the sampling areas described in Figure 1. All resin sections were 1-μm thick and stained with toluidine blue. Normal squirrel retina differed morphologically in different retinal regions (**A**, **D**, **G**). Differences were observed in photoreceptor density and thickness of the INL. Normoxic retina detached for 3 days (**B**, **E**, **H**). IS and OS degeneration and many dark nuclei were observed within the ONL. Hyperoxic retina detached for 3 days (**C**, **F**, **I**). Photoreceptor degeneration was less extreme, and the number of remaining photoreceptor nuclei in the ONL was greater than in normoxic detached retinas. Darkly stained nuclei normally occur in the INL of the ground squirrel retina (**A**, **D**, **G**). These cells were not pyknotic, nor did they label by TUNEL. Magnification, ×300.

rods with the antibody to rod opsin (blue). A comparison of Figures 7A and 7B shows the complete absence of cone labeling in this area of the normoxic detached retina, but the presence of a reduced population of rods. Figure 7C demonstrates the preservation of PNA and S-cone labeling in the detachments in hyperoxia. In addition, the number of rods labeled with the anti-rod opsin antibody showed a more normal population of these cells.

Anti-EAAT1. EAAT1 immunoreactivity was observed throughout the retina, but with much more intense staining in the inner retina (Fig. 8A). These data are in agreement with results reported for rat retina.[22] The pattern shown in Figure 8A is characteristic of Müller cell staining and double labeling with the antibody to vimentin indicated that, indeed, this antibody labeled the Müller cells (data not shown). In the 3-day detachments, the labeling was markedly increased throughout the retina (Fig. 8B). By comparison, in the detachments treated with oxygen, there was less of an increase in the labeling than in the normoxic detachments, but not to the level found in control retina.

Anti-GS and PNA. In normoxic attached retina, anti-GS labeling was prominent in radial Müller cell processes extending from the end feet and into the IPL (Fig. 8D). PNA labeled all cones as well as the OPL (Fig. 8D). When the retina was detached for 3 days, the PNA labeling associated with cone OS collapsed totally, and the GS immunoreactivity was markedly increased in the Müller cells (Fig. 8E). In contrast, hyperoxia during the detachment maintained PNA staining and reduced the GS increase in the INL, OPL, and ONL (Fig. 8F).

Anti-CRALBP and PNA. Immunolabeling with the antibody to CRALBP occurred throughout the Müller cell cytoplasm and the RPE cytoplasm in normoxic attached retina (Fig. 8G). After detachment for 3 days, the signal was increased in the Müller cell processes, especially noticeable where these filled in the ONL as photoreceptor cells were lost (Fig. 8H). In hyperoxic detached retina, there was more PNA labeling of

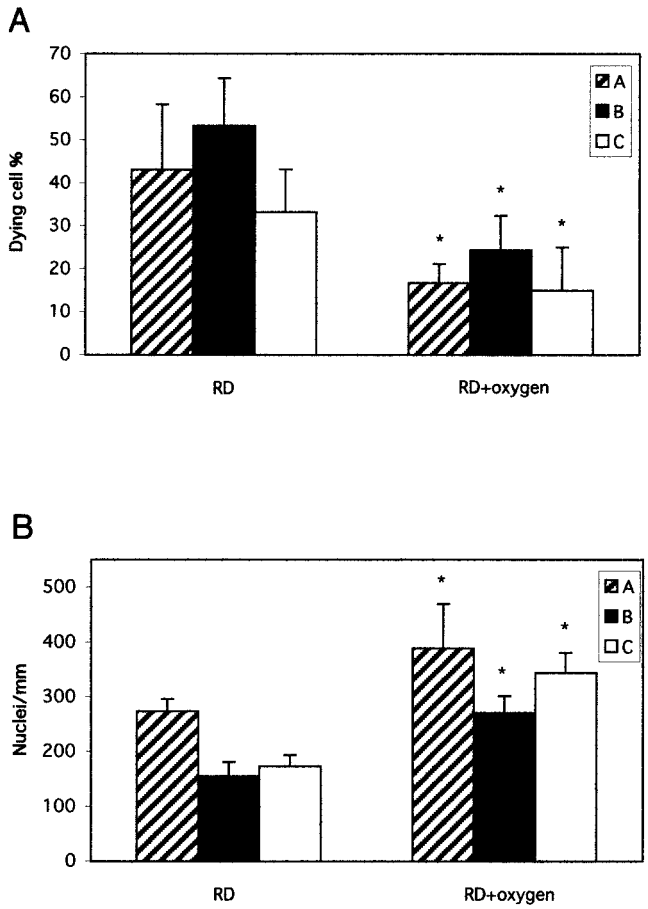

FIGURE 3. The percentage of densely stained, presumably dying photoreceptor cells (**A**), and the total number of nuclei (**B**) in the ONL of normoxic and hyperoxic detached retinas counted from resin sections. The results from each sampling area identified in Figure 1 are shown. The oxygen-treated retinas showed a significant decrease in the proportion of dying photoreceptor cells and a larger number of nuclei in all areas. Data are mean ± SD. $^{*}P < 0.01$, compared with normoxic detached retinas.

cone matrix and less labeling with the anti-CRALBP antibody (Fig. 8I).

DISCUSSION

The first studies to show that hyperoxia could mitigate the cellular effects of retinal detachment were performed in a feline model system.[1,2] The feline retina is heavily dominated by rods,[3,23] and thus we developed the present model in a species in which we could more critically evaluate the effects on cone photoreceptors. The results show hyperoxia to have a protective effect on cone degeneration induced by detachment by reducing cone cell death and improving the overall structure of cones and their expression of some molecules after detachment. Furthermore, we provide evidence that hyperoxia can mitigate the active responses of Müller cells in a retina where their response is very different from that in the feline retina.[15]

In the present study, we compared the use of the TUNEL assay on agarose sections and toluidine blue staining of pyknotic nuclei in resin sections as ways of evaluating cell death. As another measure of cell death, we counted the remaining total nuclei in the ONL on both types of sections. The use of these different quantitative methods provided us with confidence in our ability to evaluate the effects of hyperoxia on cell death and survival in the ground squirrel retina. Three days' treatment in hyperoxia had marked effects on reducing cell death as assessed by TUNEL, or morphology, or simply by the size of the remaining population of photoreceptor cells. The efficacy of this treatment was dramatic in all regions, regardless of height of the detachment and rod distribution. It may be somewhat surprising that the oxygen supplementation was useful in rescuing cells in the highest detached region, because recent modeling analysis predicted that hyperoxia could rescue photoreceptors during small and low detachments.[7,8] Certainly, we found the best evidence for rescue in the shallower detached areas (Figs. 5, 6) but saw some effect even in the region of highest detachment. These results from the ground squirrel detachment model may differ from the predicted modeling outcome for several reasons. First, as Linsenmeier and Padnick-Silver[8] point out, convection in subretinal fluid during eye movements may enhance the effects. Second, we do not rule out the possibility that some flattening of detached retina may occur during an episode of detachment. Third, oxygen supply from the retinal circulation may supplement the demand during the detachment.[7,8,24] Finally, the effects on photoreceptors may be indirect, mediated through other retinal cells such as Müller cells, which border both the subretinal space and the retinal circulation. Although retinal and choroi-

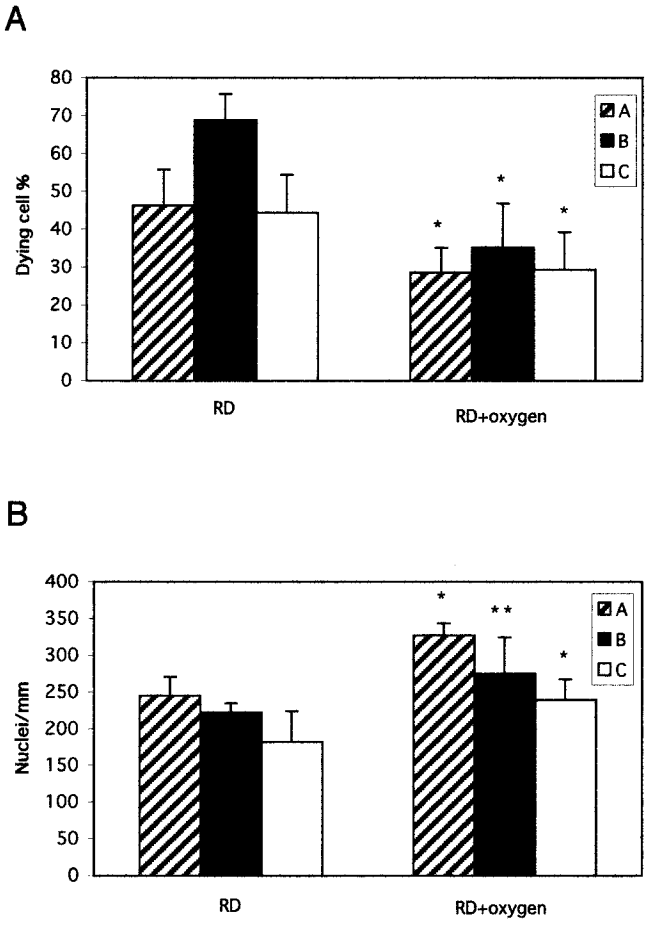

FIGURE 4. Percentage of TUNEL-positive cells (**A**) and the total number of nuclei (**B**) in the ONL of normoxic and hyperoxic detached retinas (RD) counted in agarose-embedded tissue. The results from each of the three sampling areas are shown. In hyperoxic detached retina, the percentage of TUNEL-positive cells was greatly reduced, and the nuclear counts were higher in all areas. Data are mean ± SD. $^{*}P < 0.01$, $^{**}P < 0.05$, compared with normoxic detached retinas.

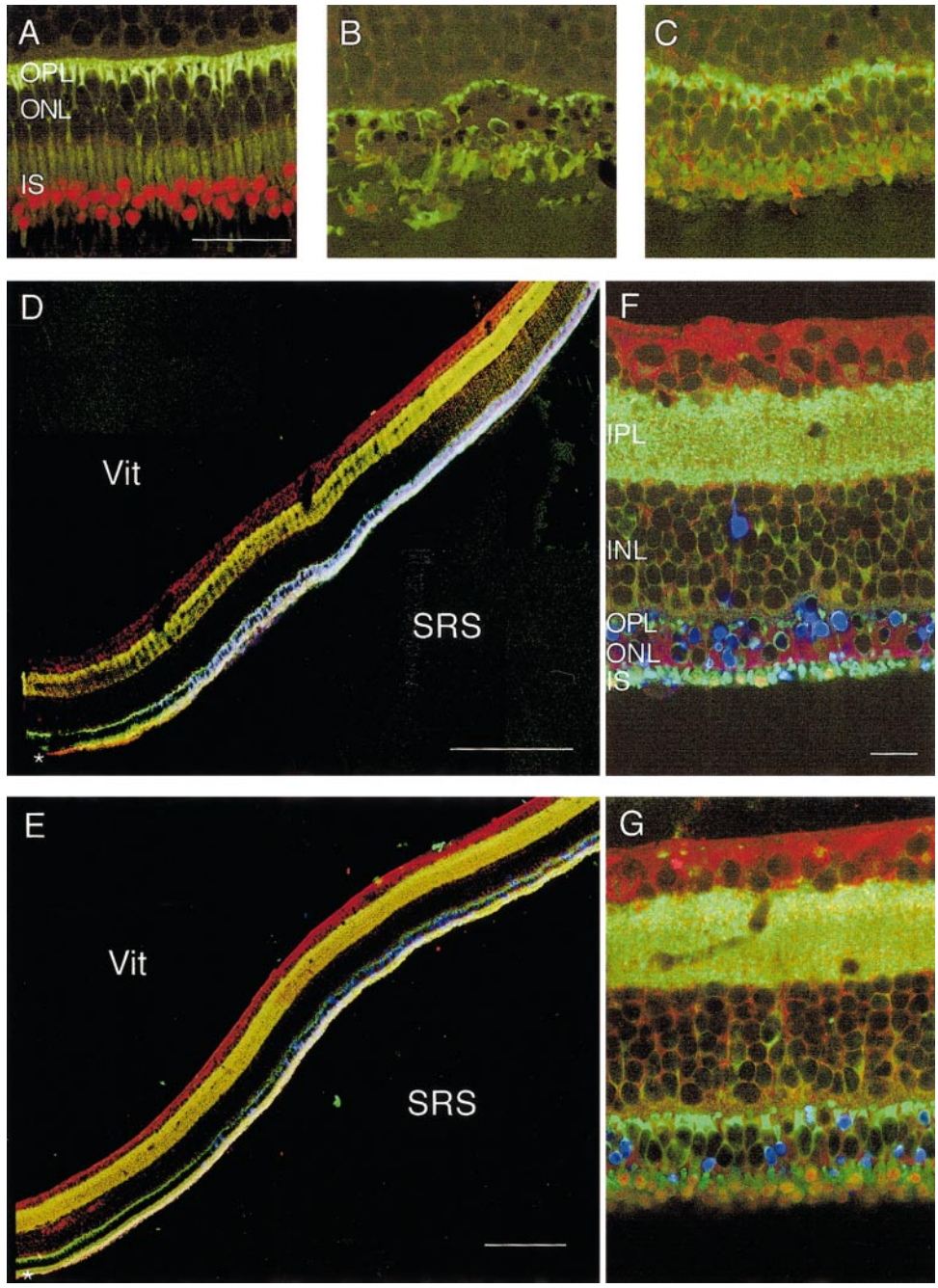

FIGURE 5. Laser scanning confocal micrographs showing the immunolabeling pattern for the antibody to cytochrome oxidase (**A**–**E**; *red*) and synaptophysin (**A**–**E**; *green*) in normoxic attached (**A**), normoxic detached (**B**), and hyperoxic detached retinal sections (**C**). The reduction of anti-cytochrome oxidase and anti-synaptophysin labeling induced by the detachment was attenuated by treatment with oxygen. Laser scanning confocal micrographs of TUNEL (**D**, **E**; *blue*) and immunostaining for the antibody to cytochrome oxidase (*red*) and synaptophysin (*green*) in normoxic detached (**D**) and hyperoxic detached retinal sections (**E**), showing the transition from the edge (✻) of the detachment to high detachment. The increase of TUNEL positivity and the reduction of anti-cytochrome oxidase and anti-synaptophysin staining showed a positive relationship with the height of the detachment in normoxic detached retina (**D**). The hyperoxia-treated retina showed a decrease of TUNEL positivity and minimal reduction of anti-cytochrome oxidase and anti-synaptophysin labeling (**E**). Higher magnification better showed the respective differences of labeling (**F**, **G**) using the triple-labeling protocol. (✻), Edge of the detachment. Vit, vitreous side; SRS, subretinal space side. Scale bar, (**A**, **B**, **C**, **F**, **G**) 20 μm; (**D**, **E**) 200 μm.

dal circulation and their respective contribution to the ONL have not been studied in ground squirrels, taken together, the present findings suggest that supplemental oxygen reaches the photoreceptors of the detached retinas generating a positive effect in the 3-day detachments.

Our combined immunocytochemical and TUNEL data in this model suggest that most rods do not show apoptosis in the 3-day detachments; indeed, rods in this species may be more resistant to the effects of detachment than the cones. The result indicates that most of the dying photoreceptors we observed were cones, and that oxygen supplementation had a marked effect on reducing cone cell death. These data would seem to indicate that oxygen deprivation may be one of the major factors influencing the survival of cone photoreceptors after detachment.

Although some previous studies suggest it,[25–28] we have shown for the first time quantitative evidence that photoreceptor cell death has a positive relationship with the height of the detachment. Therefore the present study provides specific evidence that cone cell death is related to retinal elevation during an episode of detachment.

Based on our morphologic, immunocytochemical, and wholemount data, oxygen supplementation appears to better maintain many aspects of photoreceptor cells in a cone-dominant retina, just as it does in the rod-dominated feline retina.[2] This result suggests that oxygen supplementation may improve cone metabolism through one or more effects and, as in the case with cell death, there is also a positive relationship with the height of the detachment. Cytochrome oxidase is a key enzyme involved in adenosine triphosphate (ATP) generation within the mitochondria and is an indicator of neuronal oxidative capacity.[29,30] The synaptic vesicles found in the synaptic terminals of photoreceptors each contain approximately 10,000 glutamate molecules.[31,32] Thus, our results may pro-

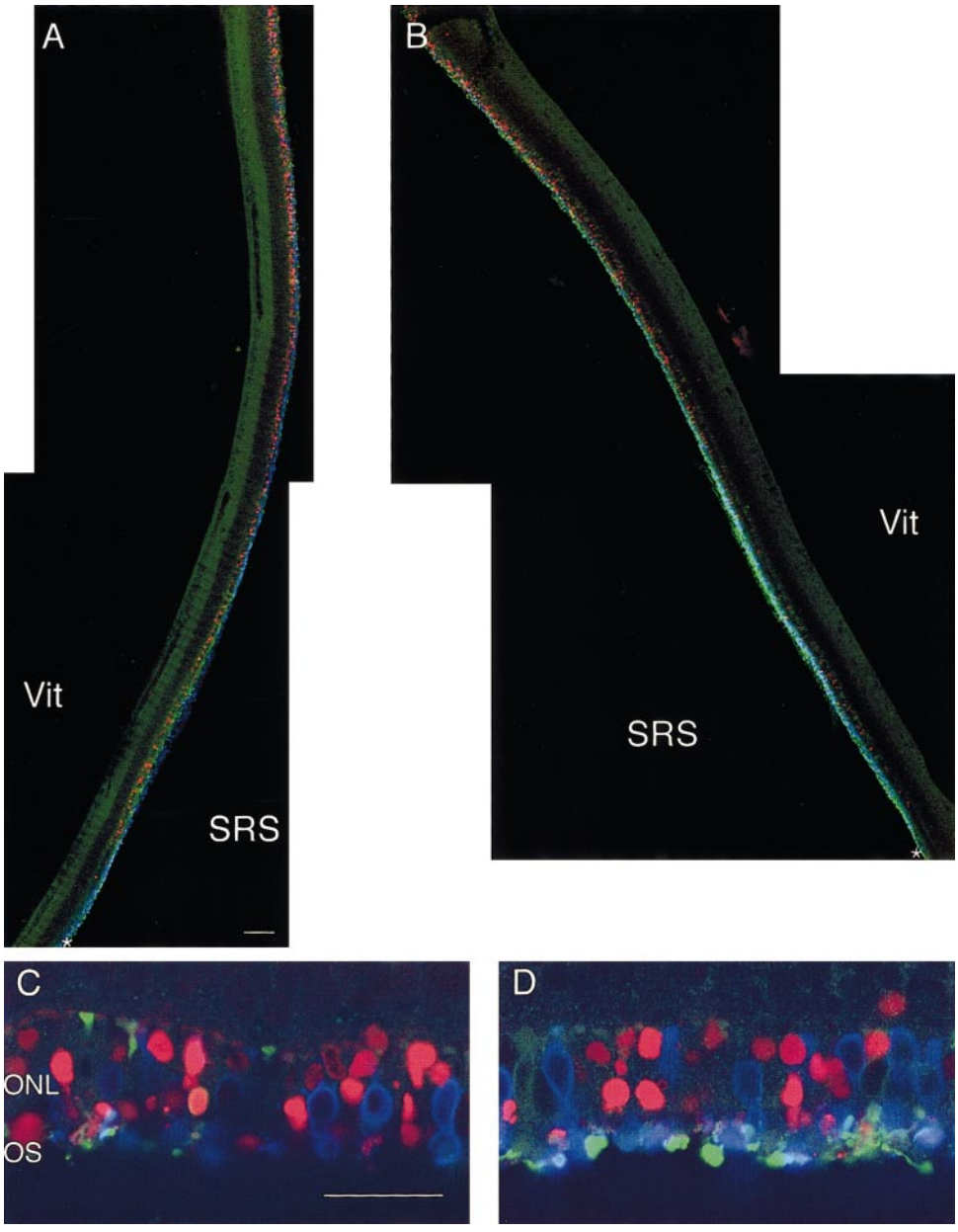

FIGURE 6. Laser scanning confocal micrographs of TUNEL (**A–D**; *red*) and immunostaining with antibodies to M/L cone opsin (**A–D**; *green*) and rod opsin (**A–D**; *blue*) in normoxic detached (**A**, **C**) and hyperoxic detached retinal sections (**B, D**). In normoxic detached retina, TUNEL positivity and the collapse of cone OS directly correlated with the height of the detachment (**A**). In hyperoxic detached retina, there were fewer TUNEL-positive cells and much more labeling of cone opsins, even in higher detached regions (**B**). The nuclei of rods (*blue*) did not demonstrate TUNEL-labeling, although they do show the redistribution of opsin to the cell body, a characteristic sign of photoreceptor degeneration (**C**). In the oxygen-treated retina, cones appear less degenerated (**D**). (✻), Edge of the detachment. Vit, vitreous side; SRS, subretinal space side. Scale bar, (**A** through **D**) 20 μm.

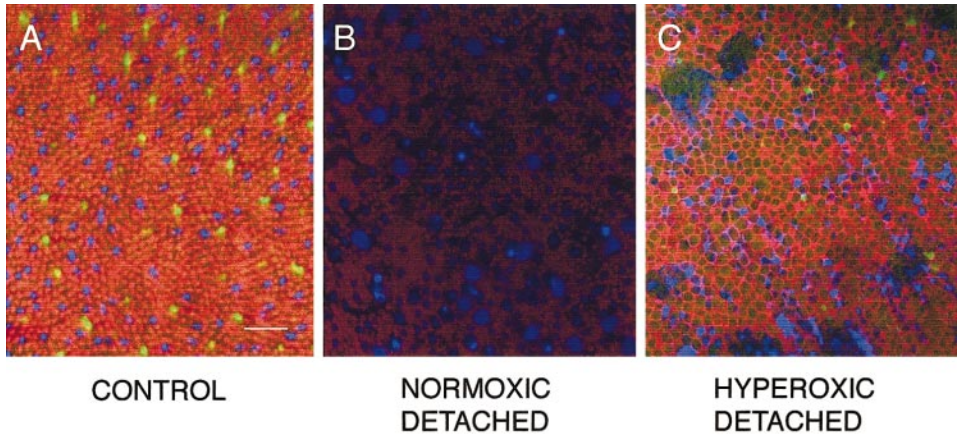

FIGURE 7. Retinal wholemount labeled with the peanut agglutinin (PNA) lectin (*red*), antibodies to short-wavelength (S) opsin (*green*; *yellow* shows colocalization) and rod opsin (*blue*). These images were sampled 20 μm distal to the OPL in sample area B (Fig. 1). (**A**) Normal retina. There was intense labeling with PNA, which stained the cone sheaths around all cones. The cones labeled by anti-S opsin were double labeled with PNA and appeared *yellow*. (**B**) Normoxic retina detached for 3 days. There were some rods present, but the PNA and S-cone opsin signals had virtually disappeared. (**C**) Hyperoxic retina detached for 3 days. There was a dramatic preservation of the PNA, S opsin, and rod opsin labeling. Most PNA labeling appeared around IS. Scale bar, (**A** through **C**) 20 μm.

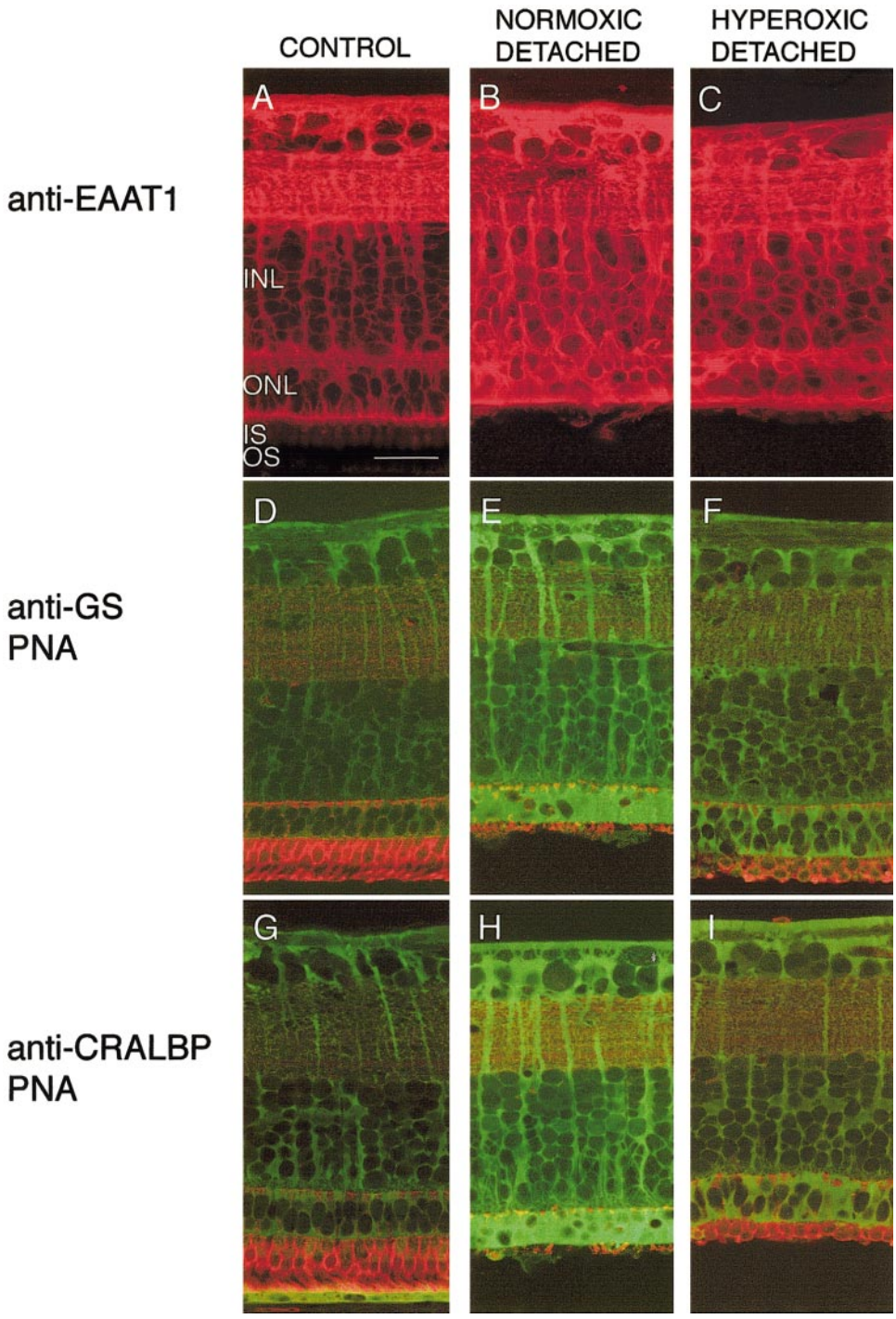

FIGURE 8. Laser scanning confocal micrographs showing the labeling pattern for antibodies to EAAT1 (**A–C**; *red*), GS (**D-F**; *green*), CRALBP (**G–I**; *green*), and PNA (**D–I**; *red*) There was a dramatic increase in Müller cell labeling with anti-EAAT1 in 3-day normoxic detached retina (**B**). In 3-day hyperoxic detachments (**C**), such labeling within the INL, OPL, and ONL was partially reduced. Although anti-GS labeling intensity in Müller cells was increased in normoxic detached retina (**E**), the distribution of labeling was nearer normal in hyperoxic detached retina (**F**). In normoxic attached retina, anti-CRALBP labeling was found in the Müller cells and RPE (**G**). In normoxic detached retina, the immunolabeling increased in the Müller cells (**H**). Hyperoxia during the detachment mitigated the increase in CRALBP in the Müller cells (**I**). PNA labeling of cone sheaths was completely disrupted in the IS and OS of normoxic detached retina, but more preserved in those of hyperoxic detached retina. Each image is a two-dimensional projection of a series of five consecutive images with a single-image plane equal to 1 μm in depth. Scale bar, (**A** through **I**) 20 μm.

vide further evidence that the major effect of detachment is to induce hypoxia in the photoreceptor layer resulting in disturbance of mitochondrial ATP production which could lead to cone metabolic death and may also result in abnormal glutamate cycling. The data suggest that hyperoxia could generate its dramatic effect on cone survival by maintaining ATP production in the mitochondria and/or maintaining functional capacity in physiological processes such as glutamate cycling.

Recently, we reported a distinct and dramatic difference in the reactivity of Müller cells in the rod-dominant feline retina compared with the cone-dominant squirrel retina.[15] By comparison with cat Müller cells, those in ground squirrel retina appear to react in reverse with respect to proliferation and the expression of GS, CRALBP, carbonic anhydrase C, and intermediate filament proteins after detachment.[33] In the current study, we focused on EAAT1 and GS, because of their important roles in glutamate cycling in the Müller cells. EAAT1 acts to remove glutamate from the synaptic cleft,[34] and GS is the primary enzyme for glutamate metabolism,[34] converting glutamate to glutamine in Müller cells. It has been suggested that the level of GS in mature Müller cells may be determined by cell interactions at the OPL and ONL.[35] Thus, EAAT1 and GS may be jointly related to glutamatergic neurotransmission and cycling.[34] Our results showing that EAAT1 and GS immunoreactivity were upregulated in normoxic detached retina but not in the hyperoxic detached retina, may suggest that the Müller

cells have an active response to changes in retinal glutamate levels that occur as a result of detachment. A previous report indicated that RPE cells, photoreceptor cells, and bipolar cells also possess a glutamate transporter to take up glutamate from the extracellular space.[36–39] Thus, in normal outer retina, there is a well-organized glutamate uptake system to eliminate excess glutamate. The increase in EAAT1 and GS that appeared to occur in the ground squirrel Müller cells may indicate an acceleration of glutamate cycling in them in normoxic detached retinas, perhaps induced by changes in glutamate balance in the outer retina. The suppression in hyperoxic detached retinas may result from the maintenance of that system in the surviving photoreceptors. It is interesting that in the feline retina there are also apparent shifts in glutamate balance after detachment[40] but in that case, there is a dramatic decrease in the GS content of Müller cells. It is significant that in both animal models the effectiveness of oxygen is to return GS toward normal levels of expression.

There is increasing evidence that Müller cells are linked to the visual cycle through CRALBP,[41,42] a retinoid-binding protein, found both in Müller cells and RPE. It has been suggested that cones may regenerate their visual pigment through Müller cells in the absence of RPE.[10] This suggests that CRALBP upregulation in the Müller cells may help maintain cone metabolism through the regeneration of cone visual pigments. The improved overall cone survival and presumed metabolic capacity due to hyperoxia would explain the absence of CRALBP upregulation in the oxygen treated eyes.

In conclusion, we have shown clearly that oxygen supplementation has a protective effect on cone degeneration during retinal detachment in a second species, one whose retina is cone dominated. It reduces cell death and improves the overall structural integrity and probably the metabolic state of the photoreceptor cells. This in turn appears to be accompanied by a metabolic change in glutamate cycling and perhaps the visual cycle between the photoreceptors and the Müller cells. We believe that this study strengthens the case for hyperoxia as a candidate for improving the ability of the cone photoreceptors to recover in reattachment. In addition, this study suggests that there may be special metabolic interactions, which are related to visual function, between cone photoreceptors and Müller cells in the macula. Hyperoxia may also be an important adjunct to surgical procedure that involves the production of a macular detachment, such as macular translocation in the treatment of AMD.[43–46]

Acknowledgments

The authors thank Brian Matsumoto, Maura Jess, William P. Leitner, and Derrick L. Mann for assistance; and Pharmacia for providing the Healon.

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