The Ability of Rapid Retinal Reattachment to Stop or Reverse the Cellular and Molecular Events Initiated by Detachment

Geoffrey P. Lewis,[1] *David G. Charteris,*[2] *Charanjit S. Sethi,*[2] *William P. Leitner,*[1] *Kenneth A. Linberg,*[1] *and Steven K. Fisher*[1,3]

Purpose. To determine the effects of reattachment on the molecular and cellular events initiated by a retinal detachment lasting 1 hour or 1 day.

Methods. Experimental retinal detachments were created in the right eyes of nine cats. Reattachments were performed 1 hour ($n = 3$) or 1 day ($n = 3$) after the detachment, and the animals were killed 3 days after detachment. Three-day detached ($n = 3$) and normal ($n = 3$) retinas were used for comparisons. Agarose-embedded sections were double labeled with a panel of antibodies. Some sections were also probed with the TUNEL technique to detect apoptotic cells. Wax-embedded sections were labeled with the MIB-1 antibody to the Ki67 protein to detect proliferating cells.

Results. The 1-hour and 1-day detachments followed by reattachment showed a very similar and consistent reduction in photoreceptor deconstruction and the Müller cell gliotic response when compared with 3-day retinal detachments without reattachment. Light microscopy and immunolabeling with opsin antibodies showed a significant reduction in both rod and cone outer segment (OS) degeneration, even though OS length was shorter than normal. The reattachments also showed a reduction in opsin redistribution, retraction of rod terminals, TUNEL-labeled photoreceptors, loss of cytochrome oxidase staining in photoreceptors, neurite outgrowth from second-order neurons, the number of proliferating cells, and the increase in intermediate filaments and loss of soluble proteins from Müller cells. The apparent re-ensheathing of the OS by the apical processes of the retinal pigment epithelium had begun but was not completely normal.

Conclusions. These data indicate that, even though the length of the OS is less than normal, retinal reattachment within 1 day of detachment can either greatly retard or reverse many of the molecular and cellular changes initiated by detachment. (*Invest Ophthalmol Vis Sci.* 2002;43:2412–2420)

Retinal detachment is a relatively common retinal injury that remains a significant cause of blindness.[1] Although the anatomic success rate for retinal reattachment surgery has increased over the years to approximately 90%,[2] functional recovery, in many cases, can be less than perfect. Indeed, if the macula has been detached for even short periods, successful reattachment surgery leads to a final visual acuity of 20/50 or better in only 39% of the cases.[3,4] More recent studies using ERG measurements indicate that recovery after reattachment is a slow process, with acuity still improving up to 10 years later.[5] Other parameters, such as photopigment recovery, color matching, and metamorphopsia recover even more slowly than visual acuity in foveal detachments.[6] Although the exact cause for this slow recovery is not known, the extent of changes occurring in photoreceptor cells before reattachment most certainly affects both the final visual outcome and probably the speed with which that outcome is reached. Several studies have shown that rod and cone outer segments (OS) degenerate shortly after detachment, but that they retain some capacity to regenerate if the retina is reattached to the retinal pigment epithelium (RPE).[7–12] Furthermore, some photoreceptors die after detachment,[13–16] and in photoreceptors that survive, there are many molecular and structural changes that have the potential to affect visual recovery. The most notable changes are the general disruption of cellular organelles, including mitochondria in the inner segments, along with the retraction and/or remodeling of synaptic terminals.[13] It is presumably the changes in synaptic terminals and their loss through photoreceptor cell death that leads to plastic changes in the dendrites of second-order neurons to which the photoreceptors connect.[17] There are also changes in Müller cells that may have effects on visual recovery. The potential effects of some are obvious, such as subretinal and preretinal proliferation, and some are not so obvious, such as changes in the expression of various proteins[18–20] or the amino acid profiles of these cells.[21]

Retinal reattachment studies in the cat[10] and primate[12] have shown that the morphology of the RPE–retina interface does not return to normal, even after recovery periods of up to 6 months. In those studies, 7 days was generally the shortest detachment time used. Because foveal detachments are usually repaired very quickly, in this study, we focused on detachments of only 1 hour or 1 day and then studied the early events associated with retinal reattachment. The goal was to determine whether very early reattachment could halt the structural and molecular changes that begin within the first day of detachment. This has become even more relevant in recent years, because short-term retinal detachment occurs as part of experimental therapies in use or proposed for retinal degenerative diseases (e.g., retinitis pigmentosa, age-related macular degeneration) including retina–RPE transplantation,[22,23] foveal translocation,[24] or the introduction of trophic factors or vectors for transfection of retinal cells.[25] Thus, it is essential that we gain an understanding of the consequences of inducing a detachment of short duration if we are going to optimize the regenerative capacity of the retina.

In this study, we chose 3 days as the end point of these studies, because many important events, such as photoreceptor deconstruction, the loss of cone marker molecules, apoptosis, neurite outgrowth, proliferation, and changes in gene ex-

From the [1]Neuroscience Research Institute and [3]Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, Santa Barbara, California; and [2]Moorfields Eye Hospital, London, United Kingdom.

Supported by National Eye Institute Grant EY00888, The Medical Research Council UK, the Cripplegate Foundation of Moorfields Eye Hospital, and the Bogue Foundation, University of London.

Submitted for publication November 13, 2001; revised February 19, 2002; accepted March 5, 2002.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Geoffrey P. Lewis, Neuroscience Research Institute, University of California, Santa Barbara, Santa Barbara, CA 93106; g_lewis@lifesci.ucsb.edu.

pression in Müller cells, can be reliably identified at that time.[26,27] We compared the results in retinas that had either remained detached or had been reattached after 1 hour or 1 day.

Materials and Methods

Retinal Detachments and Reattachments

Detachments were created in the right eyes of domestic cats. After the removal of the lens and vitreous, a balanced salt solution (Alcon, Fort Worth, TX) was infused between the neural retina and the RPE with a glass micropipette. At either 1 hour ($n = 3$) or 1 day ($n = 3$) after the detachment, the retina was reattached. First a fluid–gas exchange was performed, with care taken to drain the fluid from under the retina. After the retina was flat, 20% sulfur hexafluoride (Alcon) in filtered room air was flushed through the eye. Detachments in the eyes of three cats were not reattached. The left eyes of all animals served as the normal control. All animals were killed 3 days after the detachment, at which time the eyes were divided for analysis by histology, light microscopy, immunohistochemistry (either by transmitted light or laser scanning confocal microscopy), and TUNEL. At least three different regions from each eye were analyzed. All procedures adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation

Retinal samples were prepared for histologic analysis by fixing the tissue in 1% paraformaldehyde and 1% glutaraldehyde (both from Electron Microscopy Sciences, Fort Washington, PA) in sodium phosphate buffer (PBS; 0.086 M, pH 7.3) overnight at 4°C. The tissue was then fixed in osmium tetroxide (2%) for 1 hour, dehydrated in increasing concentrations of ethanol and embedded in Spurrs resin (Polysciences, Warrington, PA). Each retinal location was sectioned at 1 μm and counterstained with saturated aqueous *p*-phenylenediamine (PPDA), a lipophilic stain that enhances the appearance of the OS.

To examine the distribution of specific proteins in the retina using laser-scanning confocal microscopy, we fixed the eyes and stored them in 4% paraformaldehyde in sodium cacodylate buffer (0.1 M; pH 7.4; Electron Microscopy Sciences). Before sectioning, pieces of retina approximately 2 mm^2, were excised from the eyecup, rinsed in PBS, and embedded in 5% agarose (Sigma) in PBS. One-hundred-micrometer–thick sections were cut using a tissue sectioning system (Vibratome; Technical Products International, Polysciences) and incubated in normal donkey serum (1:20; Jackson ImmunoResearch, West Grove, PA) in 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) overnight at 4°C on a rotator (PBS+BSA+Triton+azide; PBTA). The next day the blocking serum was removed and the primary antibodies were added. Five sets of double-label combinations were used: anti-glial fibrillary acidic protein (GFAP; 1:500; Dako, Carpinteria, CA) with anti-rod opsin (1:50; gift of Robert S. Molday, University of British Columbia, Vancouver, BC); anti-medium-to-long–wavelength (M/L) cone opsin (1:2000; gift of Jeremy H. Nathans, Johns Hopkins, Baltimore, MD) with anti-vimentin (1:500; Dako); anti-calbindin D (1:1000; Sigma) with anti-short-wavelength (S) cone opsin (1:2000; gift of Jeremy H. Nathans); anti-synaptophysin (1:100; Dako) with anti-cytochrome oxidase (CO; 1 μg/mL; Molecular Probes, Eugene, OR); anti-cellular retinaldehyde binding protein (CRALBP; 1:400; gift of John C. Saari, University of Washington, Seattle, WA) and biotinylated peanut agglutinin (PNA; 1:50; Vector Laboratories, Burlingame, CA). Single labeling was performed using antibodies to CRALBP (1:400), glutamine synthetase (GS), carbonic anhydrase C (CAC; both used at 1:600; gifts of Paul J. Linser, University of Florida, St. Augustine, FL), and protein kinase C (PKC; 1:100; Biomol Research Laboratories, Plymouth Meeting, PA). All probes were diluted in PBTA. After rotating overnight at 4°C, 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) were used. Streptavidin-Cy3 was used with the biotinylated PNA. All secondary reagents were used at a 1:200 dilution. The sections were then rinsed, mounted in *n*-propyl gallate in glycerol, and viewed on a laser scanning confocal microscope (model 1024; Bio-Rad, Hercules, CA).

To evaluate cellular proliferation in the retina, retinal samples were fixed in 4% paraformaldehyde in sodium cacodylate buffer (0.1 M; pH 7.4) overnight at 4°C. The tissue was then dehydrated in increasing concentrations of ethanol and embedded in paraffin (Paraplast X-tra; Fisher Scientific, Pittsburgh, PA). The tissue was sectioned at 4 μm and placed on capillary gap slides (Fisher Scientific) at which time they were dewaxed in xylene, rehydrated in graded ethanol, and stained with the MIB-1 antibody (1:100; Immunotech, Westbrook, ME) to the Ki67 protein, with an automated tissue stainer (Techmate 1000; Ventana, Tuscon, AZ).

To determine the extent of cell death in the retina, retinas were embedded in agarose as described earlier and stained using the TdT-dUTP terminal nick-end labeling (TUNEL) method. Briefly, 100-μm-thick 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 incubated in 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. TUNEL-labeled cells were counted, and the section length was measured to give the number of cells labeled per millimeter of retina.

OS measurements and counts of nuclei in the ONL were made on 1 μm-thick resin embedded sections stained with PPDA. For sampling, sections were taken from three different regions from three eyes within each condition. The width of the OS layer was measured with 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

Retinal Morphology

Typically, in a retina detached for 3 days there was significant inner and OS degeneration as well as photoreceptor cell loss from the outer nuclear layer (ONL; Fig. 1B; compare with normal, Fig. 1A). The morphology of the retinas reattached after 1 hour (Figs. 1C–E) or 1 day (Figs. 1F–H), and examined at 3 days, however, showed considerably less evidence of degeneration. Although some variability was present both between and within animals after reattachment, photoreceptor OS were usually longer and more organized than control detachments. Figures 1C, 1D, and 1E (1-hour detachments) and Figures 1F, 1G and 1H (1-day detachments) demonstrate the extent of the variability observed. In most cases OS appeared remarkably normal (compare Figs. 1C and 1F with 1A), whereas in other cases some shortening of the OS was apparent (Figs. 1D, 1E, 1G, 1H). Regardless of the extent of the shortening, however, the ONL always appeared remarkably homogeneous in the reattached retinas, showing none of the uneven borders and gaps characteristic of detached retina. Rarely, some regions of retina had small folds, most likely occurring as the retina settled down during the reattachment procedure (not shown). No measurements or analyses were performed in these regions.

Photoreceptor Cell Counts and OS Length

In the normal retina, in the regions sampled for the above studies, we found, on average, 257 photoreceptor nuclei per millimeter of retina (Fig. 2). After 3 days of detachment, there was a reduction to an average of 207 nuclei/mm ($P = 0.03$ when compared with normal retina with a two-sample *t*-test,

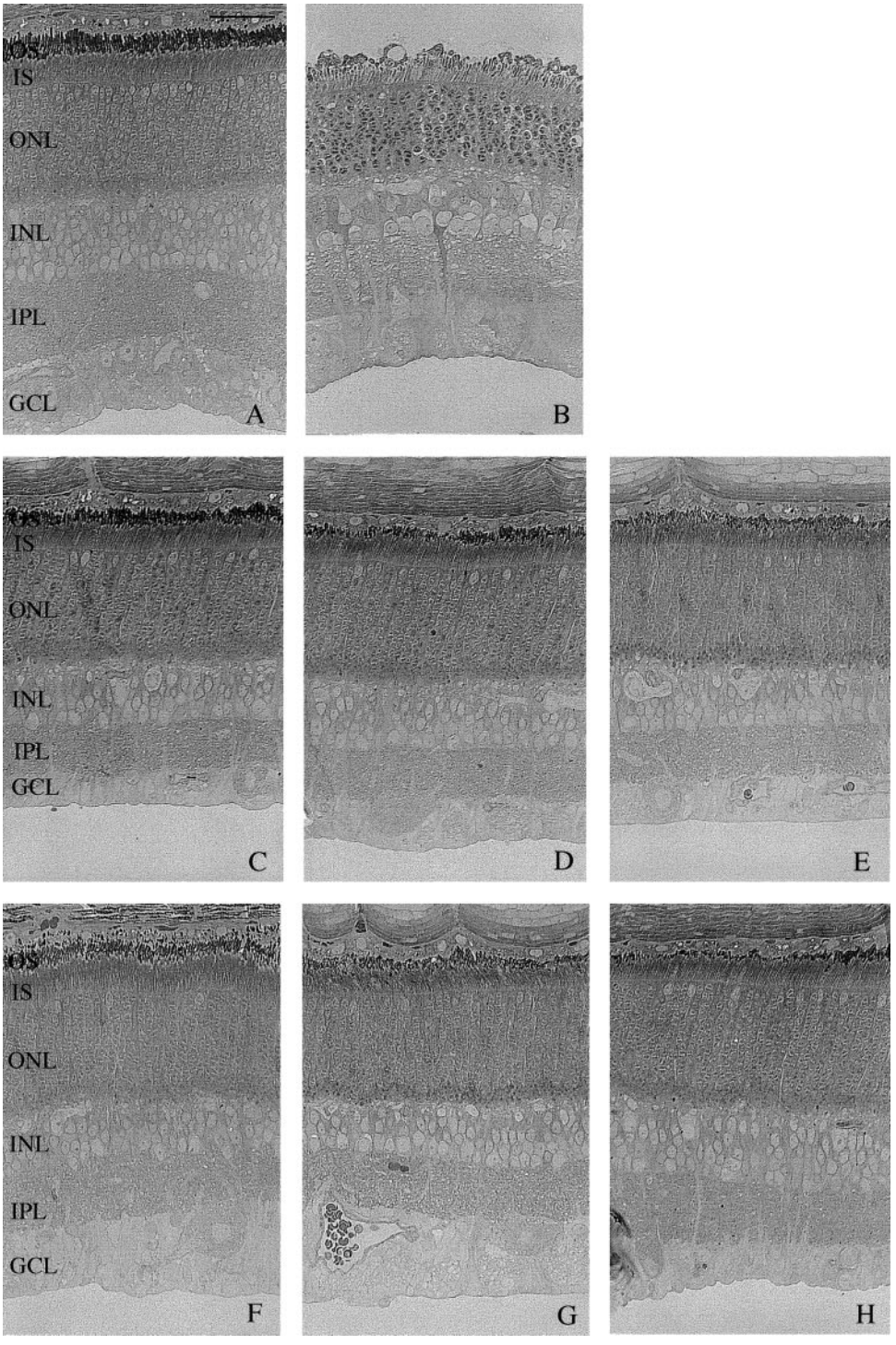


FIGURE 1. Light micrographs of normal (**A**), 3-day detached (**B**), 1-hour detached/3-day reattached (**C–E**), and 1-day detached/2-day reattached (**F–H**) retinas. Some shortening of OS occurred in the reattached retinas (**D**, **E**, **G**, **H**); however, they usually appeared longer and more organized (**C**, **F**) than those in the 3-day detached retinas (**B**). The ONL of the reattached retinas was also more organized compared with the 3-day detached retina. There was no significant difference in the appearance of the retinas between the 1-hour and 1-day detachments followed by reattachment. OS, outer segment; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 50 μm.

assuming equal variance). In the reattached retinas, the reduction was smaller (232 nuclei/mm in both the 1-hour and 1-day detachment–reattachment experiments). The same *t*-test yields $P = 0.09$ when these data are compared with the counts in normal retina.

The OS layer in normal eyes averaged approximately 17 μm thick in the region of retina used for these studies (Fig. 3). By 3 days of detachment the thickness had decreased to approximately 10.3 μm. In the 1-hour detachment–reattachment experiment, it averaged 9 μm and in the 1-day detachment–reattachment experiment, 8.2 μm. All three of these measurements are highly significant when compared with normal ($P < 0.0001$) but are not significantly different from each other.

Apoptosis

Cell death in the retina after detachment occurs mainly by apoptosis, peaks at the 3-day time point, and continues at lower levels, as long as the retina is detached.[15] At 3 days after detachment, there were, on average, 54.2 TUNEL-labeled photoreceptor cells per millimeter of retina (Fig. 4, 3d RD). The number was dramatically lower (0.21 and 0.05 cells/mm) in retinas that had been detached for 1 hour or 1 day, respectively, and then reattached and harvested at 3 days (Fig. 4,

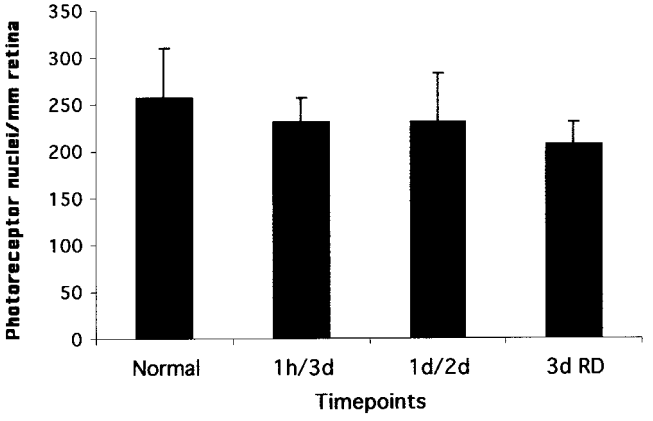


FIGURE 2. Number of photoreceptor nuclei counted per millimeter of retina in normal, 1-hour detached/3-day reattached (1h/3d), 1-day detached/2-day reattached (1d/2d), and 3-day detached retinas (3d RD). The outer nuclear layer photoreceptor counts in reattached retinas were not significantly different from those in normal retina. The 3-day detachments showed a significant decrease in photoreceptor number compared with normal retina. Error bars, 1 SD.

1h/3d, 1d/2d). No TUNEL-positive cells were found in normal retina. The reattachments was not significantly different from each other ($P = 0.28$) or from normal retina (1 hour, $P = 0.16$; 1 day, $P = 0.07$) in the number of TUNEL-positive cells, but they are significantly different from the 3-day detachments ($P < 0.0001$).

Immunohistochemistry

Results from four sets of antibodies used to characterize changes in the retina are shown in Figure 5. Typical 1-hour and 1-day detachments with reattachment can be compared with normal retina and retina that had been detached for 3 days. In the normal retina, anti-GFAP labeled intermediate filament proteins in the end foot portion of Müller cells and, to a lesser degree, horizontal cells; anti-rod opsin labeled rod OS (Fig. 5A). At 3 days after detachment, anti-GFAP labeling occurred throughout the entire Müller cell, extending to the outer limiting membrane (OLM); anti-rod opsin labeling was still present in the truncated and disorganized OS but also occurred throughout the plasma membrane of the entire cell (Fig. 5B). In the reattached retinas qualitatively similar but greatly attenuated changes were observed. Anti-GFAP labeling stopped in the inner portion of the ONL (Figs. 5C, 5D). Anti-rod opsin labeling occurred predominantly in the rod OS and surrounding only an occasional photoreceptor cell body in the ONL (Fig. 5D, arrows). This pattern of anti-GFAP and anti-opsin labeling was remarkably consistent in all the reattachment regions examined; the only variability being in the OS length, as illustrated in Figures 1 and 3.

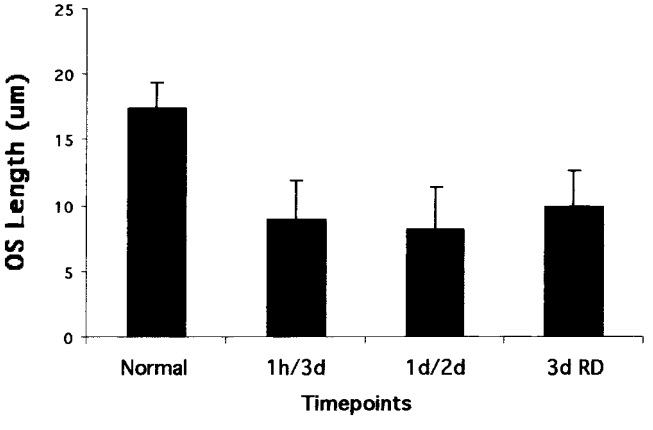


FIGURE 3. Length of OS in normal, 1-hour detached/3-day reattached (1h/3d), 1-day detached/2-day reattached (1d/2d), and 3-day detached retinas (3d RD). Both the reattached and 3-day detached retinas showed a similar decrease in OS length compared with normal retina, although they were not significantly different from each other. Error bars, 1 SD.

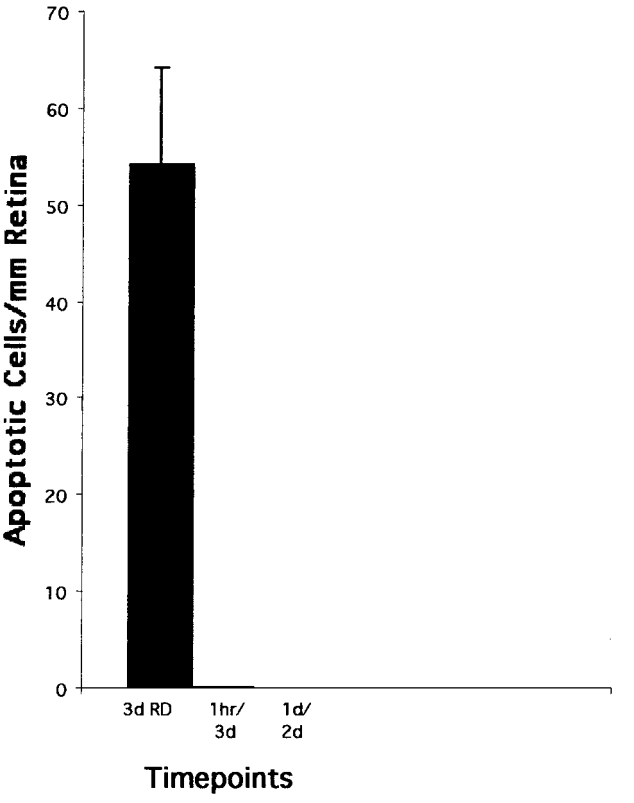
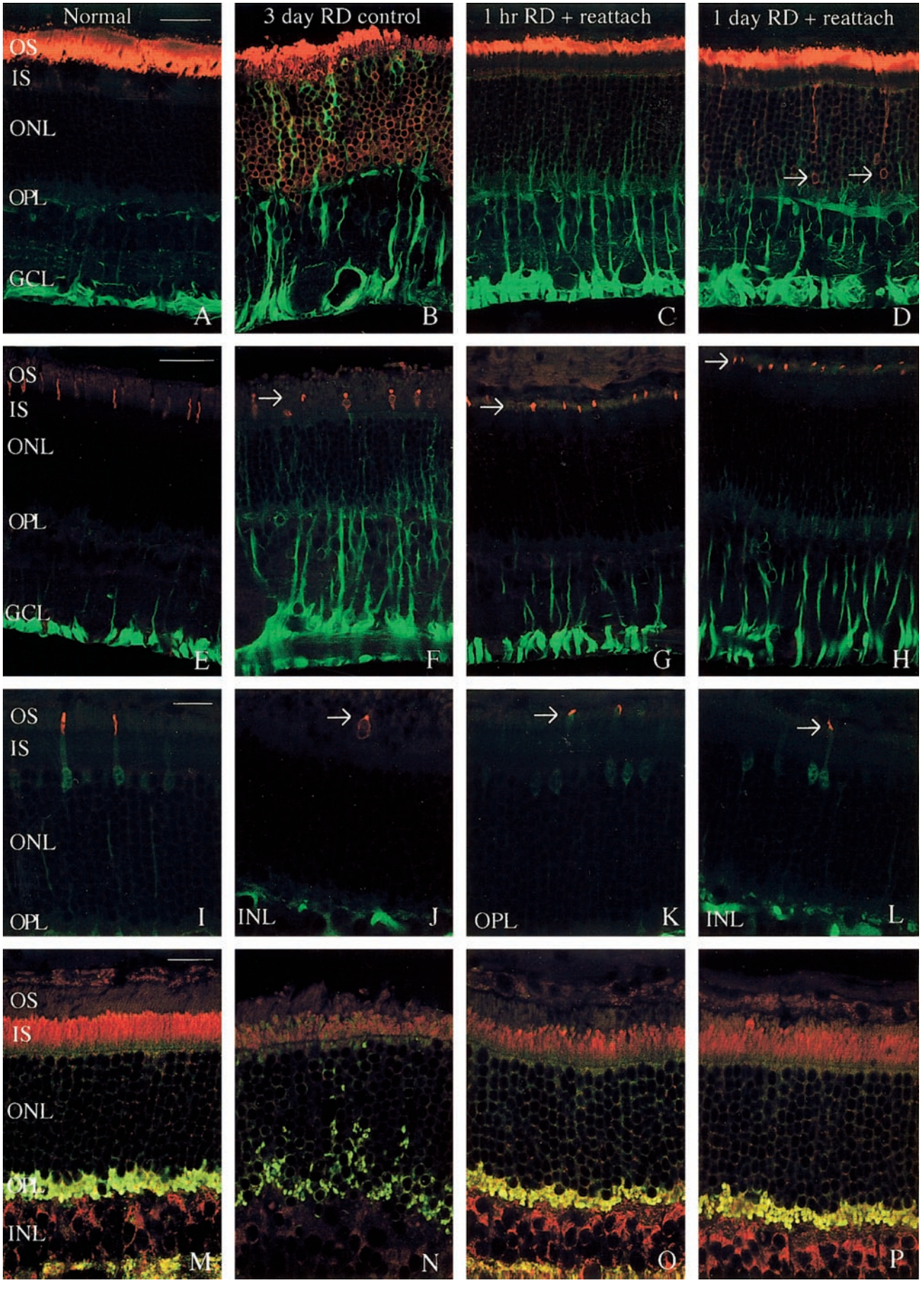


FIGURE 4. Extent of TUNEL labeling at day 3 in 3-day detachments (3d RD), and 1-hour (1h/3d) or 1-day (1d/2d) detachments followed by reattachment. Essentially no TUNEL labeling was observed in the reattachments. Error bar, 1 SD.

Anti-vimentin and anti-M/L cone opsin labeling showed changes similar to those observed with anti-GFAP and anti-rod opsin (Figs. 5E–H). In retinas that had been detached for 1 hour or 1 day and then reattached, the anti-vimentin labeling showed a small increase, stopping at the border of the outer plexiform layer (OPL; Figs. 5G, 5H). Very little variability was observed in the pattern of anti-vimentin staining in the reattached retinas. Unlike the 3-day detachments, the anti-M/L cone opsin labeling was restricted to the OS, with little or no redistribution (Figs. 5G, 5H; arrows). As with the rod labeling, the most variability was seen in the length of the cone OS.

To examine specifically the response of the S-cones, sections were labeled with anti-S cone opsin and anti-calbindin D. The S cones responded similarly to M/L cones (Figs. 5I–L). In the normal retina anti-S cone opsin was observed only in the OS, and anti-calbindin D labeled all cones in their entirety (Fig. 5I). At 3 days after detachment, very little S-cone opsin and calbindin D labeling was present on the sections. When S-cone opsin was observed, it was present in very truncated OS (Figs. 5J, arrow) and was redistributed to the inner segment (IS) and cell body. In the reattachments, however, many anti-S cone opsin–labeled OS (Figs. 5K, 5L, arrows) were observed, with these cells rarely showing opsin redistribution. Anti-calbindin D labeling was present in the entire cone cell with an intensity similar to normal. As with the M/L cones and rods, most of the


Normal
3 day RD control
1 hr RD + reattach
1 day RD + reattach
OS
IS
ONL
OPL
GCL
INL
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

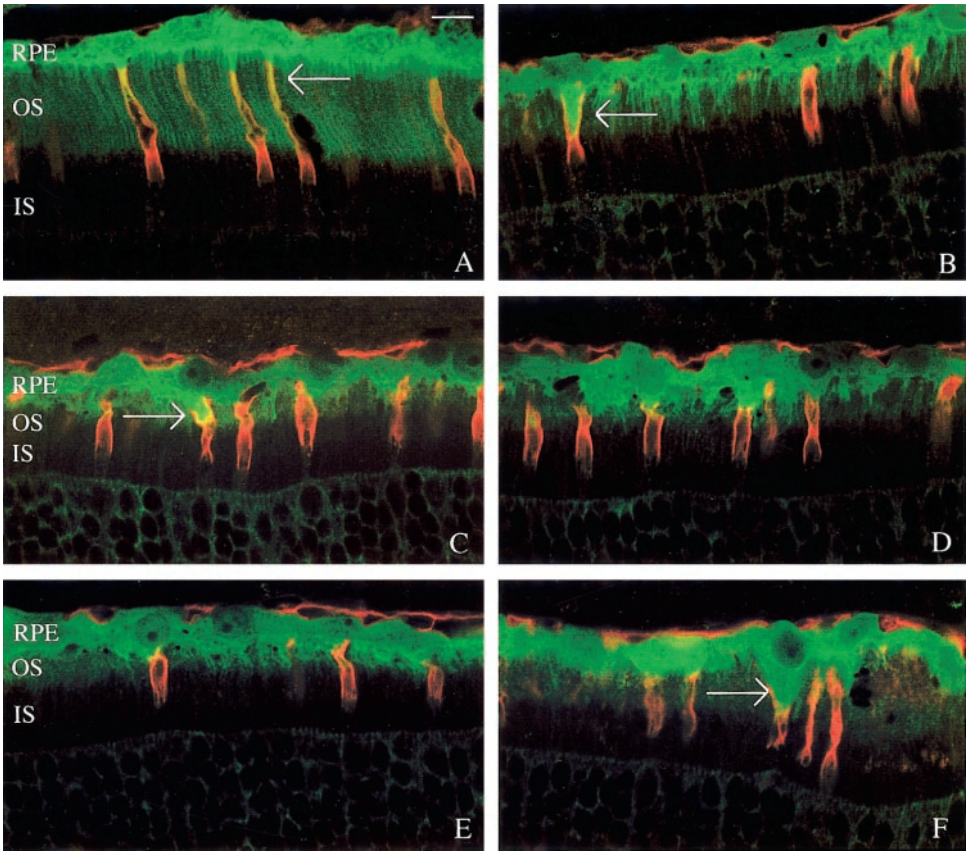


FIGURE 6. Double-label immunohistochemistry with probes to CRALBP (*green*) and biotinylated PNA (*red*). In the normal retina (**A**) anti-CRALBP labeling was present in the RPE cytoplasm and in the fine apical microvilli that projected into the cone matrix sheath, labeled with PNA (*arrow*). In detachments of 1 hour (**B**, **C**, **D**) or 1 day (**E**, **F**) followed by reattachment and examined at 3 days, anti-CRALBP labeling was still present in the RPE and in large processes extending toward the photoreceptors. In some cases, these processes projected into the PNA-labeled sheath (**B**, **C**, **F**; *arrows*). In other cases the RPE had yet to form apical projections, so that the sheath only abutted the RPE (**D**, **E**). The PNA labeled cone sheaths were also shorter and less organized in the reattachments compared with the normal appearance. In some cases, a few RPE cells appeared to label more intensely than other RPE cells in the same section (**F**). The *red* staining at the *top* of some figures represents PNA binding to the basement membrane. This staining was not altered by reattachment. OS, outer segment; IS, inner segment; RPE, retinal pigment epithelium. Scale bar, 10 μm.

variability between and within animals with anti-S cone opsin labeling was in the length of the OS.

Anti-CO and anti-synaptophysin were used to examine the population of mitochondria in the photoreceptor IS and the organization of photoreceptor terminals in the OPL, respectively (Figs. 5M–P). In the normal retina, anti-CO labels the IS robustly and anti-synaptophysin labeling clearly demonstrates the highly organized layer of photoreceptor terminals in the OPL (Fig. 5M). At 3 days after detachment, CO antibody labeling of the IS decreased, whereas the synaptophysin antibody labeling pattern showed substantial disruption of the OPL, as rod terminals were retracted into the ONL (Fig. 5N). Rarely was either of these responses observed in any of the reattached retinas (Figs. 5O, 5P). Very little variability occurred with these two probes, and in most cases, the retina simply appeared normal, although in rare cases, some retraction of photoreceptor terminals, as well as a decrease in anti-CO labeling of the IS, was observed (data not shown).

Anti-PKC and anti-calbindin D were used to examine the extent of neurite outgrowth from rod bipolar and horizontal cells, respectively. At 3 days of detachment, neurite outgrowth extending into the ONL was observed in many, but not all, sections.[17] In the reattached retinas, this response was never observed (data not shown).

To further explore the response of Müller cell proteins after reattachment, sections were labeled with antibodies to GS, CAC, and CRALBP. At 3 days of detachment, the intensity of the Müller cell labeling dramatically decreased with all three antibodies.[19] After reattachment in either detachment duration,

FIGURE 5. Double-label immunohistochemistry using antibodies to GFAP and rod opsin (**A–D**), vimentin and M/L cone opsin (**E–H**), calbindin D and S cone opsin (**I-L**), and cytochrome oxidase (CO) and synaptophysin (**M–P**). Comparisons were made between normal (**A**, **E**, **I**, **M**), 3-day detached (**B**, **F**, **J**, **N**), 1-hour detached/3-day reattached (**C**, **G**, **K**, **O**), and 1-day detached/2-day reattached feline retinas (**D**, **H**, **L**, **P**). GFAP and rod opsin: In the normal retina, GFAP (*green*) was restricted to the inner portion of the Müller cell and rod opsin (*red*) was localized to the rod OS (**A**). At 3 days of detachment, GFAP increased in Müller cells to the level of the OLM. Rod opsin was present in the truncated OS and was redistributed to the ONL (**B**). In detachments of both 1 hour (**C**) or 1 day (**D**) followed by reattachment, GFAP increased within Müller cells to the level of the ONL. Very little opsin redistribution was present, although labeled cell bodies were occasionally observed (*arrows*; **D**). Vimentin and M/L cone opsin: In the normal retina, vimentin (*green*) was restricted to the inner portion of the Müller cell, and M/L cone opsin (*red*) was present in the cone OS (**E**). At 3 days of detachment, vimentin increased in the Müller cells to the level of the OLM (**F**). M/L cone opsin was present in the truncated cone OS (*arrow*) and was redistributed to the IS and cell bodies (**F**). In detachments of 1 hour (**G**) or 1 day (**H**) followed by reattachment, vimentin increased to the level of the OPL/ONL. Cone opsin was present only in the shortened OS (*arrows*). Calbindin D and S cone opsin: In the normal retina, calbindin D (*green*) was present in the entire cone cell, and S cone opsin (*red*) was present in the OS of S cones (**I**). At 3 days of detachment, no calbindin D labeling was present. S cone opsin was present in the truncated cone OS (*arrow*) and was redistributed to the IS and cell bodies (**J**). In detachments of 1 hour (**K**) or 1 day (**L**) followed by reattachment, calbindin D labeling appeared normal. S cone opsin was present only in the shortened OS (*arrows*). (Note: the calbindin D–stained cells in the INL of (**J**) and (**L**) were horizontal cells, which were not always in the picture, and therefore are not shown in (**I**) and (**K**).) CO and synaptophysin: In the normal retina, CO (*red*) was present in mitochondria in the photoreceptor IS (as well as other cell types), and synaptophysin (*green*) was present in the photoreceptor terminals (**M**). At 3 days of detachment, the intensity of CO decreased in the IS. Synaptophysin was present in the rod terminals that had retracted into the ONL (**N**). In detachments of both 1 hour (**O**) or 1 day (**P**) followed by reattachment, the decrease in intensity of CO in the IS was less. Anti-synaptophysin labeling was more normal, showing an organized OPL. Abbreviations as in Figure 1. Scale bar: (**A–H**) 50 μm; (**I–P**) 25 μm.

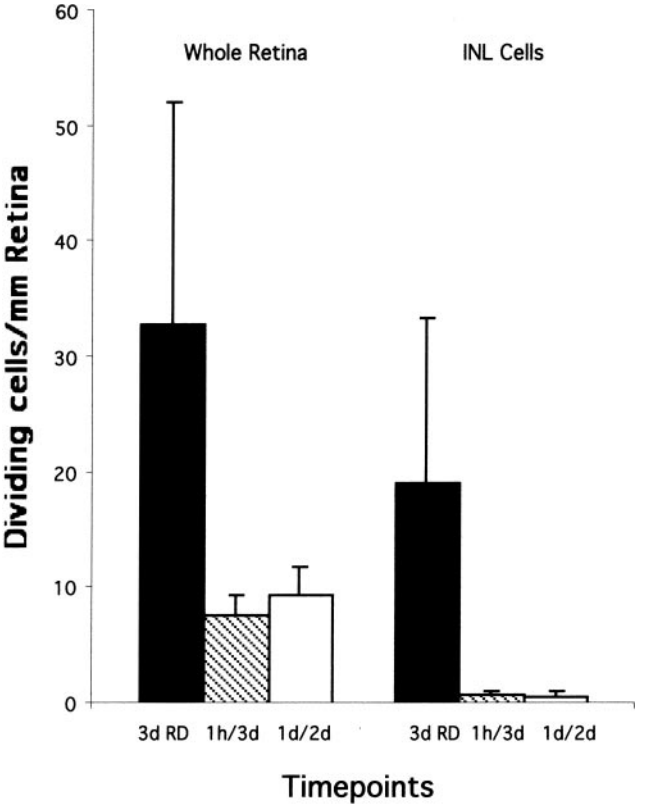


FIGURE 7. Number of nonneuronal cells dividing at 3 days (3d RD) in the entire retina (Whole Retina) and only in the inner nuclear layer (INL Cells), as detected by the MIB-1 antibody. Cell proliferation was much lower in the reattached retinas (1h/3d; 1d/2d), compared with the 3-day detachments. The proliferating cell types included Müller cells, astrocytes, endothelial cells, microglia, and RPE. Reattachment appeared to reduce the amount of cell proliferation substantially in the INL (primarily Müller cells), indicating that other retinal cell types (primarily astrocytes) were responsible for most of the proliferation after reattachment. Error bars, 1 SD.

very little change in labeling intensity was observed compared with normal (data not shown).

The RPE-OS interface was examined by the double labeling of sections with anti-CRALBP and biotinylated PNA. In the normal retina, long, slender apical projections of the RPE microvilli (labeled with anti-CRALBP) extend into the cone matrix sheath (labeled with biotinylated PNA; Fig. 6A). Detachment separated and disrupted these two structures but on reattachment, this relationship showed signs of beginning to re-form (Figs. 6B–F). In some cases, these newly formed apical projections from the RPE appeared very wide, yet they appeared already to be enveloped by the cone matrix sheath (Figs. 6B, 6C, 6F, arrows). In other areas from the same sections, however, the RPE appeared simply to abut the PNA-labeled cone matrix sheath, apparently having not yet formed the specialized apical processes (Figs. 6D, 6E). The 1-hour (Figs. 6B–D) and 1-day detachments (Figs. 6E, 6F), followed by reattachment, showed a similar response.

Proliferation

After retinal detachment, proliferation began in all nonneuronal retinal cell types, including astrocytes, Müller cells, endothelial cells, and microglia.[28,29] The MIB-1 antibody, which detects dividing cells in all phases of cell division, was used to determine the effect of reattachment on this proliferative event. In past experiments it has been estimated that the peak of proliferation occurs at 3 to 4 days after detachment. In the retina detached for 3 days we counted an average of 32.86 MIB-1 labeled cells/mm of retina (Fig. 7, 3d RD, Whole Retina). Reattachment after either 1 hour or 1 day of detachment reduced these numbers to 7.65 and 9.38 cells/mm retina, respectively (Fig. 7, 1h/3d, 1d/2d, Whole Retina). When the labeled cell types were tabulated separately, the number of dividing cells in the INL (presumed Müller cells) was reduced from 19.13 labeled cells/mm of retina in the 3-day detachment to 0.55 and 0.7/mm of retina, in the 1-hour and 1-day detachments with reattachments, respectively (Fig. 7, INL Cells). Thus, the number of labeled cells across the whole retina was reduced approximately 4-fold in the reattachments, whereas the number of labeled Müller cells was reduced by nearly 30-fold.

In 3-day detachments without reattachment, approximately 0.71 RPE cells/mm retina labeled with the MIB-1 antibody (Fig. 8, 3d RD). The number of labeled RPE cells after reattachment in either the 1-hour (Fig. 8, 1h/3d, 0.72 cells/mm) or 1-day (Fig. 8, 1d/2d, 0.26 cells/mm) detachments with reattachment, however, was almost equivalent to the number found in the retinas with 3-day detachments.

DISCUSSION

Previous experimental studies of retinal reattachment have focused primarily on the morphology of the photoreceptor–RPE interface, because of the primary role of OS in the visual process.[8,10–12] In this study, we used several different molecular probes to examine the effects of very early reattachment on protein expression, cell death, and proliferation, as well as photoreceptor recovery. An overview leads to the conclusion that reattachment within 24 hours is remarkably effective at halting many of the cellular changes induced by detachment that may pose a threat to the return of normal vision (Table 1). The reattached retinas also show variability in their responses, particularly in OS length and in the OS–RPE interface. It is this

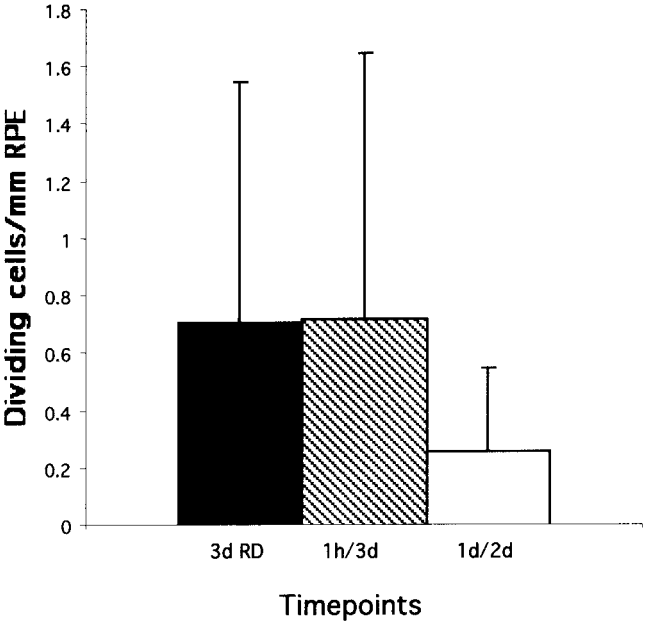


FIGURE 8. Number of dividing RPE cells at 3 days in 3-day detachments (3d RD) and 1-hour (1h/3d) or 1-day (1d/2d) detachments, followed by reattachment, as detected by the MIB-1 antibody. There was no significant difference between the groups, indicating that RPE cell proliferation continued, albeit at a low rate, after reattachment. Error bars, 1 SD.

TABLE 1. A Summary of the Effects of Early Retinal Reattachment

Photoreceptors
Does not prevent the shortening of OS induced by detachment
Prevents opsin redistribution in both rods and cones
Preserves the amount of cytochrome oxidase in IS.
Prevents rod synaptic terminal retraction
Second-order neurons
Prevents rod bipolar neurite outgrowth
Prevents horizontal cell neurite outgrowth
Müller Cells
Slows the increase in intermediate filament protein expression
Greatly reduces proliferation
Preserves the expression of soluble proteins GS, CAC, CRALBP
Retinal pigment epithelium
Does not prevent shortening of apical processes
Does not completely reestablish ensheathment of outer segments
Does not stop proliferation
Astrocytes, endothelial cells, microglia
Reduces the proliferative response

variability that may explain some of the imperfections in vision that can result, even after rapid and successful reapposition of neurosensory retina to the RPE. Indeed, these variations appear to form the beginning of "patchwork" regeneration described across retinas reattached for very long times.[26] "Patchy" recovery may have little effect in the periphery, but it could be highly significant in the small area encompassed by the fovea. The continued responsiveness of Müller and RPE cells may provide a mechanism for certain long-term complications after reattachment, in which cells can grow on either surface of the retina in a condition known as proliferative vitreoretinopathy (PVR).

The remarkable similarity between the retinas that were reattached after being detached for 1 hour or 1 day is striking and suggests that reattachment at any time up to 24 hours after detachment produces similar results. This, in turn, suggests that molecular processes induced during the first hour of detachment are not halted immediately by reattachment. We have recently shown that the FGF receptor-1 and the extracellular signal-regulated kinase (ERK) become phosphorylated within 15 minutes of detachment.[30] Yoshida et al.[31] showed an induction of c-*fos* mRNA 30 minutes after detachment. Members of the activator protein-1 (AP-1; c-Fos and c-Jun) complex are then highly induced at 2 hours of retinal detachment.[30] Because c-Fos and c-Jun are potentially important regulators of downstream events after detachment, it is reasonable to assume that reattachment at 1 hour did not stop their induction. However, it is also reasonable to assume that reattachment modifies events associated with these signaling pathways, because all the cellular events associated with detachment were attenuated by reattachment. Based on other studies of reattachment,[10–12] longer detachment intervals make these events more difficult to stop or reverse, thus probably producing more variability in the recovery process and a greater likelihood of complications such as PVR.

Although average OS length is similar in both sets of reattached retinas and in the 3-day detached retinas, one of the clearest indications that the cells in the reattached retinas are different from those that remain detached are the data on opsin redistribution. When the retina is detached, rod and cone opsins redistribute into the plasma membrane surrounding the inner parts of the cell.[32–34] This reaction has been observed in a number of retinal degenerations[35–40] and seems to be a reliable sign of OS degeneration. We interpret the absence of significant opsin redistribution in the reattached retinas to mean that the photoreceptors have recovered the mechanisms responsible for normal disc morphogenesis and OS construction. That the OS are no greater in length in the reattached retinas than in the 3-day detached retinas may indicate that these cells undergo a period of heightened disc shedding after reattachment. Alternatively, it may mean that the process of OS membrane assembly is much slower than normal. Primate retinas detached for 7 days show a rod OS renewal rate that is approximately one third of normal at 7 and 14 days after reattachment.[12] However, frog retinas detached for 10 hours show essentially normal membrane assembly rates, but a significant reduction at 2 days of detachment.[41] Thus, the balance between disc morphogenesis and disc shedding may not be restored for an extended time, even if the retina is detached for as short as only 1 hour.

Although the effects on OS may be variable and long-term, it appears that other aspects of photoreceptor deconstruction were halted by reattachment. Labeling with the antibodies to synaptophysin showed almost no evidence of rod photoreceptor synaptic terminal retraction, a prominent response at 3 days of detachment.[17] Although OS can regrow, little is known about the ability of photoreceptors to reform functional synapses once those synapses become disrupted. Maintaining the integrity of photoreceptor synaptic circuitry may turn out to be one of the most important effects of early reattachment. Detachment of more than a few days' duration produces widespread and profound effects on the organization of the OPL,[13,17] and virtually nothing is known about the functional effects of this remodeling.

Equally impressive to the effect on photoreceptor deconstruction is the effect of reattachment on Müller cells. Their expression profiles for the proteins we commonly study—GS, CAC, CRALBP, GFAP, and vimentin—are close to that in normal retina. Furthermore, the robust proliferative response of Müller cells, normally at its peak 3 days after retinal detachment, was reduced to almost zero, even though the proliferation of other non-neuronal cells (mostly astrocytes) continued at a low level. Overall, these results may indicate that Müller cells still maintain their role in regulating the retinal environment (e.g., glutamate levels[21]) when the retina is rapidly reattached. The reduction in proliferation also suggests that subsequent gliotic responses are less likely to occur after rapid reattachment, at least in terms of intraretinal and subretinal hypertrophy, the two most common responses in the feline retina.[13,18,20,28] We do not yet know whether reattachment completely halts Müller cell hypertrophy over longer periods, nor do we know whether the low level of proliferation in the reattachments continues, allowing RPE cells, astrocytes, or Müller cells eventually to form epiretinal membranes, as occurs in human patients after reattachment surgery.[42–44]

We have reported that supplemental oxygen can also slow the events described herein after detachment.[16,45] The results of those experiments, as well as modeling done by Linsenmeier and Padnick-Silver,[46] point to hypoxia as playing a major role in the cellular responses to detachment. The results in this study seem to strengthen that conclusion, because one of the first effects of reattachment must be to reestablish normal oxygenation of the photoreceptor layer. Reestablishing other cell–cell interactions between the RPE and photoreceptors or photoreceptors and Müller cells, or reversing the effect of signaling cascades initiated by detachment, takes longer and may provide a mechanism for creating the variability that occurs in the recovery of the retina. Defining these other events seems to be critical to improving the results of retinal reattachment, whether detachment happens in a setting of trauma or disease or as a result of a therapeutic procedure.

Acknowledgments

The authors thank Derek Mann and Peter J. Kappel for technical assistance.

References

1. Aylward GW. Latest developments in treating retinal detachment. *Br J Hosp Med.* 1996;55:100–103.
2. Sullivan PM, Luff AM, Aylward GW. Results of primary retinal reattachment surgery: a prospective audit. *Eye.* 1997;11:869–871.
3. Burton TC. Preoperative factors influencing anatomic success rates following retinal detachment surgery. *Trans Am Acad Ophthalmol Otolaryngol.* 1977;83:499–505.
4. Tani P, Robertson DM, Langworthy A. Prognosis for central vision and anatomic reattachment in rhegmatogenous retinal detachment with macular detachment. *Am J Ophthalmol.* 1981;92:611–620.
5. 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.
6. Liem AT, Keunen, JE, Van Meel GJ, Van Norren D. Serial foveal densitometry and visual function after retinal detachment surgery with macular involvement. *Ophthalmology.* 1994;101:1945–1952.
7. Kroll AJ, Machemer R. Experimental retinal detachment in the owl monkey. III: electron microscopy of the retina and pigment epithelium. *Am J Ophthalmol.* 1968;66:410–427.
8. Kroll, AJ, Machemer, R. Experimental retinal detachment and reattachment in the rhesus monkey: electron microscopic comparison of rods and cones. *Am J Ophthalmol.* 1969;68:58–77.
9. Machemer R. Experimental retinal detachment in the owl monkey. II: histopathology of the retina and pigment epithelium. *Am J Ophthalmol.* 1968;66:396–410.
10. Anderson DH, Guerin CJ, Erickson PA, Stern WH, Fisher SK. Morphological recovery in the reattached retina. *Invest Ophthalmol Vis Sci.* 1986;27:168–183.
11. Guerin CJ, Anderson DH, Fariss RN, Fisher SK. Retinal reattachment of the primate macula: photoreceptor recovery after short-term detachment. *Invest Ophthalmol Vis Sci.* 1989;30:1708–1725.
12. Guerin CJ, Lewis GP, Fisher SK, Anderson DH. Recovery of photoreceptor outer segment length and analysis of membrane assembly rates in regenerating primate photoreceptor outer segments. *Invest Ophthalmol Vis Sci.* 1993;34:175–183.
13. Erickson PA, Fisher SK, Anderson DH, Stern WH, Borgula GA. Retinal detachment in the cat: the outer nuclear and outer plexiform layers. *Invest Ophthalmol Vis Sci.* 1983;24:927–942.
14. Wilson DJ, Green WR. Histopathologic study of the effect of retinal detachment surgery on 49 eyes obtained post mortem. *Am J Ophthalmol.* 1987;103:167–179.
15. Cook B, Lewis GP, Fisher SK, Adler R. Apoptotic photoreceptor degeneration in experimental retinal detachment. *Invest Ophthalmol Vis Sci.* 1995;36:990–996.
16. Mervin K, Valter K, Maslim J, Lewis GP, Fisher SK, Stone J. Limiting photoreceptor death and deconstruction during retinal detachment: the value of oxygen supplementation. *Am J Ophthalmol.* 1999;128:155–164.
17. Lewis GP, Linberg KA, Fisher SK. Neurite outgrowth from bipolar and horizontal cells after experimental retinal detachment. *Invest Ophthalmol Vis Sci.* 1998;39:424–434.
18. Lewis GP, Erickson PA, Guérin 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.
19. Lewis GP, Guérin 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.
20. 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.
21. Marc RE, Murry R, Fisher SK, Linberg KA, Lewis GP. Amino acid signatures in the detached cat retina. *Invest Ophthalmol Vis Sci.* 1988;39:1694–1702.
22. Bok D. Retinal transplantation and gene therapy: present realities and future possibilities. *Invest Ophthalmol Vis Sci.* 1993;34:473–476.
23. del Cerro M, Lazar ES, Diloreto D. The first decade of continuous progress in retinal transplantation. *Microsc Res Tech.* 1997;36:130–141.
24. de Juan E Jr, Loewenstein A, Bressler NM, Alexander J. Translocation of the retina for management of subfoveal choroidal neovascularization II: a preliminary report in humans. *Am J Ophthalmol.* 1998;125:635–646.
25. Lewin AS, Drenser KA, Hauswirth WW, et al. Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. *Nat Med.* 1998;4:967–971.
26. Fisher SK, Anderson DH. Cellular effects of detachment on the neural retina and retinal pigment epithelium. In: Ryan SJ, Wilkinson CP, eds. *Retina*. 3rd ed. Vol 3. *Surgical Retina.* Mosby, St Louis. MO. 2001;1961–1986.
27. Fisher SK, Stone J, Rex TS, Linberg KA, Lewis GP. Experimental retinal detachment: a paradigm for understanding the effects of induced photoreceptor degeneration. *Prog Brain Res.* 2001;131 679–698.
28. Fisher SK, Erickson PA, Lewis GP, Anderson DH. Intraretinal proliferation induced by retinal detachment. *Invest Ophthalmol Vis Sci.* 1991;32:1739–1748.
29. 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.
30. Geller SF, Lewis GP, Fisher SK. FGFR1, signaling, and AP-1 expression after retinal detachment: reactive Müller and RPE cells. *Invest Ophthalmol Vis Sci.* 2001;42:1363–1369.
31. Yoshida K, Muraki Y, Ohki K, et al. c-fos gene expression in rat retinal cells after focal retinal injury. *Invest Ophthalmol Vis Sci.* 1995;36:251–254.
32. Lewis GP, Erickson PA, Anderson DH, Fisher SK. Opsin distribution and protein incorporation in photoreceptors after experimental retinal detachment. *Exp Eye Res.* 1991;53:629–640.
33. Fariss RN, Molday RS, Fisher SK, Matsumoto B. Evidence from normal and degenerating photoreceptors that two outer segment integral membrane proteins have separate transport pathways. *J Comp Neurol.* 1997;387:148–156.
34. Rex TS, Lewis GP, Fisher SK. Rapid loss of blue and red/green cone opsin immunolabeling following experimental retinal detachment [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1997;38(4):**S**35. Abstract nr 162.
35. Nir I, Papermaster DS. Immunocytochemical localization of opsin in the inner segment and ciliary plasma membrane of photoreceptors in retinas of rds mutant mice. *Invest Ophthalmol Vis Sci.* 1986;27:836–840.
36. Usukura J, Bok D. Changes in the localization and content of opsin during retinal development in the rds mutant mouse: immunocytochemistry and immunoassay. *Exp Eye Res.* 1987;45:501–515.
37. Jansen HG, Sanyal S, De Grip WJ, Schalken JJ. Development and degeneration of retina in rds mutant mice: ultraimmuno-histochemical localization of opsin. *Exp Eye Res.* 1987;44:347–361.
38. Bowes C, van Veen T, Farber D. B. Opsin, G-protein and 48-kDa protein in normal and rd mouse retinas: developmental expression of mRNAs and proteins and light/dark cycling of mRNAs. *Exp Eye Res.* 1988;47:369–390.
39. Nir I, Papermaster DS. Immunocytochemical localization of opsin in degenerating photoreceptors of RCS rats and rd and rds mice. *Prog Clin Biol Res.* 1989;314:251–264.
40. Edward DP, Lim K, Sawaguchi S, Tso MOM. An immunohistochemical study of opsin in photoreceptor cells following light-induced retinal degeneration in the rat. *Graefes Arch Clin Exp Ophthalmol.* 1993;231:289–294.
41. Hale IL, Fisher SK, Matsumoto, B. Effects of retinal detachment on rod disc membrane assembly in cultured frog retinas. *Invest Ophthalmol Vis Sci.* 1991;32:2873–2881.
42. Van Horn DL, Aaberg TM, Machemer R, Fenzel R. Glial cell proliferation in human retinal detachment with massive periretinal proliferation. *Am J Ophthalmol.* 1977;84:383–387.
43. Hiskott PS, Grierson I, Trombetta CJ, Rahi AHS, Marshall J, McLeod D. Retinal and epiretinal glia: an immunohistochemical study. *Br J Ophthalmol.* 1984;68:698–707.
44. Guerin CJ, Wolfshagen RW, Eifrig DE, Anderson DH. Immunocytochemical identification of Müller's glia as a component of human epiretinal membranes. *Invest Ophthalmol Vis Sci.* 1990;31:1483–1491.
45. 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.* 1999;128:165–172.
46. Linsenmeier RA, Padnick-Silver L. Metabolic dependence of photoreceptors on the choroid in the normal and detached retina. *Invest Ophthalmol Vis Sci.* 2000;41:3117–3123.