Rapid Changes in the Expression of Glial Cell Proteins Caused by Experimental Retinal Detachment

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We examined the expression of several proteins normally present in Müller's glia after the production of experimental retinal detachment in adult cats. Retinas were detached for one-half to seven days, after which the tissue was processed for correlative immunocytochemistry and biochemistry. Previous studies demonstrated that the intermediate filament proteins glial fibrillary acidic protein and vimentin, increase after long-term retinal detachment (30 to 60 days), whereas glutamine synthetase, carbonic anhydrase C, and cellular retinaldehyde-binding protein all decrease to barely detectable levels. Alterations in Müller cell protein expression are rapid and specific events that can be detected as early as two days after retinal detachment. By seven days, levels of protein expression are similar to those in the long-term retinal detachments. Within the first week after injury the Müller cell processes hypertrophy and begin forming glial scars, which indicates that early intervention may be required to halt or reverse the effects of detachment.

MÜLLER CELLS, the highly specialized radial glia of the retina, play important roles in proliferative diseases of the retina, which are induced by events such as retinal detachment or penetrating wounds. A definition of what these roles are is necessary to understand their cellular mechanisms. The overall characteristics of the Müller cell response are similar to that occurring in astrocytes elsewhere in the central nervous system; that is, the cells become reactive. This state is characterized by proliferation, migration, and hypertrophy of the cells. When reactive Müller cells migrate toward, or send their processes into, the space between the photoreceptors and retinal pigment epithelium, they can cause subretinal fibrosis. When they migrate onto the vitreal surface, they have the capacity to form cellular membranes in conditions such as proliferative vitreoretinopathy.[1-3] Another less well understood aspect is their hypertrophy within the neural retina.[4-5]

In animal models of retinal detachment and reattachment,[4-5] we have shown that the presence of Müller cell processes in the subretinal space assures virtually complete inhibition of outer segment regeneration after successful surgical reattachment. An important concern that emerged from these data is how soon after detachment the Müller cells enter the reactive phase. If this occurs soon after detachment, then even successful reattachment may not be adequate to inhibit their further growth and migration. The first evidence that the proliferation and migration occur rapidly after detachment came from a study of the induction of messenger ribonucleic acid (mRNA) for glial fibrillary acidic protein.[7]

A characteristic component of the reactive state is a dramatic increase within the Müller cells' cytoplasm, of intermediate filaments 10 nm in diameter. They are a highly stable component of the cytoskeleton, which may serve to stabilize the new and enlarged processes of the reactive cells. In the normal retina these filaments, which are composed of both glial fibrillary acidic protein and vimentin, are limited to the vitreal half of the cells' cytoplasm; however, after detachment they progressively fill the Müller cell cytoplasm and extend into new processes that have grown into the subretinal

space. Our previous study demonstrated that we could detect a fivefold increase in mRNA for glial fibrillary acidic protein three days after detachment and that this increase occurred specifically in Müller cells. Additionally, there is a peak of induced Müller cell proliferation that occurs approximately three to four days after detachment, which is another indicator that the cells become reactive relatively quickly.[8]

Reactive Müller cells not only greatly upregulate the synthesis of intermediate filament proteins but also downregulate their synthesis of other proteins. Carbonic anhydrase C, glutamine synthetase, and the retinoid-binding protein known as cellular retinaldehyde-binding protein[9] become almost undetectable by ordinary immunocytochemical staining procedures or by Western blot analysis in a 30-day detachment.[10]

Immunocytochemical data from retinal detachments show that virtually all of the Müller cells in the detachment behave similarly; all show an upregulation of intermediate filament proteins and a downregulation of the other proteins mentioned above. Thus, we have used these changes as a measure of the Müller cells' reactivity, to determine how early they could be detected in the retinas of animals with experimental retinal detachments. The results show that we can detect changes within two days, a finding that agrees with our prediction that, even though events such as subretinal fibrosis or proliferative retinopathy may not be detectable as clinical entities for weeks or months after a detachment or reattachment, at least one important component of these responses, Müller cell reactivity, has its beginnings soon after the detachment event.

Material And Methods

Cat retinas were experimentally detached from the retinal pigment epithelium for one-half to seven days, as described elsewhere.[6] The *lens was* removed, and the eye was allowed to heal for at least two weeks. After removal of the vitreous, a solution of 0.25% sodium hyaluronate (Healon, Pharmacia, Piscataway, New Jersey) was infused between the retina and the retinal pigment epithelium with a micropipette. Cats were initially entrained to a 12-hour: 12-hour light cycle and were killed four hours after light onset, at which time retinas were processed for immunocytochemistry, sodium dode-cyl sulfate–polyacrylamide-gel electrophoresis, and Western blot analysis.

Retinas for postembedding immunocytochemistry were cut into quadrants and fixed for one hour with 1% paraformaldehyde and 1% glutaraldehyde, in sodium phosphate buffer. The tissue was first dehydrated in increasing concentrations of methanol with uranyl acetate (2%) added at the 70% step, and embedded in London Resin White (Polysciences, Warrington, Pennsylvania). The immunocytochemistry was done using 1-μm thick sections from normal and detached retinas. The sections were placed on slides that had been coated with aminopropyltriethoxysilane (Aldrich, Milwaukee, Wisconsin) to promote adhesion to the glass surface. All antibodies were diluted in phosphate-buffered saline containing 0.5% bovine serum albumin. Sections were blocked with normal goat serum (1:50; Vector Labs, Burlingame, California) for 30 minutes and then incubated overnight in primary antibody. Rabbit anti-bovine-glial fibrillary acidic protein (DAKO Corp., Carpenteria, California), mouse anti-porcine-vimentin (DAKO Corp., Carpenteria, California), and rabbit anti-bovine-cellular retinaldehyde-binding protein were used at a 1:400 dilution. Rabbit anti-chicken-glutamine synthetase and rabbit anti-chicken-carbonic anhydrase C were used at a dilution of 1:600. All primary immune reagents were IgG fractions. The next day the sections were rinsed in combined phosphate-buffered saline and bovine serum albumin, and the secondary antibody was added for one hour. Goat anti-rabbit or goat anti-mouse IgG conjugated to 5-nm gold spheres was used at a 1:50 dilution (Amersham, Arlington Heights, Illinois). After the sections were rinsed in phosphate-buffered saline and distilled water, the gold spheres were enlarged using a silver enhancing procedure (Amersham, Arlington Heights, Illinois). To ensure equal treatment, sections of normal and detached retinas were enhanced for the same time (25 minutes), rinsed in distilled water, and air dried. All procedures were done at room temperature.

The technique of pre-embedding immunocytochemistry was used in conjunction with the laser scanning confocal microscope, as described elsewhere.[11,12] Retinas were cut into quadrants and fixed in 4% paraformaldehyde in sodium cacodylate buffer (0.1 N; pH 7.4). Tissue approximately 2 mm square was excised and embedded in 5% agarose in phosphate-buffered saline. One hundred-micrometer-thick

sections were cut on a vibratome (Technical Products International, Polysciences, Warrington, Pennsylvania) and incubated in normal donkey serum (1:20) overnight at 4 C on a rotator. The next day the blocking serum was removed, and the primary antibodies were added. Glial fibrillary acidic protein and vimentin were used at 1:500. After rotating overnight at 4 C, the sections were rinsed in phosphate-buffered saline/bovine serum albumin and incubated in either donkey anti-rabbit or donkey anti-mouse IgG conjugated to the fluorochrome Cy3 (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) overnight at 4 C on a rotator. The sections were then rinsed, mounted in 5% n-propyl gallate in glycerol, and viewed on the laser scanning confocal microscope (BioRad, Hercules, California). All the antibodies were diluted in phosphate-buffered saline containing 0.5% bovine serum albumin, 0.1% Triton X-100, and 0.1% sodium azide.

Retinas for sodium dodecyl sulfate–polyacrylamide-gel electrophoresis and Western blot analysis were homogenized at 4 C in 0.02 M phosphate-buffered saline containing 0.1 mM phenylmethylsulfonylfluoride, 10 mM ethylenediaminetetraacetate, 0.5% Triton X-100, and 2.55% sodium dodecyl sulfate, to inhibit protein degradation. Protein concentrations were determined by the BCA (bicinchoninic acid) Protein Assay (Pierce Chemical Co., Rockford, Illinois). Fifty micrograms of protein was loaded in each lane of a 11% to 23% gradient gel (Phorcast, Amersham). The separated retinal proteins were transferred to nitrocellulose paper, were blocked in 3% bovine serum albumin in Tris-buffered saline, and were incubated overnight in primary antibody. Antibodies to glial fibrillary acidic protein, vimentin and cellular retinaldehyde-binding protein were used at a 1:400 dilution, whereas antibodies to glutamine synthetase and carbonic anhydrase C *were used* at 1:600, all in 1% bovine serum albumin in Tris-buffered saline. Blots were washed in Tris-buffered saline containing 0.5% Tween and incubated with goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (1:1,000; Bio-Rad) for one hour. After washing in Tween Tris-buffered saline and Tris-buffered saline, the blots were placed in horseradish peroxidase color development reagent (Bio-Rad) for 15 minutes, rinsed in distilled water, and air dried.

Results

When the postembedding immunocytochemical technique was used, antibodies to glial fibrillary acidic protein (Fig. 1, top left) and vimentin (Fig. 1, top right) labeled intermediate filament proteins only in the endfoot region of the Müller cell cytoplasm in the normal retina; labeling rarely extended past the inner plexiform layer. By two days after the production of a retinal detachment, immunolabeling with both of these antibodies was often observed into the outer plexiform layer (Fig. 1, middle left, anti-glial fibrillary acidic protein; middle right, anti-vimentin). By seven days, as a result of a hypertrophy of the Müller cells, labeling could be observed in enlarged processes that extended through the outer nuclear layer and onto the exposed surface of the photoreceptors (Fig. 1, bottom left, anti-glial fibrillary acidic protein; bottom right, anti-vimentin).

When the laser scanning confocal microscope and the pre-embedding technique was used, more subtle changes in the distribution of the intermediate filament proteins could be detected. Labeled Müller cell processes could be observed occasionally reaching the subretinal space by three days after detachment (Fig. 2, middle, anti-vimentin). By comparison, in a normal retina, anti-glial fibrillary acidic protein and anti-vimentin labeling within the Müller cells extended only to the outer plexiform layer (Fig. 2, left, anti-vimentin). By day 7, the entire Müller cell cytoplasm contained intermediate filaments as the cell hypertrophied both within the retina and onto the surface of the photoreceptors (Fig. 2, right, anti-vimentin).

Immunolabeling with antibodies to glutamine synthetase, carbonic anhydrase C, and cellular retinaldehyde-binding protein in normal retina occurred throughout the Müller cell cytoplasm with the heaviest labeling present in the endfoot region (Fig. 3, top left, anti-glutamine synthetase; middle left, anti-carbonic anhydrase C; bottom left, anti-cellular retinaldehyde-binding protein). Anti-cellular retinaldehyde-binding protein labeling was also present in the retinal pigment epithelium, and anti-carbonic anhydrase C labeling occurred in the photoreceptor nuclei. In Müller cells, the labeling intensity, with all three antibodies, began to decrease two days after retinal detachment, compared with time-matched con-
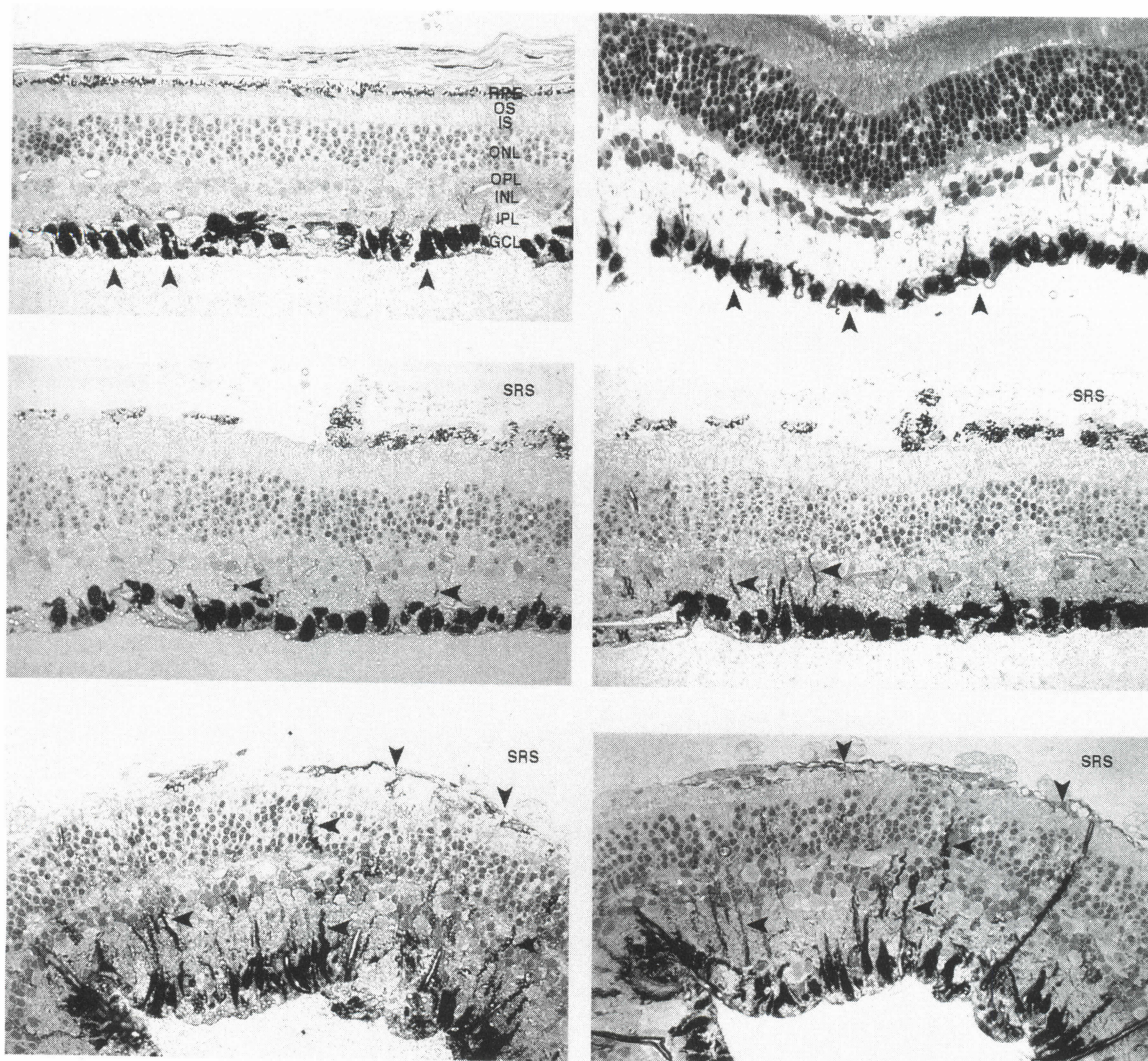
Fig. 1 (Lewis and associates). Histologic sections labeled with anti-glial fibrillary acidic protein (left) and anti-vimentin (right) (labeling appears black). The retinal pigment epithelium (RPE) is at the top of each photograph, and the vitreous is at the bottom. Top, In the normal retinas, these antibodies label intermediate filaments only in the most vitreal component of the Müller cell (arrowheads). Middle, Two days after detachment, intermediate filaments increase into the outer plexiform layer (OPL; arrowheads). Bottom, Seven days after detachment, intermediate filaments within the Müller cell cytoplasm pass through the outer nuclear layer (ONL) into the subretinal space (SRS) forming a glial scar (arrowheads). The cells in the subretinal space in the middle panels are pigmented cells and are not labeled by the antibodies. These cells are most likely retinal pigment epithelium cells that have proliferated from the retinal pigment epithelium monolayer and attached to the photoreceptor outer segments; they are not the result of retinal pigment epithelium detachment. Sections were counterstained with basic fuchsin. OS indicates outer segment; IS, inner segment; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer.

trol sections. The labeling intensity was lower still at five days, and, by seven days, labeling was barely detectable (Fig. 3, top right, anti-glutamine synthetase; middle right, anti-car-bonic anhydrase C; bottom right, anti-cellular retinaldehyde-binding protein). Anti-cellular retinaldehyde-binding protein labeling was still apparent in the retinal pigment epithelium
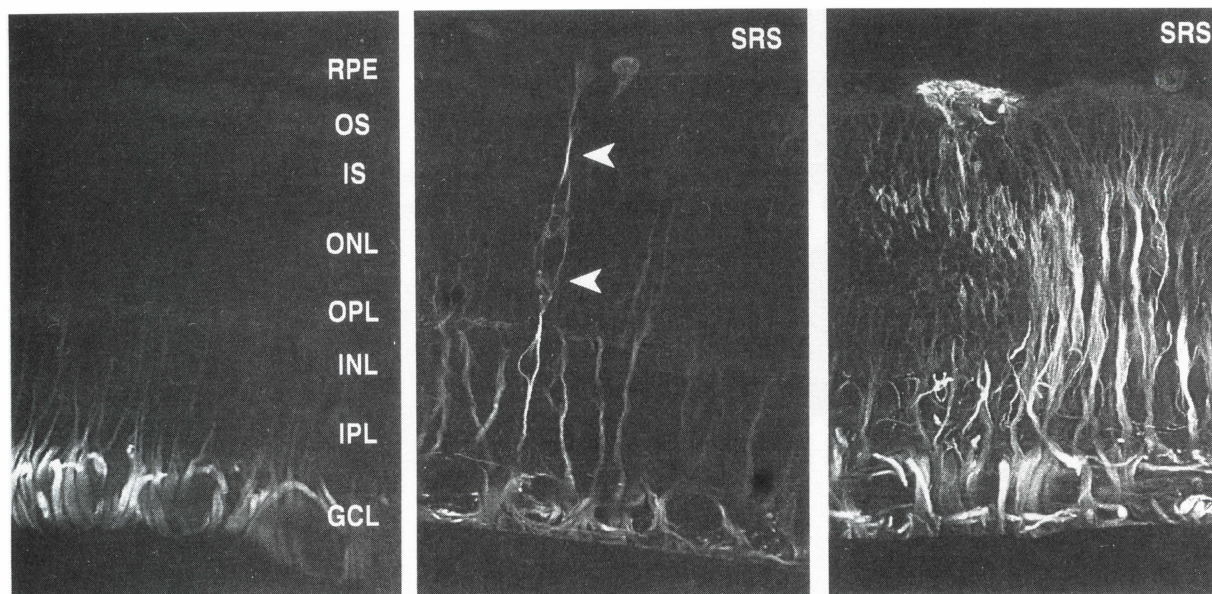
Fig. 2 (Lewis and associates). Histologic sections labeled with anti-vimentin (labeling appears white) and viewed with the laser scanning confocal microscope. The retinal pigment epithelium is at the top of each photograph, and the vitreous is at the bottom. Left, In the normal retina, vimentin intermediate filaments in the Müller cell cytoplasm extend from the endfoot region into the outer plexiform layer (OPL). Middle, Three days after retinal detachment, some Müller cells extend into the subretinal space (SRS) and label with intermediate filament antibodies. Right, By seven days after detachment, many of the Müller cells have hypertrophied both intraretinally and subretinally and are filled with intermediate filaments. RPE indicates retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer.

(not shown), and anti-carbonic anhydrase C antibodies still labeled photoreceptor cell nuclei at the seventh day, although both appeared to have reduced immunoreactivity. Control sections stained with preimmune serum showed no labeling.

Western blot analysis of retinal homogenates from normal, two-, five-, and seven-day-old detached retinas was consistent with the immunolabeling results: glial fibrillary acidic protein and vimentin levels increased, whereas glutamine synthetase and cellular retinaldehyde-binding protein levels decreased. Carbonic anhydrase C levels decreased to a lesser extent, most likely because of its continued expression in the photoreceptors (Fig. 4). Each antibody labeled a band at the appropriate molecular weight for that protein at each time point.

Discussion

Retinal detachment initiates a cascade of abnormal molecular and cellular events, which often results in permanent impairment of retinal function even after successful anatomic reattachment.[13] Understanding the cellular and molecular basis of the changes that occur after detachment and reattachment will be necessary to optimize visual recovery. Clearly the degeneration of photoreceptor outer segments is the initial cause of visual loss after detachment.[4,14-17] The death of photoreceptor cells,[5,18] and variability in the regeneration of outer segments after reattachment[4,19] may also contribute to variability in visual recovery. Our studies of animal models of detachment and reattachment indicate that major changes that occur in the Müller's glia of the retina, may also contribute to this variability of visual recovery. Some of these changes are easily understood in the context of retinal disease. The growth or migration of Müller cells into the subretinal space can generate subretinal scarring (fibrosis). A subretinal scar consisting of only a single layer of Müller cell processes intervening between the retinal pigment epithelium and the photoreceptors assures virtually no outer segment regeneration[6] in the area beneath the scar. Müller cells that migrate to the vitreal surface
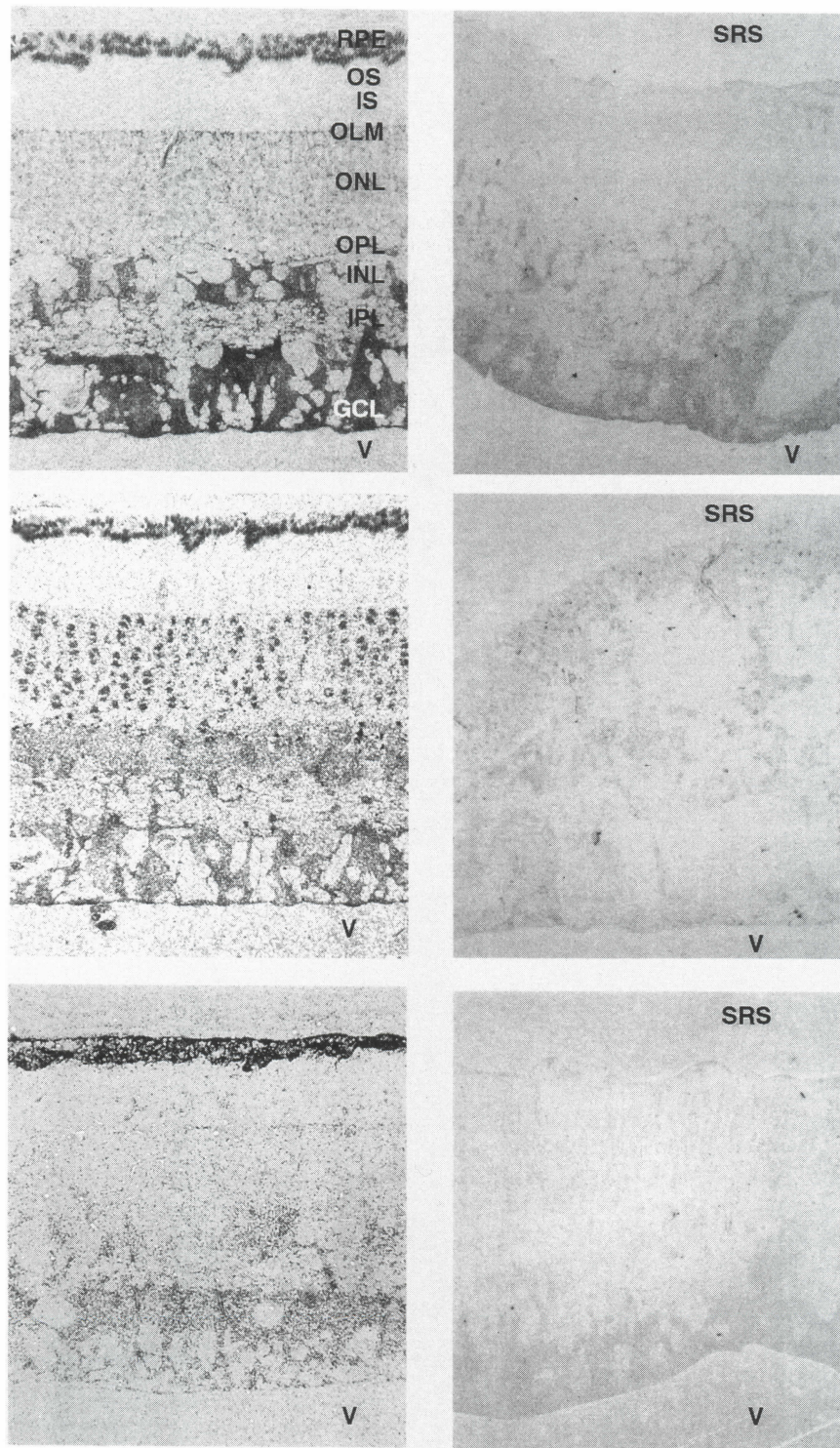
Fig. 3 (Lewis and associates). Left, Histologic sections from normal retina labeled with antibodies to glutamine synthetase (top), carbonic anhydrase C (middle), and cellular retinaldehyde-binding protein (bottom). The retinal pigment epithelium (RPE) is at the top of each photograph, and the vitreous (V) is at the bottom. Labeling occurs primarily in the Müller cell cytoplasm, with carbonic anhydrase C also present in photoreceptor cell bodies and cellular retinaldehyde-binding protein also in the retinal pigment epithelium. Right, Seven days after retinal detachment, the labeling for all antibodies is reduced in the Müller cells. Sections are unstained so that the faint labeling is not obscured. OS indicates outer segment; IS, inner segment; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; and GCL, ganglion cell layer.
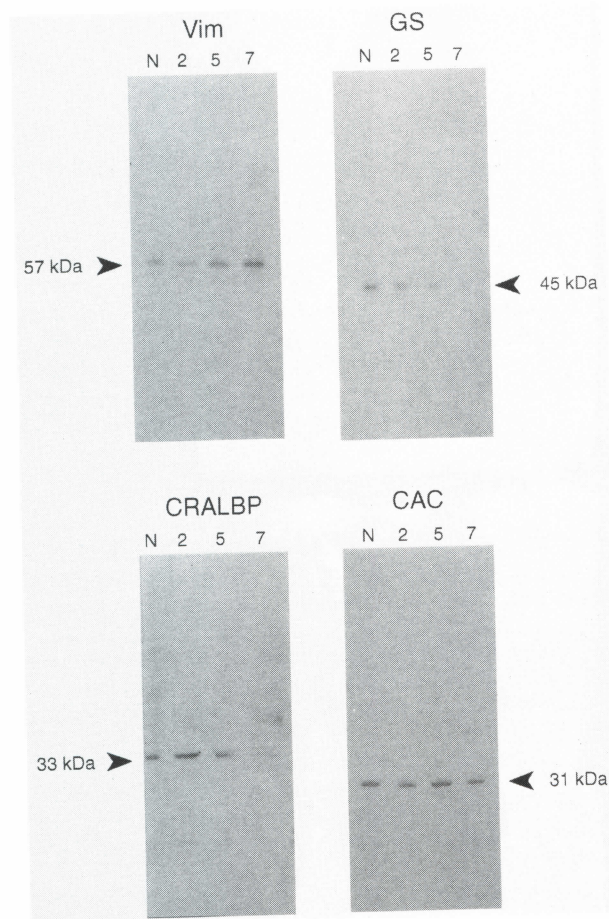
Fig. 4 (Lewis and associates). Western blots of tissue from normal retina and retinas detached for two, five, or seven days. Anti-vimentin (Vim; top left) shows an increase in staining, whereas anti-glutamine synthetase (GS; top right) and anti-cellular retinaldehyde-binding protein (CRALBP; bottom left) show a decrease beginning two days after detachment. Anti-carbonic anhydrase C (CAC; bottom right) shows less of a decrease, probably because of its continued expression in the photoreceptors. Each antibody labels a band at the appropriate molecular weight for that protein at each time point.

may eventually participate in the production of proliferative vitreoretinopathy membranes with the well-established consequences of this disease, including subsequent redetachment of the retina.[20-23] There are, however, other dramatic changes that occur in Müller cells that may contribute to variability in recovery in ways that are not yet understood. A drop in the expression of molecules such as carbonic anhydrase C, glutamine synthetase, or cellular retinaldehyde-binding protein may affect the nor-

mal physiologic characteristics of these cells, altering their capacity to function in the retina. For example, acetazolamide reduces carbonic anhydrase C activity[24] and leads to a reduction in the amplitude of the b-wave of the electroretinogram. Müller cells are thought to scavenge the transmitter molecule glutamate from the extracellular space. Glutamate, a toxic molecule, is immediately converted to glutamine by the enzyme glutamine synthetase.[25] A decrease in glutamine synthetase may impair this function in Müller cells, leaving retinal neurons susceptible to the effects of glutamate. Similar decreases in glutamine synthetase levels have been detected in rat brain astrocytes after central nervous system injury,[26] and in dystrophic retinas of mice with inherited retinal degeneration[27] and may represent a characteristic response of glial cells to injury. The role of retinoid-binding proteins in Müller cells is still not well understood, and at this point a change in the expression of the protein cellular retinaldehyde-binding protein can only be regarded as an indication that these cells are undergoing fairly major transformations in their normal functional activities.

Müller cell proliferation and the hypertrophy of their processes within the retina, coupled with the filling of these processes with arrays of intermediate filaments,[5,10,28,29] results in the production of what are the equivalent of intraretinal scars that may affect the physiologic characteristics of the retina. The hypertrophy of astrocytic processes, along with a concomitant increase in their complement of cytoplasmic intermediate filaments, is a well-recognized response to central nervous system injury,[30-33] resulting in the formation of central nervous system scar tissue that may impair the regrowth of severed axons. We know from earlier morphologic studies in our animal model of detachment that at least some photoreceptors retract their synaptic terminals.[5] Thus, adequate regeneration may require not only the regeneration of photoreceptor outer segments, but also the reestablishment of neuronal connections within the retina. The hypertrophy of Müller cell processes within the outer plexiform layer may inhibit the reformation of these connections. Although the intermediate filament response is dramatic in central nervous system astrocytes and Müller cells, specific functions of these intracellular filamentous structures are still under intensive investigation. There is reasonable evidence that they are involved in the differentiation and maintenance of cell shape[34]

and that they are required for the formation of stable cellular processes.[35] In other words, they are generally associated with basic changes in cell architecture, such as those occurring in Müller cells after detachment.

The data from the current study show that we can detect numerous changes in Müller cells within two days after detachment, and our earlier study[8] showed that Müller cell proliferation is certainly underway within this same two-day time frame. It seems certain that the molecular events that enable these changes to appear begin even earlier than two days after detachment. We do not know whether reattachment arrests or inhibits the progression of these events, or both. If data from the animal model are extrapolated to detachments present in human patients, then it is likely that at least some Müller cell processes, which would not be visible by ordinary examination of the retina, would already have grown into the subretinal space within a few days of detachment. If these processes continue to grow and expand after reattachment, then the prognosis for optimal visual recovery is diminished. Similarly, if the changes occurring within the retina also affect retinal function and if these events are not arrested by reattachment, then they too may continue to affect visual outcome adversely.

Our results indicate that Müller cells respond quickly to retinal injury. We have proposed elsewhere[6] that reactive gliosis in the retina may be a clinically significant limiting factor in the recovery of vision after retinal injuries such as detachment. In the absence of gliosis and subsequent scar formation after injury, improvements in overall visual recovery may be possible. The results from this study demonstrate clearly that these gliotic events can be recognized and detected much earlier than they could before. Therefore, future attempts to prevent or arrest the glial response in the retina should take into account the fact that the initiating molecular events probably have their origin within hours, rather than days, after the injury.

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