Müller Cell Outgrowth after Retinal Detachment: Association with Cone Photoreceptors

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Purpose. Subretinal gliosis is a relatively common occurrence after retinal reattachment. Because Müller cell processes only intermittently penetrate the outer limiting membrane (OLM) beneath extensive detachments, this study was conducted to determine whether this was preferentially associated with rod or cone photoreceptors.

Methods. Cat retinas were detached from the retinal pigment epithelium and 3 days later were fixed in 4% paraformaldehyde, embedded in 5% agarose, sectioned at 100 μm, and processed for standard immuohistochemistry. The retinas were double labeled with either anti-vimentin and anti-long/medium wavelength–sensitive (anti-L/M) cone opsin or anti-glial fibrillary acidic protein (GFAP) and biotinylated peanut agglutinin (PNA).

Results. The hypertrophy of Müller cells was readily traced using antibodies to vimentin and GFAP. When labeling with these antibodies was combined with labeling by either antibodies to cone opsins or biotinylated PNA, a consistent relationship was observed between the Müller cell processes growing through the OLM and cone photoreceptors.

Conclusions. The growth of Müller cell processes into the subretinal space forms a fibrotic layer that completely inhibits the regeneration of outer segments. The current results show that there appears to be a highly specific interaction between growing Müller cell processes and cone photoreceptors during the earliest phase in this process. (*Invest Ophthalmol Vis Sci.* 2000;41: 1542–1545)

Injury to the retina often results in severe visual impairment or blindness. Although damage to photoreceptor cells plays a significant role in this process, numerous secondary events can also affect the loss of vision. For example, after retinal detachment, retinal pigment epithelial (RPE) cells, Müller cells, and astrocytes can proliferate and migrate onto the retinal surfaces, causing conditions known as proliferative vitreoretinopathy (PVR) and subretinal fibrosis.[1] Both of these conditions have severe consequences. In PVR, cells attach to the internal limiting membrane and proliferate. Subsequent contraction of these cells often produces recurrent retinal detachment and results in poor prognosis for visual recovery. In subretinal fibrosis, the formation of "cellular membranes" between the photoreceptors and RPE prevents the regeneration of photoreceptors after reattachment surgery.[2]

Müller cells are one of the two main cell types involved in the formation of subretinal cellular membranes.[1–6] After experimental detachment, these cells quickly begin to hypertrophy, filling space left by dying photoreceptor cells, and then grow along the exposed photoreceptor surface of the retina. During this time, they show increased levels of the intermediate filament (IF) proteins, glial fibrillary acidic protein (GFAP), and vimentin.[7] Both hypertrophy and increased levels of IFs are of particular interest, because they are the defining features of glial scar formation in subretinal fibrosis.

To better understand the initial events in the development of subretinal fibrosis, we used a feline model of experimental retinal detachment where Müller cells become highly reactive immediately after detachment. Within minutes, proteins become phosphorylated and transcription factor production is stimulated.[8] Müller cell proliferation begins within a day after detachment,[9] and by 3 days Müller cell hypertrophy is obvious both within the retina and in the subretinal space.[7] Although these events are observed quite reliably in eyes with retinal detachments, there have been no indications of what stimulates this outgrowth. By using immunocytochemistry with laser scanning confocal microscopy we observed that when Müller cells grow into the subretinal space, they are always adjacent to cone photoreceptors. This suggests that cones may produce factors that initiate the Müller cell outgrowths into the subretinal space, one of the earliest events in the formation of subretinal membranes.

Materials and Methods

Retinal Detachments

Detachments were created in the right eyes of domestic cats. After the removal of the lens and vitreous, a solution of 0.25% sodium hyaluronate, (Healon; Pharmacia, Kalamazoo, MI) was infused between the neural retina and the retinal pigment epithelium using a glass micropipette.[2] Animals were killed 1 (n = 2), 3 (n = 6), 7 (n = 2), and 28 (n = 2) days later. All procedures adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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Immunohistochemistry

Retinal samples were prepared for laser scanning confocal microscopy by fixing the tissue in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in sodium cacodylate buffer (0.1 M [pH 7.4]; Electron Microscopy Sciences). The tissue was then rinsed in phosphate buffered saline (PBS) and embedded in 5% agarose (Sigma, St Louis, MO) in PBS. One hundred-micrometer-thick sections were cut (Vibratome; TPI Polysciences, Warrington, PA). The sections were first 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 (Boehringer–Mannheim, Indianapolis, IN) and 0.1% sodium azide (Sigma) overnight at 4°C on a rotator (PBTA contains PBS, BSA, Triton, and azide). The next day the blocking serum was removed, and the primary antibodies were added. Anti-GFAP and anti-vimentin (Dako; Carpinteria, CA) were used at 1:500 dilution, anti-long/medium (L/M) and short (-S) wavelength–sensitive cone opsin (gifts from Jeremy H. Nathans, John Hopkins Medical School, Baltimore, MD) at 1:2000, and biotinylated-peanut agglutinin (PNA; Vector, Burlingame, CA) at 200 μg/ml. All probes were diluted in PBTA. After incubation overnight at 4°C on a rotator, the sections were rinsed in PBTA and incubated in the secondary antibody overnight at 4°C on a rotator. Donkey anti-mouse and donkey anti-rabbit, conjugated to Cy2 (both from Jackson ImmunoResearch), were used to label vimentin and GFAP, respectively. Donkey anti-rabbit and streptavidin, conjugated to Cy3 (both from Jackson ImmunoResearch), were used to label L/M cone opsin and PNA, respectively. All secondary reagents were used at a 1:200 dilution. The sections were then rinsed, mounted in *n*-propyl gallate in glycerol and viewed with a laser scanning confocal microscope (model 1024; Bio-Rad, Hercules, CA).

Results

A retinal detachment of 3 days' duration was the earliest time point at which Müller cells were consistently observed extending beyond the outer limiting membrane (OLM). Although an increase in IFs in Müller cells could be detected as early as 1 day after detachment, there was very little evidence of growth past the OLM. By 7 and 28 days, Müller cell outgrowths covered large areas in the subretinal space,[7] making it difficult to correlate the site of Müller cell penetration through the OLM with any specific region. Moreover, many of the cone markers we have used fail to label cones after longer duration.[10] For these reasons, we concentrated our efforts in this study on data from 3-day detachments.

In the normal retina, the anti-GFAP antibody labels IFs in Müller cells from the end feet to the inner nuclear layer and astrocytes in the ganglion cell layer (Fig. 1A). The lectin PNA labels both plexiform layers as well as the cone sheaths in the outer segment region, as previously described by Johnson and Hageman (Fig. 1A).[11] At 3 days after detachment, a dramatic increase in immunostaining of IFs is already apparent, and these fill the cytoplasm of many of the Müller cells (Fig. 1B). In areas where processes containing IFs penetrated the OLM, they were always superimposed on PNA-labeled cone sheaths (Figs. 1B, 1C, 1D). Intermediate filament-containing processes frequently accumulated in the region of cone cell bodies in what may have been a prelude to penetration through the OLM (Fig. 1E).

Although PNA is an indicator of the location of cones, it labels only the sheath surrounding the outer segments. We therefore used cone opsin antibodies to label the cones more specifically. Because the cone opsin antibodies are rabbit polyclonal antibodies, we double labeled these sections for vimentin using a mouse monoclonal anti-vimentin, rather than anti-GFAP, which is a rabbit polyclonal antibody.

In the normal retina, anti-vimentin labeled Müller cell processes from their end feet into the INL; the anti-L/M cone opsin antibody labeled cone outer segments (Fig. 1F). At 3 days after detachment, the anti-L/M cone opsin antibody labeled the degenerating outer segments as well as the plasma membranes of the inner segments and to a lesser degree the cell bodies (Fig. 1G, right arrow); anti-vimentin labeling appeared throughout the entire Müller cell. In areas where anti-vimentin labeling extended beyond the OLM, it was, without exception, in register with cone cells (Figs. 1G through 1K). In some cases, anti-vimentin labeling surrounded the base of the inner segment (Figs. 1H, 1I), traversing the edge of the inner segment plasma membrane (Fig. 1J), or accumulating around the cell body (Fig. 1K). Although the Müller cell processes invading the subretinal space were always associated with cones, not all cones had Müller cell processes growing along their inner and outer segments.

We also used an anti-S cone opsin antibody in a similar set of experiments with anti-vimentin and observed several examples of Müller cell outgrowths in association with the blue-sensitive cones (data not shown) with no apparent preference of the Müller cell outgrowths to one cone cell type over the other.

Discussion

After retinal detachment in humans, subretinal fibrosis is often present but is not often identified as a component of PVR.[12] More recent data indicate, however, that subretinal fibrosis is in fact a prominent component of retinal detachment. In one study of 37 eyes that had reattachment surgery and PVR, subretinal fibrosis was noted in 16 of them.[13] Although permanent retinal reattachment can be achieved in eyes with subretinal fibrosis, the prognosis for visual recovery is poor. Bonnet et al.[14] showed that postoperative visual acuity of 20/40 or better was obtained in only 34% of eyes with subretinal fibrosis, compared with 58% of retinal detachments without fibrosis. Ultimately, methods for preventing or arresting the subretinal growth of Müller cells may depend on identifying molecular regulators of this processes; the identification in the present study of a relationship between cones and Müller cells may facilitate this processes significantly.

The mechanism through which the cones initiate or attract the growth of Müller cells into the subretinal space after detachment remains to be elucidated. In a previous study,[15] it was observed that when animals were kept in an increased (70%) oxygen environment after retinal detachment, cones showed increased expression of basic fibroblast growth factor (bFGF), a growth factor known to initiate Müller cell proliferation and hypertrophy.[16] Furthermore, it has been demonstrated that Müller cells have the receptor for bFGF[17] and can bind and internalize the factor in vivo.[18] It is possible that

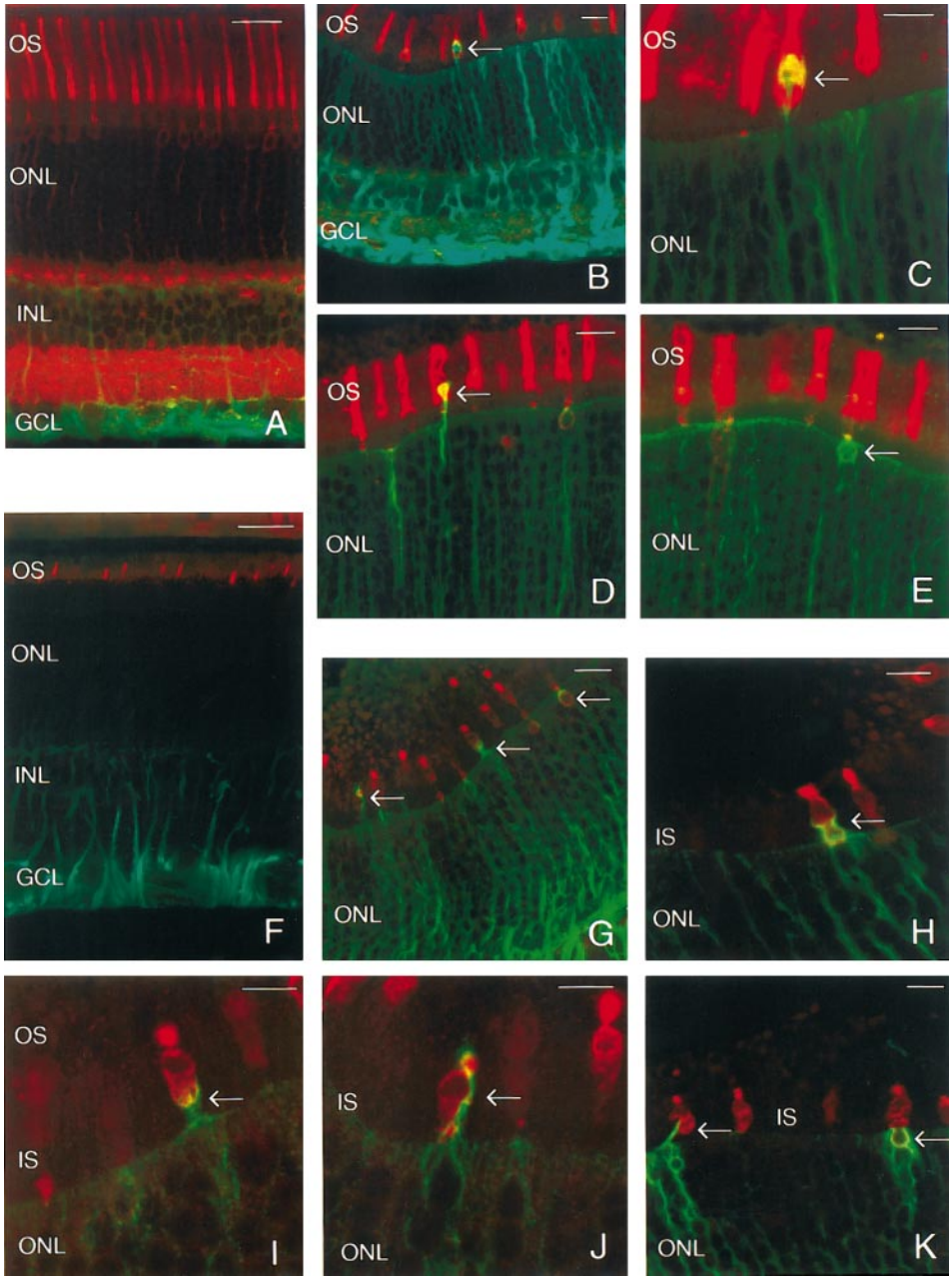

FIGURE 1. (**A** through **E**) Double-labeled images using PNA (*red*) and GFAP (*green*). (**A**) Normal retina: PNA labels the long cone sheaths in the outer retina as well as the outer plexiform layer and inner plexiform layer; anti-GFAP labels astrocyte processes and Müller cell end feet in the ganglion cell layer (GCL) and to a lesser degree, Müller cell processes extending through the inner nuclear layer (INL). (**B**, **C**, and **D**) Three-day detached retina. (**B**) Low-magnification image showing an increase in anti-GFAP labeling throughout the retina and in one area extending past the OLM at the site of a PNA-labeled cone (*arrow*). (**C, D**) Higher magnification images showing anti-GFAP labeling at the site of PNA-positive cones (*arrows*). (**E**) Anti-GFAP labeling is present in a Müller cell processes overlying a cone cell body (*arrow*) in the outer nuclear layer (ONL). (**F** through **K**) Double-labeled images using anti-L/M cone opsin (*red*) and anti-vimentin (*green*). (**F**) Normal retina: Anti-L/M cone opsin labels only the outer segment (OS) region of the L/M wavelength–sensitve cone photoreceptor; anti-vimentin labels Müller cells from the end foot region through the INL. (**G**) Low-magnification image showing several sites at which anti-vimentin labeling occurs past the OLM in association with cone photoreceptors (*left 2 arrows*) as well as labeling at the site of a cone cell body (*right arrow*). (**H**, **I**, and **J**) Higher magnification images showing anti-vimentin labeling in close association with cone inner segments (IS; *arrows*), in some cases appearing to traverse along the plasma membrane of the cone IS and truncated OS (**J**, *arrow*). (**K**) Anti-vimentin labeling is shown at the site of a cone cell body (*right arrow*) and extending past the OLM at the site of a cone inner segment (*left arrow*). Bars, (**A**, **B**, **F**, **G**) 20 μm; (**C**, **D**, **E**, **H**, **I**, **J**, **K**) 10 μm.

Müller cells may be responding to increased levels of bFGF production by cones after retinal detachment. Although the increase of bFGF by cones was observed only in animals that were kept in hyperoxia for 3 days after the retinal detachment, it is possible that an increase also occurs in normoxia but that it is simply below the level of sensitivity for detection by immunocytochemistry. Although all cones appear to show an increase in anti-bFGF immunolabeling in high oxygen,[15] not all cones have Müller cells growing adjacent to them. This implies that it may not be bFGF alone that is causing the outgrowth. An alternative mechanism could involve the expression of surface molecules on cones that then somehow permit the growth of the Müller cell processes. Indeed, the pattern of growth of the Müller cells around cone cell bodies and along the surface of the cone inner segments as shown in the micrographs in this article support this idea. A similar observation was made by Li et al.[19] in eyes with retinitis pigmentosa in which rod neurites were observed growing along the plasma membrane of Müller cells. In that case it was suggested that increased levels of neural cell adhesion molecule, which occurs in eyes affected by retinitis pigmentosa, may be the preferable substrate for rod neurites. Although no evidence for such surface molecules has been reported on cone photoreceptors, it cannot be ruled out as a possible mechanism for the outgrowth of the Müller cells.

Clinically, PVR and subretinal fibrosis do not appear in the retina for quite some time after the initial insult. Wallyn and Hilton[20] showed that the incidence of preoperative subretinal fibrosis varied in a linear fashion with the duration of the detachment, varying from 0.8% in cases less than 1 month old to 22% in cases estimated to be more than 2 years old. The initial stage of the disease process, however, may well begin within the first days after retinal injury. Alternatively, there may

be species differences. Müller cell outgrowths were consistently observed beyond the OLM by 3 days after detachment in the cat, but this has not been investigated in primate species. Thus, similar mechanisms may occur in human detachments but on a different time scale. Although such may be the case, our observations suggest that strategies to inhibit the Müller cell (and probably RPE) response to detachment or other retinal injuries should begin soon after the injury to be effective. Although it is not known whether reattachment stops or slows the outgrowth of Müller cells, the fact that subretinal fibrosis is a serious complication after successful retinal reattachment surgery indicates that reattachment alone may not be enough to inhibit the process.

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References

1. Fisher SK, Anderson DH. Cellular effects of detachment on the neural retina and the retinal pigment epithelium. In: Glaser B, Ryan SJ, eds. *Retina*. *Surgical Retina*. Vol. 3. St. Louis: Mosby; 1994: 2035–2061.
2. Anderson DH, Guerin CJ, Erickson PA, Stern WH, Fisher SK. Morphological recovery in the reattached retina. *Invest Ophthalmol Vis Sci.* 1986;27:168–183.
3. Van Horn DL, Aaberg TM, Machemer R, Fenzl R. Glial cell proliferation in human retinal detachment with massive periretinal proliferation. *Am J Ophthalmol.* 1977;84:383–393.
4. 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.
5. Hiscott 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.
6. Guerin CJ, Wolfshagen RW, Eifrig DE, Anderson DH. Immunocytochemical identification of Muller's glia as a component of human epiretinal membranes. *Invest Ophthalmol Vis Sci.* 1990;31:1483–1491.
7. 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.
8. Geller SF, Lewis GP, Fisher SK. Map kinase phosphorylation and CREB, STAT3, and NF-kappaB activation following experimental retinal detachment [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1999;40(4):**S**476. Abstract nr 2511.
9. Fisher SK, Erickson PA, Lewis GP, Anderson DH. Intraretinal proliferation induced by retinal detachment. *Invest Ophthalmol Vis Sci.* 1991;32:1739–1748.
10. 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.
11. Johnson LV, Hageman GS. Structural and compositional analysis of isolated cone matrix sheaths. *Invest Ophthalmol Vis Sci.* 1991;32: 1951–1957.
12. Sternberg P, Machemer R. Subretinal proliferation. *Am J Ophthalmol*. 1984;98:456–462.
13. Lewis H, Aaberg TM. Causes of failure after repeat vitreoretinal surgery for recurrent proliferative vitreoretinopathy. *Am J Ophthalmol.* 1991;111:15–19.
14. Bonnet M, Hajjar C, Fleury J. Subretinal proliferation and rhegmatogenous retinal detachment. *J Fr Ophtalmol.* 1993;16:235–240.
15. Mervin K, Valter K, Maslim J, Lewis GP, Fisher SK, Stone J. Limiting the death and deconstruction during retinal detachment: the value of oxygen supplementation. *Am J Ophthalmol*. 1999;128:155–164.
16. Lewis GP, Erickson PA, Guerin CJ, Anderson DH, Fisher SK. Basic fibroblast growth factor: A potential regulator of proliferation and intermediate filament expression in the retina. *J Neurosci.* 1992; 12:3968–3978.
17. Guillonneau X, Regnier–Ricard F, Laplace O, et al. et al. Fibroblast growth factor (FGF) soluble receptor 1 acts as a natural inhibitor of FGF2 neurotrophic activity during retinal degeneration. *Mol Biol Cell.* 1998;9:2785–2802.
18. Lewis GP, Fisher SK, Anderson DH. Fate of biotinylated basic fibroblast growth factor in the retina following intravitreal injection. *Exp Eye Res.* 1996;62:309–324.
19. Li ZY, Kljavin IJ, Milam AH. Rod photoreceptor neurite sprouting in retinitis pigmentosa. *J Neurosci.* 1995;15:5429–5438.
20. Wallyn RH, Hilton GF. Subretinal fibrosis in retinal detachment. *Arch Ophthalmol.* 1979;97:2128–2129.