Expression of Glial Fibrillary Acidic Protein in Rabbit Müller Cells After Lensectomy-Vitrectomy

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Purpose. To assess retinal response after lensectomy-vitrectomy, the authors analyzed glial fibrillary acidic protein (GFAP), which retinal glial cells express in several pathologic conditions.

Methods. GFAP expression was examined by means of immunohistochemistry, Western blot analysis, and enzyme-linked immunosorbent assay (ELISA) in rabbit retina after lensectomy-vitrectomy.

Results. Müller cells had detectable GFAP immunoreactivity in peripheral retina 3 days after surgery. GFAP immunoreactivity was present in both peripheral and posterior regions 7 days, 14 days, and 6 months after surgery. The authors confirmed that anti-GFAP antibody recognized the 51 kDa protein specifically by Western blot analysis. Time-dependent increase of GFAP in peripheral retina was also obtained by ELISA.

Conclusions. Such progressive GFAP accumulation in Müller cells, which are sensitive to microenvironmental changes, may reflect some underlying retinal response after lensectomy-vitrectomy. Invest Ophthalmol Vis Sci 1993;34:3154-3160.

Vitreous is a clear hydrogel that fills the posterior part of the eye and provides a semisolid support for it. It allows light to reach the retina and nutrients to diffuse from the ciliary body to the retina. Vitrectomy is often required for proliferative diabetic retinopathy and vitreoretinopathy. Some reports have described morphologic, metabolic, and electrophysiologic changes in rabbit retina after vitrectomy. Shimada demonstrated morphologically that retinal pigment epithelial cells migrated into subretinal space, and outer segments were destroyed 2 weeks after vitrectomy. It also was reported that b-wave electroretinographic responses decreased in amplitude at 2 and 5 days after vitrectomy. Durlu et al indicated that Müller cells showed temporarily increased glucose-6-phosphatase and glutamine synthetase activities that decreased 12 hours after lensectomy-vitrectomy. These findings suggest that some pathogenic substance may be released in the retina after lensectomy-vitrectomy.

Retinal Müller cells have expressed glial fibrillary acidic protein (GFAP) in response to several pathologic conditions, including glaucoma, retinal injury, retinal detachment, and degeneration of visual cells. To assess retinal response, GFAP appeared to be an appropriate marker. As previously reported, we found GFAP expression in Müller cells after lensectomy-vitrectomy. In this article, we describe increased expression of GFAP and report on the distribution, time course, and quantitative analysis of GFAP expression in rabbit retina after lensectomy-vitrectomy. In addition, a quantitative comparison of GFAP expression was made after two different types of vitreous surgeries were performed.

MATERIALS AND METHODS

The care and treatment of animals in this investigation were in compliance with the ARVO Resolution on the Use of Animals in Research. Lensectomy-vitrectomy was performed in the right eye of domestic albino rabbits, each weighing 2.0 to 2.5 kg, according to the technique of Durlu et al. Anesthesia was administered by intramuscular injections of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (4.7 mg/kg) and a retrobulbar injection of 1% xylocaine (0.5 ml). Total operating time was about 30 minutes; intraocular irrigation time was 20 minutes. As a control, only retrobulbar anesthesia with 0.5 ml of 1% xylocaine was...
performed on the right eyes of four additional rabbits. All lensectomies and vitrectomies were performed through a pars plana approach. The surgical wounds were closed with 8-0 nylon sutures. For the sham operation, we created a scleral wound 2 mm posterior to the limbus in the right eye, punctured into the vitreous cavity, and illuminated the eye with the light source of the operating microscope for 20 minutes using four rabbits. During the follow-up period, eyes with vitreous hemorrhage and retinal detachment were excluded from this study. For immunohistochemical evaluation, the rabbits were anesthetized and killed with an overdose of pentobarbital sodium. Eyes were enucleated at 0 hour (immediately after surgery) (n = 3), and at 4 hours (n = 3), 3 days (n = 4), 7 days (n = 3), 14 days (n = 3), and 6 months (n = 2) after surgery. The control eyes that underwent retrobulbar anesthesia or sham operations were enucleated at 3 and 7 days after treatment. We fixed all eyes for 3 days with periodate-lysine-paraformaldehyde preparation. After embedding in paraffin, 3-μm-thick cross-sections of the retinas containing visual streaks were made.

Immunohistochemical studies were carried out with use of the avidin-biotin peroxidase technique. Endogenous peroxidase was blocked by immersing the sections in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. Next, sections were incubated overnight at 4°C with 1:100 diluted monoclonal mouse anti-human GFAP, clone GF-2 (Dako, Glostrup, Denmark). Then the sections were treated with a second antibody, biotin-conjugated horse anti-mouse IgG (1:50 diluted) (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. The sections were incubated with avidin-biotin-peroxidase complex for 30 minutes at room temperature. The primary antibody was omitted from the control sections. All antibodies were dissolved in PBS containing 0.05% Tween 20 and 1% bovine serum albumin (TW-BP). Between each incubation, we washed the sections with PBS containing 0.05% Tween 20 (TPBS) three times. Next, the plates were incubated with anti-rabbit IgG conjugated with peroxidase (1:50 diluted) (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. The primary antibody was omitted from the control sections.

To confirm the specificity of anti-human GFAP antibody and the results of immunohistochemical testing, we carried out Western blot analysis according to the method of Towbin et al.14 We performed lensectomy-vitrectomy, as described, and enucleated eyes at 0 hour (immediately after surgery) (n = 2), 3 days (n = 3), 7 days (n = 3), 14 days (n = 3), and 30 days (n = 3) after surgery. After removing the anterior segment, retinas were separated into peripheral, midperipheral, and posterior regions and visual streaks. The peripheral retinas were homogenized with four volumes of PBS containing 3% SDS and centrifuged at 3,000 rpm for 20 minutes. The protein content of the supernatant was examined by the method of Lowry et al.15 We applied the samples (10 μg protein) onto SDS-polyacrylamide gel electrophoresis using 7.5% separation gel. Proteins of known molecular weights (BioRad, Richmond, CA) were run as standards. The retinal proteins were transferred electrophoretically to nitrocellulose membrane. The membranes were blocked with 3% gelatin and incubated with 1:200 diluted monoclonal mouse anti-human GFAP at 37°C for 60 minutes. After washing with TBPS for 10 minutes three times, the membrane was treated with 1:200 diluted alkaline phosphatase-conjugated, affinity-purified goat anti-mouse IgG (CAPPEL, West Chester, PA). Then the membrane was washed with TBS solution (150 mM NaCl and 0.02% NaN3 in 50 mM Tris-HCl, pH 9.5) for 10 minutes three times. Color development was done by treating the membrane with 5 ml of developing solution that contains 100 mM NaCl, 5 mM MgCl2, 0.1M Tris-HCl, pH 9.5, 33 μl of NBT solution (nitroblue tetrazolium, 50 mg/ml in 70% dimethylformamide), and 16.5 μl of BCIP solution (5-bromo-4-chloro-3-indolyl-phosphatase-p-toluidine salt, 50 mg/ml in dimethylform-amide). For the sandwich method of ELISA, we added 50 μl of mouse anti-GFAP antibody diluted with 0.05 M carbonate-bicarbonate buffer, pH 9.6 (1:2000), to Nunk-ImmunoPlate (Nunk, Denmark). After washing the plates with TBPS and PBS three times, the plates were incubated with 1% BSA-PBS at 37°C for 30 minutes. We washed the plates with PBS and incubated the plates with 50 μl of retinal extract diluted with TB-BP at 37°C for 30 minutes. Next, the plates were incubated with 50 μl of rabbit polyclonal anti-GFAP antibody (Dako) diluted with TB-BP (1:200). Final incubation was performed with anti-rabbit IgG conjugated with peroxidase (1:500 dilution) at 37°C for 30 minutes. The reaction was performed by incubating the plates with 50 μl of citrate-phosphate buffer, pH 5.0, containing 0.04% o-phenylenediamine and 0.006% hydrogen peroxide. Color development was stopped with the addition of 50 μl of 2.4 M sulfuric acid. Absorbance was examined at 492 nm (reference absorbance: 610 nm).

To compare the retinal responses between lensectomy-vitrectomy and vitrectomy alone, vitrectomy was performed in the right eye of three rabbits with use of the Octome 10,000 (Alcon, Fort Worth, TX) with a 3-port method. Seven days after surgery, enucleation and preparation of retinal samples were performed, and GFAP content of the peripheral retina was measured by the sandwich method.

**RESULTS**

Müller cells at 7 days after retrobulbar anesthesia (n = 4, data not shown) or sham operation (n = 4) (Fig. 1a)
FIGURE 1. GFAP expression in rabbit retina. Seven days after sham operation (a) and 4 hours after lensectomy-vitrectomy (b), retinas exhibited no GFAP immunoreactivity. No GFAP immunoreactivity was seen in the posterior region 3 days after surgery (c). GFAP started to express from 3 days after lensectomy-vitrectomy: (d) midperipheral region, (e) peripheral region (arrowheads reveal immunoproduct). At 14 days after lensectomy-vitrectomy, retinas had apparent GFAP immunostaining: (f) posterior region, (g) midperipheral region, (h) peripheral region. Peripheral GFAP immunoreactivity was stronger than posterior GFAP immunoreactivity. Visual streak (VS) usually expressed GFAP immunostaining. Six months after surgery (i, peripheral region), Müller cells still revealed GFAP immunoreactivity. Bar = 50 μm.
exhibited no GFAP immunoreactivity. However, the specimens of visual streak and optic nerve from all the retinas expressed GFAP. Retinas fixed immediately and at 4 hours (Fig. 1b) after experimental lensectomy-vitrectomy showed no GFAP immunoreactivity in Müller cells. In three of four cases, GFAP immunoreactivity was observed in the peripheral retina 3 days after surgery (Fig. 1e), whereas no GFAP was present in Müller cells of the posterior region. The immunoreactive product was present from the end feet of the Müller cells to the outer plexiform layer of the retina. Müller cells expressed GFAP immunoreactivity in both the peripheral and posterior regions 14 days after surgery (Figs. 1f, 1g, 1h). At this time, peripheral retina had stronger GFAP-immunoreactivity than posterior retina. No GFAP immunoreactivity was present in the control sections without the primary antibody. Figure 2 shows diagrammatically the GFAP immunoreactive changes in Müller cells.

As shown in Figure 3, 51 kDa protein in retinal extracts 7 days after surgery was demonstrated with monoclonal anti-GFAP antibody by Western blotting and immunostaining techniques. There was no GFAP band without monoclonal anti-GFAP antibody.

The GFAP content of peripheral retinal extracts was measured by the sandwich method. The retinal extracts of the control eyes and at 0 hour after surgery had no GFAP; however, GFAP was detectable at 3 days. Increased GFAP content was observed after day 3 (Fig. 4). In addition, we compared the retinal GFAP content between lensectomy-vitrectomy and vitrectomy alone on postoperative day 7. In the peripheral retinal region, GFAP content of the retinas after lensectomy-vitrectomy was higher than in those that underwent vitrectomy alone (P < 0.05) (Fig. 5).

DISCUSSION

In the present study, we reported on the distribution and time course, and evaluated quantitatively GFAP expression in retinal Müller cells of the rabbit after lensectomy-vitrectomy. We found that GFAP immunoreactivity first appeared in the peripheral retina 3 days after surgery and increased thereafter, as confirmed by ELISA. Even with simple vitrectomy, the increase in GFAP is nearly as large as for combined lensectomy-vitrectomy. So it is possible that simple vitrectomy causes the same damages to the retina as those that lensectomy-vitrectomy causes.

The ability to quantify such a marker protein may make it possible to evaluate the effects of other surgical methods. In addition, it may also be possible to assess retinal toxicity and damage with antibiotics, antimicotics, and silicon oil using GFAP expression.

**FIGURE 2.** Time course of GFAP expression in Müller cells. Striped zones show the existence of GFAP immunoreactive product. Müller cells started to express GFAP from the peripheral region 3 days after lensectomy-vitrectomy. At 14 days and 6 months after surgery, Müller cells showed GFAP in both the peripheral and the posterior regions.

**FIGURE 3.** Results of Western blot analysis of GFAP. Ten micrograms of protein of peripheral retina obtained on postoperative day 7 were analyzed. Lane a shows the result of silver staining. Mouse monoclonal anti-GFAP antibody detected 51 kDa protein (lane b, arrowhead).
FIGURE 4. Quantitative analysis of GFAP by the sandwich method. (a) The relationship between the amount of retinal extract and absorbance at 492 nm. The retinal extract was obtained from the peripheral retina after lensectomy-vitrectomy. Each symbol shows unoperated retina (■) immediately after surgery (○) and at 3 days (●), 7 days (▲), and 14 days (★) postoperatively. Absorbance of unoperated retinas (0.16 ± 0.02 ± SD) was almost the same as that of retinas immediately after surgery. (b) Time-dependent changes of GFAP content. A total of 500 ng of retinal extract was used for the assay. GFAP content of peripheral retina increased and reached a plateau at 14 days after surgery.

The mechanism of GFAP expression in Müller cells from the pathologic retina is not well understood. In our study, GFAP appeared early in Müller cells of the peripheral retina and late in the entire retina. Our findings indicate two possible explanations. First, the peripheral retina may be more fragile than the posterior retina after surgery. Mechanical stimuli may induce GFAP expression in the Müller cells. Second, destruction of the blood-ocular barrier may result in the release of GFAP-inducing factor from serum or other ocular tissues. In our sham operation control, breakdown of the blood-ocular barrier occurred as a result of scleral puncture, but no GFAP expression was found in the retina. This finding makes the second explanation less likely.

Until now, other changes occurring in the eye after lensectomy-vitrectomy were described. Durlu et al reported changes of activities on glucose 6-phosphatase and glutamine synthetase, which are key enzymes of glycogenolysis and nitrogen metabolism, respectively, after lensectomy-vitrectomy. They showed that 12 hours after surgery, both enzymes temporarily increased and then decreased after 3 days. Mito et al reported increased Na-K ATPase activity of the iris and ciliary body from 7 hours after lensectomy-vitrectomy. Breakdown of the blood-ocular barrier appears immediately after lensectomy-vitrectomy. The barrier restores in 24 hours, but the second breakdown of the barrier occurs 3 days after surgery. Tsuchiya et al observed decreased levels of ascorbic acid in the vitreous immediately after surgery to 4 weeks postoperatively. In these previous studies, Müller cells, the ciliary body, and the blood-ocular barrier responded within 3 days. In the present study, the GFAP increase was detected from 3 days after surgery. The time required for GFAP expression was long compared with other cellular responses described in previous reports. This long latency may support the hypothesis of Burns and Robles.

Burns and Robles reported that in light- and photocoagulation-damaged rat retina, Müller cells showed a gradient pattern of GFAP immunoreactivity. They thought that the primary injury lead to progressive degeneration in adjacent cells, and that the degenerative cells modified the immediate environment in a way that induced GFAP expression. In our study, a weak gradient pattern of GFAP immunoreactivity was found in 14 days after retina surgery. In addition, we found that GFAP appeared early in the Müller cells of peripheral retina and expanded later to the posterior...
GFAP Expression After Vitreous Surgery

Figure 5. Comparison of GFAP content (mean ± SD) between vitrectomy alone (n = 3) and lensectomy-vitrectomy (n = 3) at 7 days after surgery. Retinal extracts (1 μg protein) of the peripheral region from the lensectomy-vitrectomy group had significantly higher GFAP than the vitrectomy group alone (P < 0.05, Wilcoxon rank-sum test). Even with simple vitrectomy, GFAP content is 80% of that for combined lensectomy-vitrectomy.

The significance of the increased GFAP immunoreactivity in operated retinas is unclear. Müller cells of rabbit retina express little GFAP normally, whereas astrocytes show massive amounts of GFAP in the optic nerve in various species. It is reasonable that astrocytes require abundant GFAP to play a role as supportive cells in the optic nerve, which is susceptible to mechanical damage. Lens and vitreous support the retinal structure directly or indirectly in the ocular tissue. After lensectomy-vitrectomy, the retina may become more fragile than normal retina to mechanical stimuli. Expression of GFAP was found in retinal Müller cells from 3 days to 6 months after lensectomy-vitrectomy. This finding indicates that even 6 months after surgery, Müller cells would continue to respond. We cannot, however, exclude the possibility that a subtle activation of astrocytes has occurred as well after lensectomy-vitrectomy. Increased GFAP in glial cells may prevent mechanical damage in operated retinas.

Erickson et al have suggested that GFAP-containing filaments correlate with Müller cell nuclear relocation and mitosis in the study of GFAP expression in Müller cells of detached cat retina. Kerns and Hinsman reported that reactive astrocytes increased the number of GFAP filaments and formed glial scars in the central nervous system. These studies indicate that increased GFAP expression in retinal Müller cells may have some relation to proliferation and the scarring process caused by lensectomy-vitrectomy.

Key Words
- glial fibrillary acidic protein
- enzyme-linked immunosorbent assay
- rabbit
- Müller cells
- lensectomy-vitrectomy

Acknowledgments
The authors thank Maxine A. Gere for her helpful review of this manuscript.

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