Cytochemical Studies on Pathological Müller Cells after Argon Laser Photocoagulation

Hiroshi Ishigooka, Akira Hirato, Takashi Kiraoka, and Saroki Ueno

The cytochemical localization of G6Pase activity, which is specific to the endoplasmic reticulum (ER) of Müller cells, was studied after argon laser photocoagulation in the guinea pig retina. After argon laser radiation, Müller cells exhibited enlargement of the cytoplasm, an increase of reactive ER and the nuclei, dislocation of the nuclei and diagonal stretching of the cytoplasm. However, cell attachment between Müller cells and the proliferated pigment epithelial cells or Bruch's membrane differed with the degree of retinal coagulation. This histo- and cytochemical method may be useful for examining Müller cells under various pathological conditions. Invest Ophthalmol Vis Sci 30:509-520, 1989

Photocoagulation, using argon, krypton or YAG laser, is routinely performed in the treatment of various ocular diseases. The basic studies indicate that retinal glial and/or pigment epithelial cells are involved in such pathological changes of the retina. Müller cells are sometimes difficult to identify and differentiate cytologically even at the electron microscopic level because of their complicated distribution.2

We reported that the localization of ouabain-insensitive, potassium-independent para-nitrophenylphosphatase (non K-NPPase) activity was restricted to the endoplasmic reticulum (ER) of Müller cells in the normal guinea pig retina.4 Also, Kuwabara5 reported high G6P-dehydrogenase activity in Müller cells in the rabbit and cat retina after mechanical damage. In the current study, we examined whether this histo- and cytochemical method was useful for identifying Müller cells in the coagulated retina, and examined the pathological changes after argon laser coagulation.

Materials and Methods

The retina of adult pigmented normal guinea pigs, each weighing about 500 g, were coagulated through the pupil under general (ketamine hydrochloride 20 mg/kg, I.M.) and surface anesthesia (2% lidocaine) using an argon laser (Coherent, Palo Alto, CA; System 900) at an energy of 50 mW (mild coagulation) or 100 mW (moderate coagulation), duration of 0.1 sec and 200 μm in diameter. The following experiments were carried out 1, 3, 5, 7, 10, 14, 28 and 56 days after coagulation in the experimental and control animals. Under ketamine and diethyl ether anesthesia, the guinea pig was perfused with a cold mixture of 0.25% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, with 8.0% sucrose, from the left ventricle for 5 min. The eyeballs were removed and sliced, and the specimens were then immersed in the same fixative for another 25 min. After being washed in 0.1 M cacodylate buffer, pH 7.4, with 8.0% sucrose, from the left ventricle for 5 min. The eyeballs were removed and sliced, and the specimens were then immersed in the same fixative for another 25 min. After being washed in 0.1 M cacodylate buffer, pH 7.4, with 8.0% sucrose for 2–3 hr, the specimens were sectioned into 20 μm thick sections for light microscopy and 40 μm for electron microscopy with a vibratome (Oxford, England) or cryotome (Yamato Kohki, Tokyo). Then the sections were incubated in the standard or control reaction medium for G6Pase by Wachstein and Meisel6 for 20–60 min.

After being rinsed in distilled water, the incubated sections were treated with 2.0% ammonium sulfide for light microscopic observation. For electron microscopic observation, the incubated sections were rinsed in a 0.1 M cacodylate buffer, pH 7.4, with 8.0% sucrose, and postfixed with 1.0% osmium tetroxide for 60 min. Serial dehydration in a graded alcohol series was followed by embedding in Spurr's epoxy resin and ultrathin sections were made with an LKB ultratome. The sections were observed under a Hitachi HS-9 electron microscope after double staining with uranyl acetate and lead citrate.
Fig. 1. (a) A lesion 1 day after mild argon laser photocoagulation. Relatively demarcated retinal edema and disturbance of cellular arrangement were observed mainly in the outer portion of the lesion (×310). (b) A lesion 3 days after mild laser coagulation. The reaction products were increased in the whole outer retina of the coagulated area (×310). (c) A lesion 7 days after mild laser coagulation. The cytoplasm became markedly enlarged in the peripheral part of the lesion, and staining extended from the periphery to the center (×310).

Control Studies

Transitional area and optic nerve of the guinea pig were coagulated by argon laser at an energy of 200 to 300 mW, duration of 0.1 sec and 200 μm in diameter. The optic nerve was also coagulated by YAG laser (Coherent, system 9900) at an energy of 1.5 to 2.0 mJ. These areas were examined after 3, 7 and 10 days.

The investigations involving animals conformed to the ARVO Resolution on the Use of Animals in Research.

Results

In the guinea pig retina, the localization of the activity of non K-NPPase was closely associated with that of G6Pase. Therefore, only the localization of the G6Pase activity was studied. In the normal retina of the guinea pig, G6Pase activity was intense on the ER of Müller cells in the inner retina, from the inner limiting membrane to the inner nuclear layer, but slight in the outer retina, from the outer nuclear layer to the outer limiting membrane, under light and electron microscopy.
Mild Coagulation

On the first day after mild coagulation, destruction was observed mainly in the outer retina up to the outer plexiform layer of the lesion (Fig. 1a). The enzyme activity in the inner retina of the lesion was similar to that in the normal retina. Within a few days, the entire outer retina showed diffuse enzyme activity (Fig. 1b), but no change was seen in the inner retina. After 1 week, staining of the cytoplasm of Müller cells was more distinct in the peripheral part of the lesion and extended diagonally to the central part of the lesion (Fig. 1c).

By electron microscopy, although most of the photoreceptor cells were destroyed on the first day, the reaction products on the ER of the Müller cells enlarged in the peripheral part of the coagulated area (Fig. 2). On the third day, the nuclei of the Müller cells in the outer retina exhibited reaction products on the nuclear envelope, and the Müller cells were markedly enlarged. The enzyme reaction on the ER in Müller cells was marked, and the cells occupied most of the outer retina at the center of the lesion (Fig. 3). After the tenth day, both Müller cells showing enzyme activity and proliferating pigment epithelial cells were observed in the subretinal space (Fig. 4).
Fig. 3. Outer nuclear layer (ON) near center of lesion, 3 days after mild coagulation; inset—normal outer nuclear layer. The enlarged cytoplasm and nuclei of Müller cells (M) were present at the level of the outer nuclear layer. Only few photoreceptor cell nuclei (P) were present. In the inset, there were only narrow areas of cytoplasm. *OL: outer limiting membrane (bar = 1 μm).

Fig. 4. A lesion 14 days after mild coagulation. Some Müller cells (M) extended into the subretinal space and were directly in contact with proliferated pigment epithelial cells (PE) (bar = 1 μm).

Fig. 5. A lesion 10 days after mild laser coagulation. Hypertrophic cytoplasm was distributed diagonally from the peripheral (right side) to the central part of the lesion (left side) with the transfer of Müller cell nuclei (M) to the outer retina. *P: nuclei of photoreceptor cell, ON: outer nuclear layer, OL: outer limiting membrane (bar = 1 μm).

No changes were seen in the inner retina. Müller cells appeared hypertrophic with marked enzyme activity on the ER, and occupied most of the outer nuclear layer between the destroyed photoreceptors (Fig. 5). Also, the nuclei of the Müller cells were frequently translocated from the inner nuclear layer to the outer nuclear layer.

**Moderate Coagulation**

One day after moderate coagulation, retinal edema, cell disarrangement and decrease in enzyme activity were observed (Fig. 6a). In contrast to the changes after mild coagulation, the enzyme activity on the ER of Müller cells in the inner retina was
markedly decreased. No enzyme activity was detected in the outer retina. After only a few days, diffuse staining for G6Pase was observed throughout the lesion (Fig. 6b). However, the enzyme activity in the inner retina, including the inner nuclear layer, was weaker than that in the unaffected portion. After 1 week, the enzyme activity had increased in the whole retina, and was marked in the outer retina. However, little change in enzyme activity was observed from the peripheral to the central lesion (Fig. 6c), as seen after mild coagulation.

Electron microscopy revealed necrosis of the retinal cells, but only weak enzyme reaction was noted in the ER or nuclear envelopes of Müller cells on the first day. On the third day, some staining was detected near the retinal pigment epithelial cells (Fig. 7). After 1 week, the number of nuclei in the peripheral part of the lesion was remarkably larger than that in the unaffected area, with an increased amount of cytoplasm (Fig. 8). After 10 days, the cytoplasm became much more enlarged, and Müller cells stretching in the subretinal space were in direct contact with pig-
Fig. 7. Moderate coagulation, after 3 days, shows nuclei and cytoplasm of Müller cells within the subretinal space (SS). *OL: outer limiting membrane, M: nuclei of Müller cells (bar = 1 μm).

Control Studies
When the substrate, G6P, was removed from the incubation medium, or the sections were preheated at 60°C before the incubation, no reaction products were detected in the retina (Fig. 11a).

Although hardly any other glial cells are considered to be present in the guinea pig retina because of its avascularity, the enzyme reaction in other glial cells was examined under normal and pathological conditions. The enzyme activity in the inner retina at the normal transitional area between retina and optic nerve was high, but no activity was detected in the optic nerve head under light microscopy (Fig. 11b).

No reaction products were detected in the cells of the optic nerve after the application of argon laser coagulation at the transitional area, or YAG laser coagulation at the optic nerve.

Discussion
Wallow and Tso7 produced moderate xenon arc burns in the monkey retina and suggested that Müller...
Fig. 8. Inner nuclear layer (IN) at the periphery of lesion; inset—normal inner nuclear layer. The number of Müller cell nuclei and the amount of Müller cell cytoplasm had increased. Compare with inset, where only a few Müller cell nuclei were observed. *M: nuclei of Müller cell (bar = 1 μm).

Fig. 9. A lesion 14 days after moderate laser coagulation. The cytoplasm of some of the Müller cells was in direct contact with pigment epithelium or Bruch's membrane (Br). *M: nuclei of Müller cell (bar = 1 μm).

cells were responsible for most of the retinal repair of the defect of the outer nuclear layer and outer limiting membrane facing the retinal pigment epithelium; only in the severe lesions did astrocytes appear to play a major role among the glial cells in the repair process. These assumptions were based on the morphological similarity between Müller cells and astrocytes in the brain, such as long cellular processes without pigment granules, many filaments in the cytoplasm, cell attachments by zonulae adherentes between adjacent cells and localized demarcation of the plasma membrane by a basement membrane. Also, Ishikawa8 considered Müller cells to be mainly involved in scar formation based on electron microscopic findings of nuclei in the inner nuclear layer, cytoplasm extending from inner retina to the outer limiting membrane and small processes projecting from the cytoplasm. However, these findings are not specific to either astrocytes or Müller cells. Therefore, it is difficult to identify or differentiate Müller cells from other glial cells merely by morphological observation, especially under pathological conditions.

Histochemical methods such as silver staining or immunohistochemical methods, using the S-100 protein9-12 or glial fibrillary acidic protein (GFAP),13-17 have been widely used to stain glial cells in the eye. However, the S-100 protein binds to both Müller cells and astrocytes. With GFAP immunohistochemistry, only astrocytes are labeled in the normal retina. Although positive reactions were observed in Müller cells of injured retina, these cells cannot be differentiated from other glial cells.15,17 Nork et al18

Fig. 10. In the central part of a lesion, 5 days after moderate laser coagulation, the nuclei of the Müller cells (M) were sometimes seen near the inner limiting membrane (IL) (bar = 1 μm).
reported that Müller cells could be specifically identified even in the pathological retina using combined carbonic anhydrase and GFAP staining, and they showed that Müller cells play an active role in massive retinal gliosis and proliferative diabetic retinopathy.

Several monoclonal antibodies have been reported to bind specifically to the Müller cells of several species. Although these studies have so far been carried out in the normal retina and only at the light microscopic level, immunohistochemical methods using such monoclonal antibodies may be valuable for demonstration of pathological changes of Müller cells. Ruthenium red staining appears to be specific for detecting Müller cells, but this method is unstable and cannot be used for the outer sensory retina or under pathological conditions.

Using an enzyme-histochemical technique, Kuwabara found high G6P-dehydrogenase activity in Müller cells in rabbit and cat retina after mechanical damage. Also, Kuwabara reported that glycogen was accumulated and that G6P-dehydrogenase activity was increased in Müller cells of pathological retina. These findings coincide with our histochem-

Fig. 11. (a) A lesion 7 days after moderate argon laser coagulation. When the substrate, G6P, was removed from the incubation medium, the reaction products disappeared completely from the retina (compare with Fig. 6c) (x270). (b) The normal transitional area between retina and optic nerve (OP). Müller cells with enzyme reaction were easily differentiated cytochemically from astrocytes or other cells (x450).
The changes in Müller cells following cell injury induced by argon laser photocoagulation were as follows. First, the cytoplasm became enlarged and the extent of the reaction product within the endoplasmic reticulum increased. Next, the number of nuclei increased followed by dislocation of the nuclei, after which the cytoplasm extended from the edge to the center of the lesion. These changes in Müller cells were also reported by Erickson et al.\textsuperscript{24} in the detached cat retina.

Moreover, differences in the pathological changes in Müller cells were observed between mild and moderate coagulation. With mild coagulation, the initial morphological and histochemical changes were observed mainly in the outer retina of the lesion, without any notable changes in the inner retina. On the other hand, with moderate coagulation, Müller cells seemed to be damaged in all the retinal layers in the central part of the lesion. In the final stage of scar formation, after mild coagulation, almost all the Müller cells were reactive in the outer retina and some of them were in direct contact with the proliferated pigment epithelial cells. In contrast, although unreactive Müller cells at the central part of the lesion seemed to be severely damaged by moderate coagulation, Müller cells in the peripheral part of the lesion reacted by elongating and making contact with the pigment epithelial cells or Bruch's membrane.

Some of the characteristics of Müller cells in avascular retina have been reported to differ from those in vascularized retina in normal and/or pathological conditions.\textsuperscript{23,25-27} Since the guinea pig retina is avascular, glial cells, except for Müller cells, are not considered to play a role in scar formation. However, even if some other glial cells exist in the retina, they are not stained by this method, as demonstrated in our control studies, and only the changes occurring in the Müller cells were observed. Astrocytes might also be important during the scar formation in the vascular retina, but such changes were outside the scope of this study.

In the clinical application of various lasers to retinal coagulation, the changes occurring in the Müller cells in the lesion should be considered. By using our histo- and cytochemical method to identify Müller cells, small changes in the peripheral or central part of the coagulated areas can easily be observed even at an early stage.

Key words: Müller cells, G6Pase, histo- and cytochemistry, argon laser photocoagulation, guinea pig retina

References