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Müller Cells in Developing Rats with Inherited Retinal Dystrophy

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KIMURA, N., NISHIKAWA, S. & TAMAI, M. *Müller Cells in Developing Rats with Inherited Retinal Dystrophy*. Tohoku J. Exp. Med., 2000, **191** (3), 157–166 —— Morphology and enzymes of Müller cells in the developing retina of RCS (Royal College of Surgeons) rats were investigated. RCS (rdy/rdy) rats with inherited retinal dystrophy were studied and RCS (+/+) rats served as normal controls. Rats underwent intracardiac perfusion with 4% paraformaldehyde and the eyes were enucleated on postnatal days P1, 4, 10, 21, 35, and 100. Eyes were then fixed with 4% paraformaldehyde, and silver enhancing technique was applied to show glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP). For solubilized retinas, Western blot analysis and enzyme-linked immunosorbent assay (ELISA) were performed to detect GS and GFAP in the extracts. Immunohistochemistry showed GS expression first on P10. It increased later in both normal and dystrophic retinas. GFAP was not expressed in normal retinas, but Müller cells of dystrophic retinas were stained on P35 and P100. GS immunoblots were recognized on P21 and later in both normal and dystrophic retinas with similar densities, while GFAP immunoblots were observed only on P35 and P100, and only in dystrophic retinas. ELISA demonstrated increased GS concentrations with the development in both normal and dystrophic retinas, but no significant difference was observed between them. GFAP concentrations had no significant difference on P21 between both groups, those of normal ones remained unchanged later, while those of dystrophic rats were remarkably increased on P35 and P100. Müller cells might be affected following the progressive degeneration of photoreceptor cells and react to the glio-neuronal relationship. ———— development; dystrophic rat; glutamine synthetase (GS); GFAP; Müller cell

Riepe and Norenburg (1977) first localized glutamine synthetase (GS), a key enzyme of the glutamate-glutamine cycle, in Müller cells in rat retina by immunohistochemical techniques. Since then, many studies have examined the morphology and function of Müller cells, looking at GS. Müller cells are thought to play an important role both structurally and functionally in the retina, and they have

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a close relationship to the neuronal environment. Furthermore, it appears that they are involved in the pathology and repair of various diseases. Müller cells express glial fibrillary acidic protein (GFAP) when the retina is damaged by some factors or is in pathological situations, including inherited retinal dystrophy (Eisenfeld et al. 1984; Ekstrom et al. 1988; Roque and Caldwell 1990; Li et al. 1993) and other cases of retinal degeneration, injury, or light damage (Bignami and Dahl 1979; Shaw and Weber 1983; Eisenfeld et al. 1984). GFAP appears to be a pathological marker of Müller cells. Royal College of Surgeons (RCS) rats have an autosomal recessive retinal dystrophy that results in progressive degeneration of (rod) photoreceptor cells (Dowling and Sidman 1962; Bourne et al. 1983; Hartig et al. 1995). Müller cells of developing RCS rats have never been examined systematically, to our knowledge. In this study, we investigated these two markers of Müller cells in developing RCS rats with inherited retinal dystrophy using immunohistochemical techniques, Western blot analysis and ELISA.

Materials and Methods

Animals

These experiments were conducted in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research. Dystrophic RCS (rdy/rdy) rats on postnatal days P1, 4, 10, 21, 35, and 100 were examined. Age-matched RCS (+/+) rats were used as normal controls. The eyes of these rats were treated as follows.

Immunohistochemical staining

First, the rats underwent intracardiac perfusion with 4% paraformaldehyde, and eyes were enucleated on the postnatal days indicated. The eyes were fixed overnight with 4% paraformaldehyde. The 3-μm thick paraffin-embedded sections were treated with 0.3% hydrogen peroxide-methanol for 15 minutes at room temperature. Next, they were incubated overnight at 4°C with 1 μg/ml anti-GS antibody (Chemicon International Inc., Temecula, CA, USA) and GFAP antibody (ICN Biomedicals, Inc., Aurora, OH, USA) (these antibodies were used also in immunoblots and ELISA) and rinsed with phosphate buffered saline (PBS) containing 0.05% Tween 20. They were incubated with anti-mouse IgG-gold (10-nm gold particles, British BioCell International, Cardiff, UK), 1 : 200 dilution for 1 hour at room temperature, and rinsed with deionized, distilled water. Antibodies were dissolved in PBS containing 0.05% Tween 20 and 1% BSA. Staining was developed by incubation in a silver-enhancing kit (British BioCell International) for 10 minutes at room temperature. The sections were then dehydrated and mounted.

Immunoblots of extracts of rat retinas

The extracted retinas were solubilized with 1% sodium dodecyl sulfate(SDS).

Protein concentrations were measured according to the method of Lowry et al. (1953), using BSA as the standard. Western blot analysis was performed according to Towbin et al. (1979), using each 5-μg protein sample. After proteins were transferred to nitrocellulose membrane by electrophoresis, the membrane was treated with 3% gelatin for 1 hour at 37°C. We used 1 μg/ml mouse monoclonal anti-GS antibody and mouse monoclonal anti-GFAP antibody. Next, 1 : 5000 diluted anti-rabbit IgG alkaline phosphatase was used as the second antibody. The antibodies were diluted with PBS (0.14 M NaCl and 10 mM phosphate buffer, pH 7.4) containing 1% BSA and 0.05% Tween 20. Incubation time was 1 hour at room temperature. Washing was performed after each step with PBS containing 0.05% Tween 20. Color development was carried out with 5 ml of dye solution (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris-HCl buffer, pH 9.5) containing 33 μl nitroblue tetrazolium solution (50 mg/ml nitroblue tetrazolium in 70% dimethylformamide) and 16.5 μl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) solution (50 mg/ml BCIP, *p*-toluidine salt in dimethylformamide).

Assay of relative GS and GFAP contents by ELISA

Relative GS and GFAP contents were measured by ELISA. After the retinas were extracted, 50 μl of diluted samples of rat retinas was applied in each well. The binding of this antigen to microtiter wells was performed overnight at 4°C. The diluted anti-GS and anti-GFAP antibody was applied to the wells in 0.05-ml aliquots. After 2-hour incubation at room temperature, the plate was washed with PBS containing 0.1% Triton X-100 (TX-PBS) and PBS 4 times each. Peroxidase-labeled goat anti-rabbit IgG at a dilution of 1 : 1000 (0.05 ml) was added to the wells. The plates were incubated at room temperature for 2 hours and then washed with TX-PBS and PBS. Fifty microliters of 3.7-mM *o*-phenylenediamine and 0.006% H_2O_2 in citrate-phosphate buffer (pH 5.0) was added to each well. After another 2-hour incubation, the same amount of 2.4-MH_2SO_4 was added to each well. The absorbance at 492 nm was read on a two-wavelength microplate photometer.

Results

From P21 and afterward, the outer nuclear layer of the dystrophic retina is getting disarranged (degenerative) and its thickness getting thinner with the development (Fig. 1).

Immunohistochemical staining

GS was first expressed in Müller cells of both normal and dystrophic rats on P10, and staining increased afterward (Fig. 2A). In normal rats, GFAP was not expressed throughout the examined developmental stages. In dystrophic retinas, however, Müller cells were stained with anti-GFAP antibody on P35 and P100. GFAP staining was hardly observed in Müller cells on P1, 4, 10, and 21 (Fig. 2B).

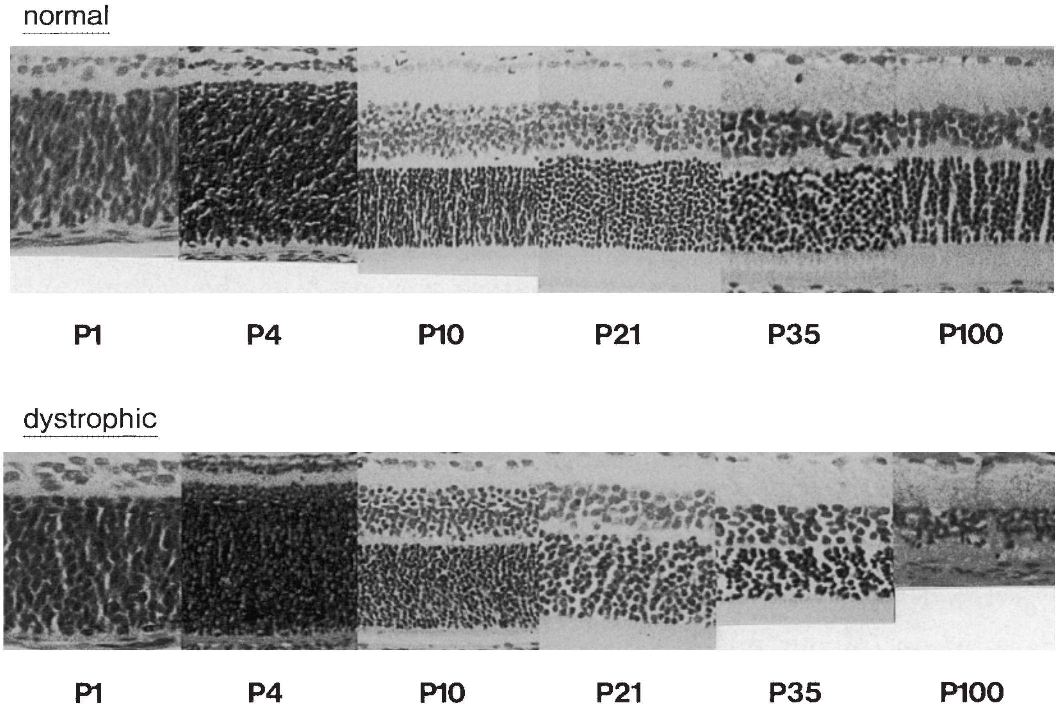


Fig. 1. Rats retinas with development (H & E staining): It is remarkable that from P21 and afterward, the outer nuclear layer is getting disarranged (degenerative) in dystrophic retina and its thickness getting thinner.

Immunoblots of extracts

In both dystrophic and normal retinas, GS immunoblots were not recognized on P1 and P10, but a single band with similar densities was found close to 44 kDa on P21, P35, and P100 of these animals (Fig. 3A). GFAP expression by Western blots showed a single band close to 50 kDa on P35 and P100 dystrophic retinas, and the intensity of P100 was stronger than that of P35. There was hardly any GFAP expression in rats examined in other stages (Fig. 3B).

Developmental profiles of GS and GFAP determined by ELISA

GS concentrations in both dystrophic and normal rat retinas increased according to development, but no remarkable difference was found between dystrophic and control retinas at any developmental stage (Fig. 4A). GFAP concentrations in both dystrophic and normal rat retinas were similar to day P21. In normal retinas, the concentrations were constant afterward, whereas in dystrophic retinas they remarkably increased on P35 and P100 (Fig. 4B).

Discussion

In 1851, Müller cells were first described (Müller 1851). Since then, many studies have examined these cells, particularly their morphology, function, and significance in the retina. Müller cells take the metabolic, rescuing, and trophic roles in retinal injury and pathology. Growth factors, such as basic fibroblast

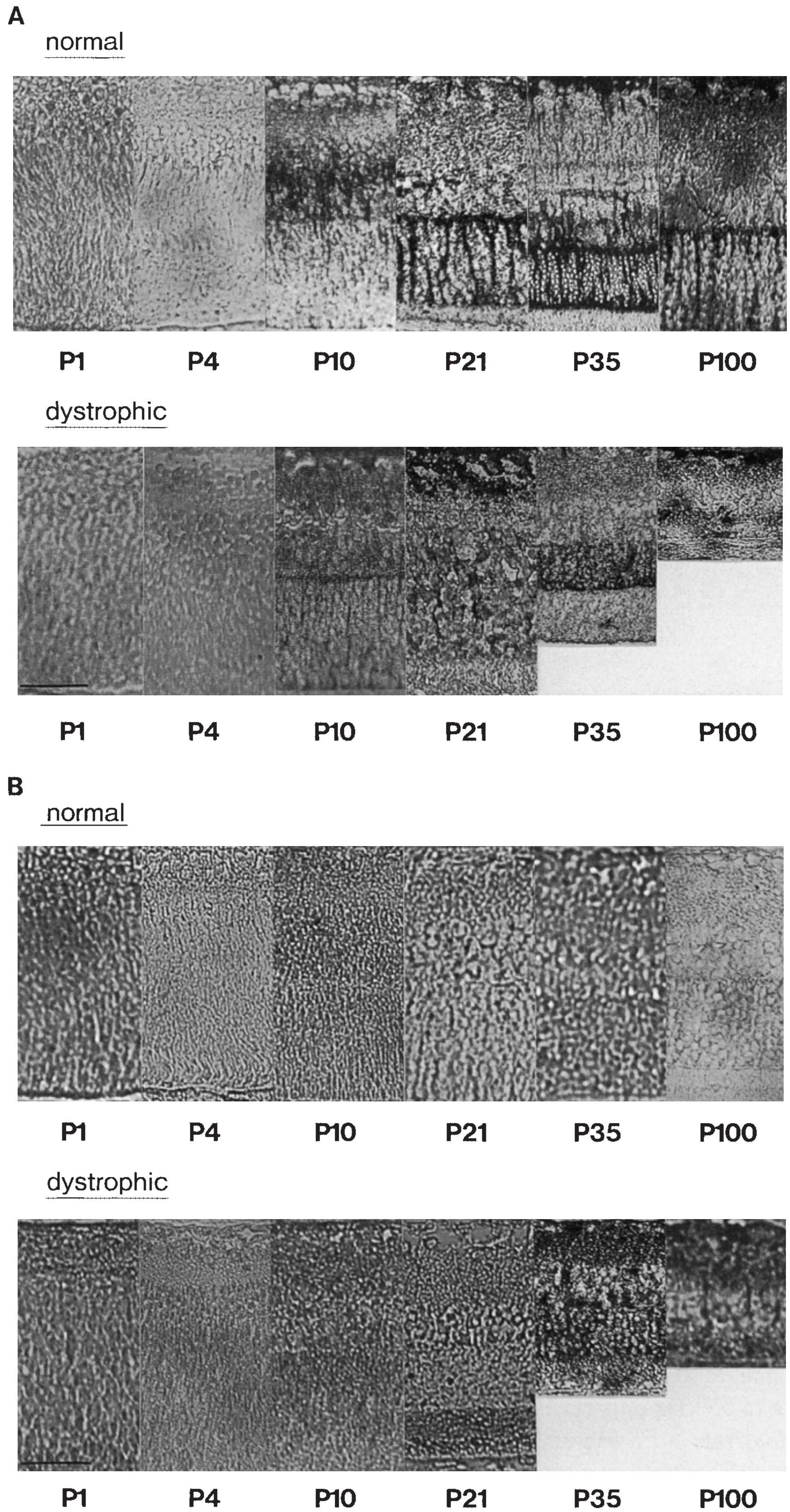
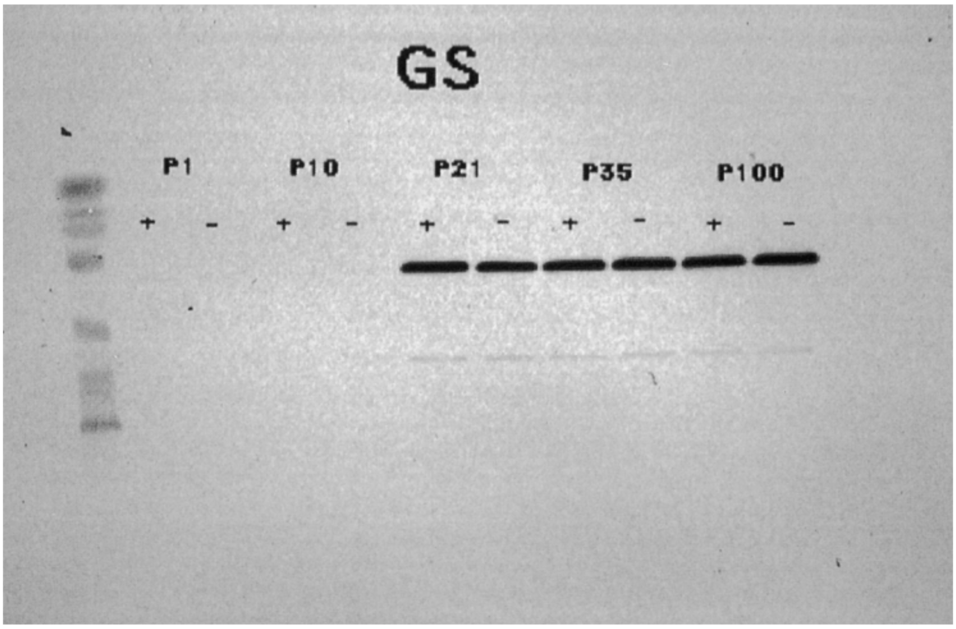


Fig. 2. Immunohistochemistry with anti-GS and GFAP antibody. A, GS immunoreactivity increased with development in both normal and dystrophic retinas. No significant differences were observed between two animals. B, No GFAP expression was seen in normal retinas. Müller cells of dystrophic retinas were immunostained on P35 and P100. Scale bar $=50\ \mu$m.

A



B

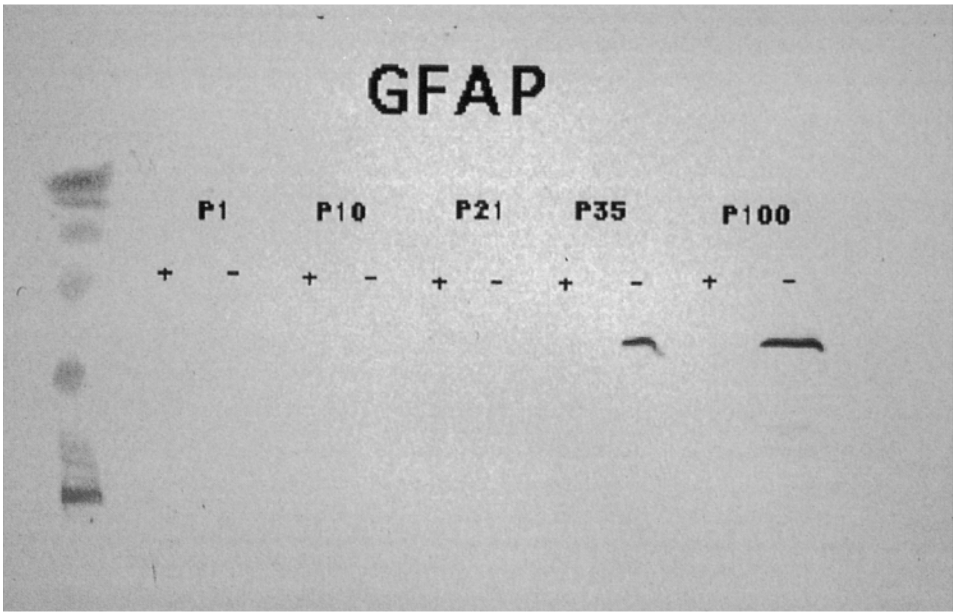


Fig. 3. Immunoblots of extracts of rat retinas with GS and GFAP. A, GS: A single band close to 44 kDa on P21, P35, and P100 in both normal and dystrophic extracts of retinas was shown. Both groups showed similar densities. B, GFAP: The extracts of dystrophic retinas showed a single band close to 50 kDa only on P35 and P100. The density was greater on P100. +, normal rat; —, dystrophic rat.

growth factor (bFGF), which is known to rescue photoreceptors after injury or light damage, have been identified in Müller cells (Distler and Dreher 1996). Furthermore, Müller cells' involvement in some clinical conditions including diabetic retinopathy (Lieth et al. 1998) has been stressed according to the development of diagnostic and treatment techniques.

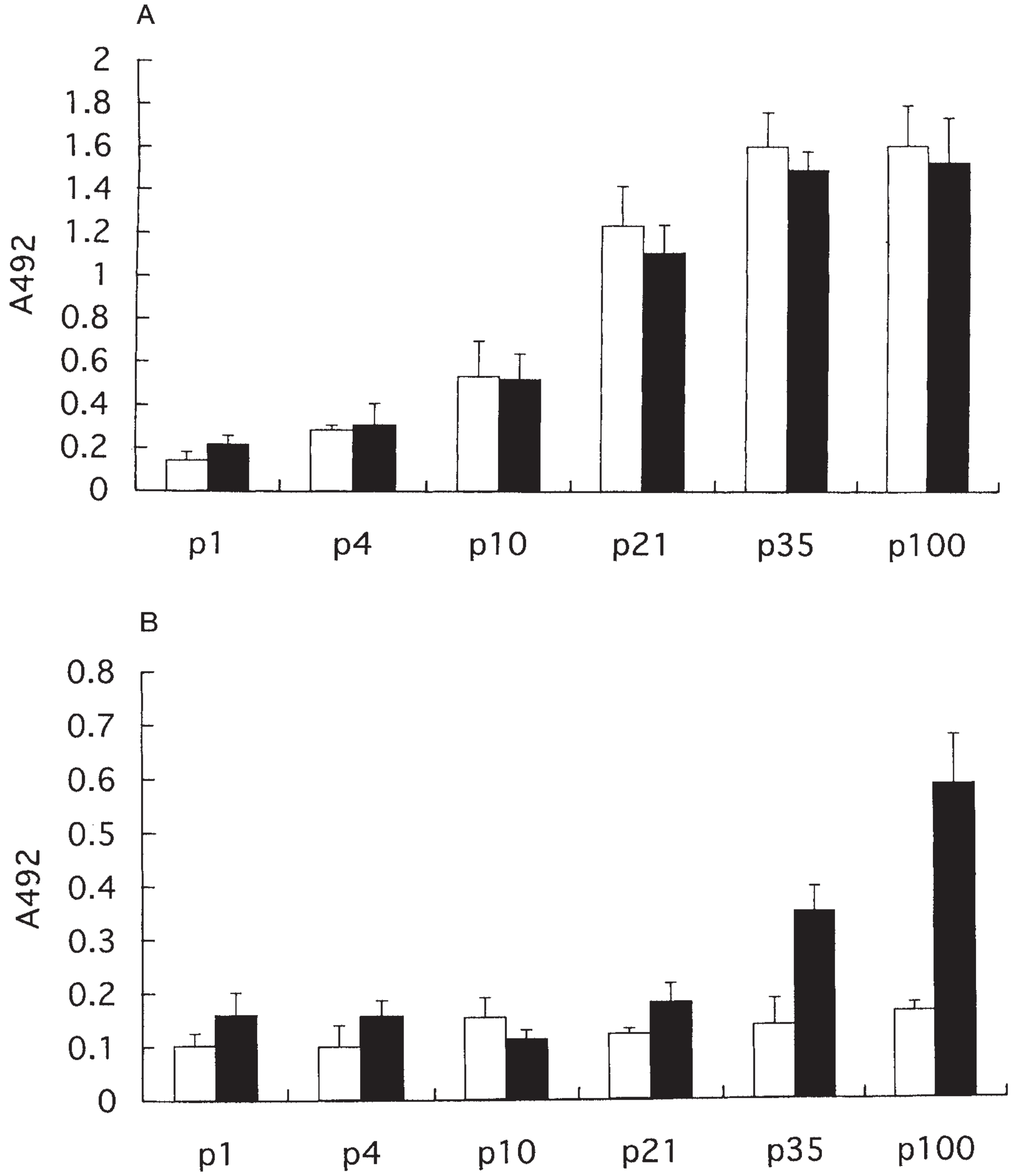


Fig. 4. Developmental profiles of GS and GFAP in rat retinas determined by enzyme-linked immunosorbent assay. □, normal; ■, RCS. A, GS: GS concentrations per protein increased with the development in both normal and dystrophic retinas, however, the difference between them was not significant. B, GFAP: In dystrophic retinas, GFAP concentrations per protein remarkably increased on P35 and P100. They were constantly at a low level in normal retinas throughout the developmental stages.

The development of Müller cells has been investigated using various animal models including rat (Riepe and Norenburg 1978), mouse (Lavail and Reif-Lehrer 1971), and rabbit (Reichenbach and Reichelt 1986; Reichenbach et al. 1991). It is reported that, in normal rat retinas, GS, a specific enzyme of Müller cells (Riepe and Norenburg 1977; Sarthy and Lam 1978; Linser et al. 1984), was first detected on P5 by immunohistochemical techniques (Riepe and Norenburg 1978). In our study, its expression was observed on P10, and no obvious staining was detected on P4. It was speculated that GS activities emerge within a week after birth, with the development of Müller cells.

There was no significant difference between GS specific activity per mg protein of normal rats and that of dystrophic ones. We believe that GS converts the neurotransmitter glutamate into glutamine, which photoreceptor cells take up and synthesize glutamate to release. That is, photoreceptor cells are the major source of glutamate in the retina. Even if the degeneration of photoreceptor cells progressed, the function of Müller cells seem not to change. But we think there is another possibility. It has been well known that the thickness and total protein of the dystrophic rat retina have decreased with age (Lavail and Reif-Lehrer 1971). We think that although there was no significant difference between GS specific activity per mg protein of two species, taking account of total amount of GS, our observation might show significant difference and the dystrophic retinas might display a rapid loss of GS. It might be possible that GS activity, the Müller cell itself, or the glio-neuronal interactions were down-regulated (Hartig et al. 1995).

Columnar arrangements of cells exist in the developing retina which can be observed by means of scanning electron microscopy. In the adult retina, each Müller cell ensheaths a columnar group of neuronal cells. These cells consist of one Müller cell, 11 rod photo-receptor cells, about 2 bipolar cells, and 1 to 2 amacrine cells. It is thought that this unit arises by migration of groups of preneurons along a common Müller (precursor) cell, and such columns might constitute metabolic and functional units. It is known that rod photoreceptor cell degeneration in RCS rats induces degenerative changes in secondary neurons of the inner retina (Dowling and Sidman 1962; Noell 1965; Hartig et al. 1995). Also in RCS rats, Müller cells are shorter than in normal rats, and their outer processes are hypertrophic (Distler and Dreher 1996). GFAP is thought to be expressed due to gliosis, and hypertrophy of macroglial cells (Reichelt et al. 1989; Hartig et al. 1995). Moreover, GFAP is thought to be expressed usually in astrocyte in the retina, but in Müller cell under pathological or stressful conditions (Hartig et al. 1995). Our results showed that GFAP immunoreactivity and its content increased in dystrophic retinas at late developmental stages. Taken together, the following possibility is suggested. As the degeneration of rod photoreceptor progresses, other neurons might become degenerative, and the neuronal relationship between Müller cell and neuronal cells might be damaged within the columnar unit. Also, Müller cells might reactively become gliotic and hypertrophic to supplement the structural defect.

In this study, we examined the changes of Müller cell in the different developmental stages, using its two markers, GS and GFAP. We believe this is the first systematic report about Müller cells in the developing RCS rat retina. In this study we tried to clarify the characteristics of Müller cells in dystrophic rats. Our observations demonstrated that Müller cells might be affected following the progressive degeneration of photoreceptor cells and react to the glio-neuronal relationship.

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