SPATIOTEMPORAL DISTRIBUTION OF PROTEOGLYCANS IN THE DEVELOPING AND ADULT MOUSE RETINA

（発生中および成マウス網膜におけるプロテオグリカンの分布）

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Contents

GENERAL INTRODUCTION

Retina; a neural layer of eye was a focus of study by anatomists for long time, however, studies were mostly confined to the cellular elements nevertheless to the extracellular matrix which was thought to work only as cement substance derived from the connective tissue. The major components of the extracellular matrix are proteoglycans, collagens, glycosaminoglycans and fibronectin. Proteoglycans are complex macromolecules composed by a core protein with one or more covalently bound glycosaminoglycan chains.

Proteoglycans have been recognized not only to play a part in providing shape and biomechanical strength of organs and tissues, but also to exhibit direct and indirect cell signaling properties. It is emphasized that the modulatory role of proteoglycans on cell proliferation cannot be separated from their participation in tissue organization in general, thereby explaining the diverse and sometimes contradictory reports on the effects of proteoglycans on cell proliferation and differentiation (Kresse and Schönherr, 2001).

Recently, many studies were done on the proteoglycans revealed that these molecules have an important biological role during development of different organs in addition to act as tissue organizers, influence cell growth and the maturation of specialized tissues, modulate growth-factor activities,

regulate collagen fibrillogenesis, and influence corneal transparency (Vogel, 1994; Izzo, 1998; Taylor and Gallo, 2006; Zhang et al., 2006).

Chodroitin sulfate is uniquely important in morphogenesis, cell division and is the carbohydrate component of chondroitin sulfate proteoglycans molecules that are spatiotemporally regulated during brain development (Knudson and Knudson, 2001; Hwang et al., 2003; Laabs et al., 2005) and upregulated after injury in the central nervous system (Chung et al., 2000; Silver and Miller, 2004). Chondroitin sulfate proteoglycans have also been found to be expressed in the retina (Hocking et al., 1998; Kresse and Schönherr, 2001). However, there are a little or no reports about localization and developmental changes of these proteoglycans in mice retina (Zako et al., 1997; Popp et al., 2004).

Small leucine-rich proteoglycans comprise at least nine members that, though structurally related, have evolved from different genes, have acquired unique functions, related to cell proliferation and extracellular matrix assembly (Kjellén and Lindhal, 1991; Ruoslahti and Yamaguchi, 1991) and play a role in the regeneration of nervous tissues in central nervous system injury. Early study on the rat retinas with inherited retinal dystrophy showed that interphotoreceptor matrix is abnormal in dystrophic retinas. Moreover,

the distribution of interphotoreceptor matrix differs from that in normal retinas (La Vail et al., 1981).

The current experiment was done on the developmental changes in the retina and optic nerve, to estimate the changes in proteoglycans parallel with retinal differentiation. At embryonic day (E) 12, the retina consists of homogenous neuroblast cells and on E18, inner retinal cells form ganglion cell layer and inner plexiform layer, differentiation of outer retinal layer occurs from postnatal day (P) 7 to P14. The outer retinal layer differentiated to inner nuclear layer, outer plexiform layer, outer nuclear layer and photoreceptor layer. The retina becomes fully mature by P21 (Kuwabara and Weidman, 1974; Sharma et al., 2003).

Thus, the present study represents an attempt to assess the developmental changes of proteoglycans in mouse retina at different stage of development from early neuroblastic stage to adult stages. Taken in consideration different stages of retinal differentiation, the diversity and timing of the expression and distribution of chondroitin sulfate proteoglycans were estimated in chapter I. In addition, developmental distributions of small leucine-rich proteoglycans in mouse retina were studied in chapter II.

CHAPTER I

Spatiotemporal Distribution of Chondroitin Sulfate Proteoglycans in the Developing Mouse Retina and Optic Nerve

ABSTRACT

The aim of the present study was to determine the developmental changes and distribution of chondroitin sulfate proteoglycans in the mouse retina and optic nerve of the prenatal and postnatal mouse by immunohistochemistry. At embryonic day (E) 18, chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S) and biglycan were detected in the retina and optic nerve. However, aggrecan was seen in the retina but not in the optic nerve. At postnatal day (P) 7, aggrecan and biglycan were clearly observed in the optic nerve, inner nuclear layer and ganglion cell layer and diffuse in the outer retina. C4S diffusely distributed in the retina and optic nerve, but C6S was mainly confined to the photoreceptor layer and optic nerve sheath. At P42, biglycan showed diffuse distribution in the retina and optic nerve with intense staining in nerve-fiber rich layers. Aggrecan showed weak staining at the inner plexiform layer with higher density in the outer and inner nuclear layers, outer plexiform layer and ganglion cell layer. Both C4S and C6S were detected in the optic nerve and retina, but C6S showed strong immunostaining in the photoreceptor layer. The distributions of these proteoglycans with respect of time course during development of the retina and optic nerve suggest that they may have unique or overlapping roles in development and maintenance of the retina and optic nerve.

INTRODUCTION

The extracellular matrix (ECM) plays an integral role in the pivotal processes of development, tissue repair, and metastasis by regulating cell proliferation, differentiation, adhesion, and migration (Ruoslahti, 1988; Iozzo, 1998; Kresse and Schönherr, 2001). The major components of the ECM are collagens, proteoglycans, glycosaminoglycans (GAGs), fibronectin, and numerous less abundant proteins and glycoproteins. Proteoglycans are complex macromolecules composed by a core protein with one or more covalently bound GAG chains. GAGs, including chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and keratan sulfate, are a widely distributed structurally diverse family of sulfated, unbranched polysaccharides that are expressed abundantly on the surface of cells and the ECM. GAGs have emerged as important regulators of the signaling involved in cell growth, tumorigenesis and inflammation (Iozzo, 2005; Parish, 2006; Taylor and Gallo, 2006).

Previously, it had been believed that proteoglycans were like cement simply filling the extracellular spaces in connective tissue and they were never the focus of research on neural tissues. As many unique proteoglycans have now been identified in neural tissues and the roles they play in cell–cell and cell–substratum interactions, cell proliferation and cell differentiation have

been clarified, their major involvement in neural network formation has been indicated (Inatani and Tanihara, 2002). Proteoglycans have recently been shown to not only provide shape and biomechanical strength of organs and tissues but also to have direct and indirect cell signaling properties (Kresse and Schönherr, 2001).

CS is uniquely important in morphogenesis, cell division and cartilage development and is the carbohydrate component of chondroitin sulfate proteoglycans (CSPGs), molecules that are spatiotemporally regulated during brain development (Knudson and Knudson, 2001; Hwang et al., 2003; Laabs et al., 2005; Sirko et al., 2007) and upregulated after injury in the central nervous system (Chung et al., 2000; Silver and Miller, 2004). CSPGs have also been found to be expressed in the retina (Hocking et al., 1998; Kresse and Schönherr, 2001). Keratan sulfate was localized in growing axons from chick retinal explants (McAdams and McLoon, 1995), however there is no report about the presence of keratan sulfate in the mouse retina and optic nerve.

However, to the best of my knowledge, there are no reports about localization and developmental changes of proteoglycans in the mouse retina and optic nerve. The present study represents an attempt to assess the diversity and timing of the expression and distribution of chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S), aggrecan and biglycan, and keratan sulfate, and

to elucidate their functional roles in the development and maintenance of the retina and optic nerve.

MATERIALS AND METHODS

Animal care and experimental procedures were approved by the Animal Research Committee, Tottori University, Japan. Approval number is 09-T-19.

Animals and tissue processing: Jc1: ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The animals were maintained in a 12:12 h light-dark cycle with free access to food and water throughout the whole experiment. One male mouse was placed to mate with many female mice overnight and pregnant females were identified by the presence of vaginal plug in the next morning. Thus, the embryonic day zero (E0) was setup on the first day the vaginal plug was observed. Mouse embryos having embryonic age of 12 days (E12, n=8) and 18 days (E18, n=8) obtained by cesarean section from these pregnant mice were enucleated. In addition, eyes of mice having postnatal age of 7 (P7, n=6), 14 (P14, n=4), 42 (P42, n=8), and 120 days (P120, n=6) were also used in this experiment.

The mice were euthanized by cervical dislocation, and both eyes of each mouse were collected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight at 4°C with gentle shaking. Serial sagittal sections of the central retina were cut at 4 μm.

Antibodies: Polyclonal anti-biglycan, polyclonal anti-aggrecan and monoclonal anti-keratan sulfate antibodies were purchased from Santa Cruz

Biotechnology (CA, USA), monoclonal anti-C4S antibody was purchased from Abnova Corporation (Taipei, Taiwan), and monoclonal anti-C6S antibody was purchased from Seikagku Biobusiness Corporation (Tokyo, Japan).

Immunohistochemistry: Four-μm-thick paraffin sections of mouse retina were deparaffinized, rehydrated, and rinsed with PBS. Indirect immunostaining was performed using a Histofine SAB-PO (M) (for mouse primary antibodies) or SAB-PO (R) (for rabbit primary antibodies) immunohistochemical staining kit (Nichirei, Japan). Endogenous peroxidase activity was eliminated with 3% hydrogen peroxide in methanol for 5 min. After washing with PBS, the retinal sections were treated sequentially with blocking serum from the same animal in which the secondary antibody was raised. Blocking times were 25 min for polyclonal antibodies and 1 h for monoclonal antibodies at room temperature. The sections were then incubated at 4°C overnight either with specific primary antibodies (1: 50 in PBS for polyclonal antibodies and 1:50 1% bovine serum albumin in PBS for monoclonal antibodies) or with a buffer solution for control sections after treating with the blocking serum. After washing with PBS, biotinylated anti-mouse and rabbit IgG was applied for 30 min at room temperature. After washing with PBS, peroxidase-conjugated streptavidin solution was applied

for 30 min. Peroxidase labeling was visualized using 0.05% 3-3′ diaminobenzidine (Sigma). Micrographs of the sections were taken with a digital camera for a microscope (DP-71, Olympus, Tokyo, Japan).

RESULTS

The present study was done to investigate the distributions of CSPGs in the retina and optic nerve of mice at embryonic, early postnatal and adult stages.

Retina (Fig. 1): At E12, the retina was composed of homogenous neuroblasts. No immunostaining for CSPGs was detected in the retina. At E18, the ganglion cell and inner plexiform layers (GCL and IPL, respectively) were distinguishable from other neuroblasts. C4S, aggrecan and biglycan showed diffused distribution throughout the retina, but C6S was detected mainly in the GCL.

At P7, all layers in the mature retina were observed, but especially the photoreceptor layer (PRL) was very thin. The distribution of CS changed with retinal differentiation. Aggrecan immunostaining was clearly observed in the GCL and inner nuclear layer (INL) and was diffuse in the outer retinal layer. C4S and biglycan showed moderate diffuse immunostaining throughout the retina. C6S showed strong immunostaining in the PRL.

At P14 and P42, the PRL increased prominently in thickness and the IPL also increased in thickness. Biglycan showed diffuse distribution in the retina with higher staining affinity in nerve fiber-rich layers, including the IPL, outer plexiform layer (OPL) and nerve fiber layer (NFL), but did not

show immunostaining in the PRL. Aggrecan showed moderate immunostaining in the GCL, INL, outer nuclear layer (ONL) and OPL, and weak immunostaining in the IPL and PRL. C4S was distributed in the GCL, INL, ONL, OPL and retinal pigment epithelium (RPE) and not detected in the PRL. C6S was moderately distributed in the PRL and RPE and faint in the ONL. The distributional pattern of these CSPGs in the P120 mouse retina was similar to that in P42 but in lesser intensity. However, aggrecan showed higher immunostaining in the ONL with faint immunostaining in the PRL and C6S showed higher immunostainig in the PRL. No immunostaining for keratan sulfate was detected in the retina from E12 to P120.

Optic nerve (Fig. 2): At E12, the optic nerve was not formed yet as the previous studies (Collello and Guiliery, 1990; Chung et al., 2000). At E18, the optic nerve had formed. The optic disc showed diffuse immunostaining for C4S and biglycan, however, C6S showed weak immunostaining at the optic disc and the optic nerve with moderate staining at the optic nerve sheath. Aggrecan showed moderate immunostaining at the optic nerve head.

At P7, C4S and biglycan were diffusely distributed in the optic disc and optic nerve with high density at the lamina cribrosa and confined to the laminar beam of the optic nerve. C6S showed weak immunostaining at the

optic nerve head and moderate-staining at the optic nerve sheath. Aggrecan showed strong immunostaining in the optic disc, lamina cribrosa and optic nerve proper (Fig. 3).

At P14 and P42, the optic nerve showed diffuse immunostaining for C4S, C6S and biglycan and stronger immunostaining for aggrecan, especially at P42. The distribution of these proteoglycans at P120 was similar to that at P42 (Fig. 2). No immunoreactivity for keratan sulfate was detected in the optic nerve at any observed stage.

DISCUSSION

There are few reports on distribution of CSPGs in the retina and optic nerve (Zako et al., 1997; Knudson and Knudson, 2001; Popp et al., 2004). In this study, the presence and distribution of C4S, C6S, aggrecan and biglycan in addition to keratan sulfate were estimated to determine the possible importance of such distributions on the development of the retina and optic nerve. It has been suggested that CSPGs may regulate the pattern of retinal ganglion cell outgrowth in the developing retina because of CSPGs having an inhibitory effect on elongation of retinal ganglion cell axons (Brittis et al., 1992; Snow et al., 1992; Smith and Strunz, 2005).

At E12, when the retina was mainly composed of homologous neuroblasts as the previous study (Sharma et al., 2003), no specific staining for CSPGs was observed in the eye but at E18, when differentiation of the NFL, GCL and IPL were distinguishable from the other neuroblasts as the previous studies (Kuwabara, and Weidman, 1974; Sharma et al., 2003), while C4S was diffusely distributed throughout the retina, C6S was confined to the GCL. At P14 and P42, C4S was not detected in the PRL. In contrast to C4S, C6S was distributed in the PRL at these ages and increased in the PRL at P120. Thus, CSPGs appeared in the retina in accord with differentiation of neuroblasts in the retina. C4S might be regulator of axonal outgrowth

(Challacombe and Elam, 1997). C6S distributes in the interphotoreceptor matrix of the rat retina (Porrello and LaVail, 1986) and modulates retinal ganglion cell body polarity and stimulates retinal axon elongation (Brittis and Silver, 1994). In addition, photoreceptor degeneration is found in mice with mucopolysaccharidosis type VII, which is a lysosomal storage disorder and caused by a deficiency of specific lysosomal enzyme required for the degradation of glycosaminoglycans including CS (Lazarus et al., 1993). Little expression of CS was also found in the interphotoreceptor matrix of rds mice which photoreceptor cells progressively loses (Tawara and Hollyfield, 1990). Thus, C6S may be involved in the maintenance of photoreceptor cells.

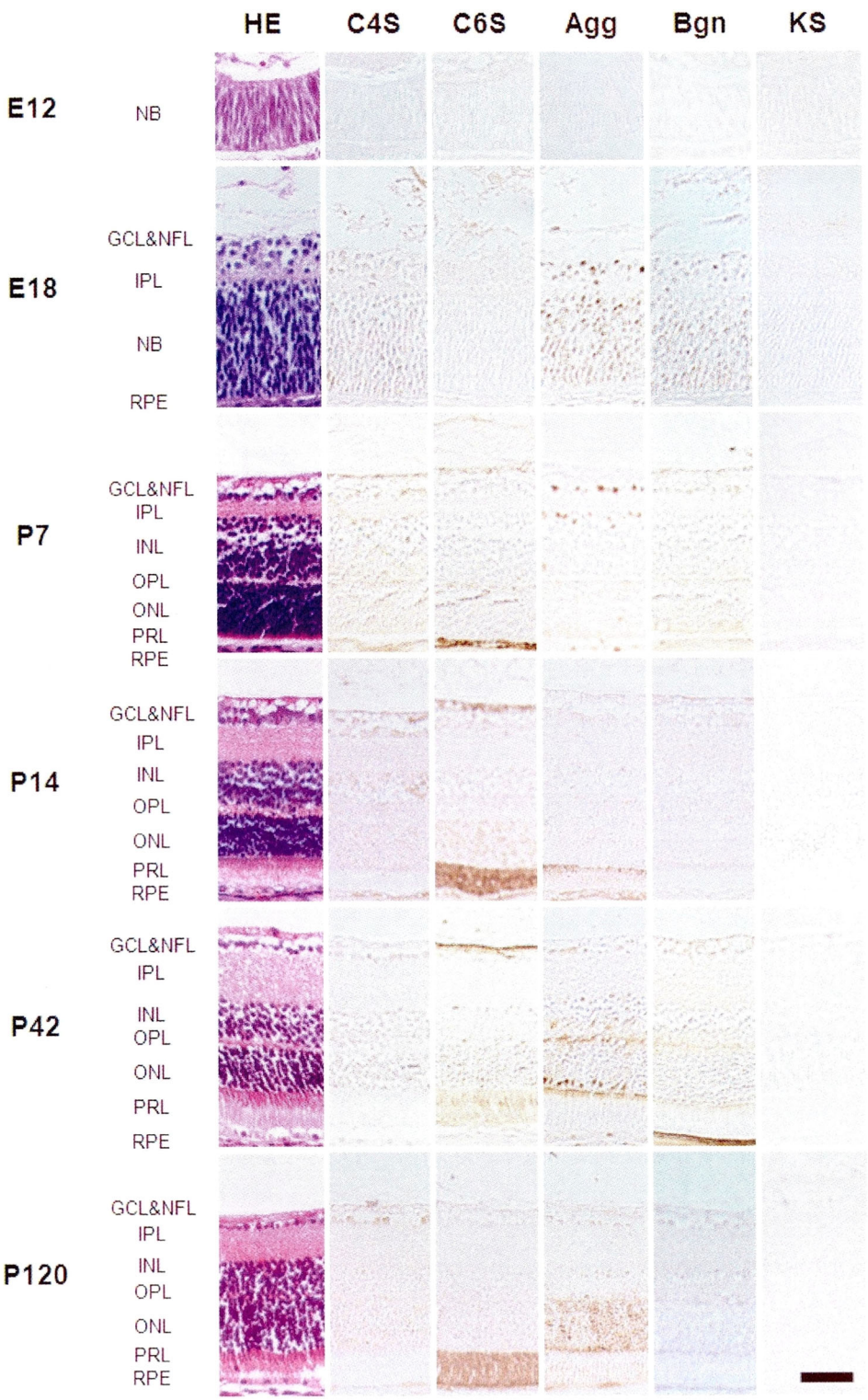
In this study, biglycan was present in the retina in a diffuse mannar at E18-P7 and showed higher affinity to nerve fiber rich layers from P14-P42. The distribution of biglycan in the mouse retina in this study was similar to that reported for decorin in the rat retina (Inatani et al., 1999). Biglycan and decorin are closely similar in molecular structure (Krusius and Ruoslahti, 1986) and are capable of interacting with collagen fibrils (Kobe and Deisenhofer, 1994; Keene et al., 2000). It is supposed that biglycan and decorin are not only neurotrophic factors for retinal cells but also relate to their differentiation.

Aggrecan is one of the major CSPGs expressed in the central and peripheral nervous system of embryos and adults (Oakley and Tosney, 1991; Schwartz et al., 1996). In this study, aggrecan appeared at E18 in the retina, was increased in the inner retina and the optic nerve at P7 and was kept in the retina at P120. Aggrecan is produced by glial precursors (Domowicz et al., 2008; Afshari et al., 2010). Astrocyte-precursor cells migrate into the mouse retina at E17 (Uemura et al., 2006). Thus, these previous studies confirm the time of appearance of aggrecan in the mouse retina. Aggrecan have multiple effects on neurite outgrowth (Snow and Letourneau, 1992; Condic et al., 1999; Chan et al., 2008) and is required for normal astrocyte differentiation (Smith and Strunz, 2005). Thus, aggrecan is a mediator in neural development and may be important in maintenance of the mature retina.

In this study C6S was detected in the GCL at E18 but showed strong to moderate immunostaining in the PRL at P7, P14 and P42. C6S is localized at the apical surface of the RPE and in adjacent photoreceptors (Porrello and LaVail, 1986). In contrast, the apical surface of the RPE of RCS rats, which inherently have a condition similar to retinitis pigmentosa, is barely immunopositive (Porrello et al., 1989). C6S plays an important role in adhesion between the neural retina and RPE (Lazarus and Hageman, 1992).

It seems reasonable, therefore, that C6S was negative in the immature PRL at E18 and positive in the developed PRL at P7 or later.

The developmental changes in the localization and levels of immunostaining of C4S, C6S, biglycan and aggrecan in the mouse eye were compared from E12 up to P120. The presence and distribution of aggrecan in the late embryonic rat retina and optic nerve have been reported (Luo and Raper, 1994), but there is no information on ocular aspects of the investigated CSPGs in mice. Although the neurobiological significance of C4S, C6S, aggrecan and biglycan in the retina and optic nerve remains to be more clearly delineated, the obtained results indicate that the individual proteoglycans probably have unique, perhaps partially overlapping, functions in the retina and optic nerve as well as in the central nervous system (Popp et al., 2003).


HE
C4S
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Agg
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P7
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Figure 1

Fig. 1. HE staining and immunostaining of CSPGs in the retina of mice at E12, E18, P7, P14, P42 and P120. C4S, chondroitin-4-sulfate; C6S, chondroitin-6-sulfate; Agg, aggrecan; Bgn, biglycan; KS, keratan sulfate; HE, hematoxylin and eosin; NB, neuroblast cell mass; GCL&NFL, ganglion cell layer and nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer; RPE, retinal pigment epithelium. Scale bar = 50 μm.

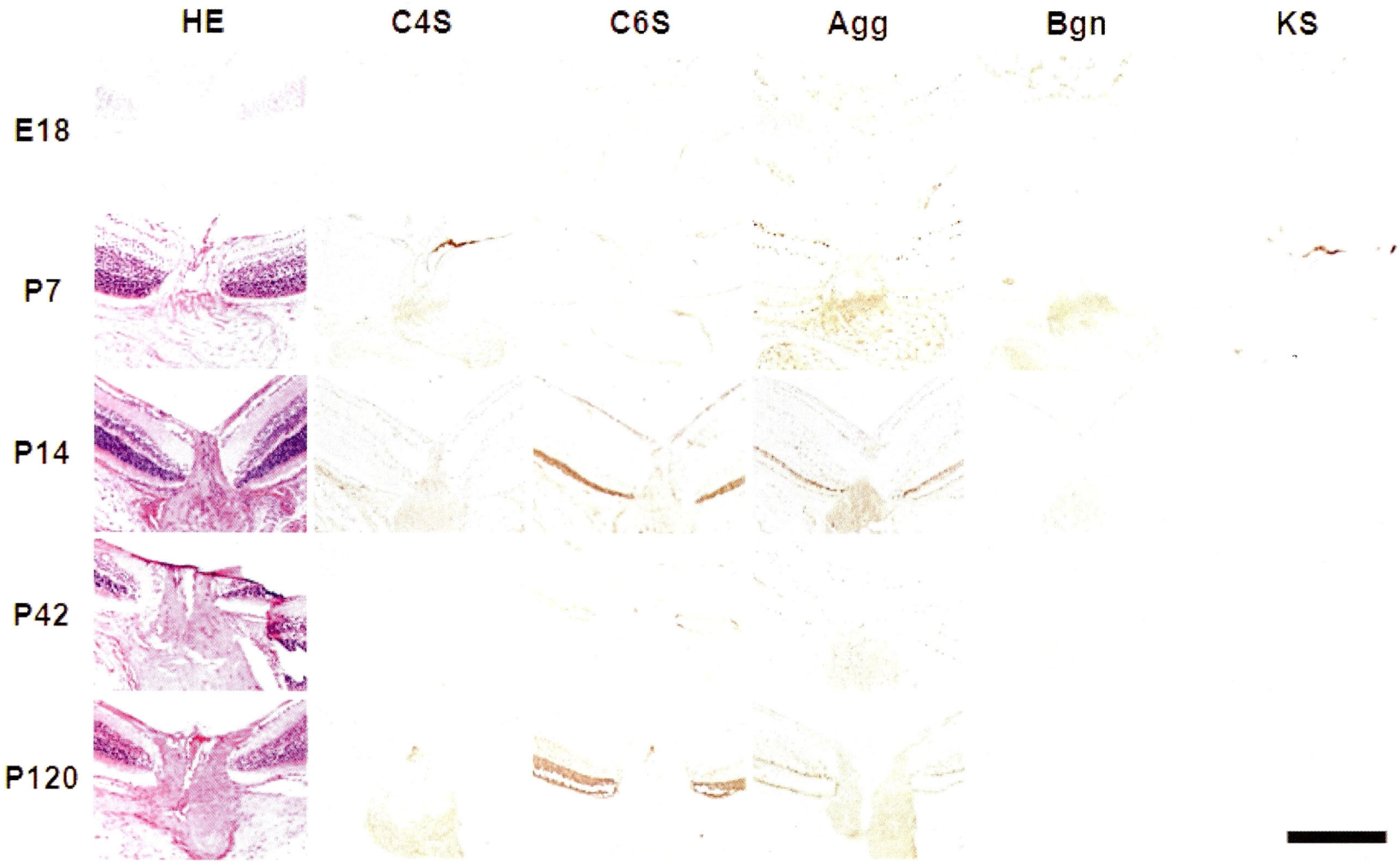


Figure 2

Fig. 2. HE staining and immunostaining of CSPGs in the optic nerve of mice at E18, P7, P14, P42 and P120. HE; hematoxylin and eosin. Scale bar = 200 μm.

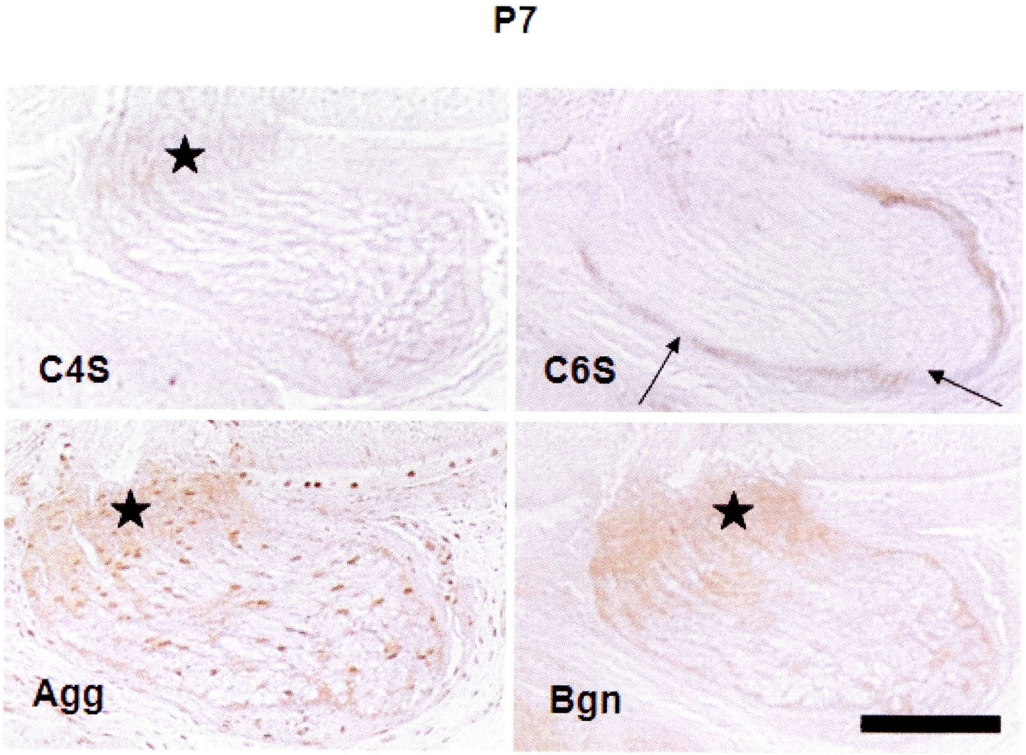


Figure 3

Fig. 3. Immunostaining of CSPGs in the optic nerve of mice at P7 showing a difference in the immunostaining pattern at the optic nerve. Lamina cribrosa (star), optic nerve sheath (arrows). Scale bar = 100 μm

CHAPTER II

Expression of Small Leucine-Rich Proteoglycans in the Developing Retina and Kainic Acid-induced Retinopathy in ICR Mice

ABSTRACT

The aim of this study was to determine the developmental changes of small leucine-rich proteoglycans (PGs), decorin, biglycan and fibromodulin, in ICR mouse retina and to elucidate their role in the adult retina using kainic acid (KA)-induced retinal damage model. Retinas of prenatal, postnatal and adult mice were collected for histological and immunohistochemical staining to investigate the changes in distribution of these PGs. Decorin-and fibromodulin-immunostainings were diffusely distributed at prenatal and early postnatal stages and were stronger in the adult retina. However, biglycan was moderately distributed in the prenatal and early postnatal stages and was faint in the adult retina. Retinas were collected at 1, 3 and 7 days after intravitreal injection of KA. Retinas of KA injected eyes underwent shrinkage accompanied by serious damage in the inner layers. Decorin and fibromodulin were upregulated in the inner retinal layers of KA-injected eyes compared to the normal ones. These results suggest that decorin and fibromodulin play key roles in retinal differentiation, and contribute to the retinal damage and repair process. However, biglycan may have a limited role in the mouse retinal development and repair process.

INTRODUCTION

Proteoglycans (PGs) play key roles in all of the fundamental biological processes and behave as potent effectors of cellular pathways. PGs consist of a core protein to which glycosaminoglycan (GAG) side chains are bound. GAG chains attached to the core protein vary markedly in length and number. These variations in GAG chain number and degree of sulfation determine the unique functions of different PGs within and between PG classes (Gilbert et al., 2005).

The families of small leucine-rich PGs (SLRPs) comprise at least nine members that, though structurally related, have evolved from different genes, have acquired unique functions, and have undergone a significant degree of structural sophistication. They can be synthesized as either glycoproteins containing N-linked oligosaccharides or as PGs containing chondroitin/dermatan sulfate or keratan sulfate chains (Iozzo, 1999; Iozzo and Danielson, 1999). The core proteins of decorin, biglycan and fibromodulin are similar in size (36-42 kDa). These core proteins have a central domain containing leucine-rich repeats and terminal domains with cysteine residues in conserved positions. Fibromodulin is a keratan sulfate PG, while decorin and biglycan are chondroitin/dermatan sulfate PGs (Vogel, 1994).

Decorin and biglycan have been isolated from mammalian connective tissues (Rosenberg et al., 1985), and it has been suggested that decorin and biglycan are small PGs related to cell proliferation and extracellular matrix assembly (Kjellén and Lindhal, 1991; Ruoslahti and Yamaguchi, 1991) and play a role in the regeneration of nervous tissues in central nervous system injury (Stichel et al., 1995). Fibromodulin, the most abundant member of the leucine-rich repeat protein family, has been shown to bind to type I collagen and to regulate fibrogenesis (Hedbom and Heinegård, 1989), and it has been also shown to be a constituent of tendons, cartilage, and sclera (Stanescu, 1990; Svensson et al., 1999). However, there are no reports on the localization and developmental changes of fibromodulin in the retina.

PGs specifically bind to many cell surface molecules and extracellular matrix molecules that are involved in various developmental events in the brain (Lander, 1989; Maeda and Oohira, 1991; Margolis and Margolis, 1993). These developmental events include proliferation and migration of neuroblasts, neurite outgrowth, and formation of specific synapses. However, there were a few studies on an involvement of PG in the retinal development. Additionally, in the neural retina, it has been inferred that alterations in the expression of PGs are involved in a number of pathologic conditions as retinal degeneration (La Vail et al., 1981; Porrello and La Vail,

1986). Although the relationships between PGs and retinal diseases have been studied, molecular biological studies on the expressional regulation of SLRP core proteins in normal and pathologic retinas are limited, and there have been only a few reports on the identification of PG core proteins expressed in the retina (Inatani et al., 1999; Ali et al., 2010).

Glutamate is the principal excitatory neurotransmitter in central nervous system and eye (Yoles and Schwartz, 1998; Meldrum, 2000). Endogenous glutamate may contribute to the brain damage occurring acutely after status epileptics, cerebral ischemia or traumatic brain injury (Meldrum, 2000). The increase of glutamate level was found in the animal model of retinal ischemia and optic nerve crush (Yoles and Schwartz, 1998). It has been shown that increased glutamate in neuro-degenerative disease in the retina was similar to that occurred after the intravitreal injection of kainic acid (KA), a glutamate receptor agonist (Li et al., 1999; Insua et al., 2008). In retinal degeneration, decorin is also found to be upregulated in a retinal ischemic model (Inatani et al., 1999), but an involvement of other SLRPs in retinal degeneration has not been clarified yet.

In this study, relationships of three SLRPs, decorin, biglycan and fibromodulin, in the retina were investigated through their immunohistochemical distributions in the developing and mature retina and

in the retina with KA-induced injury of ICR mice. Furthermore, the gene expression of these SLRPs was examined in the injured retina.

MATERIALS AND METHODS

All animal care and experimental procedures were approved by the Animal Research Committee, Tottori University, Japan (Approval number: 09-T-19).

Animals and tissue processing for developmental studies: ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The animals were maintained in a 12:12 h light-dark cycle with free access to food and water throughout the experiment. One male mouse was placed to mate with female mice overnight and pregnant females were identified by the presence of a vaginal plug the next morning. Thus, embryonic day zero (E0) was set on the first day the vaginal plug was observed. Mouse embryos at embryonic age of 18 days (E18, n=8) obtained by cesarean section from these pregnant mice were enucleated. Eyes of mice at postnatal ages of 7 (P7, n=4), 14 (P14, n=4), and 42 (P42, n=6) days were also used in this experiment. The mice were euthanized by cervical dislocation, and both eyes in each mouse were collected and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight at 4°C with gentle shaking. Serial sagittal sections of the central retina were cut at 4 μm for histological and immunohistochemical studies.

Animal model of KA-induced retinal damage: Three groups of five ICR mice at P14, P42 and P120 were used. The mice were anesthetized by intraperitoneal injection of 1.25% Avertin (2, 2, 2-tribromoethanol in tert-amyl alcohol; 17 μl/g body weight; Aldrich, WI, USA). Intravitreal injection of KA was performed as previously described [18]. Throughout this study, intravitreal injections were performed in a final volume of 2 μl using a heat-pulled glass pipette connected to a microsyringe (Microdispenser; Drummond Scientific Company, PA, USA). In this experiment, right eyes of each stage were injected with 2 μl of 10 mM corresponding to 20 nmol of KA (Tocris Bioscience, MO, USA) prepared in PBS. The left eyes were injected with 2 μl of PBS and used as controls. Animals were euthanized by cervical dislocation at 1, 3 and 7 days after injection for morphological and molecular studies.

Morphological analysis: Retinal sections were stained with hematoxylin and eosin (HE) at a distance of 1 mm from the optic disc. HE stained sections were observed and thickness of the retina was measured using Image J software. To distinguish changes in the nuclei of retinal layers of normal and KA-injected eyes, some sections were stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, OR, USA) in PBS for 10 min. Then the slides were washed 3 times for 5 min each time in PBS. To

reduce photobleaching, the sections were mounted with Vectashield mounting medium (Vector Laboratories, CA, USA) and examined under a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

Antibodies: The antibodies used in this study were purchased from different companies; polyclonal anti-biglycan and polyclonal anti-fibromodulin antibodies from Santa Cruz Biotechnology (CA, USA), polyclonal anti-decorin from R&D Systems (Abingdon, UK) and polyclonal anti-Pax6 antibody from Covance (CA, USA). Pax6 antibody was used to identify the cells in the GCL, amacrine cells and horizontal cells in the INL of normal retina and compared to that of KA-injected retinas.

Immunohistochemistry: Paraffin sections of mouse retina were deparaffinized, rehydrated, and rinsed with PBS. Indirect immunostaining was performed using a Histofine SAB-PO (G) (for goat primary antibodies; fibromodulin and decorin) or SAB-PO (R) (for rabbit primary antibodies; biglycan and Pax6) immunohistochemical staining kit (Nichirei, Tokyo, Japan). Endogenous peroxidase activity was eliminated with 3% hydrogen peroxide in methanol for 5 min. After washing with PBS, the retinal sections were sequentially blocked with normal blocking serum for 25 min at room temperature. The sections were then incubated at 4°C overnight with specific primary antibodies (1: 50 in PBS for fibromodulin and biglycan, 1: 300 for

Pax6 and 0.15 μg/ml for decorin). Control sections were incubated with a buffer solution after treatment with the blocking serum. After washing with PBS, biotinylated anti-goat or rabbit IgG was applied for 30 min at room temperature. After washing with PBS, peroxidase-conjugated streptavidin solution was applied for 30 min and peroxidase labeling was visualized using 0.05% 3-3' diaminobenzidine (Sigma, Tokyo, Japan). Micrographs of the sections were taken with a digital camera for a microscope (DP-71, Olympus).

RNA isolation and reverse transferase-polymerase chain reaction (RT-PCR): Retinas were removed and kept at -80°C until use. Total RNA was isolated from the retina by using Trizol (Invitrogen, Tokyo, Japan). For RT-PCR analysis, complementary DNA (cDNA) was prepared by ReverTraAce (Toyobo, Osaka, Japan,). The cDNA samples were then used for RT-PCR of decorin, biglycan, fibromodulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The cDNA was amplified by using Quick Taq (Toyobo) for 35 cycles in a thermal cycler (Takara, Osaka Japan) according to the manufacturer's protocol. Each cycle consisted of denaturation for 15 sec at 94°C, primer annealing for 30 sec at 59 °C and extension for 1 min at 68°C. The amplified products were electrophoresed in 2.0% (w/v) agarose gels at 100 V for 30 min in TAE buffer (40 mM Tris–

acetate, 1 mM EDTA, pH 8.0). Sequences for primers (Sigma Genosys, Hokkaido, Japan) used in this study are listed in Table 1.

Statistical analysis: The data were analyzed by ANOVA, followed by Fisher's protected least significant difference (LSD) test. All values are presented as means ± SD. $P<0.05$ was considered statistically significant.

RESULTS

Distributional changes in SLRPs during retinal development: At E18 and P7, the GCL and IPL were distinguishable from other neuroblast cell mass. Decorin and fibromodulin were diffusely distributed throughout the retina (Fig. 1). The PRL had formed at P14, and the retina was in a mature state at P42. Decorin was detected diffusely in the retina with higher affinity confined to cell consistent with ganglion cells in the GCL. Fibromodulin was moderately distributed in the retina with higher affinity confined to astrocytes and ganglion cells in the GCL and cells in the inner side of INL; supposed to be amacrine cells, in addition to nerve fiber rich layers; nerve fiber layer and IPL. Biglycan showed a moderate distribution throughout the retina at E18 and P7 and with weak staining at P14 and P42.

Morphological analysis of KA-induced retinal damage model: Intravitreal injection of KA at three stages of retinal development (P14, P42 and P120) resulted in progressive damage and shrinkage of the retinas comparing to normal ones. The damage was confined to the inner retina. Moreover, these retinas were detached from the retinal pigment epithelium and there were deformities in retinal shape in many parts resulting in a tortuous shape. The results obtained from morphological studies of HE samples from these stages revealed that the effects of KA on retinas at P14, P42 and P120 were

almost similar (Table 2). Hence, the P42 mouse was used as a model of KA-induced injury to the retina and the effects on the expression and distribution of SLRPs was evaluated.

The inner retinal layers were seriously damaged in the experimental eyes after KA injection. The time course of morphological changes in the retinas showed that ganglion cell damage occurred at an early stage (1 day after injection) followed by shrinkage of the IPL and loss of retinal cells in the INL (3-7 days after injection), whereas the outer layers of the retina remained almost intact for 7 days (Fig. 2). In the normal retina, Pax6 was distributed diffusely from the GCL to ONL, and there was prominent staining of cells consistent with ganglion cells located in the GCL, and cells in the INL supposed to be amacrine cells and horizontal cells. With damage of the GCL 1 day and 3 days after KA injection, Pax6-immunopostive cells were confined to the INL. At 7 days Pax6 was distributed diffusely throughout the retina. DAPI staining result showed that progressive loss of cells occurred in the inner retinal layer of KA-injected eyes.

Distribution and expression of SLRPs of KA-induced retinal damage model: One day after KA injection, the distributions of decorin and fibromodulin showed the same pattern as that in the control retina but with stronger immunostaining levels, especially in the inner retinal layers (GCL,

IPL and INL). Three days after KA injection, when progressive loss of inner retinal cells was observed, strong immunoreactivity for decorin and fibromodulin was detected in inner retinal layers, and decorin was also detected diffusely in outer retinal layers. However, in retinas at the late stage (7 days after injection), when significant loss of inner retinal cells was observed, decorin and fibromodulin immunostaining was distributed throughout the retina including the PRL (Fig. 3A). Immunostaining for biglycan was weak in both the normal retina and the retina after KA injection. In addition to the immunohistochemical results, all retinas of normal and KA-injected eyes at 1, 3 and 7 days after injection expressed *decorin* and *fibromodulin* mRNA throughout the experimental periods. However, the expression level of *biglycan* mRNA was very low in retinas of both normal and KA-injected eyes (Fig. 3B).

DISCUSSION

Comparison and discussion on immunohistochemistry and RT-PCR of the prenatal, postnatal of normal retina to that with KA-induced injury in the postnatal mice would be beneficial for the more understanding in the kinetics and importance of these three PGs to the development and pathogenesis of the retina. PGs play pivotal roles in various cellular processes such as proliferation, migration, cell adhesion, and survival (Hocking et al., 1998). Their influence on neurite extension (Verna et al., 1989; Snow et al. 1990; Oohira et al., 1994) makes them potential candidates for interactions with growing axons during development and after injury of the mammalian central nervous system. PGs have been shown to play an important role in neurite outgrowth of retinal ganglion cells (Snow et al. 1991; Brittis and Silver, 1995). Moreover, altered expression of PGs has been reported in retinal degenerative diseases (Hewitt and Newsome, 1985; Porrello and La Vail, 1986; Newsome et al., 1987). Thus, in the present study, the expression of PGs was investigated, especially SLRPs, in developmental and in injured situations in an attempt to elucidate their roles.

The results showed that decorin and fibromodulin are distributed diffusely in the retina at the prenatal stage. The distribution of decorin and fibromodulin becomes more obvious in early postnatal to adult stages.

Astrocytes maintain the extracellular matrix in the lamina cribrosa consisting of collagens, elastic fibers and glycoproteins such as laminin and PGs. Astrocytes express a wide variety of growth factors and receptors, many of which serve as trophic and survival factors (Ullian et al., 2004). It is suggested that astrocytes serve the same function in the retina. A previous study showed that chondroitin sulfate PGs can be neurotrophic factors for retinal ganglion cells (Schultz et al., 1990), suggesting that decorin, a chondroitin/dermatan sulfate PG, may be one of the neurotrophic factors surrounding retinal ganglion cells and their neurite. Therefore, the distribution pattern of decorin and fibromodulin in the retina suggests that these PGs are related to the differentiation of retinal cells in addition to their survival. It was reported that SLRP-deficient mice have a compensatory deposition of other SLRPs from the respective SLRP class, i.e., more biglycan in cartilage and bone in decorin-deficient mice (Zhang et al., 2006). In this study, biglycan was weakly distributed in the mouse retina compared with the distribution of decorin. This finding suggests that a decorin-biglycan compensation system is also present in the retina.

The retina of KA-injected mice underwent shrinkage confined mainly to the inner retina, GCL and INL. It has been suggested that KA-induced shrinkage in the retina is accompanied by loss of the GCL and IPL (Honjo et

al., 2000). Results obtained from cultured retinal ganglion cell (RGCs) suggest that these cells are highly sensitive to glutamate induced excitotoxicity Interestingly, a recent study showed that RGCs, in the presence of neurotrophic factors, are relatively resistant to glutamate excitotoxicity (Taylor et al., 2003), whereas retinal amacrine cells were found to be highly sensitive to elevated glutamate levels. These investigators suggest that the decrease in the number of cells observed *in vivo* in the ganglion cell layer after intravitreal glutamate injections might have resulted from a loss of amacrine cells and from a lack of trophic support for RGCs (Zhong et al., 2007).

KA induces activation of retinal astrocytes and Müller cells, which reflects a metabolic change of the cells in response to degenerative changes of their neighboring neurons, which may give evidence of repair process in KA-induced damage (Chang et al., 2007). Previous studies have shown that *Pax6* mRNA was expressed during development of the mouse retina and in the adult mouse retina (Marquardt et al., 2001) and was upregulated during retinal regeneration (Insua et al., 2008). In the present study, Pax6 protein in the retinas of KA-injected eyes showed lower density than that in retinas of normal eyes, suggesting weak or no regeneration up to 7 days post-injection.

Decorin is known to be involved in the development of certain eye structures, namely the cornea and sclera, by modulating collagen fibrogenesis. Decorin expression decreased in response to ischemia in a rat model of oxygen-induced retinopathy (Park et al., 2010). Moreover, in transient retinal ischemia, expression level of decorin mRNA decreased in the early stage (6-28 h) and recovered to near-normal levels in the late stage at 4 days to 1 week (Inatani et al., 1999). Taken together, these findings suggest that decreased decorin reflects cellular damage and death and that recovery of the level of decorin may occur in response to a regenerative process and/or the secretion of numerous cytokines by inflammatory cells and activated retinal glial cells.

This report is the first report on distribution and expression of fibromodulin in the mammalian retina. In recent studies using fibromodulin-deficient mice, multiple areas of retinal detachment were observed (Chakravarti et al., 2003). These retinal detachments were attributed to the increased ocular axial length and biomechanical weakness due to collagen fibril-matrix defects in lumican-fibromodulin double-null mice (Bonilha et al., 2004). Therefore, fibromodulin in the injured retina might promote retinal adhesion to the retinal pigment epithelium and/or guide of axon in retinal cells. However, due to the limited study, it is difficult to make

conclusion regarding the significance of the distribution and upregulation of fibromodulin after retinal injury.

The distribution of decorin and fibromodulin after KA injection was confined mainly to the inner retinal layers, and RT-PCR results revealed that these SLRPs are important neurotrophic factors for retinal layers. However, results of previous studies have shown that infusion of human recombinant decorin core protein can promote axon growth across acute adult rat spinal cord injuries (Davies et al., 2004; Minor et al., 2008). Based on the immunohistochemical and RT-PCR results, biglycan may have a limited role in the retina.

In conclusion, the results suggested that dynamic changes in the distribution of these PGs occurred in the inner retinal layers, which were mainly damaged by KA. That is, decorin and fibromodulin might contribute to retinal damage and repair process, but biglycan would not. These findings provided new insights into the role of fibromodulin in the mammalian retina and the differential roles of decorin and biglycan during development and after retinal injury.

Table 1. Primers ued for RT-PCR

Gene (Genbank)	Size (bp)	Primer Sequence
Decorin (BC132521)	149	5'-GCC TTC CAG GGA CTG AAG AGT-3' 5'-AGT CCT TTC AGG CTG GGT GC-3'
Biglycan (L20276)	171	5'-TGT CAC ACC TAC CTT CAG TG-3' 5'-TTG AAG TCA TCC TTG CGA -3'
Fibromodulin (BC064779)	149	5'-GCT ACC AAC ACC TTC AAC TC-3' 5'-GTG CAG AAG CTG CTG ATG-3'
GAP DH (NM 008084)	193	5'-GAG AGG CCC TAT CCC AAC TC-3 ' 5'-GTG GGT GCA GCG AAC TTT AT-3'

Table 2. Retinal thickness after KA injection (μm)

	Day 0	Day 1	Day 3	Day 7
P14	871.3 ± 8.2^{a}	708.0 ± 18.5^{b}	645.3 ± 5.9^{c}	517.3 ± 31.2^{d}
P42	884.7 ± 34.5^{a}	646.0 ± 21.9^{b}	590.7 ± 9.8^{b}	483.3 ± 14.5^{c}
P120	805.7 ± 6.2^{a}	560.7 ± 5.2^{b}	504.3 ± 17.1^{c}	423.3 ± 10.7^{d}

Values represent mean ± S.D. Different letters indicate significant difference among the same age groups: $p<0.05$

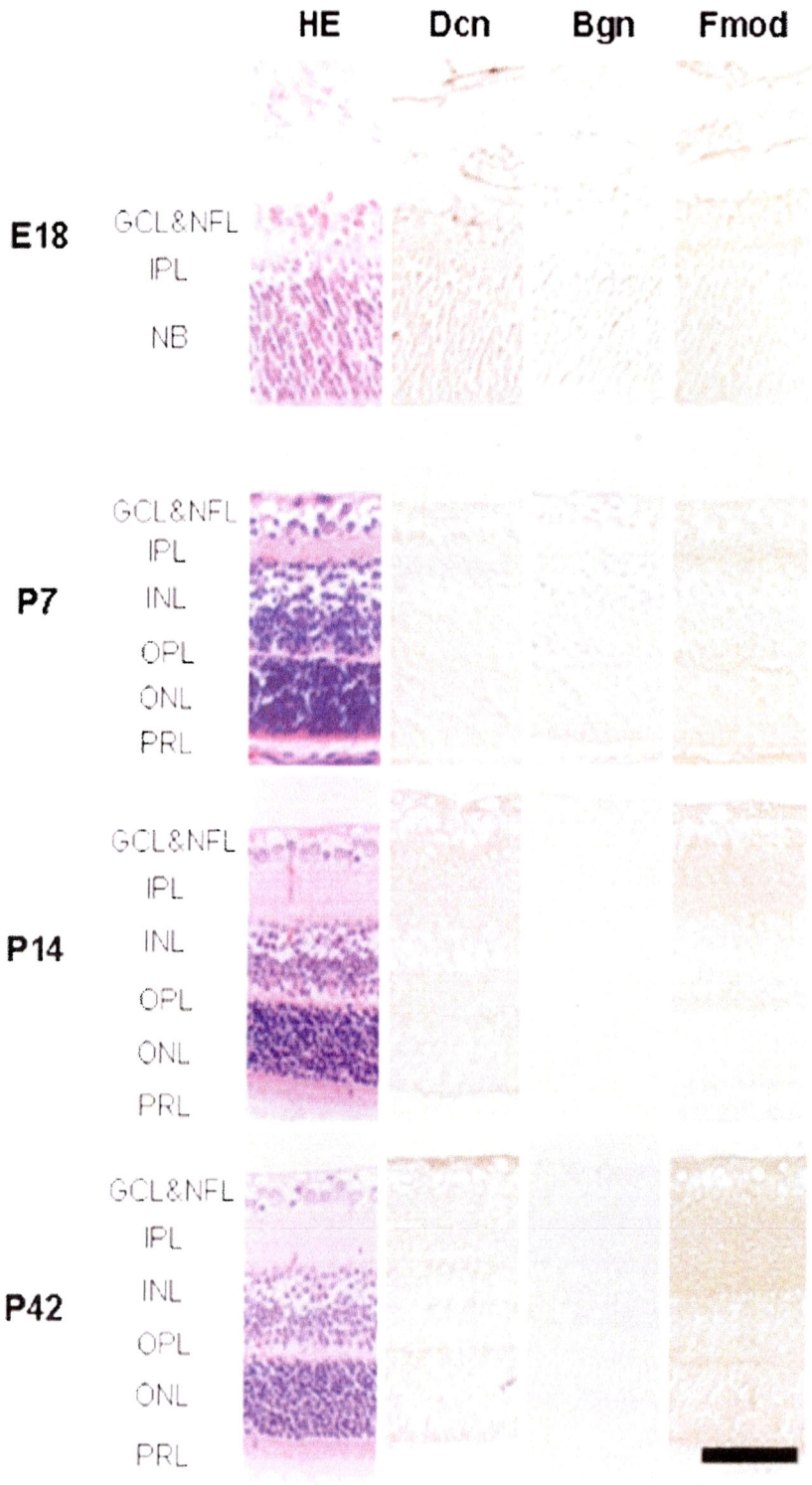

HE
Dcn
Bgn
Fmod
E18
GCL&NFL
IPL
NB
P7
GCL&NFL
IPL
INL
OPL
ONL
PRL
P14
GCL&NFL
IPL
INL
OPL
ONL
PRL
P42
GCL&NFL
IPL
INL
OPL
ONL
PRL

Figure 1

Fig. 1. HE staining and immunostaining of decorin, biglycan and fibromodulin in the retinas of mice at E18, P7, P14, and P42. HE, hematoxylin and eosin; Dcn, decorin; Bgn, biglycan; Fmod, fibromodulin; GCL&NFL, ganglion cell layer and nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; NB, neuroblast cell mass; PRL, photoreceptor layer. Scale bar = 50 μm.

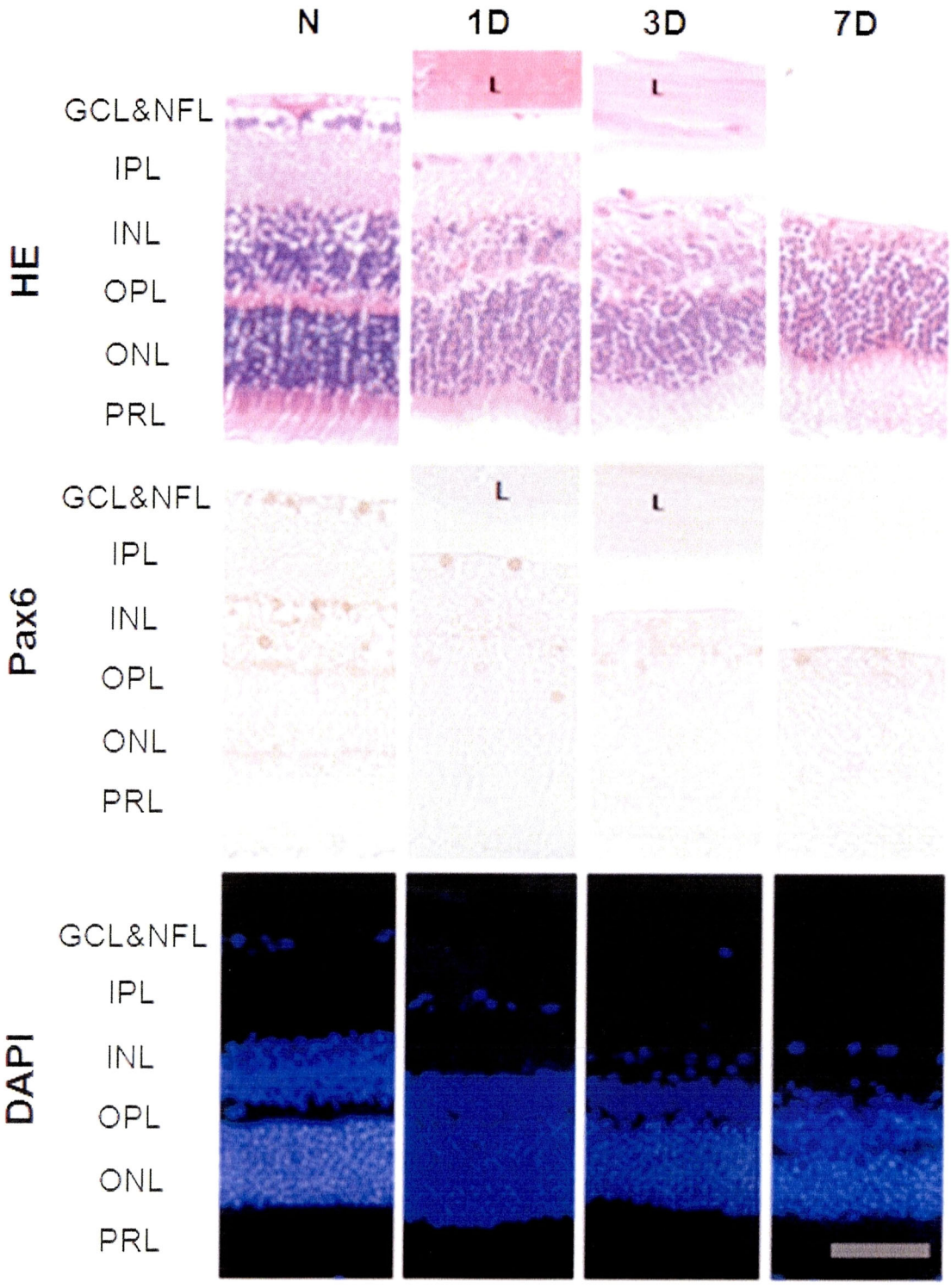
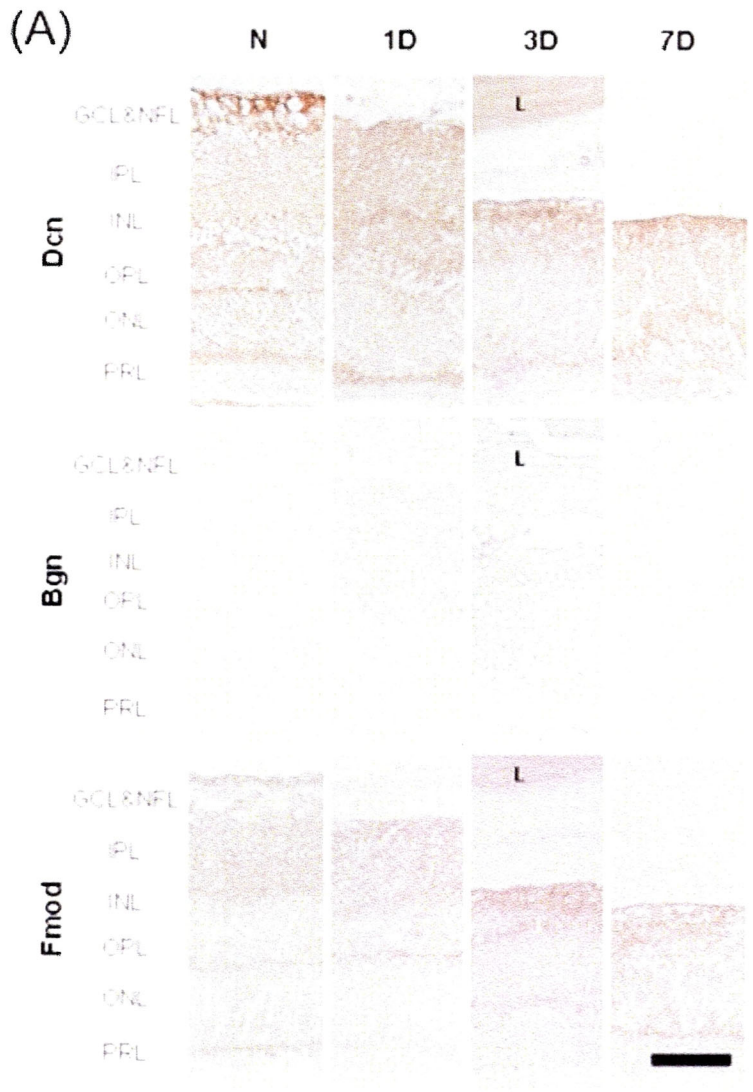


Figure 2

Fig. 2. HE staining, Pax6 immunostaining and DAPI staining of P42 mice retinas in the kainic acid (KA)-injured model. HE, hematoxylin and eosin; N, control; 1D, 3D and 7D: 1, 3 and 7 days after KA injection, respectively; L: lens; arrows: interface between vitreous body and retina. Scale bar = 50 μm.


(A)
N
1D
3D
7D
Dcn
Bgn
Fmod
GCL&NFL
IPL
INL
OPL
ONL
PRL
L

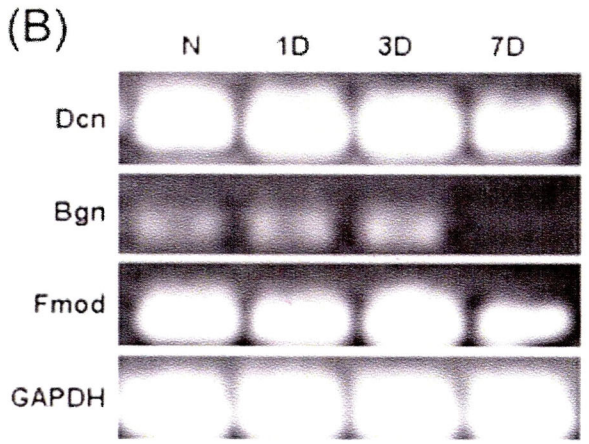

(B)
N
1D
3D
7D
Dcn
Bgn
Fmod
GAPDH

Figure 3

Fig. 3. Immunostaining (A) and RT-PCR (B) results for decorin (Dcn), biglycan (Bgn) and fibromodulin (Fmod) in the P42 mice retinas of kainic acid (KA)-injected model. N, control; 1D, 3D and 7D: 1, 3 and 7 days after KA injection, respectively; L: lens. Scale bar = 50 μm.

GENERAL CONCLUSION

Retinal matrix contains many proteoglycans which act as cement substance between the retinal cells, providing shape, biomechanical strength and implicated in the retinal development and act as neurotrophic factor for retinal cells.

In chapter I, the distribution of chonriotine-4-sulfate (C4S), chondriotin-6-sulfate (C6S), aggrecan and biglycan was estimated. I have therefore compared developmental changes and localization of C4S, C6S, biglycan and aggrecan in the mice eye from E12 up to P120. Developmental patterns for each of the proteoglycans and the changes in the distribution in the retina and optic nerve was demonstrated. Although the neurobiological significance of C4S, C6S, aggrecan and biglycan in the retina and optic nerve remains to be more clearly delineated; the obtained results indicate that the individual proteoglycans probably serve unique perhaps partially overlapping, functions in retina and optic nerve as well as the central nervous system (Popp et al., 2003). Moreover, distributions of chondroitin sulfate proteoglycans in the retinal pigment epithelium from late prenatal stage to adult stage, in addition to intense distribution of C6S in the photoreceptor layer, these results recommend that these proteoglycans have

important role not only for the retinal layers development but also for preventing retinal detachment.

In chapter II, the relationships of three small leucine-rich proteoglycans (SLRPs), decorin, biglycan and fibromodulin, to development of the retina were investigated through their immunohistochemical distributions in the developing and mature retina and in the retina with kainic acid (KA)-induced injury of ICR mouse. Furthermore, the gene expression of these proteoglycans was examined in the injured retina. Decorin and fibromodulin immunostaining was diffusely distributed at prenatal and early postnatal stages and was stronger in the adult retina with higher affinity to the inner retina. Decorin showed higher affinity to cells consistent with ganglion cell, fibromodulin was obviously stained astrocytes, ganglion cells and cells supposed to be amacrine cells in addition to nerve fiber rich layers. However bicylcan immunostaining was weaker in the adult retina comparing to decorin. Retinas of KA- injected eyes underwent shrinkage accompanied by serious damage in the inner layers. Moreover, these retinas were detached from the retinal pigment epithelium. Decorin and fibromodulin were upregulated in the inner retinal layers of KA-injected eyes compared to the normal ones.

In conclusion, the distributions of the proteoglycans during development of the retina suggest that they have roles in the retinal development and act as neurotrophic factor for retina cells. Moreover, the upregulations of decorin and fibromodulin after KA-injection suggest that they contribute to the retinal damage and repair process of adult mouse retina. In addition, these proteoglycans may be involved in preventing the detachment of the neural retinal from the retinal pigment epithelium.

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