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Localization and developmental expression patterns of CSPG-cs56 (aggrecan) in normal and dystrophic retinas in two rat strains

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ABSTRACT

Proteoglycans have a number of important functions in the central nervous system. Aggrecan (hyaluronan-binding proteoglycan, CSPG-cs56) is found in the extracellular matrix of cartilage as well as in the developing brain. We compared the postnatal distribution of CSPG-cs56 in Long Evans (LE) and Royal College of Surgeons (RCS) rat retinas to determine if this proteoglycan played a role in the development of dystrophic retinas. CSPG-cs56 expression was examined in rat retinas aged between birth (postnatal day 0, P0) and P150 using immunofluorescence and Western-blots. Immunofluorescence was quantified using ImageJ. GFAP staining was used to compare Müller cell labeling and the distribution of CSPG-cs56. Both rat strains showed a significant rise in total retinal CSPG-cs56 between P0 and P21; values peaked on P21 in LE rats and P14 in RCS rats. CSPG-cs56 then significantly decreased to lower levels (P35) in both strains before reaching significantly higher levels by P90–P150. CSPG-cs56 positive staining was present in the ganglion cell layer at birth and clear layering of the inner plexiform layer was seen between P7 and P21 due to dendritic staining of retinal ganglion cells. Staining was less intense and diffuse within the outer plexiform over a similar time-course. Light CSPG-cs56 labeling in the region of the outer segments was present at (P14) and became more intense as the retina approached maturity. CSPG-cs56 in the outer segments was the main contributor to the higher expression in older animals. Substantial differences in CSPG-cs56 labeling were not seen between LE and RCS rats. There was no evidence to suggest that Müller cells were the source of CSPG-cs56 in either rat strain, although their staining distributions had a degree of overlap. The lack of significant differences between LE and RCS rats indicates that CSPG-cs56 may not be involved in the degenerative process or the reorganization of the RCS rat retina. We suggest that the main role of CPSG-cs56 is to maintain retinal ganglion cell dendritic structure in the inner plexiform layer and is closely related to providing adequate support and flexibility for the photoreceptor outer segments, which is necessary to maintain their function.

Introduction

Proteoglycans consist of a group of proteins that covalently bind large carbohydrate glycosaminoglycans, which consist of four main sulfate groups: chondroitin, dermatan, keratan and heparan sulfate (for refs see Prydz and Dalen, 2000). Glycosaminoglycans play important regulatory roles by binding to several extracellular matrix (ECM) proteins and growth factors, and are active during cell adhesion, migration and differentiation (Carulli et al., 2006; Chacko et al., 2003; Daley et al., 2008; Esko et al., 2009; Inatani and Tanihara, 2002; Lazarus and Hageman, 1992; Leung et al., 2004). Glycosaminoglycans and oligosaccharide chains can also vary depending on the developmental stage of the tissue (Inatani and Tanihara, 2002). Proteoglycans are generally believed to be multifunctional because their core proteins consist of multiple domains that are structurally different and can interact with various biologically active molecules (Erlich et al., 2003; Esko et al., 2009; Prydz and Dalen, 2000). Proteoglycans may be secreted from cells and form part of the ECM or exist as transmembrane components. It is increasingly clear that these molecules have a more complex function than merely acting as a connective tissue cement or filler, and play a major role in neural network formation (Daley et al., 2008; Esko et al., 2009; Prydz and Dalen, 2000).

It was hoped that stem cell or photoreceptor precursor replacement therapy might restore partial vision in degenerative abnormalities such as retinitis pigmentosa (Ahmad, 2001; Chacko et al., 2003; Jones and Marc, 2005; Leveillard et al., 2007; MacLaren et al., 2006; Tian et al., 2009). However, transplantation has achieved only limited success to date (Huang et al., 2008; Liu et al., 2009a; Pinilla et al., 2007; West et al., 2010; Wojciechowski et al., 2002). One of the main reasons for this may be due to transplanted cells encountering migration barriers within the host retina that result in a low efficiency of cell integration. A recent report suggested that chondroitin sulfate proteoglycan (CSPG), an ECM protein, inhibited entry and axonal

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outgrowth of grafted neural cells into the host retina, although the mechanism(s) for this inhibition were unclear (Singhal et al., 2008). Reports detailing spinal cord injury models reported that CSPGs played a positive role in the immediate post-injury acute phase, but played a negative role during the later subacute phase (Jones et al., 2003b; Lemons et al., 2001). This suggests that the timing of cell transplantation may be a critical factor for neuronal repair after injury (Chacko et al., 2003; Cuenca et al., 2005; Jones and Marc, 2005) and that acute injury-induced cues play a significant role in promoting the migration and incorporation of ocular stem cells/progenitors in the retina (Chacko et al., 2003; Sullivan et al., 2003). This raises questions as to why dystrophic retinas, a model of chronic injury, do not appear to contain factors that promote acceptable levels of transplanted cell migration.

CSPGs have also been implicated in the formation of cortical and cerebellar perineuronal nets (Asher et al., 1995; Bignami et al., 1993b; Carulli et al., 2006; Zheng et al., 2008). One hypothesis suggests that the maturation of perineuronal nets occurs with the consolidation of cortical synaptic connections and leads to the end of the visual critical period (Zheng et al., 2008). The retina undergoes considerable reorganization between birth and the end of the first postnatal month. This period coincides with eye opening, retinal ganglion cell maturation, and functional rod/cone photoreceptors (Chalupa and Günhan, 2004; Johnson et al., 1999; Yamasaki and Ramoa, 1993; Yamasaki et al., 1999). Retinal ganglion cells (RGCs) also show a critical period after which dendritic reorganization is less likely to occur (Eysel et al., 1985). In Royal College of Surgeons (RCS) rats the degeneration of the photoreceptor layer leads to a considerable amount of synaptic reorganization within the plexiform layers (Cuenca et al., 2005; Jones and Marc, 2005; Jones et al., 2003a, 2011; Marc et al., 2008). The role that CSPGs play under these conditions has not been fully elaborated.

A large number of different CSPGs have been localized within the retina (Inatani and Tanihara, 2002; Inatani et al., 1999a, b; Koga et al., 2003). Aggrecan (hyaluronan-binding proteoglycan, CSPG-cs56) is a major structural proteoglycan found in the ECM of cartilage, but is also found in the developing and adult CNS (Asher et al., 1995; Bignami et al., 1989, 1993a,b; Lemons et al., 2001). It is the largest of four molecules within a family of proteoglycans that also consists of neurocan, brevican, and versican, all of which have been identified in the CNS (for refs see Asher et al., 1995; Bignami et al., 1993b; Inatani et al., 1999b, 2000; Inatani and Tanihara, 2002). Despite a number of similarities between these proteoglycans, it is likely that they have differential expression and distinct functions in the CNS (Bignami et al., 1993b; Inatani and Tanihara, 2002; Koga et al., 2003; Lemons et al., 2001; Prydz and Dalen, 2000). The localization and developmental expression patterns of CSPGs in the RCS rat retina are poorly represented in the literature. We asked whether the retinal distribution of CSPG-cs56 in Long Evans (LE) rats showed developmental differences when compared to dystrophic RCS rats, which might help to explain the function of this CSPG within the retina. The results were examined in relation to possible CSPG-cs56 involvement in perineuronal nets, retinal 'rewiring' in the RCS rat retina or the creation of a physical or chemical barrier to transplanted cell migration.

Materials and methods

All procedures were approved and monitored by the Third Military Medical University Animal Care and Use Committee. Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animals and tissue preparation

LE and RCS rats at postnatal day 0 (P0), P7, P14, P21, P30, P35, P60, P90, and P150 were supplied by the Third Military Medical University Animal Care facilities. To ensure uniformity across different ages, all retinal tissues were fixed using the same protocol (Zhang et al., 2010) and retinas of different ages were processed in parallel to ensure uniformity in the immunofluorescent labeling procedures. Rats were sacrificed with a lethal dose of pentobarbital, and the eyeballs enucleated. The cornea was perforated with a needle at the corneoscleral junction, and the eye then immersion fixed in 4% (w/v) paraformaldehyde in 0.1 M PB (pH 7.4) for 2 h at 4 °C. After rinsing in 0.1 M PB and removing the cornea, iris and vitreous humor, the eye cups were cryoprotected in 30% sucrose PB at 4 °C overnight, then embedded in optimum cutting temperature (OCT) compound, and flash frozen above liquid nitrogen. Vertical sections, 10–12 μm thick, were cut on a cryostat, mounted on coated slides and then stored at −20 °C until further processing.

Immunofluorescence

The mouse monoclonal anti-chondroitin sulfate clone, CSPG-cs56 (Sigma, St Louis, Missouri, USA), was used and is immunospecific for chondroitin sulfate containing proteoglycans (determined by indirect immunofluorescent labeling) and can be used to study the distribution of CSPG and its relationship to specific cell–substrate contacts. The rabbit polyclonal anti-glial fibrillary acidic protein, GFAP (Abcam, Cambridge, MA) was used to detect Müller cells. Detection of the primary antibody to CSPG-cs56 was done with a goat anti-mouse IgG conjugated to indocarbocyanine (Cy3, Molecular Probes, Eugene, Oregon) and a donkey anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC; Molecular Probes, Eugene, Oregon) was used as the secondary antibody to GFAP.

Retinal sections were treated with 0.5% Triton X-100 in PB for 5 min to improve membrane permeability, rinsed three times (5 min each) in 0.01 M PBS (pH 7.4), blocked with a solution containing 2% normal goat serum for 30 min at 37 °C, and then incubated with the primary antibody in blocking solution overnight at 4 °C. After incubation, the sections were rinsed as before, then incubated in blocking solution containing the secondary antibody for 2 h at room temperature (RT), and washed in PB (as above). The sections were counterstained with 4, 6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, China) for 5 min at room temperature, washed, and then coverslipped with 75% glycerol. Double-labeling experiments were done using a mixture of primary antibodies against CSPG-cs56 and GFAP (as above) followed by a mixture of the appropriate secondary antibodies. Negative controls were performed for every set of experiments by omitting the primary antibody. Primary and secondary antibodies were diluted in blocking solution at the following concentrations: anti-CSPG-cs56 (1:200), anti-GFAP (1:1000), Cy3 (1:1000), and FITC (1:500).

Western blots

Rat retinas were frozen in liquid nitrogen then ground to a powder (n = 5 animals/age group), and dissolved in radioimmunoprecipitation assay (RIPA) lysate (Beyotime Biotechnology, China). The solution was then differentially centrifuged at 15 000 *g*, at 4 °C for 5 min. The protein concentration of the supernatant was determined using a Micro-BCA kit (Pierce, Rockford, IL). The supernatant containing the total protein was diluted to 4 mg/ml, denatured with a loading buffer containing 10% sodium dodecyl sulfate (SDS, Beyotime Biotechnology, China) and 0.5 M Tris buffer, and subsequently sub-packaged and stored at −20 °C.

The samples (30 mg protein/sample) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using a 12% separation gel system. Gels were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) in a transfer buffer (192 mM glycine, 25 mM Tris-Cl, pH 8.3, and 15% methanol) for 2 h at 50 V using a BioRad Trans-Blot tank apparatus