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A CHONDROITIN SULPHATE PROTEOGLYCAN FROM HUMAN CULTURED GLIAL CELLS AGGREGATES WITH HYALURONIC ACID

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SUMMARY: Media harvested from cultures of glial cells grown in the presence of ^{35}S-sulphate were shown to contain ^{35}S-labelled proteoglycans. One of the components was a chondroitin sulphate proteoglycan that had an apparent monomer size similar to that of cartilage-derived chondroitin sulphate proteoglycan. The glial proteoglycan formed aggregates in the presence of hyaluronic acid; aggregation was abolished in the presence of deca- to tetradecasaccharides derived from hyaluronic acid or by previous reduction and alkylation of the proteoglycan. It is concluded that the ability to produce large chondroitin sulphate proteoglycan molecules capable of binding to hyaluronic acid is not confined to cartilage cells.

INTRODUCTION

Brain extracts contain chondroitin sulphate in the form of proteoglycan (1,2). Human glial cells in culture retain an ability to produce sulphated glycosaminoglycans, including chondroitin sulphate, most of which are secreted to the extracellular medium (3). The present experiments were undertaken to examine the presence of chondroitin sulphate proteoglycan in glial cell secretions and to study some basic properties of the glial product in relation to those of cartilage proteoglycan. The results show that a big monomer size and ability to bind hyaluronic acid is shared by the two types of chondroitin sulphate proteoglycan and thus may not be a cartilage specific feature.

MATERIALS AND METHODS

MATERIALS

Carrier-free ^{35}S-sulphate was obtained from the Radiochemical Centre, Amersham, U.K. Sepharose CL-2B, Sepharose CL-4B, Sephadex G-50 and Sephadex

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G-25 were from Pharmacia Fine Chemicals, Uppsala, Sweden. Guanidinium hydrochloride, obtained from Fluka AG, Buchs SG, Switzerland, was purified by treatment with active coal and filtration through glass filter. The absorbance of the resulting guanidinium hydrochloride solution was less than 0.1 at 280 nm and less than 0.2 at 260 nm, at a concentration of 4 M. Dithiothreitol and iodoacetamide were from Sigma Chemical Co., St. Louis, MO., USA. Bacterial chondroitinase AC (E.C. 4.2.2.5) was purchased from Miles Laboratories Inc., Elkhart, IN., USA. Testicular hyaluronidase (E.C. 3.2.1.35) was obtained from AB Leo, Helsingborg, Sweden. Papain (E.C. 3.4.4.10) was obtained in crude form from Sigma Chemical Co. and purified by the procedure of Kimmel and Smith (4). Carrier chondroitin sulphate proteoglycan (A1-D1, prepared from rat chondrosarcoma (5)) was a generous gift from Dr. J. Kimura, National Institute of Health, Bethesda, MD., USA. This preparation under appropriate conditions formed aggregates with hyaluronic acid (5). The appearance of excluded material on Sepharose CL-2B without addition of hyaluronic acid indicated that the A1-D1 also contained small amounts of hyaluronic acid. Hyaluronic acid and ^{3}H-hyaluronic acid were available in our laboratory (6;3).

METHODS

Cell culture and labelling conditions. The normal human glial cell line U-787 CG was kept under conditions described elsewhere (3). Dense cultures of glial cells were incubated with ^{35}S-sulphate in the presence of Ham's F-10 medium, having a reduced concentration of inorganic sulphate, and 10% calf serum (3). In one experiment calf serum was left out from the incubation medium. The culture media were harvested after 48 h and concentrated (approximately 10 times) under reduced pressure.

Radioactivity was measured in a Packard Model 2002 liquid scintillation counter using Insta-Gel as the scintillation medium.

Uronic acid was determined by the method of Bitter and Muir (7).

Reduction and alkylation was performed in the presence of 4 M guanidinium hydrochloride, 1 M tris-HCl pH 8.0. Dithiothreitol was added to a final concentration of 10 mM and the mixture was left at 37° for 4 hours; then iodoacetamide was added to a final concentration of 40 mM and incubation was continued at room temperature for 6 hours, with end over end mixing. After reduction and alkylation the A1-D1 proteoglycan carrier lost its ability to form high molecular weight complexes with ^{3}H-labelled hyaluronic acid, as demonstrated by Sepharose CL-2B chromatography.

Alkali degradation was carried out by treatment with 0.5 M NaOH, 0.5 M $NaBH_4$ over night at 4°C (8). Samples were then neutralized by the addition of HCl.

Chondroitinase AC digestion was carried out according to Yamagata et al. (9) after removal of guanidinium hydrochloride from test samples by dialysis against 0.5 M acetate, pH 7.0.

Gel chromatography. Sephadex G-50 chromatography was carried out on a 1 cm x 140 cm column, equilibrated with 1 M NaCl. Sepharose CL-4B chromatography was performed on a 1 cm x 140 cm column, equilibrated with 4 M guanidinium hydrochloride. Sepharose CL-2B chromatography was either on a 1 cm x 85 cm column equilibrated with 4 M guanidinium hydrochloride or a 1 cm x 80 cm column equilibrated with 0.5 M acetate, pH 7.0.

Hyaluronic acid products. High molecular weight hyaluronic acid was prepared by chromatography of hyaluronic acid (14 mg) on Sepharose CL-4B in 4 M guanidinium hydrochloride; the excluded portion was pooled and dialysed against 0.5 M acetate, pH 7.0. This preparation was used throughout the present investigation. - Hyaluronic acid oligosaccharides were prepared by partial digestion of hyaluronic acid with testicular hyaluronidase followed by chromatography on Sephadex G-50 (10) in 1 M NaCl. The material migrating with a K_{av} of 0.33-0.46, consisting of 5 to 7 disaccharide repeat units (HA_{10-14}) was pooled, desalted on Sephadex G-25 and lyophilized.

Aggregation assay. Hyaluronic acid was added to test samples (^{35}S-labelled proteoglycan) under dissociative conditions (4 M guanidinium hydrochloride) to give a weight ratio of added hyaluronic acid to proteoglycan carrier of about 1:100 (as uronic acid)(11). The mixture was then transferred to associative conditions by dialysis against 0.5 M sodium acetate, pH 7.0. The formation of high molecular weight complexes was monitored by chromatography on Sepharose 2B in 0.5 M acetate, pH 7.0 (12). In some experiments hyaluronic acid oligosaccharides (HA_{10-14}) were included; they were added under associative conditions (0.5 M acetate, pH 7.0) at a final concentration (as uronic acid) of 100 times that of hyaluronic acid.

RESULTS

Cultures of human glial cells secrete a mixture of ^{35}S-labelled polysaccharides in the presence of ^{35}S-sulphate (3). Concentrated medium from such cultures was chromatographed on Sepharose CL-4B in 4 M guanidinium hydrochloride, in the presence of carrier proteoglycan (reduced and alkylated A1-D1 proteoglycan from rat chondrosarcoma (5)(Fig. 1)). The high molecular weight material separated into two peaks of radioactivity, one migrating with the void volume (I) and one partially retarded (II). In the absence of carrier proteoglycan a similar pattern was observed; however the yield was lower and less reproducible. Alkali treatment converted peak I and II to one more retarded peak of radioactivity, migrating like free polysaccharide chains (Fig. 2). A similar result was obtained after digestion with papain (not shown). These findings indicate that the ^{35}S-labelled polysaccharides were in the form of proteoglycans. Treatment of peak I material with chondroitinase AC converted the main part to low molecular weight products, chromatographing like disaccharides on Sephadex G-50 (Fig. 3). It was concluded that peak I consisted mainly of chondroitin sulphate proteoglycan. (The chondroitinase-resistant portion of peak I was essentially heparan sulphate proteoglycan). Chromatography of peak I on Sepharose CL-2B in 4 M guanidinium hydrochloride indicated that the monomer size of the chondroitin sulphate proteoglycan (K_{av} 0.28) was similar to that of authentic, cartilage-derived chondroitin sulphate proteoglycan (11,13)(Fig. 4).

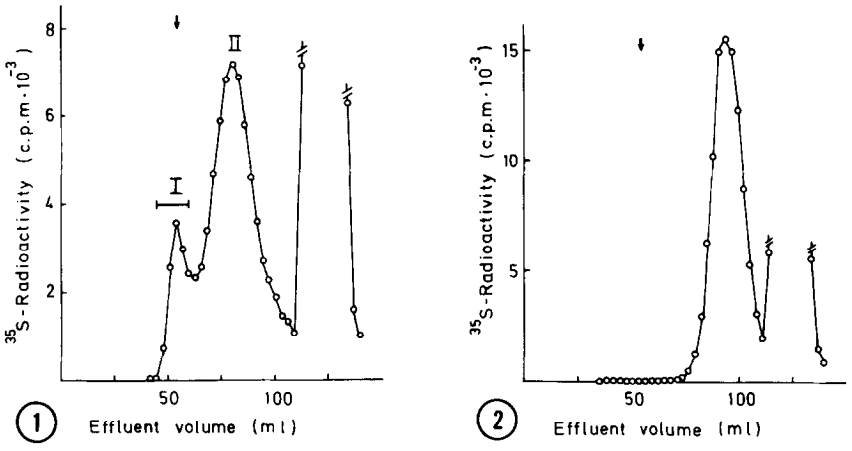


Fig. 1. Chromatography on Sepharose Cl-4B in 4 M guanidinium hydrochloride of medium, obtained from glial cell cultures after incubation with ^{35}S-sulphate. The concentrated medium (1.5 ml) was mixed with reduced and alkylated proteoglycan carrier (2 mg) before application to the column (1 cm x 140 cm). Effluent fractions were analysed by liquid scintillation counting. The void volume of the column is indicated by an arrow. Peak I was pooled as shown by the bar.

Fig. 2. Chromatography on Sepharose CL-4B in 4 M guanidinium hydrochloride of alkali-treated medium from ^{35}S-labelled glial cell cultures. Concentrated medium (0.1 ml) mixed with 0.13 mg of carrier proteoglycan was treated with NaOH as described in Materials and Methods and applied to the column (1 cm x 140 cm). Effluent fractions were analysed by liquid scintillation counting. The void volume of the column is indicated by an arrow.

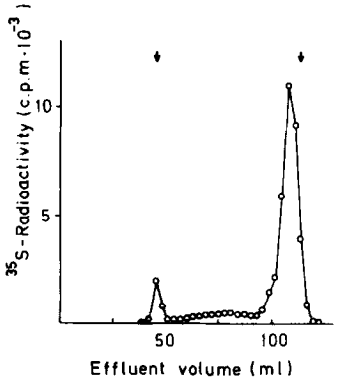
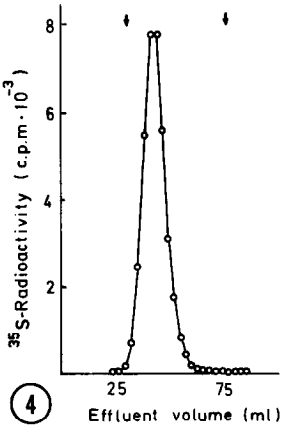


Fig. 3. Chromatography on Sephadex G-50 in 1 M NaCl of ^{35}S-labelled glial chondroitin sulphate proteoglycan (approximately 1.0 x 10^5 cpm of peak I; Fig. 1) after digestion with chondroitinase AC. Effluent fractions were analysed by liquid scintillation counting. The void volume and the total volume of the column are indicated by arrows.



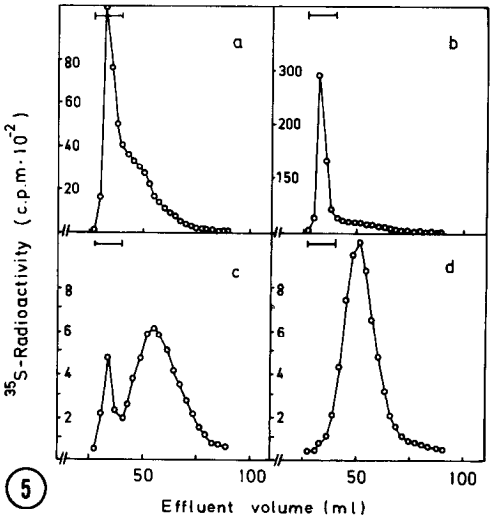


Fig. 4. Chromatography on Sepharose CL-2B (1 cm x 85 cm) in 4 M guanidinium hydrochloride of ^{35}S-labelled glial chondroitin sulphate proteoglycan (approx. 1.0 x 10^5 cpm of peak I; Fig. 1). Effluent fractions were analysed by liquid scintillation counting. The void volume and the total volume of the column are indicated by arrows.

Fig. 5. Chromatography on Sepharose CL-2B (1 cm x 80 cm) in 0.5 M sodium acetate pH 7.0, of ^{35}S-labelled glial chondroitin sulphate proteoglycan (approximately 1.0 x 10^5 cpm (a and b) or 1.0 x 10^4 cpm (c and d) of peak I; Fig. 1) without addition of extra hyaluronic acid (a); after the addition of hyaluronic acid (b); after the addition of hyaluronic acid plus and excess of hyaluronic acid oligosaccharides, HA_{10-14} (c); after the addition of hyaluronic acid to a preparation of reduced and alkylated ^{35}S-proteoglycan (d). Effluent fractions were analysed by liquid scintillation counting. The fractions represented by bars were taken as the void volume material.

Combination of peak I material with hyaluronic acid led to an increase in the apparent size of the labelled material as judged by chromatography on Sepharose CL-2B in 0.5 M acetate buffer (Fig. 5). About 77% of the radioactivity migrated with the void volume of the column, as compared to 55% in the control, to which no extra hyaluronic acid was added. It was concluded that the chondroitin sulphate proteoglycan was converted into a more high molecular weight form by interaction with hyaluronic acid. The reason why some chondroitin sulphate proteoglycan appeared in the excluded fraction in the control experiment is probably that the carrier proteoglycan contained small amounts of hyaluronic acid (J. Kimura, personal communication). Moreover hyaluronic acid is produced within the glial cell cultures (a few per

cent of total glycosaminoglycan production)(3). This may also contribute to the displacement of chondroitin sulphate proteoglycan to the void volume.

In contrast, ^{35}S-labelled material was not excluded from Sepharose CL-2B at 60°, in the presence or absence of hyaluronic acid. This finding agrees with the inability of chondroitin sulphate proteoglycan to form aggregates with hyaluronic acid at elevated temperatures and would argue against the possibility that the apparent high molecular weight of glial chondroitin sulphate proteoglycan was due to self-association (14). The reduced and alkylated carrier chondroitin sulphate proteoglycan was incapable of forming high molecular complexes with hyaluronic acid (see Materials and Methods). Further, ^{35}S-chondroitin sulphate proteoglycan produced under serum free conditions yielded a similar result as that shown in Fig. 5a and b. Therefore, self-aggregation between carrier proteoglycan or serum-derived proteoglycan and glial chondroitin sulphate proteoglycan could not explain the changed elution of the latter in the presence of hyaluronic acid.

In the presence of an excess of hyaluronic acid oligosaccharides (HA_{10-14}; approximately 100 times the amount of added hyaluronic acid) only a minor V_0 peak was demonstrable on Sepharose CL-2B chromatography; the main part of the ^{35}S-radioactivity was transferred into the included volume (Fig. 5c). This may indicate that hyaluronic acid oligosaccharides compete with polymeric hyaluronic acid for interaction with glial chondroitin sulphate proteoglycan. Reduced and alkylated ^{35}S-chondroitin sulphate proteoglycan failed to form high molecular weight radioactive components (V_0 material) in the presence of hyaluronic acid (Fig. 5d). These properties of glial chondroitin sulphate proteoglycan are shared by the analogous proteoglycan from cartilage.

DISCUSSION

Human glial cells in culture synthesized chondroitin sulphate proteoglycan with a monomer size equivalent to that of cartilage derived chondroitin sulphate proteoglycan. In further agreement with cartilage chondroitin sulphate proteoglycan the glia derived product bound to hyaluronic acid; the binding was inhibited by an excess of hyaluronic acid oligosaccharides and abolished by reduction and alkylation of the proteoglycan. These properties are previously unknown for proteoglycans of noncartilaginous cells. Rather it has been suggested that a big monomer size of chondroitin sulphate proteoglycan should be considered as a cartilage specific feature,

contrasting to the comparatively low molecular weight of the 'ubiquitous' type of proteoglycan, displayed by other tissues (15). Further the ability to form aggregates with hyaluronic acid has been thought to be confined to chondroitin sulphate proteoglycan of cartilage only (17). These ideas may be questioned by the present results. Other aspects on the binding of glial proteoglycan to hyaluronic acid, such as minimal chain length of hyaluronic acid for binding, or involvement of link in the formation of aggregates need, however, to be examined to permit further comparison with the established model for chondroitin sulphate proteoglycan - hyaluronic acid interaction.

Since cultured glial cell cultures produce both chondroitin sulphate proteoglycan and hyaluronic acid the formation of aggregates may be expected to take place within the cultures. In fact, chromatography on Sepharose 2B in 0.5 M guanidinium hydrochloride of concentrated medium from glial cultures after labelling with ^{35}S-sulphate revealed significant amounts of high molecular weight material, migrating with the void volume of the column (not shown). This material was dissociated in 4 M guanidinium hydrochloride and may represent endogeneous chondroitin sulphate proteoglycan - hyaluronic acid aggregates. Chondroitin sulphate proteoglycan is present in brain tissue but it is not known whether it can aggregate with hyaluronic acid. If glial cells secrete aggregating proteoglycan *in vivo* as well as *in vitro* this may influence the extracellular space of brain; recognition of hyaluronic acid by chondroitin sulphate proteoglycan may have importance for the organization of the extracellular matrix and may mediate contacts between the cells and their environment.

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