PARTIAL CHARACTERIZATION OF A HEPARAN SULFATE PROTEOGLYCAN RELEASED FROM PRECHONDROGENIC RAT LIMB BUDS BY HEPARIN

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ABSTRACT

Prechondrogenic rat limb buds contain two different molecular species of heparan sulfate proteoglycan, a smaller one (HSPG I-A) and a larger one (HSPG III-A). Although a part of HSPG I-A was solubilized with phosphate-buffered saline (PBS) from the limb bud, HSPG III-A and a large part of HSPG I-A were resistant to extraction with the same solution. To examine whether these PBS-insoluble proteoglycans exist in a different way in the limb bud, proteoglycans were extracted from the PBS-insoluble fraction of the limb bud, which had been labeled with [³⁵S]sulfate, successively with the following solutions in the presence of protease inhibitors; heparin (100 µg/ml) in PBS, 0.8% nonanoyl-*N*-methyl-glucamide (MEGA-9) in PBS, and 4 M guanidine HCl in 50 mM Tris-HCl, pH 7.5. About 40% of [³⁵S]labeled material in the PBS-insoluble fraction were solubilized with the heparin solution. About 25%, 30% and 5% were recovered in MEGA-9 extract, guanidine HCl extract, and the extracted residue. Heparin extract and MEGA-9 extract contained a heparan sulfate proteoglycan with structural parameters, such as overall size, electric charge and length of heparan sulfate side chains, almost identical to HSPG I-A. On the other hand, guanidine HCl extract contained another heparan sulfate proteoglycan structurally similar to HSPG III-A, in addition to HSPG I-A. These results suggest that HSPG I-A and HSPG III-A exist in a different way in the limb bud, and that about a half of HSPG I-A may be associated with tissue via heparin-like domain in the heparan sulfate side chains.

Heparan sulfate proteoglycans are located at the cell surface, basement membrane and nucleus in various tissues, and are supposed to be involved in various cellular events including cell adhesion and cell proliferation (for reviews, see references 2 and 14).

We previously reported that prechondrogenic rat limb buds synthesized three distinct types of proteoglycans; a chondroitin sulfate proteoglycan called PG-M and two types of heparan sulfate proteoglycan, a smaller one (HSPG I-A) and a larger one (HSPG III-A), and that HSPG I-A and HSPG

III-A were different from each other in the overall size and in the structure of heparan sulfate side chains (11). We also demonstrated that PG-M was easily solubilized with a physiological salt solution such as phosphate-buffered saline (PBS), while HSPG I-A was partially (about 30%) solubilized with PBS. HSPG III-A was virtually insoluble in PBS (11).

The functions of these proteoglycans are unknown. However, there is a possibility that heparan sulfate proteoglycans are involved in chondrogenesis of predifferentiated mesenchymal cells. For example, San Antonio *et al.* have reported that exogenous heparan sulfate or low concentration (0.5–1 µg/ml) of heparin stimulated chondrogenesis of chick limb bud mesenchyme in micromass culture,

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Abbreviations: MEGA-9, nonanoyl-*N*-methyl-glucamide; PBS, phosphate-buffered saline

although higher concentration ($\geq 50 \mu\text{g/ml}$) of heparin decreased [^{35}S]sulfate incorporation into the micromass cultures (15). They speculated that the binding of heparan sulfate proteoglycans to specific cell surface receptors via heparan sulfate chain could be an important step in chondrogenesis. To examine this possibility, existence of heparan sulfate or heparan sulfate proteoglycan which is associated with the cell surface via heparan sulfate chain must be demonstrated in limb bud tissue.

In the present study, we demonstrate that about a half of PBS-insoluble HSPG I-A, synthesized by rat limb buds, can be solubilized with PBS containing heparin. This population of HSPG I-A may be associated to the cell surface via heparan sulfate chain.

MATERIALS AND METHODS

Materials

Sprague-Dawley female rats (Slc:SD strain; SLC, Shizuoka) were mated with males of the same strain as described previously (10). The day when a vaginal plug was observed was considered to be day zero of gestation.

Heparin was obtained from Fluka AG (Buchs SG, Switzerland). Commercial glycosaminoglycans (chondroitin 4-sulfate, chondroitin 6-sulfate and heparan sulfate) were purchased from Seikagaku Kogyo, Tokyo. Nonanoyl-*N*-methyl-glucamide (MEGA-9) was obtained from Wako Pure Chemical Industries, Osaka. Carrier-free $\text{Na}_2[^{35}\text{S}]\text{O}_4$ was purchased from Japan Radioisotope Association, Tokyo.

Differential Extraction of Proteoglycans

Limb buds dissected from rat embryos on day 13 of gestation were labeled with $\text{Na}_2[^{35}\text{S}]\text{O}_4$ and homogenized 3 times in PBS containing 20 mM EDTA, 10 mM *N*-ethylmaleimide, 0.1 M 6-aminoheptanoic acid, 0.05 M benzamidine hydrochloride and 2 mM phenylmethylsulfonyl fluoride as described before (11). About 40 embryos were used for one preparation. The residue of PBS extraction was homogenized in 10 μl (per limb) of ice-cold PBS containing 100 $\mu\text{g/ml}$ of heparin and the protease inhibitors (heparin-PBS). The homogenate was stirred for 1 h at 4°C and then centrifuged at 27,000 *g* for 1 h at 4°C. The pellet was rehomogenized two more times in heparin-PBS. The residue of the heparin extract was then treated with 10 μl (per limb) of

PBS containing 0.8% MEGA-9 and the protease inhibitors (MEGA-9-PBS). After 3 times of homogenization in MEGA-9-PBS, insoluble materials were homogenized 3 times in 10 μl (per limb) of 4 M guanidine HCl-50 mM Tris-HCl, pH 7.5, containing the protease inhibitors. The final insoluble material was removed by centrifugation. To estimate the amount of [^{35}S]sulfated glycosaminoglycans recovered in each extract (1st-3rd heparin extracts, 1st-3rd MEGA-9 extracts and 1st-3rd guanidine-HCl extracts), [^{35}S]labeled materials were precipitated by adding 3 vol of 95% ethanol containing 1.3% potassium acetate to an aliquot of each extract. The precipitate was digested with Pronase-P (Kakenkagaku, Tokyo) in 50 mM Tris-HCl, pH 7.5, containing 3% ethanol at 50°C overnight. The digest was treated sequentially with 0.2 N NaOH, 5% trichloroacetic acid, 75% ethanol containing 1% potassium acetate to prepare [^{35}S]labeled glycosaminoglycans as described previously (13). [^{35}S]Sulfated glycosaminoglycans were also prepared from the final insoluble residue by pronase digestion (13).

Extraction of [^{35}S]Labeled Proteoglycans with Exogenous Glycosaminoglycans

In some experiments, the residue of PBS extract was suspended in 10 μl (per limb) of PBS containing the protease inhibitors by homogenization. To an aliquot of this suspension, heparin, heparan sulfate or chondroitin sulfates (a mixture of the same amounts of chondroitin 4-sulfate and chondroitin 6-sulfate) was added to a concentration of 100 $\mu\text{g/ml}$. The suspensions were stirred overnight at 4°C and centrifuged at 27,000 *g* for 1 h at 4°C. [^{35}S]Sulfated glycosaminoglycans were prepared from the supernatant as described above, and their radioactivities were measured. To estimate the composition of labeled glycosaminoglycans, glycosaminoglycans were separated by two-dimensional electrophoresis on cellulose acetate film (Sepraphore III, Gelman Sciences, Ann Arbor) by the method of Hata and Nagai (3). Each radioactive spot of glycosaminoglycan on cellulose acetate was cut out, and the radioactivity was measured with a liquid-scintillation counter.

Sepharose CL-4B Column Chromatography

The first to the third extracts with heparin-PBS, MEGA-9-PBS or guanidine-HCl were combined

Fig. 1 The amount of [^{35}S]sulfated glycosaminoglycans extracted with each solution. Limb buds, which had been labeled with 7.4 MBq/ml of $\text{Na}_2[^{35}\text{S}]\text{O}_4$ for 3 h, were treated successively with heparin-PBS (a), with MEGA-9-PBS (b), and with guanidine HCl (Gu-HCl) (c). Each extract and final insoluble material were treated with pronase and alkali to prepare [^{35}S]labeled glycosaminoglycans, and the radioactivities of the glycosaminoglycans were measured.

and concentrated to 500 μl on a Diaflo YM-10 membrane. Each extract was chromatographed on a column (1.0 $\times$ 48 cm) of Sepharose CL-4B (Pharmacia, Uppsala) equilibrated with 2 M guanidine HCl-50 mM Tris-HCl, pH 7.5, containing 0.4% MEGA-9, 2 mM EDTA, 1 mM *N*-ethylmaleimide and 0.2 mM phenylmethylsulfonyl fluoride at a rate of 6 ml/h. Peak fractions eluted from Sepharose CL-4B column were pooled separately, and the sulfated materials were precipitated from aliquots of the pooled fractions with ethanol. [^{35}S]Sulfated glycosaminoglycans were prepared from the precipitates as described above. Composition of labeled glycosaminoglycans was determined by the method described above after separation of glycosaminoglycans by two-dimensional electrophoresis on cellulose acetate film. In some experiments, peak fractions eluted from Sepharose CL-4B column were chromatographed separately on a column (1.0 $\times$ 48 cm) of Sepharose CL-6B before and after sequential treatments with pronase and alkali or after nitrous acid treatment as described previously (11). Treatment with nitrous acid was done under the conditions described by Lindahl *et al.* (8).

Ultracentrifugation on a CsCl Density Gradient
Ultracentrifugation on a CsCl density gradient was

Fig. 2 The amount and composition of [^{35}S]sulfated glycosaminoglycans released either by heparin or by PBS without any glycosaminoglycans. [^{35}S]Labeled glycosaminoglycans were separated by two-dimensional electrophoresis on cellulose acetate film, and radioactivity of each glycosaminoglycan spot was measured. The hatched area represents the amount of heparan sulfate, the open area the amount of chondroitin sulfate, and the closed area the amount of other glycosaminoglycans, mostly dermatan sulfate.

Fig. 3 Sepharose CL-4B column chromatography of the [^{35}S]labeled materials in heparin extract (a), MEGA-9 extract (b) and guanidine HCl extract (c) of the limb buds of day-13 fetal rats. Fractions of 0.5 ml were collected. Abscissa, tube number; ordinate, arbitrary unit of ^{35}S -radioactivity; V_0 , void volume; V_t , total volume. The solid horizontal bars indicate fractions which were pooled separately for glycosaminoglycan analysis. The circular graph above each bar indicates the composition of [^{35}S]sulfate-labeled glycosaminoglycans in each pooled fraction. The hatched area represents the percentage of heparan sulfate, the open area the percentage of chondroitin sulfate, and the closed area the percentage of other glycosaminoglycans, mostly dermatan sulfate.

done by the method of Kjellén *et al.* (6) and Oohira *et al.* (12) with a modification as follows. Peak fractions eluted from Sepharose CL-4B were pooled and the sulfated materials were precipitated from the pooled fractions with ethanol. To the precipitates, 100 μl of 1% Triton X-100/50 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 1 mM *N*-ethylmaleimide and 0.2 mM phenylmethylsulfonyl fluo-

ride was added. The mixtures were stirred at 4°C for 5 h, and then subjected to ultracentrifugation in a CsCl density gradient at an initial density of 1.38 g/ml in 1 M guanidine HCl-50 mM Tris-HCl, pH 7.5, containing protease inhibitors at 10°C using a Hitachi RPS 65T rotor at 38,000 rpm for 50 h.

RESULTS

The Amount of [^{35}S]Labeled Glycosaminoglycans Extracted with Each Solution

Fig. 1 shows the amount of [^{35}S]labeled glycosaminoglycans solubilized by sequential treatments with various solutions. Thirty-seven percent of PBS-insoluble glycosaminoglycans were extracted by three times of homogenization in heparin-PBS, 26% in MEGA-9-PBS, 33% in guanidine HCl, and 4% remained unextracted.

Extraction of [^{35}S]Labeled Proteoglycans with Exogenous Glycosaminoglycans

To compare the effectiveness of proteoglycan extraction among various glycosaminoglycans, PBS-insoluble materials of limb buds were treated with PBS containing various glycosaminoglycans. Exogenous glycosaminoglycan which released [^{35}S]labeled proteoglycans most effectively was heparin (about 230 dpm/limb as shown in Fig. 1). Heparan sulfate released 110 dpm/limb, and both chondroitin sulfate and PBS without any glycosaminoglycans released 70 dpm/limb (data not shown).

To compare the amount and composition of [^{35}S]labeled glycosaminoglycans released by heparin with those released by PBS without any glycosaminoglycans, the glycosaminoglycan preparations were separated by two-dimensional electrophoresis on cellulose acetate film. Both heparin and PBS released almost the same amount (about 30 dpm/limb) of chondroitin sulfate (Fig. 2). However, the amount of heparan sulfate released by heparin was more than 4-fold that released by PBS. This result suggests that heparan sulfate proteoglycans are displaced specifically by heparin.

Gel Filtration on Sepharose CL-4B

We previously reported that HSPG I-A was eluted at a position with K_{av} =0.38 from a Sepharose CL-4B column and HSPG III-A was excluded from the same column. It is of interest to clarify which pro-

Fig. 4 Sepharose CL-6B column chromatography of heparin-released proteoglycan which was eluted from Sepharose CL-4B column at a position with K_{av} =0.40 (see Fig. 3a). Proteoglycan preparation was applied to a column of Sepharose CL-6B before (—) and after sequential treatments with pronase and alkali (----) or after nitrous acid treatment (.....). Fractions of 0.5 ml were collected. V_o , void volume; V_t , total volume

teoglycan can be released by heparin. To examine this, [^{35}S]labeled material solubilized by heparin was subjected to gel filtration on Sepharose CL-4B. Most radioactivity was eluted as a peak with K_{av} =0.40, closely similar to the elution position of HSPG I-A (Fig. 3a). MEGA-9 extract gave an elution pattern essentially the same as that of heparin extract (Fig. 3b), whereas guanidine HCl extract contained an excluded peak in addition to a peak with K_{av} =0.40 (Fig. 3c). In either of these peaks, heparan sulfate was the predominant glycosaminoglycan. These findings suggest that the proteoglycan released by heparin could be HSPG I-A, and that some other population of HSPG I-A would be recovered in both MEGA-9 extract and guanidine HCl extract. HSPG-III seemed to be recovered only in guanidine HCl extract.

Characterization of Heparin-Released Proteoglycan

To examine the structure of heparan sulfate side chains of the proteoglycan released by heparin, the peak fraction with K_{av} =0.40 on Sepharose CL-4B was chromatographed on Sepharose CL-6B before and after sequential treatments with pronase and alkali or after nitrous acid treatment. Before proteolysis, the sulfated material was eluted from a Sepharose CL-6B column as a single peak with K_{av} =0.18, while, after proteolysis, it was eluted at a position with K_{av} =0.45 (Fig. 4). Treatment with nitrous acid degraded it into small molecules as expected. Judging from the elution position of the heparan

sulfate on Sepharose CL-6B, its molecular weight was about 27 K, almost identical to that of HSPG I-A. Analyses, including proteolysis and nitrous acid treatment, of the peak fraction with K_{av} =0.40 of Sepharose CL-4B either in MEGA-9 extract or in guanidine HCl extract gave virtually the same results as those of heparin extract (data not shown). These observations support the idea that some population of HSPG I-A can be released from the limb bud by heparin.

Ultracentrifugation on a CsCl Density Gradient

Ultracentrifugation of various HSPG I-A preparations on a CsCl density gradient was done in the presence of Triton X-100, to examine if HSPG I-A released by heparin is different from other populations of HSPG I-A in hydrophobicity. Proteoglycan with hydrophobic property should have affinity to a detergent such as Triton X-100, and a complex between proteoglycan and Triton X-100 should have a low buoyant density. As shown in Fig. 5a, HSPG I-A released by heparin had a high buoyant density even in the presence of Triton X-100, suggesting that this population of HSPG I-A is less hydrophobic. About a half of the MEGA-9 solubilized HSPG I-A was recovered in the top fraction (Fig. 5b). This proteoglycan would have a hydrophobic domain in the core protein, which may be intercalated into plasma membrane. Alternatively, this proteoglycan may have a phospho-inositol-lipid membrane anchor (4). We do not know the reason why the guanidine HCl-extracted proteoglycan had a low

Fig. 5 CsCl density gradient ultracentrifugation of [^{35}S]labeled HSPG I-A from the heparin extract (a), from the MEGA-9 extract (b), and from the guanidine HCl extract (c). HSPG I-A, pretreated with 1% Triton X-100 solution, was centrifuged under dissociative conditions as described in Materials and Methods.

buoyant density as shown in Fig. 5c. This proteoglycan may be associated not only with insoluble collagenous matrix by an ionic bond but also with the plasma membrane by a hydrophobic structure.

DISCUSSION

In our previous paper (11), we demonstrated the occurrence of two distinct heparan sulfate proteoglycans; HSPG I-A and HSPG III-A. HSPG I-A was shown, in the present work of differential extraction, to be further divided into three populations such as a heparin-released, a detergent-solubilized, and a guanidine HCl-extractable molecule, although HSPG III-A was recovered only in the guanidine HCl extract (Fig. 3). Of the three populations of HSPG I-A, a heparin-released proteoglycan is the major and is homogeneous in its molecular density (Fig. 5a). The existence of a heparin-displaceable (hydrophilic) heparan sulfate proteoglycan has been reported in some other tissues (1, 5-7). This type of heparan sulfate proteoglycan is believed to be associated with receptors in plasma membrane specific to a heparin-like structure via heparan sulfate side chains.

San Antonio *et al.* (15) have suggested that heparin-displaceable heparan sulfate proteoglycans play an important role in chondrogenesis of limb bud mesenchymal cells. They showed that higher con-

centration ($\geq 50 \mu\text{g/ml}$) of heparin reduced cartilage nodule size in micromass cultures of chick limb bud mesenchyme obtained from Hamburger-Hamilton (HH) stage 23/24 chicken embryos, and that it also decreased [^{35}S]sulfate incorporation into the micromass cultures. However the inhibitory effects by heparin were not observed in micromass cultures which contained an enriched population of chondroprogenitor cells derived from the wing-tip subridge mesoderm of the HH stage 25 embryo. We observed, using an organ culture of prechondrogenic rat hindlimb bud obtained from day-13 embryo, that the chondrogenesis of distal mesenchyme was inhibited by $100 \mu\text{g/ml}$ of heparin although the inhibition was not seen in chondrogenesis of proximal mesenchyme whose developmental process is more advanced than that of distal mesenchyme (9). These results suggest that high concentration of heparin exerts its inhibitory effect to less differentiated mesenchymal cells, and imply that a step prerequisite for chondrogenesis, for example mesenchymal cell condensation, may be inhibited by heparin. There is a possibility that heparin may interfere with an association between the heparan sulfate proteoglycan and cell surface receptor via heparan sulfate chains, which is needed for mesenchymal cell condensation.

At this moment, we do not know what are the structural differences among these three populations of HSPG I-A. Recently, Brandan *et al.* (1) have shown that a hydrophobic heparan sulfate proteoglycan synthesized by rat hepatocytes is converted to a hydrophilic heparan sulfate proteoglycan probably at the surface of plasma membrane. In the prechondrogenic rat limb bud, one may consider that some part of hydrophobic HSPG I-A recovered in MEGA-9 extract or in guanidine HCl extract is converted to a hydrophilic form by removal of a hydrophobic domain of its core protein. To examine this possibility, a precursor-product relationship should be analyzed among various populations of HSPG I-A, in addition to detailed structure of the core protein moiety.

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