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ARTICLES

Isolation of Heparan Sulfate Proteoglycan From Beneath the Monolayers of Rat Hepatocytes and Its Binding to Type IV Collagen

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Abstract Primary cultures of rat hepatocytes maintained as monolayer in a serum-free medium synthesise and secrete sulphated proteoglycans. Nearly 5% of the total ^{35}(S)-sulphated material was obtained in a soluble form from beneath the cell layer. A shift in gel filtration pattern on β-elimination with alkali suggested that it is a sulphated proteoglycan. On ion exchange chromatography over Dowex AG 1x2, the major fraction was eluted with 1.25 M NaCl. Further, nearly 80% of the ^{35}(S)-labeled material was susceptible to nitrous acid degradation and more than 90% of the material was resistant to chondroitinase ABC digestion suggesting that it is predominantly a heparan sulphate proteoglycan (HSPG). Since HSPG is a major component of basement membrane, its binding with collagen was studied by a solid phase binding assay. About 75% of the ^{35}(S) HSPG bound to wells coated with type IV collagen whereas only about 20% bound to type I collagen at physiological pH. Binding to collagen IV was reduced by about 50% when free GAG chains were used indicating that the protein core is also involved in interaction with the collagen. These results indicate the possible role of this basal extracellular heparan sulphate proteoglycan in the basal lamina formation.

Key words: HSPG synthesis, isolation of basal extracellular HSPG, primary cultures, serum-free medium

Heparan sulfate proteoglycans (HSPGs) are a heterogeneous group of macromolecules within the large family of proteoglycans. They are composed of linear sulphated polysaccharide chains, the heparan sulfate moieties, that are covalently linked to protein. In animal tissues they have been shown to be present both at the plasma membrane and in the extracellular matrix [1,2]. HSPGs are components of the basement membrane in glomeruli [3], mammary epithelia [4,5], and that of a basement membrane-producing tumour [6]. Co-distribution of heparan sulfate proteoglycans with actin and association with pericellular matrix proteins in cells in culture have been demonstrated by immunological techniques [7]. Studies with transformed cells in culture suggested that HSPG may be essential for matrix assembly [8].

Rat hepatocytes maintained in a serum-free medium synthesise and secrete heparan sulfate in a protein-bound form [9]. The proteoglycans isolated from rat liver plasma membrane was reported to consist of 3–4 heparan sulfate chains linked via xylose to core protein [10,11]. Part of the HSPG has been shown to be intercalated in the plasma membrane in hepatocytes in culture [12]. This paper reports the presence of a soluble extracellular proteoglycan accumulating beneath the basal surface of rat hepatocytes maintained in a serum-free medium and its interaction with type IV collagen.

MATERIALS AND METHODS

All chemicals used were high purity analytical grade reagents. Minimum essential medium (Eagle), and collagenase type IV were purchased from Sigma (St. Louis, MO). Fetal bovine serum was a product of Gibco. Tissue culture plastic wares were from M/s NUNC (Roskilde). Sephacryl-S-300 was obtained from Pharmacia (Uppsala). ^{35}(S)-SO_4 was a product of BARC (Bombay). Type IV collagen prepared from EHS tumour was kindly provided by R.C. Hughes, NIMR (London). Type I collagen was kindly provided by J. Rauterberg, Munster, Germany.

Cell Culture

Hepatocytes were isolated from normal rat (Sprague-Dawley strain) liver by collagenase per-

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fusion according to the procedure of Seglen [13] as described before [14]. Cells were suspended in Eagle's MEM supplemented with 10% fetal bovine serum, pencillin (100 mg/L), streptomycin (100 mg/L) and were seeded on 60 mm NUNC plastic petri dishes. Culture was maintained in a CO_2 incubator (Que system) for 4–5 h. The unattached cells were removed and the cell monolayer was washed with serum-free medium (Waymouth).

Metabolic Labeling With ^{35}(S)-SO_4 and Extraction of Proteoglycan

The cells were metabolically labeled with ^{35}(S)-SO_4 (10 μCi/ml) as described before [14]. At the end of the incubation period the medium was removed and the cell layer was washed 4 times with 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and the basal extracellular proteoglycans were extracted by the procedure of Koda and Bernfield [4]. Accordingly, the cell layer was harvested by scraping them from their substratum with a rubber policeman in 1 ml PBS at 4°C containing 0.5 mM EDTA, 0.5 mM PMSF, and 2 mM benzamidine hydrochloride. The cells were sedimented at 600*g* and the supernatant containing the basal extracellular proteoglycan was collected.

Isolation and Characterisation of Extracellular Heparan Sulfate

^{35}(S)-proteoglycans were purified by ion exchange chromatography over Dowex AG (1 × 2) column (0.5 × 5 cm) in 4 M Urea in Tris-HCl (50 mM pH 7.6) and the bound material was eluted with different concentration of NaCl in the same buffer. Glycosaminoglycans were released from proteoglycan by digestion with crystalline papain in phosphate buffer pH 7 containing 5 mM EDTA and 5 mM cystein hydrochloride or by treatment with 0.15 M NaOH at 37°C for 4 h. Isolation of ^{35}(S)-glycosaminoglycans from medium was done by precipitation with N-cetyl pyridinium chloride as described before [15].

Digestion of (^{35}S)-glycosaminoglycans with chrondroitinase ABC and separation of the products by paper chromatography was done by the method of Saito et al. [16]. Nitrous acid degradation was carried out by the method of Shiveley and Conrad [17].

Solid Phase Binding Studies

Binding of ^{35}(S)-GAG to collagen type I and type IV was studied in small polyvinyl multiwell plates. The wells were passively coated with respective materials by incubating for 2 h at room temperature in PBS. After removal of the nonadsorbant solution, the wells were incubated with 1.5% BSA for another hour. The solution was removed and the wells were washed with phosphate-buffered saline. Each well was filled with (^{35}S)-labeled proteoglycans samples in a final volume of 250 μl and were kept at room temperature for 1 h. At the end of the incubation period the samples were removed and the wells were washed with PBS. Both the samples and washed solutions (in PBS) were mixed and quantitated. Each well was again filled with 0.2% SDS, to solubilize the bound radioactive material and it was quantitated by measuring the radioactivity.

Gel Filtration Over Sephacryl-S-300

The molecular size of the ^{35}(S)-heparan sulfate was determined by gel filtration over sephacryl-S-300 column in 4 M guanidine hydrochloride in Tris-HCl buffer (50 mM pH 7.6).

Other Methods

Protein was estimated by the method of Lowry et al. [18]. Radioactivity was measured in an LKB liquid scintillation counter.

RESULTS

Isolation and Characterisation of Basal Extracellular Proteoglycans

To prepare the newly synthesised proteoglycans that are in the basal extracellular space, monolayers of hepatocytes were labeled for 24 h with ^{35}(S)-SO_4. The medium was removed and the cell layer was washed with PBS (until the supernatant was free of radioactivity) and scraped off the plastic into PBS. Cell pellet was removed by centrifugation at 600*g* for 3 min and the supernatant was further cleared by centrifugation at 12,000*g*, dialysed against PBS, and ^{35}(S)-PG were isolated. The basal proteoglycans account for nearly 5% of the total ^{35}(S)-labeled glycosaminoglycans synthesised by the cells. On ion exchange chromatography of the ^{35}(S)-PG in PBS supernatant over Dowex AG (1 × 2), nearly 75% of the labeled material was eluted from the column by 1.25 M NaCl (Fig. 1). The ^{35}(S)-GAG were released from the 1.25 M NaCl eluate and were subjected to chondroitinase ABC digestion and nitrous acid degradation. More than 90% of the ^{35}(S)-labeled material was resistant to chon-

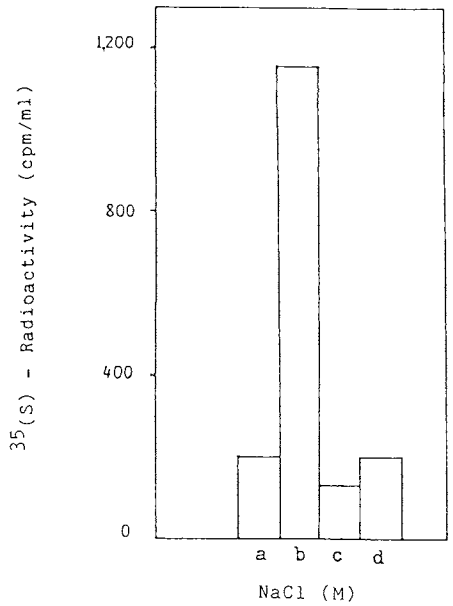

Fig. 1. Ion exchange chromatography of ^{35}(S)-PG over Dowex AG 1 × 2. ^{35}(S)-proteoglycan isolated from basal extracellular space was applied over Dowex AG 1 × 2 (0.5 × 5 cm) in Tris/HCl buffer (pH 7.6, 0.05 M) containing 4 M Urea. The bound ^{35}(S)-PGs were eluted with (3 × 2 ml each) 0.5 M (**a**), 1.25 M (**b**), 1.5 M (**c**), and 2.5 M (**d**) NaCl.

droitinase ABC digestion. Nearly 75–80% of the ^{35}(S)-SO_4-labeled material was susceptible to nitrous acid degradation. These results indicate that the ^{35}(S)-labeled material deposited by liver cells in the basal extracellular space was heparan sulfate. Gel filtration of this ^{35}(S)-labeled material over sephacryl-S-300 in 4 M guanidine hydrochloride showed its elution in the void volume while after treatment with 0.15 M NaOH there was a shift in the elution volume of the radioactive material (Fig. 2) indicating that the ^{35}(S)-SO_4-labeled heparan sulfate deposited in the basal extracellular space was a proteoglycan.

Binding of Proteoglycan to Matrix Protein

Since heparan sulfate proteoglycans are associated with the basement membrane, we have studied the nature of interaction between other components such as collagen and the basal soluble extracellular heparan sulfate PG isolated from primary cultures of hepatocytes. The basal extracellular ^{35}(S)-PG eluted in the void volume on gel filtration was further purified by ion exchange chromatography over Dowex AG 1 × 2 and eluted at 1.25 M NaCl. The partially purified PG was used for binding studies with type IV collagen and type I collagen. Multiwells were passively coated with different matrix proteins and the binding of ^{35}(S)-labeled HSPG was studied.

About 70–75% of the added proteoglycan bound to wells coated with type IV collagen whereas only less than 20% of the added proteoglycan bound to type I collagen-coated wells (Fig. 3). A concentration-dependent increase in the amount of HSPG binding to collagen-coated wells was observed (Fig. 4). This was found to follow a saturation pattern indicating the presence of saturable binding sites on type IV collagen for this HSPG. When wells coated with different concentration of type IV collagen was used there was a steady increase in the amount of HSPG bound, reaching a maximum probably as a result of limiting concentration of the radioactive proteoglycans. The binding of (^{35}S)-PG increased with time during the first 20 min and reached a maximum at 60 min at room temperature. The binding at room temperature (25°C) initially increased rapidly and began to plateu after 20–30 min. The binding at 0°C was only less than 10% of that at room temperature at earlier time intervals and slowly increased to reach nearly 30–35% of the maximum binding found at room temperature at 60 min.

To determine the contribution of the glycosaminoglycans and the protein core of the PG in the binding to collagen, GAG chains were released from PG by papain digestion or alkali treatment and purified by ion exchange chromatography. About 35% of the added (^{35}S)-GAG bound to collagen IV whereas about 70% of the added (^{35}S)-PG bound to wells coated with the same amount of collagen IV indicating that the heparan sulfate in its proteoglycan form is more effective (Fig. 5). This is probably due to a cooperative effect of multiple GAG chains on the protein core, and/or the protein core may also be involved in the binding of the proteoglycans to collagen.

DISCUSSION

The primary cultures of rat hepatocytes synthesise and deposit proteoglycan in its basal extracellular space. Susceptibility to nitrous acid degradation, elution from Dowex AG 1 × 2 at a salt concentration of 1.25 M, and resistance to chondroitinase ABC digestion suggest that the ^{35}(S)-glycosaminoglycan is predominantly heparan sulfate; whereas more than 90% of the ^{35}(S)-labeled material was resistant to chondroitinase ABC digestion, only about 75–80% of the material was susceptible to nitrous acid degrada-

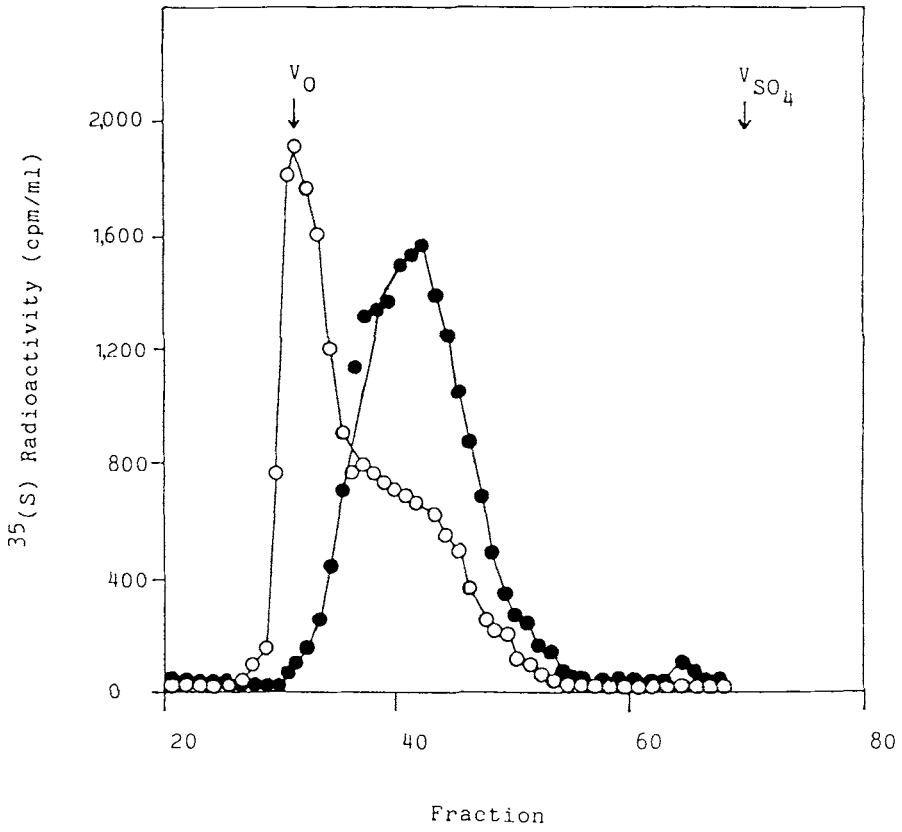

Fig. 2. Gel filtration over Sephacryl-S-300. The ^{35}(S)-labeled polysaccharides isolated from the basal extracellular PG before (○) and after (●) alkali cleavage were applied on a column of Sephacryl-S-300 (1 × 96 cm) equilibrated and eluted with 4 M guanidine hydrochloride in Tris/HCl buffer (pH 7.6, 0.05 M). V_O and V_{SO_4} are the elution volume for dextran blue and ^{35}(S)-sodium sulphate, respectively. Average fraction size was 1.25 ml.

tion. The nitrous acid-resistant material could not be characterised.

Primary cultures of rat hepatocytes have been shown to synthesise and distribute almost exclusively heparan sulfate in a proteoglycan form. The cell-associated form of HSPG has been solubilised on treating the cells with trypsin [9]. This has been termed pericellular HSPG. Part of the cell membrane-associated HSPG has been shown to be released by treatment with heparin and the remaining by digestion with trypsin [19]. It is possible that the soluble extracellular HSPG now identified might have been collected along with the cell membrane-associated HSPG while harvesting the cells with trypsin, and might have been grouped along with pericellular HSPG referred to therein [9]. This apparently may not be associated with the cell membrane at the basal surface as the cell membrane-associated material was not solubilised by treatment with isotonic salt solution. Solubility characteristics and the binding pattern to collagens suggested that this is different from the cell membrane-associated HSPG [12] or from that secreted into the medium. The possibility that it represents a separate pool with weak affinity to cell surface and/or substratum cannot be excluded. It may be a basement membrane type HSPG, as described by Bernfield and colleagues, wherein mouse mammary epithelial cells have been shown to deposit at their basal surface an extracellular proteoglycan which contained nearly 85% heparan sulfate and about 15% chondroitin-dermatin sulfate [4,20].

Since heparan sulfate proteoglycans are one of the major components of the basal lamina and since HSPG has been shown to interact with various other components of the laminae such as collagen, fibronectin, and laminin, the interaction of this PG deposited in the basal surface of hepatocytes with collagen has been studied. The proteoglycan bound greatly to type IV collagen and less to type I collagen. Since the binding occurs under physiological conditions of pH and ionic strength and there is substantial reduction in binding at low temperature, it suggests that

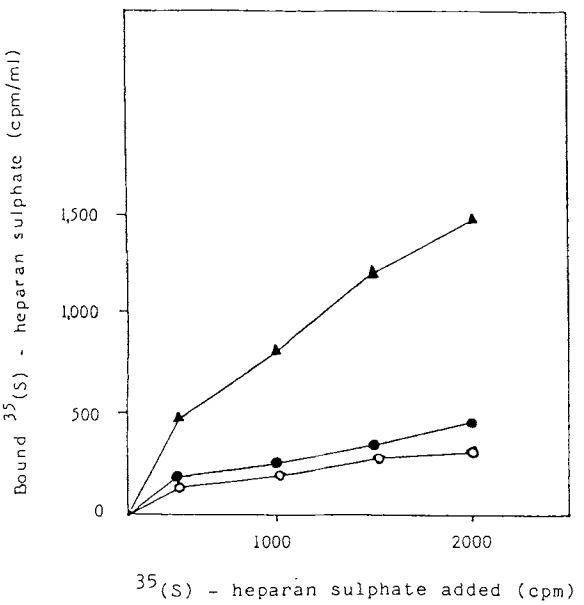

Fig. 3. Binding of basal extracellular ^{35}(S)-HSPG to collagen type IV and type I. Multiwell plates were coated with native collagen type IV or type I (20 μg/ml) at room temperature as described in text. Different amount of purified ^{35}(S)-HSPG was added to the multiwells and incubated at room temperature for 1 h. The unbound material was removed, washed, and the bound ^{35}(S)-radioactivity was solubilised and quantitated. BSA-coated wells served as control (▲, type IV collagen; ●, type I collagen, ○, control).

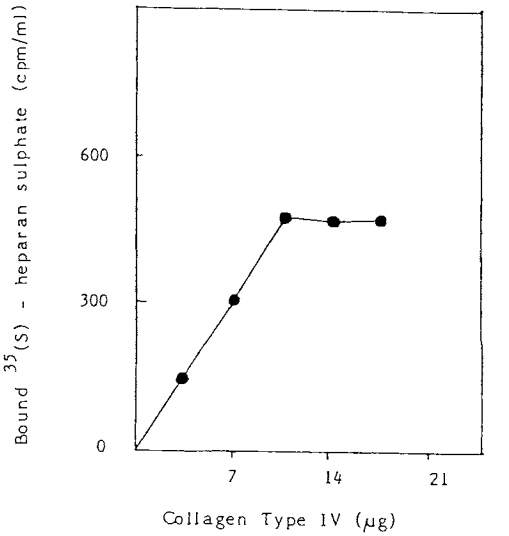

Fig. 4. Binding of ^{35}(S)-HSPG to type IV collagen-effect of concentration of collagen. Multiwell plates were passively coated with type IV collagen of different concentration as described in text. Binding of basal ^{35}(S)-HSPG to these wells was studied by adding a known amount of purified radioactive material as described in the legend to Figure 3.

the binding of heparan sulfate PG to collagen IV may have some physiological relevance. Reduction in the binding to collagen on removal of the core protein of the HSPG also suggests that the native proteoglycan is much more effective in the interaction with collagen.

In intact liver, the basal surface of the parenchymal cells is in contact with a basement membrane which contains type IV collagen, type I collagen, laminin, fibronectin and some other glycoproteins [21]. HSPG present in the basement membrane has been shown to co-distribute with collagen and fibronectin [7]. The binding of HSPG to collagen IV and fibronectin forms stable ternary complexes. Therefore the high-affinity binding of the soluble extracellular HSPG deposited in the basal surface of the hepatocytes with type IV collagen may be relevant in the biogenesis of basement membrane. Further studies are in progress to see whether this HSPG is related to the HSPG that is associated with cell membrane or to that present in basement membrane in liver.

Basal lamina underlying epithelial cells in many tissues contain type IV collagen, fibronectin, and laminin [22] while reticular lamina which separate basal lamina from stromal cell layer contains type I collagen [23]. The greater binding of the HSPG produced by hepatocytes to type IV collagen and relatively low binding to type I collagen probably indicate the possible role of the HSPG in the formation of basal laminar structure by binding with type IV collagen and also with other proteins such as fibronectin and laminin.

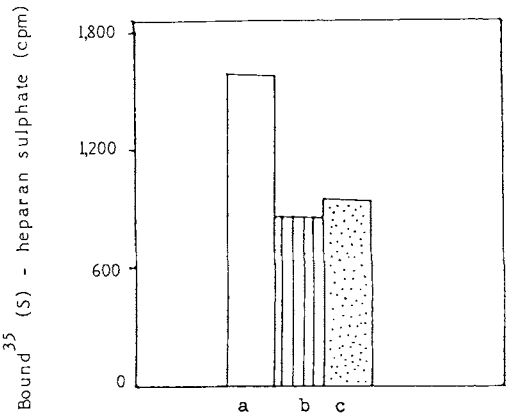

Fig. 5. Binding of ^{35}(S)-HSPG and ^{35}(S)-GAG to type IV collagen. Multiwell plates were coated with type IV collagen (20 μg/ml). ^{35}(S)-HSPG (**a**) or free GAG chains released by protease digestion (**b**) or alkali digestion (**c**) were added (~2,000 cpm) and the bound radioactivity was measured as described in the legend to Figure 3.

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