BINDING OF HEPARIN AND HEPARAN SULPHATE TO RAT LIVER CELLS

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SUMMARY: The binding of pig mucosal heparin and rat liver heparan sulphate to rat liver cells is demonstrated. The process is shown to be time dependent, reversible and saturable. The maximal amount of heparin bound to the cells exceeds that of heparan sulphate, on a molar basis.

The binding of both polysaccharides is specific, in that excess amounts of glycosaminoglycans other than heparin-related do not affect the binding reaction.

The binding of heparin to cells was markedly reduced when incubations were performed at low temperature or after trypsin treatment of the cells.

INTRODUCTION

The presence of heparan sulphate at the surface of cells in tissue culture is now well documented (1-4). However, the physiological role of this glycosaminoglycan remains obscure. Speculations have been put forward as to a role for cell surface polysaccharides in the regulation of cell proliferation (5), cell-cell communication (2), and the accessibility of underlying membrane receptors to external agents (6). Addition of exogenous heparin-related polysaccharides to cells in culture have been shown to modify the behaviour of the cells (for review see Ref. 7). Of particular interest in this context is the recent finding that heparan sulphate, isolated from normal rat liver cells, restores density dependent inhibition of cell growth of a rat hepatoma cell line (8).

MATERIALS AND METHODS

Materials

^{3}H-Glucosamine (12 Ci per mmol) and ^{3}H-acetic anhydride (500 mCi per mmol) were purchased from the Radiochemical Center, Amersham, England.

Heparin (prepared from pig intestinal mucosa) was obtained from Inolex Pharmaceutical Div., Park Forest South, Ill., U.S.A., and purified as described by Lindahl et al. (9). Chondroitin sulphate (isolated from bovine nasal septa) was kindly given by Dr. Å. Wasteson of this Department. Hyaluronic acid was prepared from rooster combs according to a method described by Laurent et al. (10). Dermatan sulphate (isolated from pig intestinal mucosa) was obtained from Dr. L. Rodén, Birmingham, Ala., U.S.A.,

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and purified as described (11). Heparan sulphate was prepared from bovine liver essentially according to the technique for glycosaminoglycan isolation described by Iverius (12). The details of the procedure will be reported elsewhere.

^{3}H-Heparin was prepared by chemical ^{3}H-acetylation of free amino groups of the polysaccharide. The labelling procedure has been outlined before (13) and will be described in detail in a separate communication. The final product had a specific activity of 9.2×10^4 cpm per µg of uronic acid.

^{3}H-Heparan sulphate was obtained by biosynthetic labelling of rat liver heparan sulphate. Rats were injected i.p. with 250 µCi of ^{3}H-glucosamine. Two hours later the animals were killed, the livers removed and homogenized in 50 ml of 10 mM EDTA, 10 mM cysteinium hydrochloride, 2 M sodium chloride in acetate buffer, pH 5.5. After addition of 150 mg of crystalline papain, the mixture was incubated for 36 hours at 60°. The supernatant obtained after centrifugation of the digestion mixture was applied to a column (5 x 80 cm) of Sephadex G-50, equilibrated with 1.0 M NaCl. The macromolecular material emerging in the void volume was pooled and after desalting by dialysis the heparan sulphate was isolated by anion-exchange chromatography as previously described (14). The final product had a specific activity of 6.8×10^3 cpm per µg uronic acid[1)] and was characterized as a high-sulphated heparan sulphate (see Ref. 14).

Methods

Methods for the determination of radioactivity, protein and uronic acid were as described (15).

Isolation and culturing of rat liver cells

Cells were isolated from rat livers after perfusion with collagenase and calcium-free buffers. The procedure adopted was essentially that of Seglen (16) and has been described in detail elsewhere (14). Cells were seeded on 60 mm petri dishes in a volume of 5 ml of Ham's F 10 medium (17), supplemented with 10% postnatal calf serum, 100 U/ml penicillin, 50 µg/ml streptomycin and 1.25 µg/ml amphotericin B at a cell density of 1.6×10^6 cells/ml. Incubations were performed in a humidified incubation chamber at 37°. Thirty minutes after seeding, the medium containing cells which had not attached to the plate was removed; the attached cells were washed with medium and then incubated with 2 ml of F 10 medium containing the labelled polysaccharide.

Quantitation of labelled polysaccharide bound to cells

Incubations were terminated by removal of the culture medium followed by repeated washing with 3 x 1 ml of 10 mM HEPES, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 0.5 mM $MgSO_4$ and 1 mM $CaCl_2$. Cells were detached and disrupted by incubation at 60° for 30 min with 1 ml of 0.1% pronase in 10 mM HEPES, pH 7.4, containing 142 mM NaCl, 7 mM KCl. The amount of cell-associated labelled polysaccharide was determined by scintillation counting, using Dimilume (Packard Instrument Co., Ill., U.S.A.) as scintillation medium.

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1) The presence in rat liver of sulphated glycosaminoglycans other than heparan sulphate (i.e. dermatan sulphate) has been demonstrated (20). These polysaccharides are probably not synthesized in the tissue (14) but are taken up by the liver prior to degradation. Since heparan sulphate is the only labelled sulphated polysaccharide in the preparation, the presence of other unlabelled, glycosaminoglycans would tend to reduce the specific activity of the preparation.

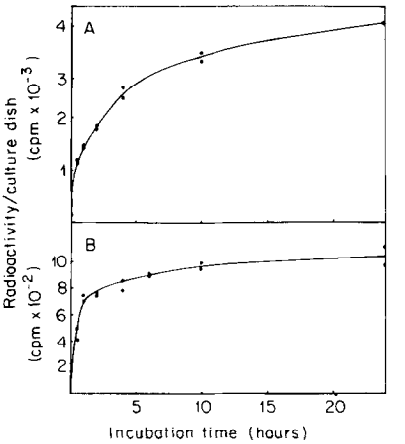

Fig. 1. Time course of ^{3}H-polysaccharide binding to cells

Cells (3 x 10^6 cells/plate) were incubated in the presence of (A) [^{3}H]heparin (2 x 10^4 cpm/plate) or (B) [^{3}H]heparan sulphate (6.8 x 10^3 cpm/plate) for the indicated periods of time and cell-associated, labelled polysaccharide was quantitated as described in the Materials and Methods section.

RESULTS

Rat liver cells (approximately 3 x 10^6) were incubated in the presence of 2 x 10^4 cpm of [^{3}H]heparin or 6.8 x 10^3 cpm [^{3}H]heparan sulphate, respectively. After incubation periods of ten hours both heparin and heparan sulphate had bound to the cells[2]; the amount of cell-associated glycosaminoglycan corresponded to 16% and 14%, respectively, of added polysaccharide.

Various experimental conditions were used in attempts to influence the binding of [^{3}H]heparin to cells. When incubations were performed at 4^{o} instead of 37^{o} or when cells were treated with trypsin (0.001% trypsin at 37^{o} for 5 minutes) prior to the incubation, the binding was markedly reduced. In contrast, the presence of metabolic inhibitors such as Na_2N_3 (0.001%) or 2,4-DNP (1 mM or 10 mM) during incubations, did not influence the binding reaction.

[2] Control incubations, performed in the absence of cells, showed that the [^{3}H]polysaccharides used do not bind to petri dishes or to serum proteins, precipitated on the dishes.

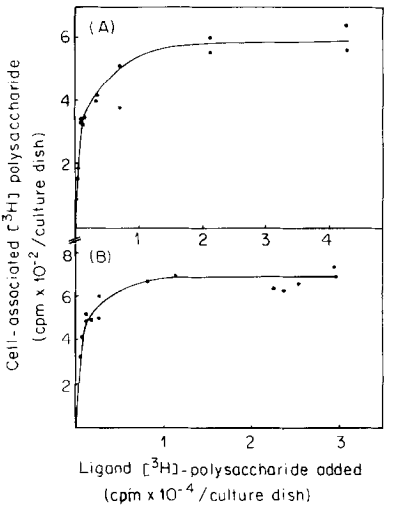

Fig. 2. Binding of ^{3}H-labelled polysaccharides to cells as a function of polysaccharide concentration

Cells (3 x 10^6 cells/plate) were incubated in the presence of varying amounts of (A) [^{3}H]heparin (diluted with unlabelled heparin to give a specific activity of 2.2 x 10^3 cpm/µg uronic acid) or (B) [^{3}H]heparan sulphate (specific activity 6.8 x 10^3 cpm/µg uronic acid). The amount of cell-bound [^{3}H]polysaccharide was quantitated after an incubation period of 4 hours (see Materials and Methods section).

The binding of [^{3}H]heparin and [^{3}H]heparan sulphate to rat liver cells was characterized with regard to:

A. Effect of incubation time

The amounts of cell-associated [^{3}H]polysaccharide increased with incubation time (Fig. 1). The binding of heparan sulphate showed a biphasic course, with a marked decrease in rate after 1 hour of incubation (Fig. 1B).

B. Effect of polysaccharide concentration

Cells were incubated for 4 hours in the presence of labelled polysaccharide at different concentrations. As shown in Fig. 2 the cell binding process of both heparin and heparan sulphate shows saturation kinetics indicating the presence of a limited number of cell-associated binding sites. The maximal amount of heparin bound (~0.3 µg/10^6 cells) exceeded that of heparan sulphate (~0.1 µg/10^6 cells; see Footnote 1). Assuming a molecular weight of 15000 for heparin and 40000 for heparan sulphate (18), an average of about 10^7 molecules of heparin and 10^6 molecules of heparan sulphate can bind per rat liver cell.

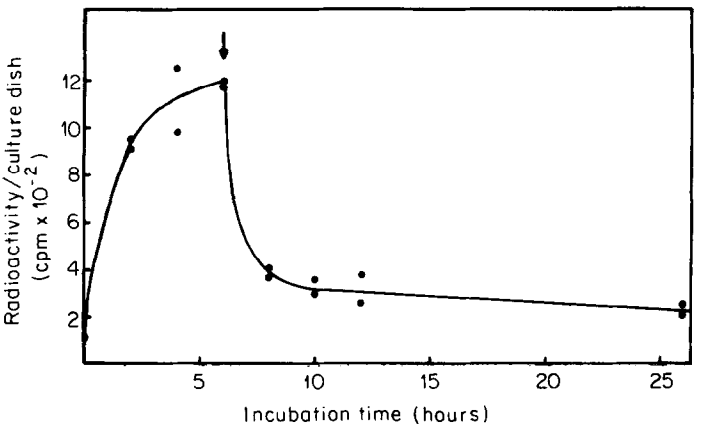

Fig. 3. Reversibility of heparin binding to cells

Cells (3 x 10^6 cells/plate) were incubated in the presence of 6.9 x 10^3 cpm of [^{3}H]heparin (corresponding to 0.075 µg of uronic acid). After 6 hours of incubation the cells were washed and fresh medium containing excess amounts of unlabelled heparin (equal to 7.5 µg of uronic acid) was added (indicated by the arrow). The cell-associated ^{3}H-labelled polysaccharide was quantitated after the indicated periods of time.

C. Reversibility of binding

Cells were incubated in the presence of [^{3}H]heparin for 6 hours, allowing the labelled polysaccharide to bind to the cells. The incubation medium was then discarded, and the cells were washed and fresh medium containing unlabelled heparin in a hundred-fold excess over the initial [^{3}H]heparin was added.

Throughout the experiment, cell-associated [^{3}H]heparin was quantitated. The results shown in Fig. 3 demonstrate that most of the ^{3}H-labelled heparin bound to the cells during the initial incubation period was released during the subsequent exposure to unlabelled heparin.

In an analogous experiment cell-associated [^{3}H]heparan sulphate was readily released on the addition of excess amount of either unlabelled heparin or heparan sulphate to the medium.

D. Specificity of polysaccharide binding

Incubations of rat liver cells with [^{3}H]heparin or with [^{3}H]heparan sulphate were performed in the presence of a 20-fold excess of chondroitin sulphate,

Table 1. Inhibitory effect of glycosaminoglycans on binding of [^{3}H]heparin and [^{3}H]heparan sulphate to cells

Ligand [^{3}H]polysaccharide	Unlabelled inhibitory polysaccharide	% Cell-bound [^{3}H]polysaccharide a)
[^{3}H]heparan sulphate	None	100
	Hyaluronic acid	102
	Chondroitin sulphate	99
	Dermatan sulphate	40
	Heparan sulphate	14
	Heparin	<10
[^{3}H]heparin	None	100
	Hyaluronic acid	96
	Chondroitin sulphate	108
	Dermatan sulphate	91
	Heparan sulphate	94
	Heparin	21

a) Binding without inhibitory polysaccharide was taken as 100%

Cells (3 x 10^6 cells/plate) were incubated with [^{3}H]heparan sulphate (6.8 x 10^3 cpm = 1 µg of uronic acid) or [^{3}H]heparin (1.6 x 10^4 cpm = 0.3 µg of uronic acid) in the presence or absence of a 20-fold excess (on uronic acid basis) of inhibitory polysaccharide. After 6 hours at 37^o the amount of cell-associated [^{3}H]polysaccharide was quantitated.

dermatan sulphate, hyaluronic acid, heparan sulphate or heparin. The binding of [^{3}H]heparan sulphate to cells was affected by the presence of iduronic acid-containing polysaccharides (dermatan sulphate, heparan sulphate and heparin), whereas the binding of [^{3}H]heparin to cells was only markedly reduced in the presence of unlabelled heparin (Table 1).

DISCUSSION

In the present investigation heparin-related polysaccharides are shown to bind to specific sites present at the surface of rat liver cells. The binding between polysaccharide and cells is analogous to the interaction between e.g. hormones and cell surface receptors (for review see Ref. 19) in being reversible, trypsin sensitive, time and temperature dependent and saturable. Furthermore, the binding of heparin and heparan sulphate to cells

possesses a certain degree of specificity as no inhibitory effect of structurally unrelated glycosaminoglycans was observed.

Binding experiments performed in the presence of competitive polysaccharides indicate that the binding sites for heparan sulphate also have affinity for heparin. The reverse, that all heparin binding sites have affinity for heparan sulphate, appears not to be valid as A) the maximum number of heparin molecules bound per cell by far exceeds that of heparan sulphate and B) the binding of [^{3}H]heparin to cells is not markedly reduced in the presence of unlabelled heparan sulphate.

Presently, only speculations can be offered as to the physiological significance of the cell surface binding of heparin-like polysaccharides. The binding of polysaccharide to cells may be a prerequisite for polysaccharide internalization and subsequent degradation. Another possibility is that the heparan sulphate-cell interaction is involved in some sort of intercellular communication, such as cell-cell adhesion.

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