DEMONSTRATION OF A PLATELET ENZYME, DEGRADING HEPARAN SULPHATE

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1. Introduction

Platelets have recently been shown to contain cell multiplication stimulating factor(s), acting on cultured mouse fibroblasts [1] monkey arterial smooth muscle cells [2] and human fibroblasts and glial cells [3,4]. We have used platelet lysate as a stimulating agent to study the metabolism of cell associated glycosaminoglycans in relation to cell multiplication. Preliminary experiments indicate that exposure of human, cultured glial cells to a platelet lysate may lead to a release of cellular heparan sulphate, in low molecular weight form, to the culture medium (unpublished results). Since this finding might indicate the presence in platelets of a heparan sulphate degrading enzyme, the present investigation was undertaken to examine the susceptibility of isolated heparan sulphate to platelet preparations. The results demonstrate that platelets contain a heparan sulphate degrading endoglycosidase. The presence of such an enzyme in platelets, lacking endogenous heparan sulphate, may indicate a previously unknown capacity of platelets to modify the heparan sulphate of other cells in the vascular space, e.g. endothelial cells.

2. Materials and methods

2.1. *Chemicals*

Carrier-free [^{35}S]sulphate was obtained from the Radiochemical Centre, Amersham, UK. Sephadex gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Whatman DEAE-cellulose (DE-52) was a product of Balston Ltd., UK. Testicular hyaluronidase was obtained from AB Leo, Helsingborg, Sweden, and bacterial chondroitinase ABC from Miles Laboratories, Inc., Elkhardt, Ind.

2.2. *Glycosaminoglycans*

^{35}S-labelled chondroitin sulphate and a chondroitin sulphate [^{35}S]hexasaccharide were available in our laboratory (Amadò et al., 1974).

Heparan sulphate isolated from human aorta (HS II, ref. [5,6]) was generously provided by Dr P.-H. Iverius of this Institute.

^{35}S-labelled heparan sulphate was obtained from cultures of human glial cells [7,8] (10×10^6 cells) incubated in the presence of radiosulphate (10 μCi per ml of Ham's F-12 [9] at 37°C for 48 h. The spent medium (50 ml) was collected and digested with crystalline papain for 20 h at 55°C, pH 5.5 [10]. The macromolecular ^{35}S-labelled material (1.5×10^6 cpm) was recovered by gel chromatography in 1 M NaCl on Sephadex G-50 and desalted by passage through a column of Sephadex G-50. After lyophilization, the ^{35}S-labelled galactosaminoglycans were eliminated from the preparation by treatment with chondroitinase ABC [11], followed by gel chromatography on Sephadex G-50 to remove the degradation products, and desalting. The resulting material (0.45×10^6 cpm) was identified as heparan sulphate by the following criteria: Treatment with nitrous acid (procedure A ref. [10]) quantitatively converted the polysaccharide to low molecular weight products, included in Sephadex G-50. High voltage paper electrophoresis of these products showed inorganic [^{35}S]sulphate and labelled oligosaccharides corresponding to an *N*-[^{35}S]sulphate to *O*-[^{35}S]sulphate ratio of 3:2 in the parent polysaccharide. In contrast the polysaccharide was resistant to digestion with

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testicular hyaluronidase or further treatment with chondroitinase ABC. Anion exchange chromatography on DEAE-cellulose [12] revealed a single component with the chromatography properties of standard monosulphated chondroitin sulphate.

2.3. *Human sera and platelet lysate*

Fresh citrated blood from three healthy donors was collected, pooled and centrifuged at 190 *g* for 2 X 15 min. The supernatant was divided into three parts which were handled individually. All operations with intact platelets were performed at room temperature; subsequent steps were made at 4°C.

Platelet-poor serum. The supernatant was recentrifuged at 1250 *g* for 30 min and the cell-free plasma was collected and dialyzed against Eagle's MEM [13]. The fibrin clot was spun down (5000 *g*; 20 min) and the serum dialysed against 0.15 M NaCl.

Platelet-rich serum. The supernatant was recentrifuged at 1250 *g* for 30 min. The pellet was suspended in 1/5 of the plasma supernatant and the resulting platelet-rich plasma was converted to serum as described above.

Platelet lysate. The supernatant was further freed from red and white blood cells by five centrifugations (400 *g*; 10 min). After the last centrifugation platelets were the only cells found in the supernatant on examination in the phase contrast microscope. The platelets were spun down (1250 *g*; 30 min), washed in phosphate buffered saline (PBS; 3.4 mM KCl, 170 mM NaCl, 10 mM phosphate, pH 7.3 and recentrifuged three times (1250 *g*; 30 min). The pellet was suspended in a small volume of PBS and freeze-thawed five times. After dialysis against 0.15 M NaCl a 100 000 *g* supernatant of the lysate was prepared.

Human sera and platelet lysates were stored at −20°C until used.

2.4. *Analytical methods*

Protein was determined by the method of Lowry et al. [14]. Uronic acid was determined with the carbazole reaction according to Bitter and Muir [15].

Liquid scintillation counting was performed in a Packard Model 2002 liquid scintillation counter, using Instagel (Packard Instruments) as the scintillation medium.

2.5. *Incubations*

^{35}S-labelled polysaccharide ([^{35}S]heparan sulphate or [^{35}S-chondroitin sulphate), 6000 cpm, corresponding to less than 5 μg of uronic acid) was incubated with platelet lysate (0.5 mg of protein) or serum (200 μl) in acetate buffer, pH 5.6, 0.075 M NaCl for 15 h at 37°C in a final volume of 400 μl. At the end of the incubation period, carrier chondroitin sulphate (0.5 mg), sodium chloride (25 mg) and sodium sulphate (1 mg) were added to the incubation mixtures. They were then heated to 100°C for 3 min and stored at −20°C until analyzed. Deviations from standard conditions are indicated in the text.

2.6. *Estimation of polysaccharide breakdown*

The incubation mixtures were centrifuged to remove insoluble material and the supernatants chromatographed on a column of Sephadex G-100 (1 X 87 cm) or G-50 (1 X 146 cm), equilibrated with 1 M NaCl. Elution was made with 1 M NaCl; eluted fractions were analyzed for radioactivity by liquid scintillation counting or uronic acid by the carbazole reaction. The void volume (V_0) and the total volume (V_t) of the columns were determined using [^{35}S] chondroitin sulphate and inorganic [^{35}S]sulphate, respectively.

3. Results and discussion

Incubation of [^{35}S]heparan sulphate with platelet lysate at pH 5.6 caused a marked depolymerization of the substrate. Gel chromatography on Sephadex G-100 indicated that the degradation products were of oligosaccharide size; inorganic sulphate or sulphated monosaccharides were not observed (fig.1). These findings indicate that the polysaccharide was degraded by an endoglycosidase, cleaving internal glycosidic bonds of the heparan sulphate chains. The observed degradation was not due to exoenzymes to any major extent since no low-molecular weight products (e.g. inorganic sulphate) were detectable. The size of the degradation products was further analyzed by chromatography on Sephadex G-50. A major portion of the material (75%) was eluted from the column with a K_{av} value [16] of 0.18–0.70. The most retarded components had an apparent size similar to that of a standard chondroitin sulphate hexasaccharide (K_{av}, 0.69).

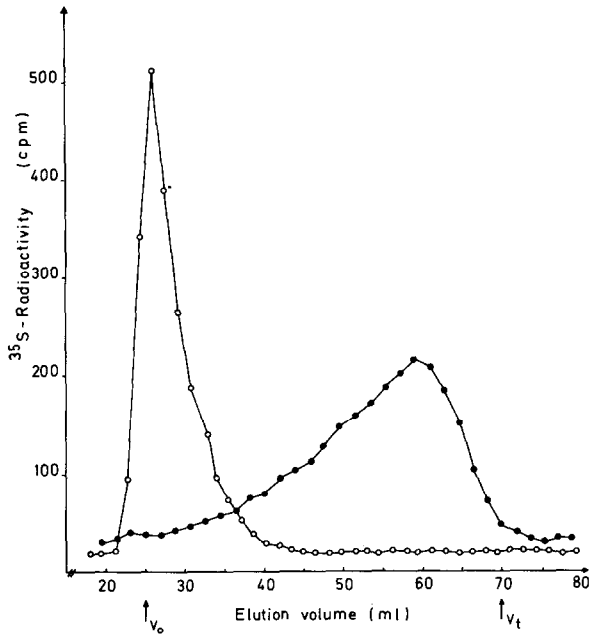

Fig.1. Gel chromatography on Sephadex G-100 of [^{35}S]heparan sulphate, incubated with platelet lysate. (●—●) Nontreated enzyme; (○—○) heat-inactivated enzyme. V_O and V_t of the column are indicated by arrows.

The gel chromatography pattern was essentially the same after reincubation with fresh enzyme. Thus, some of the glycosidic bonds in the substrate polymer were apparently resistant to the endoglycosidase. The presence of resistant fragments of different size may be ascribed to a structural heterogeneity in the heparan sulphate preparation [17].

Degradation of a standard preparation of non-labelled heparan sulphate (HS II) was also effected by the platelet endoglycosidase. Thus, after incubation of 2 mg of HS II with 4 mg of platelet protein, carbazole-positive depolymerization products were demonstrable by gel chromatography on Sephadex G-100. No monosaccharides were detected, in confirmation of the view that the degradation of heparan sulphate was achieved by an endoglycosidase rather than by exoglycosidases.

The platelet heparitinase* showed maximal activity, in acetate or phosphate buffers, at pH 5.0 to 5.6, as shown by gel chromatography of incubation products. No degradative activity, i.e. neither endo- nor exoglycosidases, could be demonstrated at pH 3.6 (acetate buffer). In contrast, appreciable endoglycosidase activity was retained at neutral pH (phosphate buffer). [^{35}S]chondroitin sulphate was resistant to degradation with the platelet enzyme preparation under standard incubation conditions. These findings clearly distinguish the enzyme from the group of hyaluronidase-like enzymes [19,20], demonstrated in other tissues.

Incubation of [^{35}S]heparan sulphate with human serum derived from platelet-poor plasma had no appreciable effect on the size of the [^{35}S]heparan sulphate chains (fig.2). In contrast, serum derived from platelet-rich plasma showed heparan sulphate-depolymerizing activity, causing a moderate shift in the size distribution of the polysaccharide molecules (fig.2). This finding suggests that the presence of heparitinase in the platelet lysate was not due to

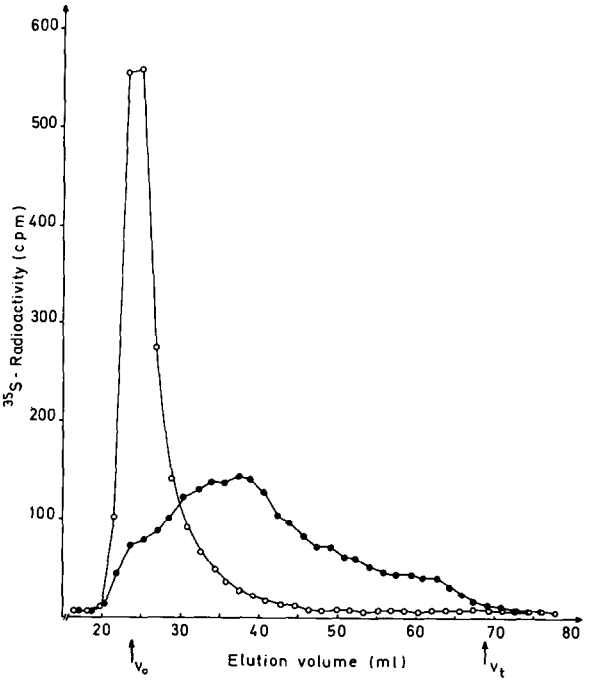

Fig.2. Gel chromatography on Sephadex G-100 of [^{35}S] heparan sulphate, incubated with human serum derived from platelet-rich (●—●) or platelet-poor (○—○) plasma. V_O and V_t of the column are indicated by arrows.

* Pending adequate nomenclature, the term heparitinase is used in analogy with the bacterial enzyme described by Linker [18].

contaminating serum proteins; rather the enzyme activity derived from the platelets.

Heparan sulphate degrading endoglycosidases from mammalian tissues have long remained unrecognized. Only recently the presence of such an enzyme activity in rat liver lysosomes was reported from this laboratory [21]. The rat liver heparitinase had a similar effect on the substrate, yielding oligosaccharides as end products, and had a similar pH-dependence, showing an optimum near pH 5.6. Further experiments will show whether the two enzymes have additional properties in common or if they are, in fact, identical.

The occurrence in platelets of a heparitinase suggests a previously unknown platelet function. Heparan sulphate does not appear to be a constituent of platelets [22] and therefore, the heparitinase is probably not involved in catabolic processes within the platelets proper. Rather, the natural substrates for the platelet heparitinase should be found in association with other cells within or near the vascular space. Heparan sulphate has been identified in a number of mammalian cells, including rabbit endothelial cells [23], and bovine arterial cells [24]. Heparan sulphate is largely associated with the plasma membrane; in fact the localization of heparan sulphate to the external surface of endothelial cells has been demonstrated by its availability to degradation with a bacterial glycosidase [25]. Preliminary experiments indicate that heparan sulphate may be released from cultured glial cells by treatment with platelet lysate, the digestion products appearing as oligosaccharides in the medium. In vivo the platelet enzyme may exert a similar effect on the heparan sulphate of endothelial cells. The removal of membrane associated heparan sulphate from these cells may significantly modify the external surface and thus influence the interaction between the endothelial cells and circulating components.

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