CHANGES IN THE LECTIN BINDING CAPACITIES OF HEPATOMA CELLS AFTER TREATMENT WITH CHONDROITINASE

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SUMMARY: Binding capacities of cells of ascites hepatoma, AH 130 FN, towards lectins were examined before and after treatment with chondroitinase AC. Chondroitin sulfate A was removed from the cells by the enzyme treatment, and the binding capacity of the cells towards Ricinus communis agglutinin (RCA) increased remarkably while the binding constant was unchanged, whereas that towards Concanavalin A (ConA) remained practically unchanged.

In a sereies of papers (1 - 4), we have been characterizing mucopolysaccharides (or their parent forms, proteoglycans) and glycoproteins of the plasma membranes of hepatocytes and ascites hepatoma cells of various types. It has been shown that plasma membranes of different types of hepatoma cells contain characteristic proteoglycans and glycoproteins with O- and N-glycosidically linked oligosaccharides. AH 66 and AH 130 cells, which are classified as island-forming cells contain heparan sulfate like hepatocytes (both resting and dividing), whereas AH 130 FN cells, which are classified as free cells, contain chondroitin sulfate A.

Using lectins that bind specifically to carbohydrate moieties of glycoproteins, we were able to demonstrate that RCA-binding capacity of AH 130 FN cells increased remarkably on enzymatic removal of chondroitin sulfate A.

MATERIALS AND METHODS

AH 130 FN cells were harvested 6 days after the intraperitoneal transfer of ascites fluid from donor rats (female albino rats, Donryu strain). Cells were isolated from the ascites fluid by centrifugation at 100Xg for 5 min, then washed repeatedly with Eagle's minimal essential medium (MEM) (5) supplemented with 10 % calf serum. Some of the rats were injected intraperitoneally with 40 μCi of [^{3}H]glucosamine (D-[1-^{3}H]glucosamine-HCl, 3.0 Ci/mmol) to label hexosamines, or with 200 μCi of [^{35}S]Na_2SO_4 (88.6 mCi/mmol) to label sulfated mucopolysaccharides, 4 hrs, or 1 hr, respectively, before harvesting the cells.

Radioactivity was counted with a Beckman model LS-100 scintillation counter.

To treat the cells with chondroitinase AC, 10^9 cells were suspended in 25 ml MEM plus calf serum and incubated with 10 units of the enzyme for 2 hrs at room temperature. Portions of the suspension were withdrawn at 20-min intervals and centrifuged, and the ^{3}H-radioactivity of the supernatant was determined. Another series of samples was used for chemical identification of the digest: The suspension was centrifuged and the supernatant was dialyzed against distilled water. The outer solution of the dialysis was fractionated on a column of Bio-Gel P-2 with water as eluant. The fractions eluted at the

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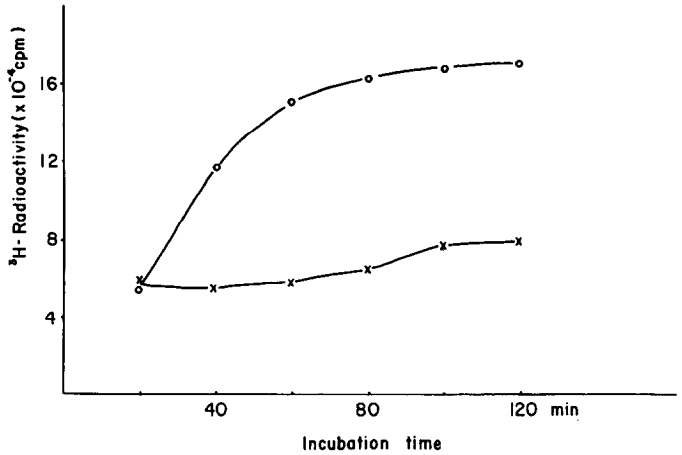

Fig. 1. Release of carbohydrates from AH 130 FN cells by treatment with chondroitinase AC. Cells were incubated with the enzyme under the conditions described in the text, and the release of carbohydrates was followed by determining ^{3}H-radioactivities due to hexosamines. o—o , Chondroitinase AC digest; ×—× , Control.

position corresponding to that of disaccharides, which contained most of the radioactivities of the dialyzable digests and were salt-free, were collected, then evaporated to dryness. The dried material was dissolved in water and submitted to paper chromatography using 1-butanol : acetic acid : 1 M aq. ammonia (2 : 3 : 1, v/v) as solvent. The chromatogram was cut into 1-cm wide segments, which were extracted with distilled water. Radioactivities of the extracts were determined.

Chondroitinase AC [EC 4.2.2.5: Chondroitin AC lyase], chondroitin sulfates A and C and hyaluronic acid were purchased from Seikagaku Kogyo Co. Ltd., Tokyo. The chondroitinase was free from protease and sialidase activities. Concanavalin A (ConA) was purchased from Sigma Chemical Co., St. Louis, and Ricinus communis agglutinin (RCA) was prepared from castor beans according to Nicolson and Blaustein (6). ConA and RCA were iodinated with ^{125}I using the chloramine T method of Allan and Crumpton (7). To prevent the labelling of the binding sites, sugars known to specifically bind to each lectin were added to the reaction mixture. Radioactivity of ^{125}I was determined using Fujitsu ATS-621 gamma ray counter of the well type. Protein was determined by the method of Lowry et al. (8).

Binding of lectins to the cells was assayed as follows. Cells treated with chondroitinase AC were washed three times with phosphate-buffered saline, pH 7.4, (NaCl-Pi) consisting of NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, $CaCl_2$ 0.132 g and $MgCl_2$ 0.1 g per liter. A suspension of the washed cells (10^5 - 10^6 cells) in NaCl-Pi with lectin added (1 - 50 μg per ml) was incubated in the absence and the presence of competing sugar (lactose for RCA and methyl α-mannoside for ConA) for 15 min at room temperature with occasional shaking. After incubation, the cells were centrifuged and washed three times with NaCl-Pi by resuspension and centrifugation, then ^{125}I radioactivity of the cell pellet was determined. The amount of specifically bound lectin was obtained by subtracting the value in the presence of competing sugar from the total value. The maximum binding and the constant were determined graphically by use of a Scatchard plot (9).

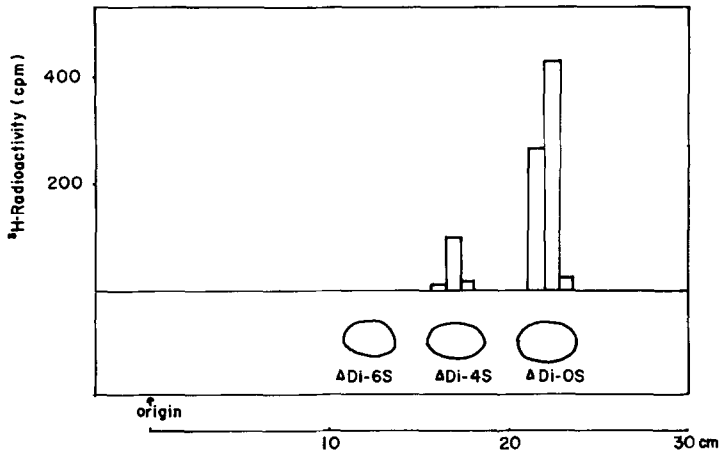

Fig. 2. Identification of disaccharides from chondroitinase AC digest of AH 130 FN cells. The sample was prepared and chromatographed as described in the text. Reference disaccharides were located by their ultraviolet absorption. An additional spot corresponding to about 20 % of the total radioactivities was detected at a position 30 cm from the point of origin.

RESULTS AND DISCUSSION

On treatment of the cells with chondroitinase AC, the enzyme-dependent release of ^{3}H-radioactivities reached a plateau within one hr, as shown in Fig. 1. More than 90 % of the cells remained viable as observed by trypan blue staining on incubation for up to 2 hrs. Enzyme-dependent release of ^{35}S-radioactivities could not be followed precisely, since substantial amounts of ^{35}S-containing compounds of low molecular weight leaked from the cells in a control experiment in which cells were incubated without chondroitinase AC.

About 80 % of the ^{3}H-radioactivities released from the cells during the first hour was recovered in the outer solution of dialysis, whereas about 30 % of the radioactivities were in the outer solution in the control experiment. The outer solution was concentrated, and the concentrate after fractionation on a Bio-Gel P-2 column to remove salts was examined by paper chromatography. As shown in Fig. 2, most of the radioactivities in the outer solution were found in the spots corresponding to ΔDi-4S and ΔDi-0S (unsaturated disaccharides derived by chondroitinase AC from chondroitin sulfate A and hyaluronic acid, respectively). Some of the radioactivities (about 20 %) migrated further than ΔDi-0S and were considered to be metabolic intermediates that had leaked from the cells. These were also detected in the control experiment, in which they accounted for all radioactivities found in the outer solution of dialysis.

The ^{3}H-radioactivities remaining in the inner solution after dialysis

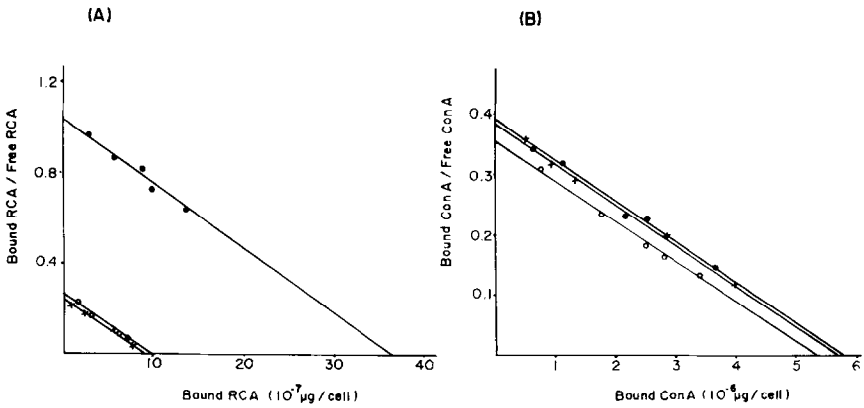

Fig. 3. Changes in lectin-binding capacities of AH 130 FN cells on treatment with chondroitinase AC. Binding experiments were carried out as described in the text. (A) RCA binding, (B) ConA binding. ×—× , Freshly harvested intact cells; ○—○ , Cells incubated without chondroitinase AC; ●—● , Cells treated with chondroitinase AC.

in the control experiment were found to be due to hyaluronic acid. Thus, the inner solution was digested with pronase, and the digest, after removing salts by gel filtration on a Sephadex G-25 column, was examined by electrophoresis on cellulose acetate strip. The ^{3}H-radioactivities were recovered at the position of hyaluronic acid.

The radioactivities which remained in the inner solution after dialysis of the chondroitinase AC digest were not due to mucopolysaccharides, judging from the results of electrophoretic analysis using cellulose acetate strip in which all the radioactivities remained at the point of origin. Their chemical nature is unknown.

The amount of hyaluronic acid released from the cells after the chondroitinase treatment, estimated by the released ΔDi-0S, was about twice as much as that in the control experiment based on radioactivity. Thus it appears that either the secretion of hyaluronic acid is enhanced by the digestion of the secreted hyaluronic acid or the permeability of the membranes increases after the removal of chondroitin sulfate A from the cell surface.

The cells which had been treated with chondroitinase AC for one hr were used for lectin-binding assays, with the results shown in Fig. 3.

The maximum amounts of RCA bound to freshly harvested intact cells and to the control cells were almost the same, amounting to about 9 µg per 10^7 cells. The binding of RCA to the chondroitinase-treated cells increased markedly, amounting to 37 µg per 10^7 cells (Fig. 3A). In contrast, the capacities of the cells to bind ConA did not change after treatment with chondroitinase AC,

as shown in Fig. 3B. These results suggest that the RCA binding sites of cells are covered with chondroitin sulfate A and the ConA binding sites are not. It is unclear whether the exposure of the binding sites for RCA on the cell surface is solely due to the removal of chondroitin sulfate A. However, the removal of chondroitin sulfate A is at least a trigger for the subsequent changes occurring in the cell membranes.

As reported elsewhere (10), trypsin released N-glycosidic glycopeptides, but no chondroitin sulfate A from AH 130 FN cells. This contrasts with the preferential release of heparan sulfate and insusceptibility of N-glycosidic glycoproteins on tryptic digestion of another type of ascites hepatoma cells, AH 66, which contain heparan sulfate instead of chondroitin sulfate A as a component of plasma membranes. After the trypsin treatment, practically no change was observed in the binding capacities of these cells for RCA. Slight decrease in the maximum amounts of bound ConA after trypsin treatment of AH 130 FN cells may be due to the release of N-glycosidic glycopeptides, the binding sites for ConA, from the cells.

There are several reports that demonstrate the occurrence of mucopolysaccharide in plasma membranes of animal cells by examining the products of treatments of the cells with proteolytic enzymes or mucopolysaccharide-degrading enzymes (11 - 15). However, this is the first to show that mucopolysaccharide is involved in cell behaviour.

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