Analyses of Rabbit Platelet Proteo-Chondroitin Sulfates Localized on the Surface and in the Granules

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(Received June 10, 1991)

Platelet proteo-chondroitin sulfates localize on the surface and in the granules. Western blotting method is able to selectively detect core-proteins prepared from proteo-chondroitin sulfates by chondroitinase ABC digestion. A very highly sensitive high-performance liquid chromatographic method was established for the determination of chondroitin sulfates as their unsaturated disaccharides. These methods were applied to rabbit platelet proteo-chondroitin sulfates. It was found that both rabbit platelet surface proteo-chondroitin sulfate and granule proteo-chondroitin sulfate consist of a predominantly homogeneous chondroitin 4-sulfate and a single core-protein having a molecular weight of approximately 26 kDa.

Keywords — proteoglycan; chondroitin sulfate; platelet; Western blot analysis; unsaturated disaccharide; core-protein

Introduction

Platelets have proteo-chondroitin sulfates (proteo-ChS) on their surface coats¹⁾ and in their granules.^{2,3)} The surface proteo-ChS was liberated during adenosine diphosphate (ADP)-induced aggregation and the granule proteo-ChS was released when the platelets were exposed to thrombin.⁴⁾ This is why concentrations of glycosaminoglycans (GAGs) in mammalian serum are higher than those in plasma.⁵⁻⁷⁾ It is demonstrated that surface proteo-ChS is an effective shield for distinct platelet receptors⁸⁾ and/or exerts an important action on the calcium metabolism of platelets,⁹⁾ and that granule proteo-ChS is a carrier for platelet factor 4 released from platelets.¹⁰⁾ Ward and Packham¹¹⁾ have shown that the surface and granule GAGs of rabbit platelets consist of 90% chondroitin 4-sulfate and 83% chondroitin 4-sulfate, respectively. It is an interesting question whether the surface proteo-ChS differs from granule proteo-ChS.

In this paper, we studied Western blotting methods for the core-proteins of proteo-ChS using anti-unsaturated disaccharide monoclonal antibodies. By using these methods and a high-performance liquid chromatographic (HPLC) method for the determination of unsaturated disaccharides established by us,¹²⁾ we compared

surface proteo-ChS with granule proteo-ChS not only with respect to GAGs but also to proteoglycans (PG).

Materials and Methods

Reagents and Materials — Standard unsaturated disaccharide, 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-D-glucose (Δ Di-HA), 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-D-galactose (Δ Di-0S), 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-4-*O*-sulfo-D-galactose (Δ Di-4S), 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-6-*O*-sulfo-D-galactose (Δ Di-6S), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo- β -D-gluc-4-enepyransyluronic acid)-4-*O*-sulfo-D-galactose (Δ Di-diS_B), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo- β -D-gluc-4-enepyransyluronic acid)-6-*O*-sulfo-D-galactose (Δ Di-diS_D) and 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-4,6-di-*O*-sulfo-D-galactose (Δ Di-diS_E), and chondroitinase ABC were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Anti-unsaturated disaccharide monoclonal antibody (Δ Di-4S, clone 2-B-6) was purchased from ICN Biomedical Inc. (IL, U.S.A.). Thrombin, pepstatine and apyrase were purchased from Sigma (MO, U.S.A.). Polyvinylidene difluoride

(PVDF) membrane was purchased from Millipore (MA, U.S.A.). All other chemicals were of reagent grade. Male albino rabbits (3–5 kg, Takasugi exp. animals, Co., Saitama) used in this study were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (CE-2, Clea Japan, Inc., Tokyo).

Preparation of Surface Proteo-ChS and Granule Proteo-ChS from Rabbit Platelets —

Surface proteo-ChS and granule proteo-ChS were prepared from rabbit platelets by a modification of the method of Ward and Packham¹¹⁾ as follows; One volume of 3.8% sodium citrate solution was added to 9 vol. of blood and then the mixture was centrifuged at $200 \times g$ for 15 min. The supernatant was centrifuged at $1200 \times g$ for 15 min. After washing platelets with 33 mM phosphate buffer solution containing 113 mM sodium chloride and 5.5 mM glucose (pH 6.5), platelets were suspended in Tyrode solution containing apyrase. Platelet suspensions were warmed at 37 °C and ADP (final concentration 500 μ M) was added with rapid stirring. After 5 min, the aggregated platelets were removed by centrifugation at $1200 \times g$ for 15 min. The supernatant contains surface proteo-ChS. The platelet pellet was resuspended in Tyrode solution containing apyrase and incubated at 37 °C for 30 min. To induce the release of granule contents, thrombin was added to the platelet suspension with rapid stirring at a final concentration of 2 unit/ml. After 5 min, one vol. of 0.1 M phenylmethylsulfonyl fluoride (PMSF) in methanol and 10 vol. of 0.2 M ethylenediaminetetraacetic acid (EDTA) solution containing 50 mM *N*-ethylmaleimide (NEM) were added to 100 vol. of the platelet suspension, and the mixture was centrifuged at $1200 \times g$ for 15 min. The supernatant contains granule proteo-ChS. The surface proteo-ChS solution and the granule proteo-ChS solution were concentrated to 50–100 μ g/ml of chondroitin sulfates by Ultracent-10 (Tosoh Co., Tokyo, Japan). We measured the platelet factor 4 activity in the surface fractions according to the method described by Harada and Zucker¹³⁾ with a slight modification.¹⁴⁾ By this method, it was recognized that ADP did not induce the release of the α -granule

contents from rabbit platelets.

Simultaneous Determination of Unsaturated Disaccharides — Unsaturated disaccharides were measured by a sensitive and specific HPLC method which we established.¹²⁾ The chromatographic conditions were as follows: column, TSK gel NH₂-60 (4.6 mm i.d. $\times$ 250 mm); eluent, 57% acetonitrile in aqueous buffer (0.1 M Tris, 0.1 M boric acid and 12 mM Na₂SO₄ adjusted to pH 7.5 with conc. HCl) at a flow rate of 0.5 ml/min.

Cellulose Acetate Membrane Electrophoresis of GAGs — GAGs were prepared from proteo-ChS by the method of Emura and Mukuda.¹⁵⁾ Electrophoresis was carried out in 0.3 M calcium acetate at 1.0 mA/cm for 3 h on a cellulose acetate strip. A strip was stained with 0.1% alcian blue in 0.1% acetic acid.

Western Blot Analysis of Core-Proteins — SDS-polyacrylamide gels (10–20% gradient gel and 4–20% gradient gel, 84 mm (w) $\times$ 90 mm (h) $\times$ 1.0 mm) were used. Digestion of proteo-ChS by chondroitinase ABC was carried out as follows; A sample solution (20 μ l) containing protease inhibitors (EDTA, NEM and PMSF) was mixed with 20 μ l of 1 unit/100 μ l chondroitinase ABC (protease-free) in 0.1 M Tris-acetic acid (pH 8.0) and the solution was held at 37 °C for 2 h. One volume of sample solution containing core-proteins was mixed with an equal vol. of 20 mM Tris-HCl buffer (pH 7.0) containing 40% (w/v) glycerol, 2% (v/v) mercaptoethanol and 2% (w/v) SDS, and the mixture was held in boiling water for 2 min. Electrophoresis was performed in 25 mM Tris–192 mM glycine solution (pH 8.3) containing 0.1% sodium dodecyl sulfate (SDS) at 60 mA. Transfer to a PVDF membrane was performed in the cold with 25 mM Tris–192 mM glycine solution (pH 8.3) containing 20% methanol in a transblot cell at 30 V for 16 h. After blocking with 3% bovine serum albumin (BSA) in 25 mM phosphate buffer saline solution, pH 7.4 (PBS) for 1 h, the membrane was exposed to anti-unsaturated disaccharide monoclonal antibody diluted 300 times with 3% BSA in PBS. The monoclonal antibody on the membrane was stained by Vectastain ABC kit (Vector Lab., CA, U.S.A.).

Fig. 1. Chromatograms of Unsaturated Disaccharides Produced Enzymatically from Rabbit Platelet Surface and Granule Proteo-Chondroitin Sulfates

A, Standard mixture of Δ Di-HA, Δ Di-0S, Δ Di-6S, Δ Di-4S, Δ Di-diS_D, Δ Di-diS_B and Δ Di-diS_E (concentration of each unsaturated disaccharide was 10 μ g/ml); B, unsaturated disaccharides produced from surface proteo-ChS; C, unsaturated disaccharides produced from granule proteo-ChS. Peaks: 1, Δ Di-HA; 2, Δ Di-0S; 3, Δ Di-6S; 4, Δ Di-4S; 5, Δ Di-diS_D; 6, Δ Di-diS_B; 7, Δ Di-diS_E.

Results and Discussion

Analysis of GAG Chains of Surface Proteo-ChS and Granule Proteo-ChS

Surface proteo-Chs and granule proteo-ChS were prepared from rabbit platelets according to the modified method of Ward and Packham,¹¹⁾ and digested to core-proteins and unsaturated disaccharides by chondroitinase ABC. Figure 1 shows analytical results of unsaturated disaccharides by the HPLC method. Both unsaturated disaccharides from surface proteo-ChS and granule proteo-ChS consist of at least 95% Δ Di-4S and a small percentage of Δ Di-0S and Δ Di-6S. However, di-sulfated unsaturated disaccharides (Δ Di-diS_D, Δ Di-diS_B, Δ Di-diS_E) were not detected.

Δ Di-4S was produced from both chondroitin 4-sulfate (chondroitin sulfate A) and dermatan sulfate (chondroitin sulfate B) by chondroitinase ABC digestion. Chondroitinase ACII or chon-

Fig. 2. Electrophoretic Patterns of Glycosaminoglycans from Rabbit Platelet Surface and Granule Proteo-Chondroitin Sulfates

Electrophoresis was carried out in 0.3 M calcium acetate at 1.0 mA/cm for 3 h on a cellulose acetate strip. A strip was stained with 0.1% Alcian Blue in 0.1% acetic acid.

droitinase B digestion of the PG, and HPLC analysis of the generated unsaturated disaccharides indicated that dermatan sulfate was not present on platelet surface and in platelet granules (results are not shown). To examine species of GAGs on surface and in granules, GAG chains were prepared through alkali-degradation, protease digestion, cetylpyridinium chloride-precipitation and ethanol-precipitation. Cellulose acetate membrane electrophoretograms of these GAGs are shown in Fig. 2. Both GAGs from surface proteo-ChS and granule proteo-ChS migrated to the same location as chondroitin 4-sulfate. These experiments confirmed the identity of the GAG chain of surface proteo-ChS and that of granule proteo-ChS as predominantly homogeneous chondroitin 4-sulfate.

Western Blot Analysis of Core-Proteins of Surface Proteo-ChS and Granule Proteo-ChS

Fig. 3. Chondroitinase ABC Digestion of Proteo-Chondroitin Sulfates

Fig. 4. Western Blot Analysis of Core-Proteins Produced from Rabbit Platelet Surface and Granule Proteo-Chondroitin Sulfates by Chondroitinase ABC Digestion

Panel A, Coomassie Brilliant Blue staining; panel B, immunostaining by anti- Δ Di-4S antibody. 1, Core-protein from surface proteo-ChS; 2, core-protein from granule proteo-ChS; 3, reagent blank (mixture of 2 units/100 μ l apyrase and 100 units/100 μ l thrombin treated with chondroitinase ABC). Arrows show core-proteins.

The GAG chains in proteo-ChS are digested by chondroitinase ABC as shown in Fig. 3. The disaccharide unit which is the closest to the oligosaccharide (glucuronosyl-(galactosyl)₂-xylose) remains on the core-protein as an unsaturated disaccharide.¹⁶⁾ The core-proteins were prepared from surface proteo-ChS and granule proteo-ChS by the chondroitinase ABC digestion in the presence of protease inhibitors such as EDTA, NEM and PMSF, and submitted to SDS-PAGE (10—20% gradient gel). After transfer to PVDF membrane, the core-proteins were selectively immunostained by the use of anti- Δ Di-4S monoclonal antibody (clone, 2-B-6). Electrophoretograms were shown in Fig. 4. Both the core-proteins of surface proteo-ChS and granule proteo-ChS migrated to the location of 26 kDa as a single band, and were immunostained by anti- Δ Di-4S antibody. However, these core-proteins did not stain well with Coomassie Blue.

As described above, platelet surface proteo-ChS have a predominantly homogeneous chondroitin 4-sulfate (sulfation ratio was more than 95% in the disaccharide unit). The core-protein of surface proteo-ChS is a single chain and its molecular weight is determined to be approximately 26 kDa. On the other hand, the molecular weight of core-protein, and the constitution of GAG chains of granule proteo-ChS exactly agreed with those of surface proteo-ChS.

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