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A PRELIMINARY ANALYSIS OF PLATELET VON WILLEBRAND FACTOR OLIGOSACCHARIDES

KAZUO KAGAMI, SYBIL WILLIAMS, MCDONALD HORNE and HARVEY GRALNICK

Hematology Service, Clinical Pathology Department, Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, MD, 20892, U.S.A.

ABSTRACT

Little is known about the carbohydrate structure of the platelet von Willebrand factor (vWf). We have analyzed N-linked oligosaccharides from both plasma and platelet vWf by Fluorophore-Assisted-Carbohydrate Electrophoresis (FACE™) and isoelectric focusing before and after digestion of the proteins with neuraminidase. We observed a greater variety of N-linked oligosaccharides derived from plasma vWf than from platelet vWf. Neuraminidase treatment reduced the oligosaccharides from both proteins to only two or three bands and produced significant increases in their isoelectric points.

Key Words: von Willebrand factor, oligosaccharides, platelets

INTRODUCTION

Although the carbohydrate of plasma von Willebrand factor (vWf) has been extensively studied,[1-3] much less is known about the glycosylation of platelet. By weight about 15% of the plasma protein is carbohydrate is distributed in 12 N-linked and 10 O-linked oligosaccharides in each vWf subunit.[4] All that is known about the carbohydrate of platelet vWf is that it includes approximately half as much sialic acid and galactose as the plasma protein.[5]

Here we report our initial efforts to characterize the N-linked carbohydrate and the isoelectric point of platelet vWf.

MATERIALS AND METHODS

1. Samples

Intermediate purity human plasma factor VIII/vWf was obtained from the American National Red Cross, Bethesda, MD. Platelet vWf was prepared from 14–18 units of fresh human platelets derived from multiple donors in the Department of Transfusion Medicine at the National Institutes of Health, Bethesda, MD.

2. Purification of Proteins

Platelet vWf was purified (purity > 92%) as previously described.[5] Plasma vWf was purified (purity > 95%) by precipitation with polyethylene glycol followed by gel chromatography on Sepharose 4B.[6] Both oligosaccharide analyses were done using 4 different purified samples of

Address: McDonald Horne, M.D., Hematology Service, Warren G. Magnuson Clinical Center, Room 2C390, Bethesda, MD, 20892 U.S.A., Phone: 01-301-496-6891 Fax: 01-301-402-2046

vWf. Protein concentrations were measured by the bicinchoninic acid method using bovine serum albumin as a standard (BCA™ Protein Assay Kit, Pierce, Rockford, IL).

3. Oligosaccharide Analysis

N-linked oligosaccharides for both plasma and platelet vWf were analyzed by Fluorophore-Assisted-Carbohydrate Electrophoresis (FACE™, Glyko, Inc., Novato, CA). Oligosaccharides were released from the purified glycoproteins by digestion with peptide N-glycosidase F. The freed oligosaccharides were labeled with fluorophore (8-aminonaphthalene-1, 3, 6-trisulfonic acid) and then electrophoresed through polyacrylamide gels, following directions provided by the manufacturer. Each electrophoresis included a lane loaded with a standard ladder of glucose polymers. A G4 band contains 50 picomoles of a fluorophore-labeled maltotetraose, which served as a standard to allow quantification of the other oligosaccharide bands. In some experiments, plasma and platelet vWf were treated with NANase III (Glyko, Inc.) in order to remove all the sialic acid before releasing the oligosaccharide with N-glycosidase F. The enzyme was used according to the manufacturer's instructions. Both analysis were done at least 4 times.

Gels were imaged and photographed using the UV light (366nm, 8W) Foto/Analyst Visionary™ with Video Copy Processor P40U by Mitsubishi (FOTODYNE Inc., Hartland, WI). Densitometry was performed with the Adobe photoshop 2.5.1 software (Adobe Systems, Inc., San Jose, CA).

4. Urea-Agarose Isoelectric Focusing

Either intact or asialo plasma vWf or platelet vWf was used in the experiments. One unit of neuraminidase was used to digest one gram of protein (Type X: from Clostridium perfringens, Sigma Chemical Co., St. Louis, MO).

Isoelectric focusing (IEF) was performed using an LKB 2117 Multiphore apparatus for flat bed IEF (LKB Instruments Inc., Los Angeles, CA) equipped with an LKB 2197 power supply and an LKB 2209 Multitemp as a water circulator. The method was based on the method used by Fulcher et al.[7]

IEF gels were run in the 4.0–6.5 pH range and they were prepared as directed by LKB. The plates were dialyzed in the dark at 4°C for 1 hour in 150 ml of freshly made 7.8M ultrapure urea (ICN Biomedicals, Inc. Aurora, OH), 10% Sorbitol and 6% ampholyte. At the end of this time the gel was gently blotted with IEF filter paper and placed on the electrophoresis chamber. Samples were diluted in the same urea solution used to equilibrate the gel and 5 ml of each sample was placed on the plate. Carbonic anhydrase (pI range 4.8–6.7) was applied as a standard for pI calibration. The electrophoresis was run as follows: 20 min at 100V; 60 min at 200V; and finally 60 min at 2500V.

Each gel (or part of it) was either stained or fixed in 250 ml of a 25% isopropanol/10% acetic acid fixing solution for 1 hr. The fixing solution and urea were then removed from the gel using a modification of the technique described by Hoyer and Shainoff.[8] vWf in the fixed gel was identified by autoradiography using a ^{125}I labeled polyclonal antibody against vWf. The stained gels were used to measure the IEF points of the standard. Each analysis was repeated 5 times.

RESULTS

1. Oligosaccharide patterns

The patterns of N-linked oligosaccharides derived from platelet and plasma vWf were differ-

ent (Fig. 1). Ten carbohydrate bands could be resolved by densitometry of the electrophoresed plasma vWf oligosaccharides, whereas only 6 bands could be detected for the platelet vWf (Fig. 2). By comparing the area under each peak with the fluoresence intensity of the standard,

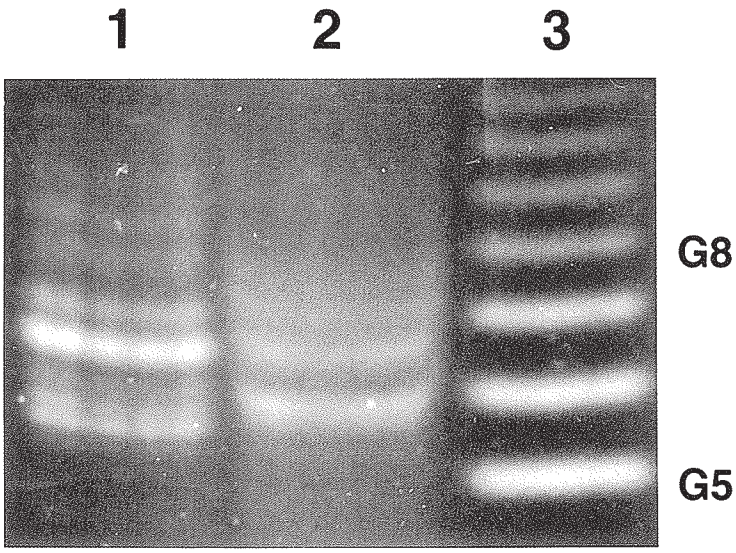

Fig. 1. Electrophoresis of N-linked oligosaccharides released from plasma vWf (Lane 1) and platelet vWf (Lane 2) labeled with fluorophore. Lane 3 contains glucose polymers of the size indicated.

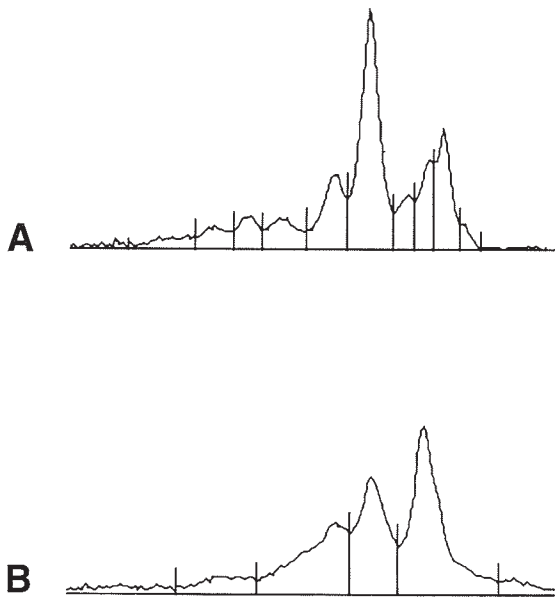

Fig. 2. Densitometry of the oligosaccharide bands shown in Figure 1: (A) from plasma vWf and (B) from platelet vWf.

it was possible to estimate the total number of picomoles of oligosaccharide contained in each electrophoresed sample. According to these calculations, 47 mg of plasma vWf yielded 670 picomoles of oligosaccharide, whereas 140 mg of platelet vWf yielded 700 picomoles of oligosaccharide.

2. Effect of Neuraminidase digestion

Digestion of plasma and platelet vWf with neuraminidase led to marked changes in the oligosaccharide bands of both proteins (Fig. 3). One band dominated in the plasma vWf sample, whereas two bands of similar intensity were derived from the platelet vWf.

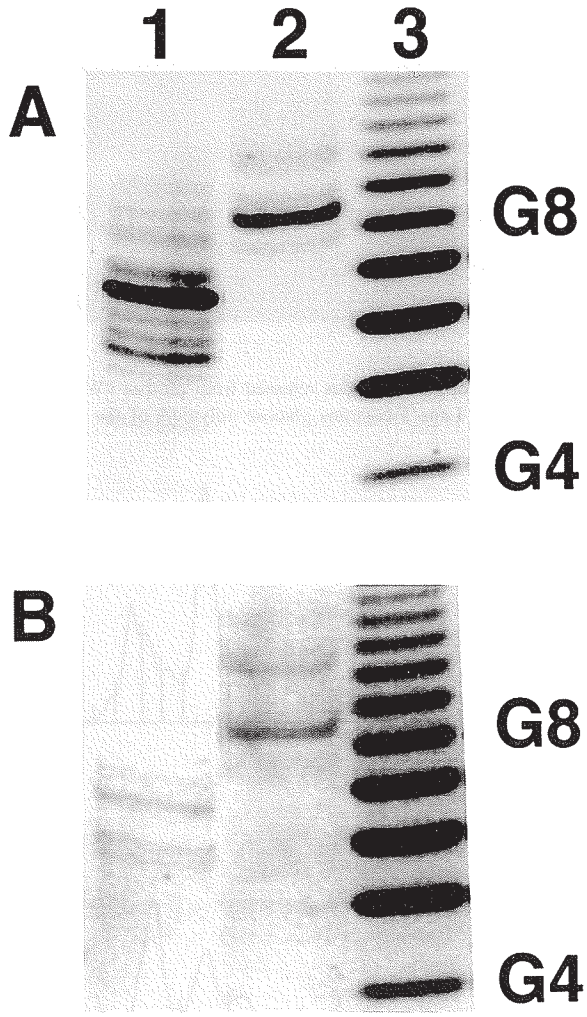

Fig. 3. Electrophoresis of N-linked oligosaccharides from plasma vWf (upper panel) and platelet vWf (lower panel) before (1) and after (2) digestion with neuraminidase. Lane 3 shows a series of glucose polymers for reference.

3. Results of isoelectric focusing

Fig. 4 shows isoelectric focusing of platelet vWf (pI 4.90–4.95), plasma vWf (pI 5.0–5.1), asialo platelet vWf (pI 5.60–5.70), and asialo plasma vWf (pI 5.65–5.70). Although the bands for the native proteins appeared diffuse, two distinct bands could be distinguished for each of the asialo proteins.

DISCUSSION

Our analyses have disclosed several structural differences between the carbohydrate of plasma and platelet vWf. A greater variety of N-linked oligosaccharides was derived from the plasma protein than the platelet protein (Fig. 1). However, treatment with neuraminidase reduced the number of separable oligosaccharides from both proteins to only two or three. It also produced significant increases in the isoelectric points of the proteins (Fig. 4). This suggests that much of the observed oligosaccharide heterogeneity is due to variable sialylation.

Furthermore, the total amount of N-linked carbohydrate was less in the platelet vWf samples than in plasma vWf. Plasma vWf yielded ~14 picomoles of oligosaccharide per microgram of protein, whereas only ~5 picomoles of oligosaccharide per microgram of protein were derived from platelet vWf. This value for plasma vWf, however, is low compared with the published information indicating there are probably 12 N-linked oligosaccharides per monomeric subunit.[4)]

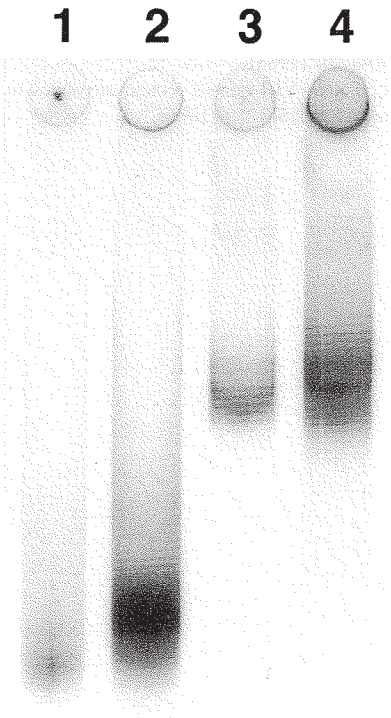

Fig. 4. IEF of (1) platelet vWf, (2) plasma vWf, (3) asialo platelet vWf and (4) asialo plasma vWf.

We found only ~3.5. This could be explained by incomplete enzymatic release of the oligosaccharide chains from the protein, by subtotal solubility of the digested sample for electrophoresis, or by fluorophore labeling inefficiency. If we assume that the technical limitations applied equally to both the plasma and the platelet proteins, however, we can still conclude that platelet vWf contains only about 33% as much carbohydrate as plasma vWf.

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