Role of carbohydrate in multimeric structure of factor VIII/von Willebrand factor protein
(asialo factor VIII/von Willebrand factor/asialo-agalacto factor VIII/von Willebrand factor)

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ABSTRACT The carbohydrate moiety of the factor VIII/von Willebrand (vW) factor protein is important in the expression of vW factor activity and the intravascular survival of the protein. Studies of normal human factor VIII/vW factor protein indicate that there is a requirement of a full complement of penultimate galactose for the maintenance of a normal multimeric structure. Release of penultimate galactose by β-galactosidase or modification by galactose oxidase results in loss of the largest molecular weight multimers and increased numbers of intermediate and smaller multimers. In contrast, terminal galactose on the factor VIII/vW factor protein does not appear to play a significant role in the maintenance of the multimeric structure. Anomalies in multimeric structure and molecular size were demonstrated by NaDodSO4/polyacrylamide/agarose gel electrophoresis, NaDodSO4/glyoxyl-agarose electrophoresis, and sucrose density ultracentrifugation. These studies indicate that the penultimate galactose plays a role in the maintenance of the largest multimers of the factor VIII/vW factor protein. This may explain why, in some patients with variant forms of vW disease, a carbohydrate abnormality also may affect the multimeric structure of the plasma factor VIII/vW factor protein.

The factor VIII/von Willebrand (vW) factor protein circulates as a series of oligomers with molecular weights estimated to be from $\sim 1.0 \times 10^6$ to $20 \times 10^6$. The subunit(s) of these oligomers has a molecular weight of $\sim 230,000$ (1, 2). The mechanism of the formation of the multimers is thought to be primarily dependent on disulfide bond linkages (3). Subunits combine to form dimers, and these dimers combine by subsequent disulfide bonds, resulting in a series of oligomers of various molecular weights. Within the oligomer populations there are significant functional differences because the vast majority of the vW factor activity both in vivo and in vitro appears to be associated with the largest multimers (4, 5). This has been demonstrated both in studies of biochemical alterations in the multimeric structure of the normal factor VIII/vW factor protein and in descriptions of the abnormalities of the factor VIII/vW factor proteins in patients with vW disease (4, 5). Further, it has been demonstrated that the binding of the vW factor protein to platelet and subsequent agglutination of platelets is related preferentially to the presence of the largest multimers compared to the intermediate or smaller multimers (4, 5). Previous reports have elucidated the importance of penultimate galactose in the expression of vW factor activity (6, 7), but these studies did not examine the multimeric structure.

We report here that the largest multimers of the normal factor VIII/vW factor protein are dependent on a full complement of carbohydrate because removal of the penultimate galactose of the factor VIII/vW factor protein or its modification by galactose oxidase results in the disappearance of the largest multimers, with a concomitant increase in intermediate and smaller multimers and a decrease in vW factor activity. These studies suggest that some of the defects of the multimeric structure described in variant forms of vW disease may be related to a decreased carbohydrate content of the protein.

MATERIALS AND METHODS

The factor VIII/vW factor protein was purified from cryoprecipitate obtained from normal blood donors by column chromatography on Sephrose 4B as described (5). The material was judged to be >95% free of other proteins by NaDodSO4/polyacrylamide gel electrophoresis with and without the presence of reducing agents. The specific activities associated with the factor VIII/vW factor protein were: vW factor, 126 units/mg; factor VIII-related antigen (Ag), 143 units/mg; and factor VIII-coagulant activity, 45 units/mg. Protein concentration was estimated by the method of Lowry (8).

Pooled normal plasma was obtained from 30 normal donors who were not taking medication. The plasma was pooled and stored at $-70^\circ$C. Plasma from two patients with severe vW disease (factor VIII coagulant activity, <5%; vW factor, <5%; and factor VIII-related Ag, <5%; bleeding time, >30 min) was obtained and stored identically to normal plasma.

Assays for factor VIII coagulant activity, factor VIII-related Ag, and vW factor and crossed antigen–antibody electrophoresis were performed as described (9).

Electrophoresis. NaDodSO4/glyoxyl-agarose electrophoresis was performed by the method of Hoyer and Shainoff (10). The antibody used was either 125I-labeled goat or rabbit anti-human factor VIII/vW factor protein antibody (9). Acrylamide/agarose electrophoresis was performed by using 2% acrylamide/0.5% agarose and was modified as described by Perret et al. (11). Electrophoresis was carried out at 8 mA per gel. The gel length was 120 mm, and the bromophenol blue marker dye usually reached the bottom within 5 hr ± 20 min.

Ultracentrifugation was performed in a Beckman L265B ultracentrifuge. A 40-m1 linear 10–40% (wt/vol) sucrose gradient was prepared in imidazole/saline buffer (0.02 M imidazole/0.14 M NaCl, pH 7.2) and was centrifuged at 100,000 × g at 4°C for 17 hr. Fifty micrograms of factor VIII/vW factor labeled with $^3$H or 50 μg of carbohydrate-modified factor VIII/vW factor labeled with $^3$H was placed in 2 ml of imidazole/saline buffer or plasma from patients with severe vW disease. The intact and carbohydrate-modified factor VIII/vW factor proteins were subjected to ultracentrifugation simultaneously. The bottom of each tube was punctured, and 1-ml aliquots were analyzed for

Abbreviations: vW, von Willebrand; Ag, antigen.

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either factor VIII/vW factor-related radioactivity or for the presence of the factor VIII-related Ag by Laurell assay. Terminal galactose of the factor VIII/vW factor protein was radiolabeled with potassium boro[3H]hydride (New England Nuclear) as described (5).

Carbohydrate Modification of the Factor VIII/vW Factor Protein. The glycosidases used were (i) neuraminidase, which was prepared from Clostridium perfringens (Worthington), purified by affinity chromatography as described (6), and stored in acetate buffer (pH 5.5) containing 3% bovine serum, and (ii) β-galactosidase, which was prepared from the Streptococcus pneumoniae cultures as described (6) (gift of G. Ashwell). The galactose oxidase was purified as described (6). All enzymes were tested for protease activity by incubation with [14C]globin, by incubation with the intact factor VIII/vW factor protein, and by measuring factor VIII coagulant activity (6).

The factor VIII/vW factor protein was incubated with the various enzymes as follows: (i) neuraminidase was at 0.02–0.03 units per mg of factor VIII/vW factor protein (1 unit of enzyme is defined as the amount that releases 1 μmol of sialic acid per min at 37°C from α, glycoprotein); (ii) β-galactosidase was at 0.002–0.004 units per mg of factor VIII/vW factor protein; and (iii) galactose oxidase was at 40 μg per mg of factor VIII/vW factor protein, and the horseradish peroxidase in this mixture was at 10 μg of enzyme per mg of factor VIII/vW factor protein. All incubations were performed at 37°C for various periods of time. Intact factor VIII/vW factor protein to which buffer was added and incubated for identical time periods served as a control for all experiments described.

Carbohydrate Assay Procedures. The total sialic acid was measured after hydrolysis of the factor VIII/vW factor protein in 0.05 M H2SO4 at 80°C for 60 min by utilizing N-acetylneuraminic acid as the standard (12). Free sialic acid was measured after treatment with the Clostridium perfringens neuraminidase. Penultimate galactose was measured by the enzymatic action of β-galactosidase on the asialo factor VIII/vW factor protein, forming asialo-agalacto protein, and the free galactose was measured in an assay with galactose dehydrogenase (13). Total galactose was determined by hydrolysis in 2 M hydrochloric acid at 100°C for 90 min. After hydrolysis of the intact factor VIII/vW factor protein, the sample was evaporated to dryness, reconstituted in half the original volume in water, reevaporated, and resuspended. The sample was then assayed for free galactose as described above.

RESULTS

The sialic acid content of the factor VIII/vW factor protein was 145 ± 12 nmol/mg or 33 mol per 230,000-dalton subunit (n = 6). Treatment with neuraminidase for 2 hr released >93% of the sialic acid (n = 6). Total galactose measured by acid hydrolysis was 205 ± 18 nmol/mg of factor VIII/vW factor protein or 47 mol per subunit (n = 6). Treatment of the intact factor VIII/vW factor protein with β-galactosidase resulted in the release of 39 ± 6 nmol/mg of factor VIII/vW factor protein or 9 mol per subunit. Treatment of the asialo factor VIII/vW factor protein with β-galactosidase released 171 ± 20 nmol/mg of factor VIII/vW factor protein or a total of 39 mol per subunit. Of the 171 nmol of galactose released, 132 nmol/mg or 30 mol per subunit were penultimate and 39 nmol/mg or 9 mol per subunit were terminal. Thus, 34 nmol/mg or 8 mol per subunit of the total galactose could not be released by β-galactosidase.

Treatment of the intact factor VIII/vW factor protein with neuraminidase, β-galactosidase, or galactose oxidase did not result in the loss of procoagulant or vW factor activity. However, treatment of the asialo factor VIII/vW factor protein with β-galactosidase resulted in a time-dependent decrease of vW factor activity that correlated well with the release of the penultimate galactose (Fig. 1). Treatment of the asialo factor VIII/vW factor protein with galactose oxidase also resulted in a time-dependent decrease in vW factor activity (data not shown). All of the enzymes used in this report were free of protease activity.

Examination of the results of the multimeric structure of the factor VIII/vW factor protein in these studies revealed marked differences between the material before and after removal or modification of the penultimate galactose. Agarose/acylamide electrophoresis of the purified protein clearly demonstrated the loss of the slowest migrating bands and the largest multimers in the asialo-agalacto material. These studies were confirmed by using glyoxyl-agarose electrophoresis in which asialo-agalacto factor VIII/vW factor definitely showed the loss of the largest multimers (Fig. 2). Not only did the largest multimeric forms of the asialo-agalacto protein disappear, but an increase

![Fig. 1. Time course of vW factor activity. The intact (●), asialo (○), and asialo-agalacto (□) factor VIII/vW factor proteins were tested for vW factor activity over 24 hr. The vW factor activity of the asialo and the intact factor VIII/vW factor proteins did not significantly change. Thirty-nine nanomoles per mg of galactose were released from the intact factor VIII/vW factor protein, and the penultimate galactose (△) released from the asialo factor VIII/vW factor protein treated with β-galactosidase at 1 hr was 33 nmol/mg, at 2 hr was 47 nmol/mg, at 4 hr was 63 nmol/mg, at 6 hr was 73 nmol/mg, and at 24 hr was 132 nmol/mg.](image-url)
of antibody-radiolabeling of the intermediate and smaller multimers was apparent in the asialo-agalacto factor VIII/v W factor protein (Fig. 3). In comparison, the intact factor VIII/v W factor protein treated only with β-galactosidase (and not neuraminidase) did not change. Similar results were seen when galactose oxidase was incubated with asialo factor VIII/v W factor protein (Fig. 3). In contrast, the galactose oxidase did not change the multimer composition of intact factor VIII/v W factor protein (data not shown).

The above-mentioned change(s) in multimeric structure were determined in the presence of NaDodSO₄. In an effort to determine whether one would see the same alterations of multimeric structure of the asialo-agalacto factor VIII/v W factor protein without the use of NaDodSO₄ ultra centrifugation was performed of the asialo, the asialo-agalacto, and the intact factor VIII/v W factor protein, and the intact factor VIII/v W factor protein treated with β-galactosidase. As shown in Fig. 4, there was a marked shift of the asialo-agalacto factor VIII/v W factor protein during ultracentrifugation, indicating that there had been a loss of the largest molecular weight structures and an increase in the intermediate and smaller molecular weight structures (Fig. 4). Analysis of the intact factor VIII/v W factor and asialo protein revealed almost identical patterns when monitored by radioactivity per tube or by factor VIII-related Ag per tube.

DISCUSSION

The carbohydrate moiety(ies) of the human factor VIII/v W factor protein has been shown to be important in the expression of vW factor activity (6, 7) and the intravascular survival (14). Removal of >90% of the sialic acid of the factor VIII/v W factor protein has been reported by some to result in a loss of ~50% vW factor activity (14), while other studies have indicated no change in vW factor activity after desialylation (6, 15). However, there is agreement concerning the role of the penultimate galactose in the expression of vW factor activity of the factor VIII/v W factor protein. Removal of penultimate galactose with β-galactosidase or modification with galactose oxidase results in >85% loss of vW factor activity (6, 7).

The only studies involving the role of carbohydrate in factor VIII/v W factor survival involve the infusion of human material into dogs (14). In these studies, infusion of human asialo factor VIII/v W factor in dogs showed a shorter intravascular survival than that of the intact protein. The studies presented here add another dimension to the role of carbohydrate in factor VIII/v W factor structure or function, or both.

We have shown that the integrity of the largest multimers of the factor VIII/v W factor protein are dependent on penultimate galactose. Removal of the penultimate galactose of the factor VIII/v W factor protein results in a dramatic change in the multimeric structure. The asialo-agalacto factor VIII/v W factor shows a loss of the larger molecular weight oligomers, a decrease in the smaller molecular weight oligomers, and a decrease of vW factor activity. These changes of multimeric structure are related to the amount of galactose released (Fig. 3). In contrast, the procoagulant activity did not change after any of the enzyme modifications of the factor VIII/v W factor protein.

We have demonstrated these changes in molecular size of the multimers by agarose/acylamide gel electrophoresis and glyoxylagarose electrophoresis with NaDodSO₄ and by ultracentrifugation without the presence of any denaturing agent. When the intact factor VIII/v W factor protein was treated with the neuraminidase, in 8 of 11 studies the asialo factor VIII/v W factor protein showed no change in its vW factor activity or multimeric structure as assessed by glyoxyl-agalacto gel electrophoresis or position after ultracentrifugation. Likewise, the subunit structure of the reduced protein in NaDodsO₄/polyacrylamide gel electrophoresis showed no change(s).

We considered the possibility that these findings were related to proteolysis of the factor VIII/v W factor protein by proteases contaminating the enzyme preparations. However, we could not detect protease activity utilizing the [125I]globin chain assay. Incubation of the β-galactosidase or galactose oxidase with the intact protein did not change the multimeric structure or the vW factor activity. These changes only occurred when these enzymes were added to the asialo factor VIII/v W factor. Other strong evidence against proteolysis is our finding that the factor VIII coagulant activity did not change after glycosidase treatment because this activity is quite susceptible to activation or destruction by proteases. Lastly, we found that the disappearance of the largest multimers from the agalacto factor VIII/v W factor protein was accompanied by an apparent increase in in-
TERMINAL and smaller multimers, and no bands of altered migration were seen (Figs. 2 and 3).

These changes in multimeric structure of the asialo-agalacto factor VIII/vW factor protein may be related to a form of ligand interaction between penultimate galactose residues within the factor VIII/vW factor protein, resulting in the formation of larger multimers; or there may be another protein not yet identified that is a structural protein to which the multimers are bound, in part, by penultimate galactose bonds. In either instance, alteration of the penultimate galactose would result in a decrease in the size of the multimers. These observations are important not only in understanding the formation of the largest oligomers in the factor VIII/vW factor protein but also in explaining some of the molecular defects that previously have been described in variants of vW disease.

We recently have described patients with vW disease whose factor VIII/vW factor protein is deficient in carbohydrate and lacks the largest multimers (16). Comparison of the multimeric structure of the vW disease factor VIII/vW factor protein with that of the normal factor VIII/vW factor protein after removal of sialic acid and penultimate galactose reveals a marked similarity. Thus, in some forms of vW disease, a deficiency of the penultimate galactose could result in the inability of the factor VIII/vW factor protein to form the largest multimers. This would be one mechanism for explaining the association of carbohydrate deficiency and an altered multimeric structure of the factor VIII/vW factor protein.

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