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Plasma Collagen Cofactor Correlates with von Willebrand Factor Antigen and Ristocetin Cofactor but Not with Bleeding Time

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Key words

Platelet – von Willebrand factor – Collagen – Collagen cofactor – Platelet adhesion

Summary

Collagen cofactor (CCo), an activity of von Willebrand factor (vWF) which increases the rate of adhesion of human fixed washed platelets (FWP) to collagen, was measured in plasma from normal individuals and individuals with von Willebrand's disease (vWD). CCo in vWD plasma was compared to vWF antigen (vWF:Ag), ristocetin cofactor (RCo), factor VIII (VIII) coagulant activity (VIII:C) and the quantitative bleeding time. There was close correlation between CCo and VIII:C (r = 0.909), vWF:Ag (r = 0.975), and RCo (r = 0.936). However, there was no correlation between CCo and the quantitative bleeding time. Plasma CCo in type IIA vWD was markedly lower than vWF:Ag and the ratio of CCo/vWF:Ag was 0.08, which was less than a mean value of 0.92 in type I vWD. CCo activity in normal plasma was completely inhibited by monoclonal antibody CLB-RAg 201, an antibody that inhibits the binding of vWF to collagen, suggesting that the binding of vWF to collagen is required for the expression of CCo. Furthermore, the partial inhibition of CCo by monoclonal antibody CLB-RAg 35 that inhibits the binding of vWF to platelet in the presence of ristocetin, suggests that CCo is partly mediated through platelet membrane glycoprotein Ib. Large multimers of vWF:Ag in normal plasma were preferentially absorbed by collagen. These studies demonstrate that CCo is another functional activity of vWF and the measurement of CCo may be useful for the detection of new variant forms of vWD.

Introduction

Von Willebrand factor (vWF), an adhesive glycoprotein synthesized by endothelial cells and megakaryocytes, is present in plasma as a series of multimers with molecular weight $0.5-20 \times 10^6$ (1, 2). vWF forms a dissociable complex with factor VIII (VIII) and acts as VIII coagulant activity (VIII:C) stabilizing factor (3). There is also evidence that vWF binds to the components of vascular subendothelium, collagen or microfibrils (4–7). We have found that vWF increases the rate of adhesion of human fixed washed platelets (FWP) to collagen (8). Recently, Perret et al. (9) have reported vWF dependent agglutination of FWP by fibrillar collagen that was extracted from bovine aorta by guanidine chloride, thus supporting our previous observations. This activity of vWF has been called vWF collagen cofactor (CCo). The relation of CCo to other properties of the vWF protein has not been described and the mechanism by which CCo mediates the adhesion of platelets to fibrillar collagen is unknown. In this report, we describe an assay for plasma CCo and examine the relationship between CCo and von Willebrand factor antigen (vWF:Ag), ristocetin cofactor activity (RCo), VIII:C and the quantitative bleeding time in individuals with von Willebrand disease (vWD). Inhibition studies with monoclonal antibody (MAb) to vWF, CLB-RAg 35 and CLB-RAg 201, suggest that the binding of vWF to collagen is necessary for the expression of CCo, and the activity is partly mediated through platelet glycoprotein Ib. This functional assay of vWF may be useful in detecting new variant forms of vWF with abnormal collagen binding domain.

Patients, Materials and Methods

Bovine serum albumin (BSA, crystallized and lyophilized) was obtained from Sigma Chemical Co., St. Louis, MO. Leupeptin was obtained from Peptide Institute Inc., Osaka, Japan. The buffer used in the adhesion experiments was 0.05 M cacodylate buffer containing 0.1 M NaCl, pH 7.3. MAbs to vWF, CLB-RAg 35 and CLB-RAg 201, were gift from Dr. van Mourik (Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). The characteristics of this antibody have been described previously (7). Other reagents were reagent grade and were obtained from Nakarai Chemical (Kyoto, Japan).

Patients

Ten unrelated patients with vWD were studied with their informed consent and all subjects were aware of the experimental nature of the studies. Eight of 10 patients were classified as type I, one as type IIA and one as acquired vWD secondary to an IgG monoclonal gammopathy (10). Platelet poor plasma was prepared as previously described (8) and stored at −80° C. One patient with severe type I vWD (VIII:C <0.01, vWF:Ag <0.03, RCo <0.03 u/ml) was infused with 1,000 units of a commercial VIII preparation (Confact F, Chemo-Sero-Therapeutic Res., Japan) for the treatment of epistaxsis. Plasma samples were collected before infusion and at 30, 60, 120 and 240 min after infusion.

Assay for RCo, vWF:Ag and VIII:C

RCo was measured by an aggregometric method (dual channel aggregometer, Payton Associates, Inc., Buffalo, NY) using FWP and ristocetin (Lundbeck Co., Copenhagen, Denmark) as previously described (11). vWF:Ag was measured by Laurell electro-immunoassay (12) using 1% Seakem ME agarose (FMC Corp., Rockland, ME) and 1:200 rabbit anti-human vWF:Ag which was prepared in our laboratory. VIII:C was measured by a one-stage method (13) using the plasma of a patient with severe hemophilia A as substrate. One unit of these activities was defined as that amount found in 1 ml of pooled normal human plasma.

Abbreviations:
von Willebrand factor (vWF) – von Willebrand's disease (vWD) – Ristocetin cofactor (RCo) – Collagen cofactor (CCo) – Monoclonal antibody (MAb)

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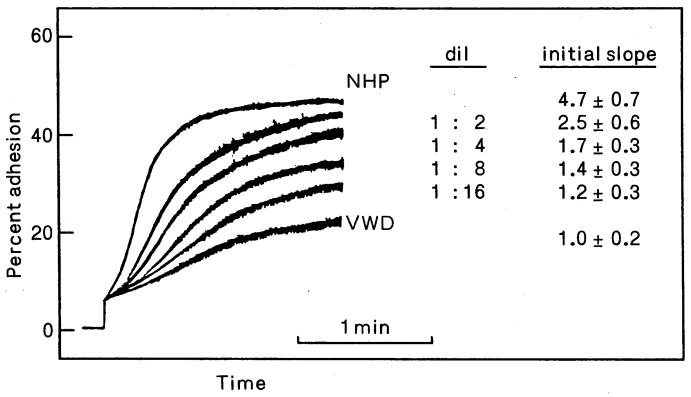


Fig. 1 Adhesion of FWP to collagen by aggregometric method. One tenth milliliter of collagen was added to a mixture of 0.2 ml of FWP and 0.2 ml of serial dilutions of normal human plasma or the plasma of severe type I vWD. Final concentration of the collagen was 200 μg/ml. Stirring setting was 600 rpm, 37° C

vWF: Ag Multimer

vWF: Ag multimeric distribution was analyzed by SDS agarose gel electrophoresis under a discontinuous system as previously reported (14). The running gel was 1.5% high gelling temperature pure (HGTP) agarose (FMC Corp.) in 0.375 M Tris buffer, pH 8.8, containing 0.1% SDS. Normal human platelets were washed and solubilized by 1% Triton X-100 and analysed for vWF: Ag multimer. The gel was stained by an immunoenzymatic method using an avidin-biotin peroxidase complex (Vector labs., Inc., Burlingame, CA) as previously described (15). The multimeric distribution was also evaluated by densitometric scanning at 570 nm using a Densitoron Model PAN-FV spectrophotometer (Johko, Japan).

Fixed Washed Platelets

FWP were prepared by a modification of the method of Cooper et al. (16). Human platelet concentrates (Aomori Red Cross Blood Center, 200–300 ml) were washed three times with 0.05 M Tris, pH 7.3, containing 0.15 M NaCl, 5 mM EDTA, 5 mM N-ethylmaleimide, 6 mM trisodium citrate and 12 μM Leupeptin. The platelets were then fixed with 1.8% formaldehyde in 0.135 M phosphate buffer, pH 6.4, overnight at 4° C. After washing the FWP three times, the platelet count was adjusted to 8×10^8/ml. FWP were stored at 4° C in 0.05 M imidazole buffer containing 1 mM EDTA and 0.02% sodium azide, pH 6.4.

Quantitation of Collagen Cofactor (CCo)

Adhesion of FWP to collagen was evaluated by a turbidimetric method using a Payton dual-channel aggregometer (Payton) as previously described (8). Dispersed bovine Achilles tendon collagen was obtained from Ethicon Inc., Somerville, NJ (a gift from Dr. R. L. Kronenthal). To obtain a reproducible fibrillar collagen reagent, the original collagen solution was diluted to 2,000 μg/ml with distilled water and mixed by rocking overnight at 4° C. Such suspensions were stable for 4–6 weeks when stored at 4° C.

Table 1 Effect of MAbs to vWF on CCo in normal plasma

	Initial slope	CCo (u/ml)
Normal mouse serum	6.9	1.00
MAb		
CLB-RAg 35	2.7	0.32
CLB-RAg 201	1.2	<0.06

Two tenth milliliters of normal pooled plasma was added by 5 μl of normal mouse serum or MAbs to vWF, and incubated for 15 min at 37° C. After 0.2 ml of FWP was added to the mixture, the adhesion was measured using 200 μg/ml collagen. Final concentration of both MAb was 10 μg/ml. Mean of duplicate determinations.

Two-tenth milliliters of either normal plasma or test materials were added to 0.2 ml FWP (8×10^8/ml in cacodylate buffer containing 5% BSA) in a siliconized glass aggregometer cuvette. The mixture was stirred by a magnetic bar at a setting of 600 rpm at 37° C. After a stable baseline was obtained, 0.1 ml of collagen suspension was added to the cuvette, and the change in light transmission over time was recorded on a Rikadenki recorder (Japan, 5 mV for 100% light transmission). For the standard curve, pooled normal human plasma was serially diluted from 1:2 to 1:16 with the plasma of a patient with severe type I vWD. The initial slope of each adhesion curve was measured and was plotted versus the percent of normal human plasma. One unit of CCo activity is defined as that amount present in 1 ml of pooled normal human plasma.

vWF-Collagen Binding Experiments

Normal human plasma, 0.3 ml was mixed with an equal volume of collagen suspension in a siliconized aggregometer cuvette and the mixture was stirred at 1,000 rpm for 10 min at 37° C. The supernatant, after removal of the collagen aggregates by centrifugation for 5 min at 3,000 rpm, was assayed for residual activities of vWF.

Measurement of the Quantitative Bleeding Time

Bleeding time was measured by a modification of the method described by Sutor et al. (18). This method allowed us to determine not only the duration of bleeding but also the amount of bleeding. Under a pressure of 40 mm Hg, a standardized incision, 1 mm deep and 4 mm wide, was made in the skin of the forearm using a spring loaded bleeding device (Simplate®, General Diagnostics, Division of Warner-Lambert Co., Morris Plains, NJ). A small plastic flow cell (2 cm in diameter) was placed over the incision and distilled water was passed through the polyvinyl tube at a constant flow rate (4 ml/min) by a peristaltic pump (SJ-1220, Atto Corp., Tokyo, Japan). The resulting hemolysate was passed through the mixing coil, and the concentration of hemoglobulin was continuously measured at 542 nm using a spectro-photometer (MFC-338, Japan) and recorded on a chart recorder. The bleeding time was defined as the time taken for the optical density (O. D.) of the hemolysate to decrease below 0.2. In addition, the amount of blood loss was quantitated by measuring the weight of a thick paper which corresponded to the area under the curve of the recorder tracing with O. D. above 0.2. A standard curve was obtained by putting samples of known amounts of hemoglobulin into the flow cell and measuring the weights of the corresponding paper tracings. The amount of blood loss was expressed in microliters after dividing the amount of hemoglobulin calculated from the standard curve by the concentration of hemoglobulin in a blood sample from the individual.

Results

FWP Adhesion as a Function of vWF

To quantitate the amount of CCo in normal plasma, FWP were suspended in cacodylate buffer containing 5% BSA and dilutions of normal plasma were made using the plasma of a severe type I vWD. As shown in Fig. 1, the initial slope of adhesion decreased with increased dilution of the pooled normal plasma. However, this dose dependency of normal plasma was not observed when the plasma dilutions were made in a buffer containing no protein.

To further investigate the specificity of vWF in the adhesion of platelets to collagen, MAbs CLB-RAg 35 and CLB-RAg 201 and a rabbit polyclonal anti-vWF: Ag were studied for their ability to inhibit the rate enhancing effect of normal plasma (Table 1). Addition of 5 μl of CLB-RAg 35 (1 mg/ml) to 0.2 ml of normal plasma decreased CCo to 32% and RCo to <3%, and the degree of inhibition of CCo was not changed by the addition of twice strong concentration of the antibody. When 5 μl of CLB-RAg 201 (1 mg/ml) was added to 0.2 ml of normal plasma and the resulting mixture was tested for CCo, the initial slope of adhesion was 1.2, a value similar to the slope of 1.1 obtained with severe type I vWD plasma. However, RCo was not inhibited by CLB-RAg 201 as previously described (7). Furthermore, the inhibition of CLB-

RAg 35 or CLB-RAg 201 was not observed when the test system contained no normal plasma. The polyclonal anti-vWF:Ag at a dilution of 1:100 also completely inhibited the CCo activity of normal plasma. Plasma RCo was also inhibited at this concentration of polyclonal antibody.

Stability of FWP During Storage

Three separate preparations of FWP were used for the assay of CCo and tested for stability. The ability of the FWP to adhere to collagen in the absence of normal plasma did not change over 1 year when stored at 4° C. vWF dependent rate of FWP adhesion to collagen was also stable for over 2 months, but tended to decrease after 1 year of storage (Fig. 2).

Plasma CCo in Normal Individuals and Patients with vWD

CCo was measured in 11 healthy individuals. The mean value was 0.96 ± 0.34 u/ml (mean ± SD), with a range of 0.35 to 1.61 u/ml.

In ten patients with vWD, VIII:C levels varied from <0.01 to 1.1 u/ml, vWF:Ag from <0.03 to 0.68 u/ml and RCo from <0.03 to 0.45 u/ml (Table 2). The plasma levels of CCo in the patients with type I vWD (n = 8) were ranged from 0.06 to 0.47 u/ml; the value (0.20 ± 0.15 u/ml) being approximately 20% of that of normal plasma. There was a significant correlation between CCo and VIII:C ($r = 0.909$, $p < 0.01$), CCo and vWF:Ag ($r = 0.975$, $p < 0.001$) or CCo and RCo ($r = 0.936$, $p < 0.01$) in the patients with type I vWD. In all patients with vWD including type IIA and acquired vWD, CCo correlated only with RCo ($r = 0.839$, $p < 0.01$). In the patient with type IIA vWD, the ratio of CCo/vWF:Ag was 0.08, markedly lower than that of type I vWD (0.92 ± 0.12).

When CCo in the plasma of a severe vWD patient was measured after the infusion of 1,000 units (20 units/kg) of a commercial factor VIII preparation, CCo increased to 0.25 u/ml and 0.33 u/ml at 60 min and 120 min, respectively. CCo then gradually decreased and returned to the preinfusion level 24 h after the infusion. When post-infusion plasma samples were included with other vWD plasmas, there was good correlation between CCo and vWF:Ag ($r = 0.854$, $p < 0.001$) or RCo ($r = 0.929$, $p < 0.001$), while no correlation was observed between CCo and VIII:C ($r = 0.483$, $p > 0.05$).

The values of the quantitative bleeding time, both the bleeding time and the amount of bleeding, were abnormal in most patients with vWD except a case of mild type I vWD. The abnormality of the quantitative bleeding time in severe type I vWD was not corrected by 1,000 units of commercial factor VIII preparation. After the infusion of 2,000 units of commercial factor VIII

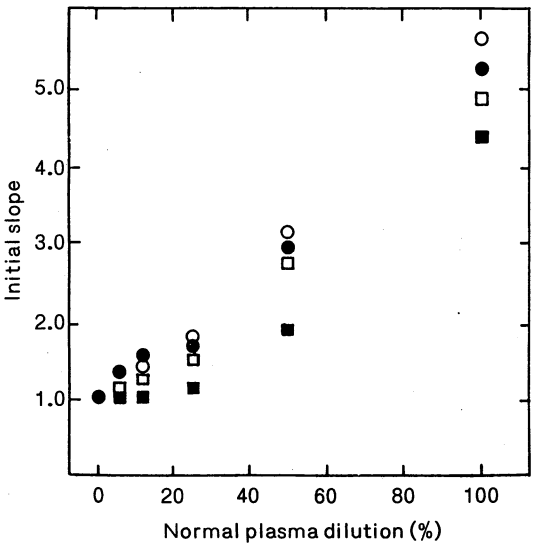


Fig. 2 Effect of storage of FWP on the assay for CCo. FWP were stored at 4° C for 1 week (○), 2 months (●), 5 months (□) and 12 months (■). The other conditions were the same as in Fig. 2

preparation, however, the bleeding time was normalized to 8.8 min at 30 min, while the amount of bleeding was still increased (274.7 µl) compared to normal (87.3 ± 86.6 µl, n = 31). There was no correlation between CCo and the duration of bleeding ($r = -0.496$, $p > 0.1$) or the amount of bleeding ($r = -0.515$, $p > 0.1$). Furthermore, the quantitative bleeding time did not correlate with the plasma level of VIII:C ($r = 0.298$, $p > 0.5$), vWF:Ag ($r = -0.410$, $p > 0.2$) or RCo ($R = -0.289$, $p > 0.5$).

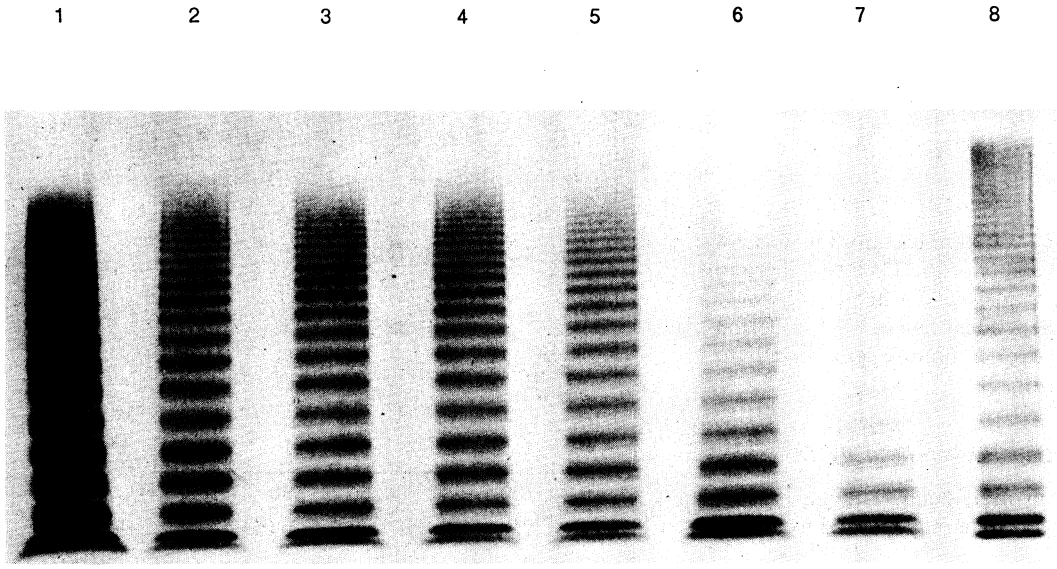
vWF:Ag Multimer

The multimeric pattern of vWF:Ag in normal plasma after mixing with collagen was studied by SDS agarose gel electrophoresis. As shown in Fig. 3A, large multimers of vWF:Ag in normal plasma were preferentially absorbed by collagen. More higher forms of vWF:Ag such as those found in platelet lysates were not observed in the supernatant plasma. With densitometric scanning, the changes in the multimeric distribution were easily evaluated and the disappearance of large multimers in the supernatants was observed below the concentration of 50 µg/ml collagen. As the concentration of collagen was increased, additional larger multimers were preferentially removed and there was a gradual decrease of RCo (Fig. 3B).

Table 2 Activities of VIII/vWF and the quantitative bleeding time in patients with von Willebrand's disease

Case	Type	CCo (u/ml)	VIII:C (u/ml)	vWF:Ag (u/ml)	RCo (u/ml)	BT (min)	Bleeding amount (µl)
1	I	0.11	0.38	0.06	0.06	*	*
2	I	0.39	1.10	0.58	0.45	24.5	*
3	I	0.47	0.80	0.54	0.36	1.5	6.5
4	I	0.28	0.52	0.38	0.36	38.5	779.4
5	I	0.14	0.40	0.07	0.07	20.8	505.2
6	I	0.06	0.18	0.03	0.03	ND	ND
7	I	0.06	0.11	0.05	0.05	*	*
8	I	0.06	<0.01	<0.03	<0.03	ND	ND
9	IIA	0.06	0.23	0.68	0.19	20.0	510.3
10	Acquired	0.22	0.10	0.05	0.05	ND	ND
(Normal)		0.96±0.34	1.0±0.5	1.0±0.5	1.05±0.5	8.1±4.4	87.3±86.6

* more than three times of normal, ND: not determined



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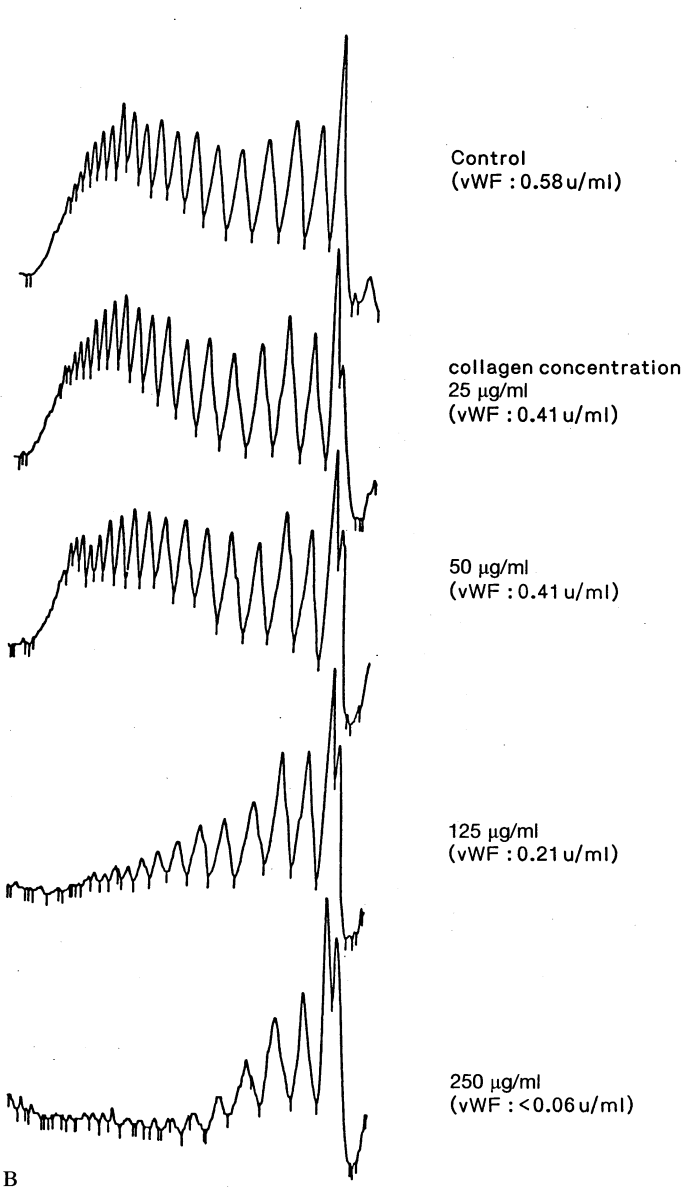

B

Figs. 3A, B A) vWF : Ag multimeric pattern by SDS 1.5% HGTP agarose gel electrophoresis. Samples; lane 1, normal plasma; lane 2, 1:2 diluted normal plasma; lanes 3–7, supernatants of normal plasma after mixing with collagen (25, 50, 100, 250 and 500 µg/ml); lane 8, 1% Triton X-100 solubilized platelets. B) Densitometric scan at 570 nm. The gel was the same as in Fig. 3A. RCo activities of vWF was measured in the supernatants of normal plasma after mixing with collagen. Control was 1:2 diluted normal plasma. The top of the gel was to the left

Discussion

Platelet adhesion to the exposed subendothelium at the site of vascular injury initiates formation of a hemostatic plug. vWF is necessary for the interaction of platelets and vascular subendothelium, especially at high shear rate conditions (19, 20). At least seven distinct types of collagen have been recognized, and the vessel wall contains four of these types of collagen: type I, III, IV, and V (21). Collagen types IV and V are thought to function partly as passive barriers to platelet interaction, while types I and III act as thrombogenic agents. We have reported that vWF has an activity that promotes the adhesion of human platelets to collagen immobilized on glass beads (11). Perret et al. (9) have recently described an activity of vWF called vWF-collagen cofactor that mediates the agglutination of FWP by collagen extracted from bovine aorta. Although collagen may not be the only binding site for vWF in the subendothelium (5, 22), vWF-collagen cofactor may play an important role in the maintenance of normal hemostasis in vivo.

In this report we have measured CCo in the plasma of normal individuals and patients with vWD. The rate of adhesion of FWP to collagen decreased with increasing dilution of normal human plasma, which dilutions were made in the plasma of an individual with severe vWD. The requirement of vWD plasma as diluent suggests the necessity of maintaining a critical non-specific protein concentration. Specificity of the CCo assay for vWF was shown by inhibition of CCo by a MAb CLB-RAg 201 and a polyclonal rabbit anti-vWF: Ag. The mean CCo in plasma from patients with vWD was 20% of normal and showed a significant positive correlation with RCo (r = 0.936). However, no correlation was observed between CCo and the duration of bleeding or the amount of bleeding. Recently, Mannucci et al. and Gralnick et al. have suggested that platelet and plasma vWF may both be important determinant of the bleeding time, and their relative role may be more or less apparent in different subtypes of vWD (23, 24). However, others have observed good correlation between plasma RCo and the bleeding time in a different sample population (25). In our study, the amount of CCo in variant type IIA vWD was markedly lower than that of vWF: Ag, a pattern similar to that previously reported for RCo/vWF: Ag ratios in type IIA vWD. This result supports our previous

observation that the adhesion rate of FWP in variant vWD plasma is consistently lower than that of FWP in normal plasma and also suggests that the higher molecular weight multimers of vWF:Ag may be necessary for optimal CCo activity.

Although it is not known how vWF interacts with platelets or collagen, larger multimers of vWF:Ag have been shown to preferentially bind to platelets in the presence of ristocetin (26) and to collagen in the absence of any cofactor (27). The high correlation of CCo and RCo suggests that platelet membrane glycoprotein Ib may be involved in platelet-vWF-collagen interactions with vWF acting as a "glue" between collagen and platelets. Recent studies have demonstrated that human vWF contains two binding sites for collagen, one residues 449–728 and the other 911–1365 (7, 28–32), although more smaller peptides of vWF were reported for monomeric type III collagen (33). The residues 449–728 are the same fragment of vWF that binds platelet membrane glycoprotein Ib (34). From the results that CCo was not only inhibited by CLB-RAg 201 but also partially inhibited by CLB-RAg 35, it is suggested that CCo is reflected by function of both collagen binding and platelet binding domain of vWF. The role of platelet membrane components in the platelet-vWF-collagen interaction has recently been studied and the partial involvement of glycoprotein Ib in this interaction has been described previously (35). Furthermore, the inhibition of CCo by CLB-RAg 201 suggests that the binding of vWF to collagen is absolutely required for the expression of CCo activity.

Recently, collagen binding quantitative assay of vWF have been described and compared to the assay for RCo or vWF:Ag (36, 37). We have also reported the decreased binding of vWF:Ag in plasma of type IIA vWD patient or commercial VIII preparations by the methods of affinity immunoelectrophoresis using collagen spacer gel (38). CCo, which is another functional activity of vWF:Ag may be useful for the detection of new variant forms of vWD.

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