Proteolytic Cleavage of Human von Willebrand Factor Induced by Enzyme(s)
Released From Polymorphonuclear Cells

By E. Anne Thompson and Margaret A. Howard

In vivo fragmentation of the von Willebrand factor antigen (vWF:Ag) molecule has been demonstrated on radio-crossed immunoelectrophoresis (CIE) in the plasma from patients with disseminated intravascular coagulation, in factor VIII concentrates, and in normal serum. Experiments reported here show that polymorphonuclear (PMN) cells contain a non-calcium-dependent protease(s) that when released and incubated with vWF:Ag results in an additional vWF:Ag peak on radio-CIE.

Production of fragments of vWF:Ag by incubation with PMN cells occurred in a time-dependent manner. The protease(s) responsible was inhibited by diisopropyl fluorophosphate, soybean trypsin inhibitor, and aprotinin, but not by benzamidine, azide, epirubicin, or hirudin. Citrate, EDTA, and leupeptin also had no effect on the PMN cell enzyme's activity, indicating that the enzyme(s) is not calcium dependent. The PMN cell enzyme responsible for vWF:Ag fragmentation is located intracellularly and released by freeze-thaw lysis or cell activation by calcium or the calcium ionophore A23187.

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MATERIALS AND METHODS

Chemicals were purchased from the following suppliers: agarose (Seakem Laboratories, Maryland, US); barbitol (sodium salt), soybean trypsin inhibitor (SBTI), phosphomethyl sulfonyl fluoride (PMSF), hirudin, leupeptin, benzamidine, sodium diatrizoate, and diisopropyl fluorophosphate (DFP) (Sigma Chemical Co, St Louis); sodium heparin (Weddell Pharmaceuticals, Wrexham, UK); apro-}

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was dissolved in 25% sucrose in 50 mmol/L Tris and 0.15 mmol/L NaCl containing 10 U/mL aprotinin and 0.02% sodium azide and subjected to centrifugation at 100,000 g at 20 °C for 30 minutes (to exclude lipids and particulate matter). A 2-ml sample of the infranatant was then applied to a Sepharose 4B-Cl column (3 cm x 80 cm), and 2.8-ml fractions were collected. Early eluting fractions of RCoF/vWF:Ag free from detectable fragments of vWF:Ag were pooled and used as a source of purified RCoF/vWF:Ag.

\[ vWF \text{ analysis. } \]
RCoF activity was measured as previously described using formaldehyde-fixed platelets. vWF:Ag was measured by electroimmunoassay (EIA). Radiolabeled vWF:Ag was incorporated into agarose as described. A 50-μL sample was electrophoresed toward the anode in the first dimension until an Evan’s blue marker migrated 7 cm from the origin. In the second dimension the sample was electrophoresed into agarose containing a mixture of whole rabbit antiserum to RCoF/vWF:Ag and immunoaffinity purified, radiolabeled rabbit IgG to RCoF/vWF:Ag. At the end of the electrophoresis, the plates were washed and dried, and autoradiography was allowed to proceed at −70 °C for up to 120 hours, depending on the batch of 125I-labeled antiRCoF/vWF:Ag IgG.

\[ vWF:Ag \text{ multimeric analysis. } \]
Purified vWF was labeled with 125I by the iodogen method (as described by the manufacturer, Pierce Chemical Co, Rockford, Ill). The multimeric composition of 125I-vWF:Ag following incubation with the lysed PMNC preparation was visualized after SDS-agarose gel electrophoresis. Slab gels (10 cm x 7 cm) containing 6 mol/L urea, 0.2% SDS, and 1% agarose at 0.075 mmol/L barbital buffer, pH 8.6, were prepared in glass molds and set at 4 °C for one hour. The gels were then transferred onto gelbond (FMC Corp. Marine Colloids Division, Rockland, Maine), and five wells (0.4 cm x 1.0 cm) were cut out, ready for use. The tank buffer was 0.075 mmol/L barbital buffer, pH 8.6, containing 0.2% SDS. In a typical experiment, 30 μL 125I-vWF:Ag (0.5 U/mL) was added to 30 μL of a lysed PMNC suspension (1.4 x 10^5 mL) or 30 μL Tris/saline buffer (control) and incubated at 37 °C for up to 60 minutes. Each sample was diluted 1/5 in gel buffer (see the preceding material) and 40 μL applied to wells in SDS/urea/agarose gel and left to set before electrophoresis at 12 mA for four hours. The gels were then dried, stained, and autoradiographed.

**RESULTS**

**Fragments of vWF:Ag in blood products.** Examination of radio-CIEs of NPP, normal serum, plasma from a patient with DIC, and a factor VIII concentrate (CSL) show the presence of an additional vWF:Ag precipitin peak (Fig 1). Figure 1 (B, C, and D) shows the presence of a precipitin peak, (indicated by the arrows) previously called VIII:FMP (fast-migrating protein). This vWF:Ag fragment is clearly visible in the radio-CIEs of DIC plasma and factor VIII concentrate, is present in smaller amounts in normal serum, and is faintly detectable in normal plasma. This antigen shows partial immunologic identity to the major vWF:Ag peak. A spur to the main vWF:Ag precipitin peak can be seen in Fig 1C (denoted by P), suggesting the presence of additional vWF:Ag fragments.

**Effect of lysed purified cell preparations on purified RCoF/vWF:Ag.** Purified vWF was labeled with 125I by the iodogen method (as described by the manufacturer, Pierce Chemical Co, Rockford, Ill). The multimeric composition of 125I-vWF:Ag following incubation with the lysed PMNC preparation was visualized after SDS-agarose gel electrophoresis. Slab gels (10 cm x 7 cm) containing 6 mol/L urea, 0.2% SDS, and 1% agarose at 0.075 mmol/L barbital buffer, pH 8.6, were prepared in glass molds and set at 4 °C for one hour. The gels were then transferred onto gelbond (FMC Corp. Marine Colloids Division, Rockland, Maine), and five wells (0.4 cm x 1.0 cm) were cut out, ready for use. The tank buffer was 0.075 mmol/L barbital buffer, pH 8.6, containing 0.2% SDS. In a typical experiment, 30 μL 125I-vWF:Ag (0.5 U/mL) was added to 30 μL of a lysed PMNC suspension (1.4 x 10^5 mL) or 30 μL Tris/saline buffer (control) and incubated at 37 °C for up to 60 minutes. Each sample was diluted 1/5 in gel buffer (see the preceding material) and 40 μL applied to wells in SDS/urea/agarose gel and left to set before electrophoresis at 12 mA for four hours. The gels were then dried, stained, and autoradiographed.

**REFERENCES**

Table 1. Distribution of Cells Present in the Isolated Cell Preparations

<table>
<thead>
<tr>
<th>Cell Preparation</th>
<th>Differential Count (%)</th>
<th>Cell Count (x 10^11/L)</th>
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<tr>
<td>MNC</td>
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</tr>
<tr>
<td>PMNC</td>
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<tr>
<td>Platelets</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>RBC</td>
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Abbreviation: ND, not determined.

**Fig 1.** Radio-CIE patterns of blood products. (A) NPP. (B) Normal serum. (C) Plasma from a patient with DIC. (D) CSL antihaeomophilic factor concentrate. The arrow indicates VIII:FMP. A spur reaction on the vWF:Ag precipitin peak is shown in (C) and indicated by P.

**Fig 2.** Radio-CIE patterns of purified RCoF/vWF:Ag (0.25 U/mL final concentration) incubated for 60 minutes at 37 °C with (A) buffer, (B) lysed RBC (concentration not determined), (C) lysed MNC (0.75 x 10^9 mL), (D) lysed platelets (8.1 x 10^9 mL), (E) lysed PMNC (0.50 x 10^9 mL), and (F) PMNC (0.50 x 10^9 mL) incubated with buffer in the absence of RCoF/vWF:Ag. All concentrations are their final concentration in the incubation mixture. This experiment was repeated twice with similar results.
logic similarity to vWF:Ag. Fragmentation of vWF:Ag was still detected if the lysed PMNC preparation was incubated with purified RCoF/vWF:Ag at a final cell concentration of 1.25 x 10^7/mL (data not shown).

**Time course of RCoF/vWF:Ag fragmentation by lysed PMNC.** The RCoF activity of a purified RCoF/vWF:Ag preparation was progressively lost during a 20-minute incubation at 37 °C with lysed PMNC, whereas the vWF:Ag level decreased only slightly during a 60-minute incubation period (Fig 3). In the absence of cells, a small but insignificant fall in both RCoF activity and vWF:Ag levels was observed. A progressive increase in the height and density of the vWF:Ag fragment peak was seen on the resultant radio-CIE autoradiograms (Fig 4) of aliquots taken from the incubation mixture at intervals during the 60-minute incubation described in Fig 3. It may also be noted from Fig 4 that as the incubation proceeded and the fragment of vWF:Ag was produced a loss of the high–molecular weight forms of vWF:Ag occurred (Fig 4E).

The loss of high–molecular weight multimers of vWF:Ag was further demonstrated by SDS-agarose electrophoresis (Fig 5). Purified vWF:Ag was radiolabeled with ^125I and incubated with PMNC for 60 minutes. Aliquots were removed from the incubation mixture at 5, 15, 30, and 60 minutes and subjected to SDS-agarose electrophoresis. A typical autoradiograph obtained from one of these experiments clearly demonstrated a progressive loss of high–molecular weight multimers and a simultaneous increase in smaller–molecular weight forms of vWF:Ag (Fig 5). The arrowhead in Fig 5 indicates the presence of multimeric forms of vWF:Ag (lanes 3 to 5) that are not present in the starting material (lane 1). These multimers would appear to correspond to the additional precipitin peaks seen on radio-CIE of similar incubation mixtures. Extension of the incubation period to four hours at 37 °C resulted in a reduction in the level of both vWF:Ag and the fragment of vWF:Ag (data not shown).

**Effect of temperature on RCoF/vWF:Ag fragmentation.** The temperature sensitivity of the PMNC protease responsible for RCoF/vWF:Ag fragmentation was investigated by incubating purified RCoF/vWF:Ag (0.29 U/mL, final concentration) and buffer or PMNC (0.5 x 10^8/mL, final concentration) for 60 minutes at 4 °C, 20 °C, 37 °C, 45 °C, and 56 °C.

Figure 6A shows that, in the presence of buffer, vWF:Ag and RCoF activity were stable to temperatures of 45 °C for 60 minutes but RCoF activity was greatly reduced above this temperature. No significant change in the radio-CIE pattern of vWF:Ag was apparent for the temperature range of 4 °C to 56 °C following a 60-minute incubation.

Incubation of the purified RCoF/vWF:Ag (0.29 U/mL, final concentration) with PMNC (0.5 x 10^8/mL, final concentration) was incubated with lysed PMNC (0.5 x 10^8/mL, final concentration) for 0 to 60 minutes. A (RCoF) and @ (vWF:Ag) were measured at various intervals during the 60-minute incubation. Similar results were obtained in an identical experiment.
Effect of activation of intact PMNC. Fragments of vWF:Ag were not detected by radio-CIE following incubation of purified RCoF/vWF:Ag with fresh intact PMNC (Fig 8A).

Inclusion of 0.2 mmol/L CaCl$_2$ or 0.17 mmol/L calcium ionophore (Fig 8B, 8D) in the incubation mixture of purified RCoF/vWF:Ag with intact PMNC resulted in production of vWF:Ag fragments following 60 minutes at 37 °C.

Effect of inhibitors on RCoF/vWF:Ag degradation by PMNC proteases. Fragmentation of vWF:Ag by PMNC was inhibited by adding either SBTI (33 mg/mL), DFP (0.6 mmol/L), or Trasylol (1.500 kIU/mL) to RCoF/vWF:Ag immediately before incubation with lysed PMNC for 60 minutes. Other inhibitors—leupeptin (1.3 mmol/L), benzamidine (15 mmol/L), sodium azide (3 mmol/L), EACA (1.5 mol/L), aprotinin (150 kIU/mL), hirudin (1.0 U/mL), EDTA (5.0 mmol/L), and trisodium citrate (13 mmol/L)—did not inhibit the action of PMNC proteases on purified RCoF/vWF:Ag. At the concentrations reported, none of these agents had any effect on the radio-CIE pattern of RCoF/vWF:Ag.

DISCUSSION

In vivo fragmentation of the RCoF/vWF:Ag molecule has been described in association with events known to cause activation of proteolytic enzymes such as DIC,7,9 urokinase therapy,7 and sickle cell crisis.16 The actual fragment of vWF:Ag demonstrated by radio-CIE appears to vary. This may depend to some extent on the antibody used in the second dimension of the radio-CIE. Using a rabbit antibody raised against vWF:Ag, we were able to demonstrate the presence of fragmentation products previously termed VIII:FMP and VIII:P9 in the plasma from patients with DIC and by in vitro manipulation of purified vWF:Ag (Figs 1 and 2). These products would appear to correspond to the VIII:Ag:fragment described by Montgomery and Johnson8 and fast-moving peak described by Lombardi et al.7

Fig 7. Radio-CIE patterns of purified RCoF/vWF:Ag (0.29 U/mL, final concentration) incubated with lysed PMNC (0.5 x 10$^9$/mL, final concentration) for 60 minutes at (A) 4 °C, (B) 37 °C, and (C) 45 °C. Radio-CIE from two identical experiments yielded similar patterns.
Experiments reported here demonstrate that PMNC contain an enzyme(s) that when released (Figs 2, 4, 6, and 7) and incubated with purified RCof/vWF:Ag results in the appearance of an additional vWF:Ag peak on radio-CIE. The relationship between this product and the formerly reported VIII:FMP or VIII:P is at present not proven. However, electrophoretic mobilities on radio-CIE indicate a similar migration distance for the PMNC-produced RCof/vWF:Ag fragment and those present in the plasma from patients with DIC and in serum formed by allowing whole blood to clot in glass tubes.

The PMNC enzyme(s) responsible for RCof/vWF:Ag fragmentation is located intracellularly. Release of the enzyme(s) was achieved by freeze-thaw lysis of the cells or by cell activation. Suitable activators were calcium or the calcium ionophore A23187. The enzyme(s)' activity is inhibited by DFP, SBTI, and high concentrations of aprotinin, but not by benzamidine, azide, EACA, or hirudin. Addition of citrate, EDTA, or leupeptin had no effect on the activity of the enzyme(s), indicating that the PMNC enzyme(s) investigated in these experiments is not calcium dependent, although calcium is able to activate PMNC to release the enzyme(s). The possibility exists that PMNC also contain CAP similar to those demonstrated in platelets that have been shown to cleave vWF:Ag. The design of the experiments reported here would have precluded demonstration of action by such enzymes since all cells were washed in a buffer containing 6 mmol/L EDTA, a concentration high enough to inactivate the platelet CAP.

The experiments comparing the temperature dependence of loss of RCof activity and vWF:Ag degradation showed a concomitant loss of RCof activity and appearance of fragments of vWF:Ag after incubation with PMNC at temperatures between 37 °C and 56 °C.

The concentration of lysed PMNC used to obtain fragmentation of vWF:Ag is more than 2.5 times that found in blood. During clot formation, PMNC cluster around the platelet/fibrin mass, and so it is not inconceivable that a local increase in PMNC concentration could occur. Using PMNC elastase as a marker for PMN leukocyte activation, Plow reported release of elastase during serum production. Further evidence of release of leukocyte constituents during coagulation is indicated by the higher lactoferrin levels found in serum than plasma in a study by Bennett and Mohla. Thus, liberation of PMNC constituents during clot formation is indicated, and the in vitro fragmentation of vWF:Ag demonstrated by lysed or calcium-activated PMNC may parallel in vivo events.

REFERENCES

Proteolytic cleavage of human von Willebrand factor induced by enzyme(s) released from polymorphonuclear cells

EA Thompson and MA Howard