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Evidence for a factor promoting the conversion of VWF from low and intermediate to high molecular mass polymers on the platelet membrane

A. Ferri, S. Guerra*, D. Gemmati and P. Chiozzi

Istituto di Chimica Biologica and **Istituto di Patologia Speciale Medica, Università degli Studi, Ferrara, Italy*

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On the membrane surface of resting platelets exists a factor capable of promoting the conversion of plasma von Willebrand factor from low and intermediate molecular mass to high molecular mass polymers. The process is accompanied by a parallel increase in the von Willebrand factor activity. This could be a regulatory system for the molecular mass distribution of von Willebrand factor during its lifetime in plasma. Glycoprotein Ib of the platelet membrane appears not to be involved in the process.

Hemostasis; Platelet; Von Willebrand factor; Ristocetin

1. INTRODUCTION

The von Willebrand disease is an autosomic coagulation disorder due to a quali- and/or quantitative deficiency of the von Willebrand factor (vWF), a large glycoprotein produced by the endothelial cells and megacaryocytes [1-3]. In plasma, vWF circulates as a heterogeneous mixture of disulfide-bridged polymers ranging from dimers (M_r 25×10^4) to multimers ($M_r > 2 \times 10^7$) and its role is to promote the initial step in hemostatic plug formation [4] by interacting with the subendothelial structures (collagen) of injured vessel and with the glycoprotein IIb-IIIa of activated platelets [5,6]. In the presence of the antibiotic ristocetin, vWF is capable of interacting with resting platelets too: in this case the platelet membrane glycoprotein Ib is responsible for the interaction with the ristocetin-vWF complex [7-11] by which platelets are activated and aggregate. Glycoprotein Ib retains the capability to interact with the ristocetin-vWF complex also when platelets are metabolically inactive such as after treatment with formaldehyde [12,13]; in this case agglutination of FFP occurs.

Both the physiological interaction of vWF with activated platelets, and the non-physiological interaction of the ristocetin-vWF complex with resting or inactivated platelets, strongly depend on the molecular mass of the polymers, the highest activity being shown by the largest molecules.

A lot of information exists indicating that the vWF molecules produced by the endothelial cells are stored into the Weibel-Palade bodies as a mixture containing all the polymeric forms, but it is not known, to date, whether this is the only way by which the HMW polymers are produced, or whether polymerization may also occur after the secretion of glycoprotein into blood [14-16]. The problem becomes more puzzling because of the recent observation [17] that the proteolytic cleavage of the precursor (pro-vWF) seems not to be essential for the multimers formation.

In the present paper we show that on the resting platelet membrane surface there is a factor capable of promoting the conversion of vWF from low and intermediate molecular mass to high molecular mass polymers with a consequent increase in the vWF activity.

Correspondence address: A. Ferri, Istituto di Chimica Biologica, Università degli Studi, Via L. Borsari 46, I-44100 Ferrara, Italy

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2. MATERIALS AND METHODS

2.1 *Materials*

All reagents were analytical grade. Human platelet poor plasma (PPP) was prepared by double centrifugation (2000×*g*; 15 min) of citrate (0.38%) added to fresh blood samples from volunteers. Platelet rich plasma (PRP) was obtained by double centrifugation of citrate added to blood (200×*g*; 8 min). Washed platelets (WP) were prepared immediately before use by centrifugation of PRP (3000×*g*; 15 min) followed by three washing cycles of the pellet with 10 mM Tris, 1 mM EDTA, pH 7.5, buffer (Tris-EDTA buffer): from 40 ml of fresh blood we usually obtained 10 ml of washed platelets ($\sim 4\times10^5$ WP/μl). Formaldehyde-fixed platelets (FFP) were prepared by one hour preincubation of PRP suspensions (10 ml) at 37°C, followed by centrifugation (2500×*g*; 15 min) and resuspension of the pellet in 10 ml of Tris-EDTA buffer containing 2% formaldehyde; after 48 h incubation at 4°C the suspension was centrifuged (2500×*g*; 15 min) and the pellet was resuspended in Tris-EDTA buffer giving 3×10^5 FFP/μl (10–12 ml) and stored at 4°C. Incubations of PPP samples with FFP or WP were performed by centrifuging (3000×*g*; 15 min) the desired amount of platelets from the storage buffer and gently resuspending the pellet in the required volume of plasma to be treated.

The experiments requiring WP were preceded by a control on the integrity of platelets by suspending them (3×10^8 WP/ml) in 200 μl of PPP in the aggregometer vessel and following the aggregation process induced by 2 μM ADP. Parallel to these experiments, aggregometric controls were routinely performed in order to check whether incidental activation of WP occurred during incubations.

2.2. *vWF activity in plasma*

200 μl of FFP suspension (3×10^5 FFP/μl), supplemented with 1 mg/ml ristocetin, were preincubated 3 min at 37°C into the aggregometer vessel under stirring, after which 50 μl of the PPP to be tested were added; the aggregometer (Menarini) recorded the increase in transmittance, due to the agglutination of platelets, vs time. The slope of the initial agglutination time course was proportional to the activity of the vWF in the sample. We assumed 100% activity to be that obtained for a PPP sample from a pool of blood samples from voluntary donors under the indicated test conditions.

2.3 *Agarose gel electrophoresis, transblotting, staining*

Gel electrophoresis was performed substantially as in [18] with some modifications. Gels, 1 mm thick, were 10×10 cm size, the stacking and the running parts were 0.75% and 1.75% agarose and were 2.5 cm and 7.5 cm in length, respectively; sample wells (2×0.2 cm) were cut in the stacking gel at 1 cm from the interface. Plasma to be investigated was diluted 5-fold with Tris-EDTA buffer adjusted to pH 8.0 and containing 2% SDS, 8 M urea and 0.01% bromophenol blue as tracking dye. After 20 min incubation at 60°C, 20 μl samples were applied to each well and electrophoresis started at 20 mA constant current. After the samples had moved out of the wells, these were filled with stacking gel suspension and the current was decreased to 4 mA. Electrophoresis was stopped when the dye reached the anode end of the gel (usually 14 h). After the run the gel was transblotted onto a nitrocellulose sheet (Bio-Rad, 10×10 cm, 0.22 μm) by a standard transblot chamber (Bio-Rad) and following the recommended procedure (24 h run; 200 mA constant current; 4°C temperature; running buffer, 15.6 mM Tris, 120 mM glycine, pH 8.3, containing 0.05% SDS, 20% methanol). The nitrocellulose sheet was treated (30 min) with fixing buffer (2-propanol/acetic acid/water, 25:10:65), washed three times with Tris-NaCl buffer (50 mM Tris, 150 mM NaCl, pH 7.5), saturated by incubation (30 min) in Tris-NaCl buffer containing 1% bovine serum albumin and washed three times with Tris-NaCl buffer containing 1% Triton X-100. Staining was obtained by a 4 h incubation of the nitrocellulose sheet with peroxidase-conjugated anti-human vWF rabbit antiserum (DAKO) diluted 1:125 with Tris-NaCl buffer containing 1% Triton X-100, followed by four washing cycles with the same buffer and four washing cycles with Tris-NaCl buffer. The last washing bath was supplemented, in the dark, with 0.5 mg/ml 4-chloro-1-naphthol (predissolved in methanol) and 5 mM hydrogen peroxide. The incubation was stopped after 20 min by repeated washings with distilled water. After air drying, the nitrocellulose sheet was photographed.

3. RESULTS AND DISCUSSION

A typical PPP from healthy donors shows the molecular mass distribution of vWF polymers represented in fig.1 (lane 1), the activity (as defined in section 2.2) of which was taken as 100%. In the presence of ristocetin and FFP, a ristocetin-vWF complex was formed capable of interacting with the membrane glycoprotein Ib of FFP [12,13] by which agglutination occurred and, consequently, the removal of vWF bound to platelets from the medium was possible by centrifugation. As shown in lane 2, by an agglutination–centrifugation treatment, the high molecular mass vWF polymers (HMW-vWF) were removed from plasma with a drop of activity to 25% with respect to sample 1. The intermediate molecular mass polymers (IMW-vWF) were removed from plasma by a second identical treatment by which only the low molecular mass polymers (LMW-vWF, i.e. dimers and tetramers) remained in the medium with an activity near zero (lane 3).

A PPP sample (fig.2, lane 1), subjected to an agglutination–centrifugation treatment to remove the HMW-vWF polymers, was incubated with WP and subsequently centrifuged. In the supernatant obtained after an hour of incubation (lane 3), the trace corresponding to the highest polymer of lane 2 completely disappeared and, after two hours of incubation, a sharp decrease in the intensity of all traces was observed. This result is interpreted as a consequence of the concurrent effects of WP and

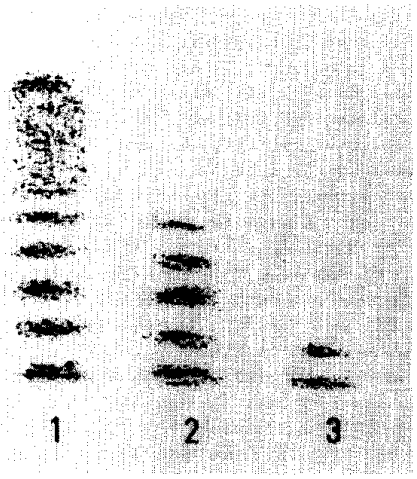

Fig.1. Molecular mass distribution of vWF in PPP before and after two subsequent agglutination treatments with ristocetin and FFP. PPP (sample 1) supplemented with 6×10^8 FFP/ml and 2 mg/ml ristocetin was incubated 3 min at 37°C under stirring and centrifuged ($3000 \times g$, 15 min). The supernatant (sample 2) was subjected to a second identical treatment and the supernatant thereof was sample 3. Aliquots of samples were employed for electrophoresis and the vWF activity assays.

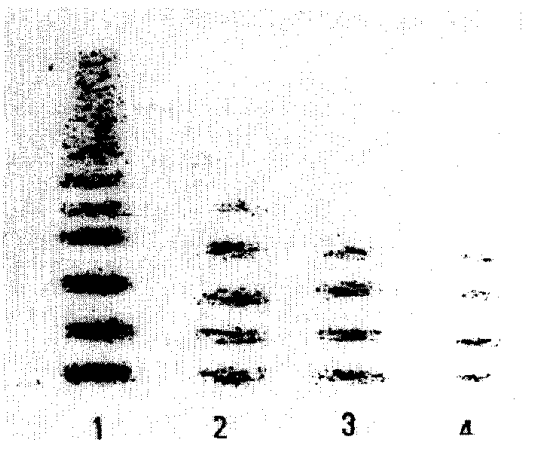

Fig.2. Effect of washed platelets in the presence of ristocetin on the molecular mass distribution of vWF in PPP. PPP (sample 1) was subjected to one agglutination–centrifugation treatment like those of fig.1. The supernatant (sample 2) supplemented with 2.5×10^8 WP/ml was incubated at 37°C for 1 or 2 h and centrifuged before assays (samples 3 and 4, respectively).

ristocetin on vWF molecules. We propose that on the membrane surface of resting platelets there is a factor capable of promoting the organization of IMW- and LMW-vWF polymers toward structures having a higher molecular mass; ristocetin, present in samples 3 and 4 because of the large excess employed in the agglutination process, on complexing with the newly formed HMW-vWF polymers allows the activitation and the consequent aggregation of WP [7–11]. This idea is in accordance with the parallel decrease in the vWF activity in the supernatants (from 25% of sample 2 to 12% and 7% after one and two hours of incubation, respectively).

Direct evidence of the capability of resting platelets to promote the association of LMW- and IMW-vWF polymers to give larger and more active species, was obtained from the experiment shown in fig.3 in which a PPP sample (lane 1, 100% activity), after an agglutination–centrifugation treatment (lane 2, 25% activity), was subjected to exhaustive dialysis to remove the ristocetin excess before incubation with WP. Under these conditions, with increasing incubation time, a progressive decrease in the concentration of LMW- and IMF-vWF polymers was observed, accompanied with the progressive appearance of significant amounts of HMW species (cf. lane 3 and 4 with lane 2) and with a dramatic increase in the vWF activity (43% in sample 3 and 65% in sample 4 corresponding to 30 and 60 min incubation, respectively). The absence of ristocetin, in this

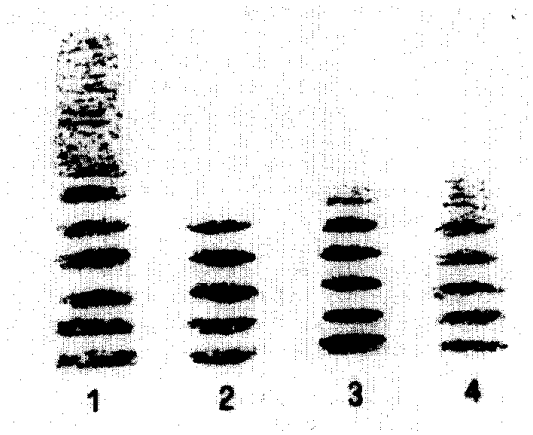

Fig.3. Effect of washed platelets in the absence of ristocetin on the molecular mass distribution of vWF in PPP. PPP (sample 1) was subjected to one agglutination–centrifugation treatment like those of fig.1. The supernatant was dialyzed against Tris-NaCl buffer (3 cycles, 1 h, 500 ml) at 4°C. The dialysate (sample 2) supplemented with 2.5×10^8 WP/ml was incubated at room temperature 30 and 60 min and centrifuged before assays (samples 3 and 4, respectively).

case, allows the accumulation of the species that, in the experiment of fig.2, were removed because of the aggregation process.

The possibility that, in the experiment shown in fig.3, the HMW-vWF polymers present in the supernatants after incubations could be due to the dismission of the content of the platelets α-granules by an incidental activation is to be excluded either because of our routine aggregometric control during incubation, and because, in this hypothesis, the platelet activation should have been followed by aggregation [7–11], by which, after centrifugation, the electrophoretic pattern and the activity assay of the supernatants should have been similar to those of the experiment represented in fig.2.

A PPP sample (fig.4, lane 1), subjected to an agglutination–centrifugation treatment (lane 2, 22% activity with respect to sample 1), was incubated for two hours in the presence of 1 mg/ml ristocetin. After centrifugation, the supernatant (lane 3) showed an electrophoretic pattern and an activity identical to sample 2, indicating that the conversion of vWF polymers observed under the conditions of experiments shown in figs2 and 3 was not due to a spontaneous association nor to a direct effect of ristocetin on the vWF molecules, the only function of the antibiotic being to complex with the

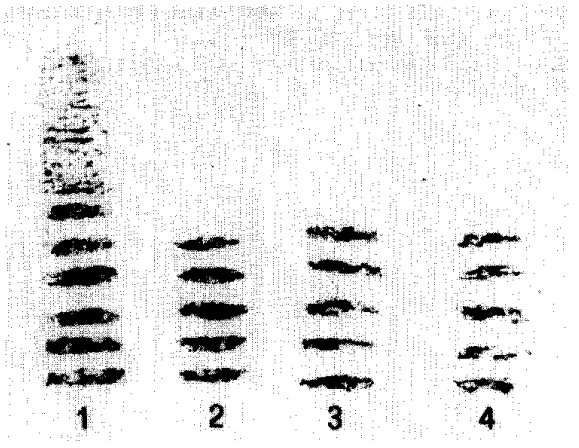

Fig.4. Effect of ristocetin and FFP on the molecular mass distribution of vWF in PPP. PPP (sample 1) was subjected to one agglutination–centrifugation treatment like those of fig.1. The supernatant, dialyzed as described in fig.3 (sample 2), was supplemented with 1 mg/ml ristocetin (sample 3) or with 6×10^8 FFP/ml (sample 4), incubated 2 h at 37°C and centrifuged before assays.

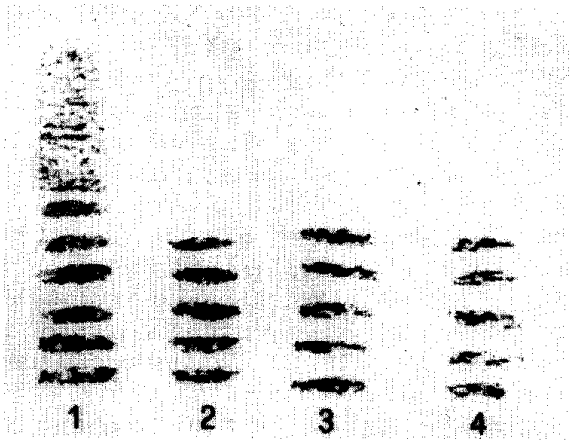

Fig.5. Effect of red and red plus white cells in the presence of ristocetin on the molecular mass distribution of vWF in PPP. PPP (sample 1) was subjected to one agglutination–centrifugation treatment like those of fig.1 and the supernatant (sample 2) was supplemented with 1 mg/ml ristocetin. Aliquots (300 μl) were incubated at room temperature in the presence of red (sample 3) or red plus white cells (sample 4) (each obtained from 750 μl of fresh blood) and centrifuged before assays.

polymers, allowing them to interact with the platelet glycoprotein Ib.

That the capability to convert the vWF polymers is a specific property of the platelet membrane surface was confirmed by the experiment shown in fig.5: aliquots of a PPP sample (lane 1), subjected to an agglutination–centrifugation treatment (lane 2, 25% activity) were supplemented with 1 mg/ml ristocetin and incubated one hour in the presence of red (lane 3) or red plus white cells (lane 4) (each obtained from 750 μl of fresh blood); after centrifugation both samples were shown to be unchanged in both the electrophoretic pattern and the vWF activity.

This property of the platelet membrane was lost when platelets were inactivated by the formaldehyde treatment: in fact, a PPP sample subjected to an agglutination–centrifugation treatment followed by dialysis to remove the ristocetin excess (fig.4, lane 2), was incubated for two hours in the presence of FFP; after centrifugation, the supernatant was unchanged in both the vWF activity (22%) and the electrophoretic pattern (lane 4).

CONCLUDING REMARKS

In the present work, it is stated for the first time

that, on the membrane surface of resting intact platelets, the conversion of vWF from LMW- and IMW- to HMW-vWF polymers occurs. It is known that the vWF content of the α-granules of platelets is rich in HMW components and that in the Weibel–Palade bodies of the endothelial cells vWF is stored as a set of polymers of varying molecular mass. Nevertheless, there are no data on the existence of a function for the LMW component in plasma. It is possible that the process occurring on the platelet membrane surface is part of a mechanism regulating the vWF molecular mass distribution during its lifetime in plasma.

Speculation on the possible involvement of the glycoprotein Ib in the process described here can be attempted. The interaction between vWF and glycoprotein Ib occurs only when the former is complexed with ristocetin. We do not know whether this property has some physiological meaning, especially, since it is retained when platelets are metabolically inactive, whereas the capability to promote the conversion of vWF polymers is lost (fig.4). Two possibilities arise: or glycoprotein Ib is not involved in the polymer conversion, or the two properties are located in two distinct domains of the protein.

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