Willebrand Factor in Hemostasis in the In Vitro Bleeding Time

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Heparinized porcine blood and plasma, at constant hydrostatic pressure, was allowed to flow through a 5-mm incision in a small piece of porcine skin. Changes in the exuded blood volume were measured, and the incision site was examined microscopically. When normal blood flowed through either normal or von Willebrand skin, the exuded blood volume decreased gradually and eventually stopped. Microscopic examination revealed a platelet plug in the incision site. This plug was positive for Willebrand factor when examined by immunofluorescence. In contrast, the blood from von Willebrand pigs continued to flow constantly, and a platelet plug was not seen. The delayed in vitro hemostasis in von Willebrand blood was corrected to the normal range by the addition of either normal plasma or partially purified Willebrand factor. Normal blood, in which the Willebrand factor was immunologically inhibited, showed delayed hemostasis. For this in vitro system, it appeared that plasmatic Willebrand factor played an essential role in hemostasis.

A PROLONGED BLEEDING TIME was the main hemostatic abnormality found in the patients reported by von Willebrand, who suspected that there may be an abnormality of the interaction between the platelets and the blood vessel. It has now been shown that patients with von Willebrand disease have a decrease or abnormality of a protein in the plasma (Willebrand factor). The protein can be detected immunologically or functionally by its ability to cause aggregation of washed platelets by ristocetin. The protein is synthesized in the endothelial cell and apparently the megakaryocyte, and it is also present in the platelet and the plasma. In the porcine endothelial cell, it is partially extracellular and appears to be fibrillar or associated with a fibrillar protein. In the plasma, the protein exists as a series of polymers, which, in the pig, range in molecular size from about 1 to 21 million daltons.

The exact hemostatic role of Willebrand factor in these various compartments is still a matter of controversy. Weiss et al. showed that in patients, the bleeding time could be correlated with the level of Willebrand factor measured by ristocetin aggregation. On the other hand, after the transfusion of plasma and various plasmatic fractions, several authors have pointed out that there is only temporary correction of the bleeding time, which becomes prolonged despite high levels of Willebrand factor.

We have investigated the role of Willebrand factor in hemostasis in our pigs with von Willebrand disease, and in this article we report these studies using a new technique that we have called the “in vitro” bleeding time. This system has been used to measure both the normal hemostasis of swine and to investigate what causes abnormal hemostasis in the von Willebrand animals.
MATERIALS AND METHODS

In Vitro Bleeding Time Method

The measurement of the in vitro bleeding time is illustrated in Fig. 1. A 5-mm cut was made through the whole thickness of the 8 × 15 mm piece of pig skin, which was then placed over the channel in a lucite block and held in position by double-sided adhesive tape. Blood was allowed to flow from the reservoir to the channel in the lucite block through vinyl tubing. A hydrostatic pressure of approximately 15 cm of water was maintained throughout each experiment. The height of the reservoir was adjusted for each experiment so that the initial volume of exuded blood would be within prescribed limits. All the blood exuding from the skin incision was collected in plastic tubes at 1-min intervals using a fraction collector. Each fraction was then analyzed for volume, hematocrit, and platelet count. Platelet-rich plasma and platelet-poor plasma were used in the same way. The vinyl tubing used in the apparatus did not affect the platelet count of the blood passing through it.

Blood and Plasma Samples

Normal heparinized blood was obtained from healthy pigs that had no bleeding tendencies. The heparin concentration was 10 U/dl (heparin sodium by Organon Company, Ltd. or Abbott Laboratories). Normal platelet-rich plasma was obtained from the heparinized whole blood by centrifugation at 135 g for 10 min. Platelet-poor plasma was obtained by centrifugation of the anticoagulated blood at 1100 g for 30 min. Platelet counts in platelet-poor plasma were below 10⁴/cu mm.

Preparation of Pig Skin

Normal pig skin was obtained surgically from the abdominal area of anesthetized normal pigs ranging in age from 6 to 12 wk. Subcutaneous adipose tissue was removed, and the skin was kept moist at 4°C for up to 5 days. von Willebrand skin was obtained from live von Willebrand pigs or from recently exsanguinated pigs up to 8 hr postmortem. The von Willebrand skin donors ranged in age from 3 days to 1 yr.

Fig. 1. In vitro bleeding time method. A piece of porcine skin measuring 8 × 15 mm is placed over the channel of a lucite block fixed in position using double adhesive surgical tape. A 5-mm incision is made in the skin, and heparinized blood from the reservoir is allowed to flow through the incision. The exuding blood is collected in the fraction collector. The initial flow rate was adjusted by alteration of the hydrostatic pressure to give a volume of between 1 and 1.5 ml/min. This same hydrostatic pressure was then maintained for the rest of the experiments.
Willebrand Factor

Willebrand factor was measured by the degree of correction of platelet agglutination using the method of Olson et al.\textsuperscript{16} It was also measured immunologically\textsuperscript{15} by the Laurell technique\textsuperscript{17} using an antibody to a preparation of Willebrand factor purified by the technique of Olson et al.\textsuperscript{16} The Willebrand factor purified by this technique was isolated by its ability to induce platelet agglutination by ristocetin.

In this article, the term “Willebrand factor” will refer to the multimeric protein that is absent, decreased, or abnormal in von Willebrand disease. The abbreviation “VIII:RWF” (VIII:ristocetin-Willebrand factor) will be used when the activity was measured by platelet agglutination induced by ristocetin. The abbreviation “VIII R:AG” (VIII:related antigen) will be used when the material was measured immunologically by the Laurell technique.

Partial Purification of Willebrand Factor

Porcine Willebrand factor was partially purified as follows. Citrated porcine plasma was adsorbed with aluminum hydroxide (final concentration: 7.3 mg/ml) for 10 min at room temperature. The supernatant plasma was made 1 M in potassium phosphate (pH 6.5) and the precipitate was redissolved in citrated saline, 0.02 M sodium citrate 0.154 M sodium chloride containing 0.05 M L-aminocaproic acid. The volume was adjusted with this solvent to 10% that of plasma volume, and 2 ml of the redissolved precipitate was applied to a 45 x 0.9 cm column of CPG 250 (Electro-Nucleonic, Fairfield, N.J.) pretreated with 1% polyethylene glycol, 20 M in 0.9% NaCl, and then equilibrated with the same buffer.\textsuperscript{18} The gel filtration was carried out at room temperature at a flow rate of 20 ml/hr, and 1-ml fractions were collected. The fractions containing the VIII:RWF were pooled and precipitated with ammonium sulfate at 25% of saturation. The precipitates were collected by centrifugation at 1000 g and redissolved in a total of 2 ml of 0.15 M sodium chloride. This concentration was then dialyzed against 0.15 M sodium chloride containing heparin at a concentration of 6 U/ml.

Immunologic Reagents

Purified Willebrand factor adsorbed to aluminum hydroxide was used to immunize rabbits against porcine Willebrand factor as described by Olson et al.\textsuperscript{19} and Brockway et al.\textsuperscript{17} Sections of the frozen skin preparations 2-\mu thick were made and washed in phosphate-buffered saline (0.01 M NaPO\textsubscript{4}, 0.15 M NaCl, pH 7.4) for 15 min. Rabbit antibody to pig Willebrand factor (IgG purified by the method of McDuffie and Brumfield\textsuperscript{21}) was diluted 1:20 in phosphate-buffered saline containing 1:20 normal goat serum. The diluted rabbit anti-Willebrand factor antibody was added to the skin sections and incubated for 45 min in a moist atmosphere at ambient temperature. The slide was washed for 15 min in phosphate-buffered saline, and a fluorescent goat anti-rabbit antibody (Behring Diagnostics) absorbed with hog liver powder, 50 mg/ml (Pel-Freeze Biologicals, Inc., Rogers, Ark.), was applied. After incubation and washing, the tissue was examined by fluorescent microscopy.

RESULTS

Normal Whole Blood Perfusion

Normal heparinized blood was perfused through an incision in normal skin. The volume of blood exuding in the first minute was taken as unity, and all other volumes were measured as a percentage of the first minute volume. Figure 2 represents the results of 12 perfusions. The actual mean volume in the first minute was 1.66 ± 0.46 ml. In all 12 cases, the amount of blood exuding from the incision decreased with time and, except in 2 instances, eventually stopped within 20 min.

In similar experiments using normal blood flowing through von Willebrand porcine skin, the results were almost identical. The initial mean volume was 1.44 ± .44 ml for 11 experiments, and all perfusions resulted in hemostasis.
von Willebrand Whole Blood Perfusion

In the von Willebrand pigs, the average VIII:C was 36 U/dl, and there was no detectable VIII:RWF or VIII R:AG. The endothelial cells and platelets of these animals do not show VIII R:AG by immunofluorescence.

When the blood from a pig with severe von Willebrand disease was perfused through normal skin, there was no systematic change in the flow rate in 6 different experiments (Fig. 3). In this case, the mean blood loss in the first minute was $1.40 \pm 0.49$ ml. Similar results were obtained (2 experiments) when von Willebrand blood was perfused through von Willebrand skin; there being no decrease in blood flow from the initial rates of 1.10 and 1.60 ml/min. The results of all these perfusions are summarized in the cumulative graphs in Fig. 4.

The volume of blood lost in the first minute is taken as unity and in the case of the von Willebrand blood, there is no diminution in the rate of blood loss during the
period of observation. The curves for the von Willebrand blood perfused through both normal and von Willebrand skin show approximately 20 volume-units of blood lost in 20 min. In contrast, the curves for normal blood through either normal or von Willebrand skin show diminution in the accumulated volume to less than 1 volume-unit/min beginning within the first 5 min. (If the blood in all the experiments had stopped flowing completely, the accumulation rate would be 0 volume-units/min, and the slope of the curves would be 0.) The individual experiments using normal blood behave in a fashion similar to the averages shown in Fig. 4, in that the flow rate progressively falls before it ceases. The major differences between the individual experiments occur in the time at which the diminution of flow begins (see Fig. 2). The mean time of in vitro hemostasis (50% stopped) for normal whole heparinized blood is 12.5 min in a sample of 30. There was no significant difference between the platelet levels in normal and von Willebrand pigs.14

The platelet count in the exuding blood decreased when normal blood was perfused through normal skin but remained the same as in the reservoir when von Willebrand blood was perfused through the normal skin. Figure 5 shows the averaged platelet count in the exuding blood of 9 experiments using normal blood and 5 experiments using von Willebrand blood. The most striking decrease in platelet count occurred just before the bleeding stopped when normal blood was perfused.

Plasma Perfusion

Hemostasis occurred when normal platelet-rich plasma was perfused through either normal or von Willebrand skin. Hemostasis did not occur with either von
Willebrand platelet-rich plasma or normal platelet-poor plasma. In eight experiments using normal platelet-rich plasma, the pattern of in vitro hemostasis was not distinguishable from that seen with normal whole blood. In two cases using von Willebrand platelet-rich plasma, the results were similar to the five experiments using normal platelet-poor plasma, i.e., no apparent hemostasis (Fig. 6). The platelet count in the normal and von Willebrand platelet-rich plasma was adjusted to 500,000/cu mm by dilution with platelet-poor plasma.

Addition of Willebrand Factor to von Willebrand Whole Blood

The delayed hemostasis seen with von Willebrand whole blood was corrected to normal by adding 1 vol of normal platelet-poor plasma to the von Willebrand blood. When partially purified porcine Willebrand factor was added to von Willebrand platelet-rich plasma, the rate of blood loss decreased and eventually stopped (Fig. 7). The Willebrand factor levels, initially present in the reservoir, were 292 U/dl. The VIII:RWF activity detected in the exuded plasma decreased to approximately half of this initial value, although factor VIII R:AG remained constant.
Fig. 7. Partially purified von Willebrand factor was added to von Willebrand platelet-rich plasma and perfused through normal pig skin. The initial VIII R:AG, VIII: RWF, and exuding volume are expressed as 100%. The exuding volume gradually decreases, and eventually the blood stops flowing. There is little change in the VIII R:AG, but the VIII: RWF, although variable shows a tendency to fall.

Fig. 8. The effect of adding the IgG faction of the rabbit antibody to von Willebrand factor to the blood of a von Willebrand carrier. The mixture was incubated for 30 min. The VIII R:AG in the initial blood measured 31 U/dl and the VIII:RWF measured 44 U/dl. Increasing amounts of immune IgG were added and the residual VIII R:AG and VIII:RWF values were as follows:

<table>
<thead>
<tr>
<th>Curve No.</th>
<th>Average Residual VIII:RWF After 30 min (U/dl)</th>
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<tbody>
<tr>
<td>1</td>
<td>Blood from carrier ($n = 6$)</td>
</tr>
<tr>
<td>2</td>
<td>Addition of non-immune IgG ($n = 2$)</td>
</tr>
<tr>
<td>3</td>
<td>Addition of immune IgG ($n = 3$)</td>
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<tr>
<td>4</td>
<td>Addition of immune IgG ($n = 4$)</td>
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<tr>
<td>5</td>
<td>Addition of immune IgG ($n = 2$)</td>
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Addition of Rabbit Antibody Against Willebrand Factor to Whole Blood From a Carrier Pig

Various amounts of a rabbit antibody (IgG) against Willebrand factor were added to the blood of a carrier pig and incubated for 30 min. The blood contained 31 U/dl of VIII R:AG and 44 U/dl of VIII:RWF. Immunologic inhibition of the VIII:RWF resulted in defective hemostasis. When VIII:RWF activity was completely neutralized by immune IgG (curve 5 in Fig. 8), the bleeding resembled that of blood from a severe von Willebrand pig.

Microscopic Findings of the Incision Site

After perfusion of the blood, the skin was fixed in Orth's fixative to which the formalin was added immediately prior to use. Paraffin-embedded skin samples were serially sectioned at 2 μ, stained with Giemsa, and inspected for adherent platelets.

In the case of normal blood flowing through normal skin (Fig. 9), platelets adhered to the edge of the dermis and in the wound channel through the dermis. In the epidermis, a platelet plug was observed. The results were similar when normal blood was perfused through von Willebrand skin. In this case, the platelets were visualized by the fluorescence of the platelet Willebrand factor after immunologic staining. The VIII R:AG was again seen in the plug and in platelets adherent to the edge of the wound channel. The von Willebrand skin is ordinarily not stained by the fluorescent reagents except where contacted by the normal plasma (Fig. 10). In contrast, normal skin showed immunofluorescence of the capillaries as well as those areas contacted by VIII R:AG-containing perfusates.

Fig. 9. (A) Light micrograph (magnification 160×) of a section through the normal skin incision after 14-min perfusion with normal heparinized blood. The platelet plug is seen in the incision through the dermis. A line of platelets is seen adherent to the corium. (B) The indicated area of the platelet plug at a magnification of 640×.
Fig. 10. The section is taken through the incision in von Willebrand skin after the perfusion of normal blood. The material has been stained immunofluorescently with a rabbit antibody to porcine von Willebrand factor. Bright fluorescence is seen in the platelet plug occluding the incision and also in the platelets coating the incision in the corium.

Fig. 11. (A) Section (mag 160×) through the skin incision (normal skin) after the perfusion of von Willebrand blood for 20 min. Small platelet clumps (arrow) are seen in the channel. No adherent platelet layer was seen along the corium. (B) Platelet clump with associated red cells and leukocytes free in the wound channel (mag 400×).
When von Willebrand blood was perfused through normal skin (Fig. 1), occasional nonadherent platelet clumps containing leukocytes were seen, but the attached platelet plug and the characteristic adhesion of the platelets to the exposed dermis were not evident.

**DISCUSSION**

The decreased rate at which blood exudes from a skin incision using the in vitro bleeding time method seemed to reflect the formation of a platelet plug to seal the incision. By microscopic examination of the incision, a platelet plug was seen to form at the epidermal end of the incision in experiments using normal blood. The platelet count in the exuding blood remained similar to that in the reservoir blood until just before the in vitro bleeding stopped, suggesting that other events that do not consume platelets occur before formation of the hemostatic plug. These findings seem to be the same as reported by Borchgrevink in his studies of the secondary bleeding time. In those studies, there was a rapid drop in the platelet concentration in the blood exuding from the wound just before bleeding stopped. This was used as a test of in vivo platelet adhesiveness. The rate of formation of the platelet plug must increase as the rate of blood loss decreases. Weiss, Turitto, and Baumgartner have shown that at low shear rates in their apparatus, even the adhesion defect of von Willebrand blood disappears.

When von Willebrand blood was perfused through the skin incision, very few platelets adhered and no clumps formed in the incision through the dermis, but occasional platelet clumps were seen in the incision through the corium. Unattached platelet clumps appearing in the wound channel of the biopsied skin of von Willebrand patients were described by Hovig and Stormorken. These findings suggest that the von Willebrand platelet can interact with other platelets to form aggregates, as would be suspected from the fact that aggregation occurs normally in vitro.

The common finding in the in vivo and in vitro bleeding time studies suggests that the in vitro bleeding time method can be used as a model of the in vivo bleeding time measurement, although the resemblances and differences between the components of the skin dermis and that of the blood vessels need to be investigated further.

Delayed hemostasis of von Willebrand blood was corrected by mixing with normal platelet-poor plasma or purified Willebrand factor. The addition of anti-Willebrand factor antibody completely suppressed the ability of blood from a carrier pig (which showed normal hemostasis) to mediate hemostasis. Willebrand factor, therefore, must play an essential role in promoting platelet adhesion to unspecified elements in the dermis.

Platelets line the wound channel when perfusates containing Willebrand factor are used. This phenomenon is seen only when Willebrand factor is perfused, suggesting that the Willebrand factor may coat the wound surface and serve as a platelet-attachment protein. It is also of interest that the VIII:RWF activity falls relative to the VIII R:AG (Fig. 8), suggesting that the fraction of Willebrand factor that is active in ristocetin aggregation is utilized during the process of hemostasis. That a particular subset of Willebrand factor molecules is involved in ristocetin-induced aggregation was demonstrated by the work of Doucet-de Bruïne et al.
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