Hemostatic Plug Formation in Normal and von Willebrand Pigs: The Effect of the Administration of Cryoprecipitate and a Monoclonal Antibody to Willebrand Factor

By Y. Sawada, D.N. Fass, J.A. Katzmann, R.C. Bahn, and E.J.W. Bowie

Hemostatic plug (HP) formation was investigated in the ear bleeding time incision in normal and von Willebrand pigs. HP volume was calculated by integrating the areas of serial sections. In normal pigs (n = 11), platelets immediately formed a layer on the surface of the cut channel. Platelet aggregates formed at the ends of transected vessels and gradually enlarged. Finally, all transected vessels were occluded by HP and bleeding stopped. In contrast, large HPs were formed in the incision in von Willebrand’s disease (vWD) pigs (n = 4); these HPs did not cover the ends of the transected vessels, which continued to bleed, allowing the formation of large hemostatically ineffective platelet aggregates in the incision. Canals traversed these HPs, and bleeding from the open vessels may have continued through them. After infusion of cryoprecipitate into a vWD pig, the bleeding time shortened, and the morphological findings of the HPs were similar to those of normal pigs. In normal pigs (n = 3) infused with an anti-Willebrand factor monoclonal antibody, which prolonged the bleeding time, a large HP formed in the incision, similar to that observed in the vWD pig. The volume of the normal and vWD HPs increased with time. These in vivo findings suggest that Willebrand factor is involved in the localization of the HP to the damaged vessel and may also play a role in platelet–platelet interaction. A computerized morphometric technique was used for measuring the volume of the hemostatic plugs and the distance of sequential points on the perimeter of the HP from the center of selected bleeding vessels.

MATERIALS AND METHODS

Definition of Terms

In this article, von Willebrand factor refers to the multimeric protein that is absent, decreased, or abnormal in von Willebrand’s disease. Factor VIII refers to factor VIII coagulant activity measured by the degree of correction of the activated partial thromboplastin time of human hemophilia A plasma. Values above 1% of normal can be reliably measured for this factor. One percent of pig factor VIII activity is equivalent to approximately 8% human factor VIII.

Von Willebrand antigen (vWF-Ag) refers to the antigenic activity detected by a rabbit antibody to purified von Willebrand factor measured by electroimmunoassay. We calibrate this assay to a lower limit of 3%

Ristocetin cofactor (R.Co.) refers to the activity of von Willebrand factor that allows ristocetin to induce platelet aggregation. This activity is missing or reduced in the plasma in von Willebrand’s disease. R.Co. is measured by the degree of aggregation of washed gel-filtered human platelets using Olson’s modification of Weis’s method. The standard curve for this assay extends from 6.25% to 50%. Values as low as 3% were measured by extrapolation.

The von Willebrand Pig Colony

For several years, we have maintained a breeding colony of pigs with von Willebrand’s disease. These animals share the impairment of primary hemostasis and other hemostatic abnormalities of the severe form of the disease in humans. The animals have a serious bleeding tendency, which is transmitted as an autosomal recessive characteristic. The abnormal tests of hemostasis include prolongation of the bleeding time, almost complete absence

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Supported in part by National Institutes of Health grant No. HL-17430B and by a grant from the Mayo Foundation.


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0006-4971/86/6705-0005$03.00/0
of von Willebrand antigen (0.25% of normal), and the lack of R.Co. in the plasma.

Hemostatic Studies in Normal and von Willebrand Pigs

Eleven normal mixed-breed pigs (Duroc, Yorkshire, and Hampshire), weighing 36 to 45 kg, were used for the study of normal morphology and two for immunoperoxidase staining to investigate the localization of von Willebrand antigen. Three normal pigs, weighing 7.2 to 8.1 kg, were used for the study with monoclonal antibodies.

Four von Willebrand pigs, weighing 41 to 45 kg, were used. The factor VIII levels varied from 32 to 44 U/dl and the levels of von Willebrand antigen and R.Co. were <3 U/dL. None of the normal or von Willebrand pigs had ever been previously infused with whole blood or any blood products.

Cryoprecipitate Infusion into von Willebrand Pig

Cryoprecipitate was made from normal pig plasma by the method of Schlichter et al.26 Cryoprecipitate (factor VIII 2350 U, vWF-Ag 1650 U, R.Co. 1800 U; 973 mL) was infused into a vein on the posterior surface of the ear. The blood samples were taken from the antecubital vein on the foreleg.

Bleeding Time

The ear bleeding time test was done by Mertz28 modification of the immersion method of Doettl and Ripke.27 The pig was placed on its back on a wooden cradle, restrained by a rope laced across its body, and allowed to lie quietly for half an hour. The ear was cleaned and shaved, and an incision was made through the thickness of the ear near the apex, using a No. 11 Bard-Parker blade 3 mm thick. The width of the incision was controlled by inserting the blade to a depth of 5 mm. The ear was immediately placed in a beaker of isotonic saline that had been warmed to 37 °C, and the time of bleeding was measured. The bleeding time was the time that elapsed between the puncture and the cessation of any visible flow of blood from the incision. Care was taken to avoid small veins on the posterior surface of the ear.

At different intervals after the bleeding time started, the incision, together with the surrounding area, was excised through the complete thickness of the ear, using a circular biopsy punch 9 mm in diameter. The biopsy procedure took less than one second.

Tissue Preparation

The biopsy specimens for histologic studies were immediately immersed in Orth’s solution for fixation. After two-day fixation, the tissues were embedded in paraffin. Serial sections (6 μ thick) were made perpendicular to the surface of the skin and the incision. Every tenth section was stained with Giemsa stain. Multiple photomicrographs (×155) were taken of each plane so that a complete photomontage of the incision could be reconstructed.

On each photomontage, cross sections of the hemostatic plugs were outlined and numbered. The area of each hemostatic plug was measured with a computer-linked planimeter (HIPAD; Houston Instrument, Austin, Tex). The number of animals, the total number of histologic planes, and average number of histologic planes studies are recorded in Table 1.

Recorded in Table 2 for each experimental procedure and for each time interval are the number of plug cross sections, the cross-sectional area of plug, the total cross-sectional area of plug, and the total volume of plug. These quantities are expressed as average values per experimental animal. The average cross-sectional area of plug is expressed as empirical relative units of area (Table 2). The average total cross-sectional area of plug was calculated by multiplying the average cross-sectional area of plug per animal by the average number of plug cross sections per animal. The average total volume of plug per animal was calculated by multiplying the average total cross-sectional area of plug per animal by the average interpolar distance. Thus, the total volume of plug per animal is expressed as empirical relative units of volume (Table 2).

The digitizing pad (HIPAD, Houston Instrument) was also used in a few selected animals to measure the distance from the center of small lacerated arteries to points on the perimeters of adjacent hemostatic plugs. The number of points collected in each photomontage ranged from 61 to 441, with an average of 220. The distances were recorded as relative linear units.

RESULTS

The Bleeding Time Incision in Relation to the Morphology of the Pig Ear

The pig ear consists of epidermis, superficial layer of the dermis, deep layer of the dermis, and cartilage. The histologic structure of the anterior surface is similar to that of the posterior surface, although the former has more hair follicles than the latter.

The dermis is composed of a network of collagen, reticular and elastic fibers, hair follicles, sweat glands, adipose tissue, and vessels. Networks of vessels are present, especially in the capillaries of the dermal papillae that form loops. Small arteries and veins exist in the deep layer close to the cartilage.
### Table 1. Results of Morphometric Studies

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### Table 2. Results of Morphometric Studies

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*Relative empirical units.
The cut channel was made through the whole thickness of the ear. Networks of the capillaries, arterioles, venules, and small arteries and veins were transected. The largest diameter of the transected small arteries was about 280 μ.

The Morphology of the Hemostatic Plug in Normal Pigs

Five experiments were performed in normal pigs and biopsies were taken at 10, 30, 60, 120, and 180 seconds.

At ten seconds after the incision. In the channel, very small hemostatic plugs (≤20 μ) were already present. They were adhering to the ends of the transected vessels or connective tissue, but others were observed free of any attachment. In two of the five experiments, no hemostatic plugs were seen. This did not necessarily mean that there were no plugs because plugs were small and only every tenth section was examined. The appearance of these plugs was homogeneous and contained neither red cells nor leukocytes. At the end of the transected vessels, aggregated platelets were observed.

The margin of the bleeding time incision was covered with a layer of platelets. This layer of platelets and the aggregated platelets were further identified by PAP staining, using an antibody to pig Willebrand factor.

At 30 seconds after the incision. At this point, more hemostatic plugs appeared. Most of the hemostatic plugs were found associated with vessels and about 20% were unattached to either the transected vessels or connective tissue. At the cut surface of the small arteries, hemostatic plugs were formed, but at this stage, they were too small to cover the entire cut area (Fig 1).

At 60 seconds after the incision. The ends of some small arteries were completely covered with hemostatic plugs (Fig 2), but some were not. At this stage, some hemostatic plugs formed near the transected vessels bridged the bleeding time incision and were attached to the connective tissue in the superficial layer of the dermis. Hemostatic plugs attached to the incised surface of the epidermis were observed.

Material suggesting fibrin fibers was observed (Giemsa stain), especially along the cut surface of the connective tissue. This material occasionally seemed to trap red blood cells and leukocytes and could be seen around the hemostatic plugs.

In general, larger hemostatic plugs were formed in the deep layer of the dermis, where a number of transected small arteries and veins were seen. Hemostatic plugs attached to the cartilage were also observed.

At 120 seconds after the incision. There was a great deal of variability in the size of the hemostatic plugs. As was in the case at 60 seconds, these large hemostatic plugs tended mainly to be adhering to small arteries or veins. In the channel of the superficial layer where papillary networks were observed, some hemostatic plugs appeared to occlude the channel. The appearance of the hemostatic plugs was occasionally different from those formed at the earlier stages. Large plugs seemed to be composed of several small plugs. Between plugs there was some material suggesting fibrin and ballooned platelets.

The Morphology of the Hemostatic Plug in von Willebrand Pigs

Three experiments were performed in von Willebrand pigs. The hemostatic plugs were found in the incision, and there was no difference between von Willebrand pigs and normal pigs at ten seconds after the incision. The structure of the cut channel was similar to that of normal pigs at 30 seconds. The hemostatic plugs were found adhering to the
surface of the cut incision, and some were adhering to the ends of the transected vessels. At 60 seconds, while the cut ends of small arteries were completely covered with the hemostatic plug in normal pigs, the arteries were only partially covered in von Willebrand pigs, despite the fact that the volume of the hemostatic plugs was the same as that of normal pigs.

At two minutes, hemostatic plugs were adhering to the ends of the transected vessels or to the surface of the cut channel, but no arteries were completely occluded (Fig 3).

At three minutes, however, some of the connective tissue area in the channel was completely covered with large hemostatic plugs, but still the ends of the transected vessels were not covered. At seven minutes, the volume of the hemostatic plugs in the incision continued to enlarge, resulting in the covering of the whole inner surface of the incision except the ends of the transected small arteries (Fig 4).

At 15 minutes, small arteries were again not covered with the hemostatic plugs, which completely filled the incision. An irregular canal filled with red blood cells and leukocytes made a track through the hemostatic plugs, as if bleeding was continuing through it (Fig 5).

The Morphology of the Hemostatic Plug After Cryoprecipitate Infusion into a von Willebrand Pig

Five minutes after cryoprecipitate infusion, bleeding time tests were done and had shortened to six and eight minutes on two occasions. Seven bleeding time incisions were made simultaneously for morphological study and they were biopsied at ten and 30 seconds and at 1, 2, 3, 7, and 15 minutes. Within ten seconds, small hemostatic plugs were already observed. At 30 seconds, hemostatic plugs were found adhering to the ends of the transected vessels, and at one minute, some vessels were completely occluded (Fig 6). At three minutes, relatively large hemostatic plugs were found attaching to the transected small arteries. The appearance of the hemostatic plug was homogeneous. At seven minutes, the transected vessels were covered with the hemostatic plugs and no canal-like structure was observed (Fig 7). At 15 minutes, when bleeding stopped in the incision, some hemo-
static plugs developed fibers like fibrin and red blood cells and leukocytes appeared to be interlaced by this fibrous material.

The Effect of Infusing Monoclonal Antibody W1-8 on the Bleeding Time and the Morphology of the Hemostatic Plug

Ear bleeding time. A normal pig was injected with 0.4 mL of anti-Willebrand monoclonal antibody W1-8, which can prolong the bleeding time to 15 minutes and has a more pronounced effect on the bleeding time than our other monoclonal antibodies. There were no changes in the levels of factor VIII, von Willebrand antigen, and R.Cu.

Morphological changes in the bleeding time incisions. Biopsy specimens were obtained while bleeding was occurring at one minute and two minutes. At seven minutes, bleeding almost stopped. At 15 minutes, bleeding was not observed.

Morphology of the hemostatic plugs at one minute was basically similar to that of the normal study observed at one minute after incision had been made. Some hemostatic plugs were formed at the ends of the transected vessels and some were adhering to the surface of the cut channel. Larger hemostatic plugs were found close to the transected vessels as seen in a normal pig. At seven minutes, biopsies were done while the incision was oozing; huge hemostatic plugs were found and the ends of the small arteries were not covered with the hemostatic plugs (Fig 8). Canal-like structures that were packed with red blood cells and leukocytes could be seen in the large hemostatic plugs, suggesting that bleeding continued through this canal. This structure is consistent with that previously described in the von Willebrand pig. At 15 minutes, huge hemostatic plugs were seen (Fig 9) and the appearance was different from that at 1, 2, and 7 minutes. Granular appearance due to individual platelets were lost and a fibrinous network appeared. In the canal-like structure, red blood cells and leukocytes seemed to be interlaced by the fibrinous tissue.

Localization of the infused monoclonal antibody. PAP staining was performed in the cryostat sections to investigate the localization of the infused monoclonal antibody W1-8 in the tissue including the bleeding time incision. W1-8, mouse antiporcine von Willebrand factor, linked to porcine von Willebrand factor was detected by adding rabbit antimouse antibody and, subsequently, mouse PAP. W1-8 was localized to the hemostatic plugs composed of platelets, individual platelets attached to the surface of the cut channel or fibrous tissue, and endothelial cells of the vessel. Platelets showed more intense staining than did endothelial cells. One minute after the bleeding time incision had been made, that is, six minutes after monoclonal antibody infusion, all above-mentioned components were positive for W1-8 and there was no increase in the reaction subsequently at 2, 7, and 15 minutes.

Morphometric Studies

The number of animals studied at various time intervals, the total number of histologic planes, and the average
Fig 7. Von Willebrand pig after administration of cryoprecipitate, seven minutes after incision. The mural arterial laceration is completely occluded by a hemostatic plug. The plug is homogenous and does not show any plexiform channels (Giemsa, original magnification x 160; current magnification x 134).

Fig 8. Normal pig after administration of monoclonal antibodies, 15 minutes after incision. Note horizontal channel of laceration distended by hemostatic plug showing plexiform pattern of channels containing erythrocytes. Lacerated wall of artery (upper middle) is not occluded (Giemsa, original magnification x 40; current magnification x 34).

Fig 9. Normal pig after administration of monoclonal antibody, 15 minutes after incision. Lacerated wall of artery (right) not occluded, but communicates with small plexiform channel of hemostatic plug (Giemsa, original magnification x 40; current magnification x 34).
number of histologic planes per animal are recorded in Table 1. The average number of hemostatic plug cross sections, the average cross-sectional area of a hemostatic plug, the average relative total cross-sectional area of hemostatic plugs, and the average relative total volume of hemostatic plugs are recorded in Table 2. Table 2 demonstrates that in all experimental groups, as time increased, the hemostatic plugs increased with respect to numbers, cross-sectional areas, and total volume. Figure 10 shows the growth of total volume of hemostatic plugs from 0 to 900 seconds in experiments involving normal pigs, von Willebrand disease pigs, normal pigs given anti-Willebrand factor monoclonal antibody, and pigs with von Willebrand’s disease given cryoprecipitate. These values varied considerably among individual animals. The average values extend over several orders of magnitude (Table 2, Fig 10). The graphs of the average total volumes of hemostatic plugs (Fig 10) demonstrates essentially no difference among the various experimental groups of animals.

While at any one time period the total volume of hemostatic plugs was essentially similar among all experimental groups, bleeding time values indicated that the normal clotting process terminated promptly. Moreover, morphological studies of normal animals demonstrates prompt and complete sealing of small arteries (Figs 1 and 2) as opposed to incomplete plugging and plexiform channels among von Willebrand disease pigs (Figs 3, 4, and 5).

The implication of these observations is that the hemostatic plug material, while of about the same total volume as in normal pigs, is distributed differently about bleeding sites of von Willebrand disease pigs. To test this hypothesis, in selected cases (Figs 3 through 9), the distance of points about the perimeters of hemostatic plugs from the center of a small lacerated artery was measured with the computerized digitizing pad. It should be noted that these quantitative data, based on measurements of the previously described photomontages, extend the above qualitative histologic observations to contiguous areas of the experimental wound, which are about five to ten times greater than the single microscopic fields shown in Figs 1 through 9.

The quantitative results are shown in Figs 11 and 12 and summarized in Table 3. Figure 11 shows that in the normal pig at 120 seconds, the hemostatic plugs are in the immediate vicinity of the arterial laceration and that essentially all the plugs are less than 6 linear units’ distance from the lacerated small artery. These findings were termed the normal pattern.

In contrast, in pigs with von Willebrand’s disease, at all time intervals, hemostatic plugs were generally not in the immediate vicinity of the arterial laceration. More than half of the perimeter points were greater than 5 units’ distance from the lacerated artery and the maximum distance at 900 seconds is 27 units. These results were termed the bleeding pattern.

Figure 12 and Table 3 indicate that administration of cryoprecipitate changed the bleeding pattern of the pig with von Willebrand’s disease to the normal pattern. On the other hand, the administration of an anti-Willebrand factor monoclonal antibody to the normal pig transformed the normal pattern to a bleeding pattern.

Table 3. Studies of the Distance of Points on the Perimeters of Hemostatic Plugs from the Center of a Small Lacerated Artery

<table>
<thead>
<tr>
<th>State</th>
<th>Time (s)</th>
<th>Maximum Distance (units)</th>
<th>Percentage of Points &gt;5 Units</th>
<th>Reference Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>120</td>
<td>6</td>
<td>1</td>
<td>1.2, 11, 12</td>
</tr>
<tr>
<td>vW</td>
<td>120</td>
<td>12</td>
<td>55</td>
<td>3, 11*</td>
</tr>
<tr>
<td>vW</td>
<td>420</td>
<td>18</td>
<td>63</td>
<td>4, 11*</td>
</tr>
<tr>
<td>vW</td>
<td>900</td>
<td>27</td>
<td>93</td>
<td>5, 11, 12*</td>
</tr>
<tr>
<td>vW + CP</td>
<td>120</td>
<td>4</td>
<td>0</td>
<td>6, 7, 12*</td>
</tr>
<tr>
<td>Normal + MC</td>
<td>420</td>
<td>23</td>
<td>91</td>
<td>8, 9, 12*</td>
</tr>
</tbody>
</table>

vW, von Willebrand’s disease; CP, cryoprecipitate; MC, monoclonal antibody.

*Photomicrographs and measurements in same animal.

**DISCUSSION**

It has been suggested that von Willebrand factor plays an important role in platelet–platelet interaction.31 The anti-biotocetin induces platelet aggregation in normal plate-
NORMAL AND VWD PIGS: HEMOSTATIC PLUGS

In the present study, the development of the hemostatic plug was investigated, and the volume of the hemostatic plug was calculated using the bleeding time incision in the pig's ear. Our findings in the normal pig can be summarized as follows: Ten seconds after the bleeding time cut had been made, small platelet plugs and platelet aggregates were already present, mainly at the ends of the transected vessels. This finding seems to be the same as reported by Wester et al., in their human study after the template bleeding time test. Immunoperoxidase staining showed a layer of platelets along the surface of the wound channel. This phenomenon has also been reported in the in vitro bleeding time system using normal pig blood and pig skin.

Thirty seconds after the cut had been made, many hemostatic plugs were formed. The volume of the individual plug also increased. About half of these hemostatic plugs were formed at the ends of the transected vessels, most of which were not completely occluded at this stage. Between 30 and 60 seconds, the size and the number of the hemostatic plugs increased dramatically, and fibrin strands began to appear.

These findings suggest the following. Immediately after bleeding starts, circulating platelets begin to adhere to the surface of the cut channel, resulting in formation of a layer of platelets. Then platelet aggregation starts mainly in the vicinity of the transected vessels. Although there were many free platelet clumps in the cut channel, it was not certain whether they were formed at the surface of the incision or whether they were displaced into the wound from the ends of the transected vessels. These aggregated platelets formed larger aggregates with time. Finally, all transected vessels were covered with hemostatic plugs and bleeding stopped.

At 120 seconds, the incisions in two of the five experiments were still bleeding slightly, but there was no difference in number and size of the hemostatic plugs between these two and the other three experiments. Despite the macroscopic arrest of the bleeding, therefore, minor bleeding continued into the incision.

In complete contrast to the normal, large hemostatic plugs were found at seven and 15 minutes in the bleeding time incisions in the von Willebrand pig. The volume of the hemostatic plugs seemed to depend on the duration of the time the tissue was exposed to the blood. However, these large hemostatic plugs did not cover the ends of the transected vessels, so that these vessels continued to bleed, allowing large platelet aggregates to form in the bleeding time incisions. These platelet aggregates were not effective in producing hemostasis, similar to the findings in humans of Hovig and Stormorken. Furthermore, in the large hemostatic plugs, canals that were packed with red blood cells and leukocytes were observed, so it is likely that bleeding continued from the open vessels through these canals.

In our study, cryoprecipitate infusion into a von Willebrand pig corrected the defect of von Willebrand's disease. The bleeding time was shortened from more than 15 minutes to six to eight minutes, and the morphological findings of the hemostatic plugs were similar to those of normal pigs, showing the transected vessels completely covered with the hemostatic plugs. This evidence suggests that the transfused von Willebrand factor could recognize some components in
the vessel wall and could mediate the binding of the platelets to the injured vessels.

In a normal pig infused with a monoclonal antibody specific for von Willebrand factor, the ends of the transected small arteries were not covered with the hemostatic plugs at seven minutes, although large hemostatic plugs were found along the surface of the cut channel. At 15 minutes, when bleeding had already stopped, the bleeding time incision was packed by huge hemostatic plugs in which canals were seen. This structure is similar to that found in a pig with von Willebrand's disease.

Localization of the infused monoclonal antibody in the skin of the pig ear was investigated by the PAP staining method. Monoclonal antibody was distributed to the hemostatic plugs, individual platelets adhering to the surface of the cut channel and fibrous tissue, and endothelial cells of the vessels. Among them, hemostatic plugs showed the strongest reaction. Although the monoclonal antibody bound to the plasmatic von Willebrand factor, this immunohistochemical study suggested that it also bound to the local von Willebrand factor in platelets and endothelial cells. Localization of von Willebrand factor in the subendothelium that might be synthesized in endothelial cells has been confirmed by immunofluorescent and immunoelectron microscopic studies, and a role for this local von Willebrand factor in platelet–subendothelium interaction has been suggested.48

Our monoclonal WI-8 antibody inhibited adhesion of platelets to the ends of the transected vessels and might bind to the epitope on von Willebrand factor that is responsible for the binding of von Willebrand factor to certain elements of blood vessels or to platelets.

These in vivo findings suggest first that von Willebrand factor is important in the interaction of the platelet and the blood vessel and is involved in the localization of the hemostatic plug to the damaged vessel and its eventual occlusion. Furthermore, large platelet aggregates form in the absence of von Willebrand factor, but these aggregates are friable and penetrated by channels through which bleeding continues. These latter observations suggest that the von Willebrand factor also plays a role in platelet–platelet interaction.

ACKNOWLEDGMENT

We thank Marylou Stewart, Laurie Zoecklein, and Gerald McGrath for their expert technical assistance.

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Hemostatic plug formation in normal and von Willebrand pigs: the effect of the administration of cryoprecipitate and a monoclonal antibody to Willebrand factor

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