**Von Willebrand Factor: Gene Dosage Relationships and Transfusion Response in Bleeder Swine—a New Bioassay**

(von Willebrand disease/genetic marker/platelet aggregation/macromolecules and hemostasis/factor VIII)

THOMAS R. GRIGGS, WILLIAM P. WEBSTER, HERBERT A. COOPER, ROBERT H. WAGNER, AND K. M. BRINKHOUS

Department of Pathology, School of Medicine, University of North Carolina, Chapel Hill, N.C. 27514

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**ABSTRACT**

Aggregation of human platelets by bovine plasma was recently recognized as a marker for the study of the antihemophilic and von Willebrand factors. A similar marker in porcine plasma is shown to be specific for the platelet-active von Willebrand factor of plasma, but not for the antihemophilic factor (factor VIII). A new quantitative assay for the von Willebrand factor is based on the dose–response relationship observed between the concentration of von Willebrand factor and platelet aggregation times determined macroscopically. Bleeder swine, homozygous for von Willebrand disease, had no detectable platelet aggregating activity, while heterozygotes, carriers of the disease, had reduced levels of approximately 50% of normal. Exact detection of non-obligate carriers, hitherto impossible, becomes readily feasible because of the gene dosage relationship to the von Willebrand factor plasma levels. Transfusion of bleeder swine with normal plasma results in an immediate post-transfusion rise of the von Willebrand factor, followed by a rapid falloff. At 24 hr post-transfusion, no von Willebrand factor is detectable at a time when the factor VIII response is at its maximum. The discordance in plasma levels of this platelet-active von Willebrand factor and factor VIII in carrier animals and in the bleeder animals after transfusion suggests a different molecular and genetic basis for these two biological activities.

A hemophilia-like disease of swine was described in 1941 (1). The disease is identical in most respects with human von Willebrand's disease (vWD) and is characterized by a severe hemorrhagic diathesis, a prolonged saline bleeding time (2), reduced levels of plasma antihemophilic factor (factor VIII) (3), a marked elevation of plasma factor VIII following transfusions out of proportion to the amount of plasma or factor VIII administered (4, 5), and impaired platelet adhesiveness (6). The disease is autosomal recessive in inheritance. Carrier swine, unlike human heterozygotes of vWD, are asymptomatic, and the carrier state in pigs cannot be determined with certainty prior to the production of affected progeny (7).

We recently reported that the platelet aggregation which occurs when bovine plasma or plasma concentrate is mixed with a suspension of human platelets may be due to the platelet-active “von Willebrand factor” (vWF) present in the animal plasma (8). This platelet aggregating factor (PAF) in the plasma was shown to be separable from plasma factor VIII activity, but it appeared to be associated with a carrier protein for factor VIII. Porcine plasma likewise contains a PAF for human platelets (9), qualitatively similar to the bovine PAF. The studies reported here examine the quantitative relationship of the porcine PAF to the heterozygous and homozygous states of porcine vWD and the comparative response of factor VIII and PAF in bleeder animals following plasma transfusions.

**MATERIALS AND METHODS**

Swine with vWD. The swine stock segregating for vWD was from the Chapel Hill colony of the University of Missouri Poland China strain. Male and female pigs, both homozygous and heterozygous for the bleeder state, and weighing 65-135 kg, were used. The bleeder swine used in the transfusion experiments had never been transfused previously or had had no transfusions in the previous 4 weeks.

**Preparation of Porcine Plasma.** Porcine blood for assay for factor VIII and PAF was drawn from the ear veins of the animals into 1/5 volume of 3.2% trisodium citrate. The blood was centrifuged at 3000 × g for 30 min at 4°. Plasma was decanted and stored in aliquots at −20° or −70° until tested. Some assays were done on fresh plasma. Normal porcine plasma for standards was usually a pool from five animals and was stored at −70°. Plasma for transfusion was obtained from normal animals by collection of whole blood into commercial Blood Pack Units containing acid-citrate-dextrose (Fenwal, Morton Grove, Ill.), followed by centrifugation at 4500 × g, 10 min, 4°. This plasma was then stored at −20°.

**Preparation of Human Platelet Suspensions for the PAF Assay.** The platelet suspensions were prepared from fresh donor blood drawn in the morning from fasting young men. The donors had abstained from using aspirin or other drugs during 1 week before venipuncture. The blood samples were drawn into 1/5 volume acid-citrate-dextrose (10) and centrifuged at 250 × g for 10 min. The platelet-rich plasma was decanted and the platelet count determined. A 10-ml aliquot was then layered onto a 2.5 × 30-cm column containing 2% agarose (Sepharose 2B) which had been equilibrated with the eluting buffer, Tris-saline-dextrose [5 mM Tris·HCl·0.15 M NaCl·0.1% dextrose (pH 7.40)] (11). Chromatography was performed at room temperature at a flow rate of 125 ml/hr. The platelets eluted with the void volume. They were used directly or were subjected to one or two washings in buffer before use. In either case, the platelet concentration was adjusted to 400,000 per mm³ with buffer for use in the assay.
**PAF Assay.** The PAF assay was based upon the macroscopic platelet aggregation test described by Brinkhous et al. (12). Porcine plasma aliquots for testing were thawed at 37°, serially diluted with Tris-buffered-saline (50 mM Tris·HCl-0.15 M NaCl (pH 7.35)), and maintained at room temperature until used. The human platelet suspension (0.2 ml) was mixed with the diluted test sample (0.2 ml) in a 10 × 75-mm glass tube and the platelet aggregation time was determined. The mixture was tapped under a bright light until platelet aggregates were observed or until 120 sec had elapsed. The pooled porcine plasma standard was assigned a value of 100% PAF.

**Factor VIII Assay.** The factor VIII assay was a modification of the partial thromboplastin time assay of Langdell et al. (13). The substrate was canine plasma obtained from the hemophili A dogs in the Chapel Hill colony; it was stored at −70° and was used within 1 month of preparation. The substrate plasma was activated with kaolin immediately before use (2.5 mg/ml of plasma, 37°, 10 min, followed by centrifugation at 3000 × g, 10 min, 4°). The cephalin preparation was Thrombax (Ortho) which was diluted 1:4 with 0.15 M NaCl. Pooled normal porcine plasma was used as a standard and was assigned a 100% factor VIII value. Results were expressed as the percent of this standard.

**Saline Bleeding Times.** Saline bleeding times were determined by the method of Mertz (2).

**RESULTS**

**Bioassay for PAF.** The assay was based on the observation that with increasing concentrations of PAF there is a shortening of the macroscopic platelet aggregation time. This relationship is shown in Fig. 1. By comparing the relative effectiveness of a standard porcine plasma and the test material, the PAF activity in the test sample could be expressed as the percent of normal. Fig. 2 illustrates the procedure used. By graphing the results semilogarithmically, we observed a linear relationship between aggregation time and normal plasma concentration in the range of 0.25–25%. By comparison of the curves, it was possible to determine the relative concentration of PAF in the unknown in relation to that in the standard, as illustrated in Fig. 2.

**PAF and Factor VIII Levels in Pigs Homozygous and Heterozygous for vWD.** PAF and factor VIII levels were determined in a group of vWD swine of both sexes from the mating A−A−·XY × A−A−·XX. A− represents the autosomal chromosome carrying the von Willebrand locus. The progeny of such matings are either carriers or bleeders. The results of the study are given in Table 1. The carrier swine had a mean factor VIII level of 115% of normal and a mean PAF level of 36%. The bleeder swine had no PAF detectable in the bioassay and the value was arbitrarily recorded as <5%. The mean plasma factor VIII level was 13%, a value that is similar to that reported in previous studies (14).

**Response of Bleeder Swine to Normal Plasma Transfusions.** Transfusion experiments were performed three times on separate bleeder animals. The results were similar in each experiment. Fig. 3 illustrates the response in one pig. The factor VIII response was characterized by a late secondary rise. The PAF rose to 17% of normal 2 hr after transfusion. At 24 hr, no PAF was detectable when the factor VIII was at its highest level, 44%. No correction of the prolonged bleeding times after transfusions was observed.

**DISCUSSION**

vWD in swine is characterized by defective hemostasis that appears related both to a deficiency of factor VIII, with the associated delayed conversion of fibrinogen to fibrin, and to a deficiency of an ill-defined plasma factor, variously known as "vascular factor" or the platelet-active vWF, which is required for proper platelet function.

We report here a deficiency in von Willebrand bleeder pigs of a factor present in normal porcine plasma which aggregates human platelets. The observation that there was a dose-response relationship between the porcine platelet aggregating factor in normal plasma and the time required for the onset of macroscopic platelet aggregation (Fig. 1) permitted the development of a quantitative bioassay for porcine PAF. The bioassay is relatively simple to perform, but meticulous attention to certain details is required. In principle, the bioassay is patterned after the one-stage assay of factor VIII (13), but with the time required for the formation of gross platelet clumps (12) serving as the endpoint rather than
formation of a fibrin clot. Reliable determination of the endpoint requires practice, but once an individual becomes an experienced observer, results of an assay as illustrated in Fig. 2 appear to be replicable within a range of about \(\pm 10\%\). Isolated human platelets in suspension remain fully responsive to the animal PAF for several hr, a finding in accord with observations made previously on the stability of platelet "receptors" to the platelet aggregating activity of bovine plasma (8).

In bleeder animals, which are homozygous for the vWF deficiency, no platelet aggregating activity was detected (Table 1). In contrast, the factor VIII level was in the range of 10–20%. Bioassay for PAF in the carrier swine gave a range of levels from 26 to 55%, with a mean of 36%. This figure, based on a small sample size, is not significantly different from the 90% value (\(P = 0.25\) using the Wilcoxon signed-rank test) which would be expected if there were a direct relationship between plasma PAF levels and wild-type gene dosage. Thus, the results of the bioassay for PAF in our colony of bleeder swine indicate that the plasma level is dependent on gene dosage and it appears evident that PAF = platelet-active vWF. The discordance between factor VIII and vWF levels was consistently noted in all of the animals, homozygous and heterozygous.

While detection of a plasma deficiency of factor VIII has long been possible by the use of a quantitative bioassay procedure, no comparable bioassay for the vWF has been available. While bleeding time tests and platelet retention tests are abnormal in the bleeder swine, they appear to be poorly reproducible and only relatively gross indicators of the levels of platelet-active vWF. With the new simple bioassay, it appears possible not only to assay vWF but also to detect carriers readily, even if they are non-obligate on the basis of mating.

Previous work from this and other laboratories (4, 5) has demonstrated that when transfusion of normal porcine plasma into bleeder swine there is a biphasic response in levels of factor VIII without correction of the bleeding time. For the first 6-hr post-transfusion, the levels of factor VIII are predictable on the basis of the amount of normal plasma infused, similar to the response in canine or human hemophiliacs. This initial phase is followed in vWD animals by a prolonged response of factor VIII, often to higher levels than observed in the initial phase. The elevated factor VIII levels persisted as long as 72 hr. In the experiments reported here, the elevation in the platelet aggregating factor did not parallel the response of factor VIII except in the immediate post-transfusion period (Fig. 3). The platelet-active vWF increased to levels of about 17%, in direct proportion to the amount of normal plasma infused, and then fell to levels of less than 5% at 6 hr. At 24 hr after transfusion, when the factor VIII level was at its peak, no vWF activity was detectable. Again, the discordance between factor VIII and vWF plasma levels suggests a different molecular basis for these two biologic activities.

The bleeding time prolongation, so characteristic of vWD, could be a function of plasma levels of vWF. Thus the bleeding time is prolonged in bleeder swine with no detectable levels of the vWF and is still prolonged when the vWF is transiently raised to nearly 20% of normal by transfusion. In contrast, the bleeding time is normal in heterozygous animals with stable levels of vWF above 25% of normal. It would appear that for normal bleeding time values, vWF levels of 25% or above would need to be maintained. These data on the bleeding time test, which is an in vivo indicator for hemostasis, would suggest that therapeutic plasma levels of vWF for control of hemorrhage would need to be above 25% of normal.

The findings presented here would indicate that the PAF of porcine plasma is a quantitative marker for vWF. In recent studies the separation of factor VIII activity from PAF (vWF) was accomplished by several procedures, including agarose gel chromatography in the presence of 0.25 M CaCl\(_2\) and selective adsorption of bovine PAF with human platelets (8). In this report the discordance between factor VIII and vWF plasma levels in the genetic and transfusion studies again suggests a different molecular basis for these two biological activities.

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**Table 1. Plasma levels of platelet aggregating factor and factor VIII in carrier and bleeder swine of the von Willebrand strain**

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Factor VIII (%)</th>
<th>Factor PAF (%)</th>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>1</td>
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<td>normal (carrier)</td>
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<td>35</td>
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<tr>
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<td>55</td>
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<td>26</td>
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<td></td>
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<tr>
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<td>bleeder</td>
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<tr>
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<td>bleeder</td>
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<tr>
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<td>A(^{+})A(^{-})-XY</td>
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<td><strong>mean</strong></td>
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<td><strong>Control</strong></td>
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