Platelet Storage Pool Deficiency in Pigs

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We report a new bleeding disease—storage pool deficiency (SPD) of platelets—in pigs from the Mayo swine colony of homozygous von Willebrand’s disease (vWD) and of heterozygous carriers of vWD. Levels of factor VIII, von Willebrand factor antigen (vWF:Ag), and ristocetin cofactor (RCof) were similar in the vWD carriers and SPD pigs. The latter pigs, however, had bleeding times of 15 minutes or more and were severe bleeders, in contrast to clinically normal vWD carriers. Platelet aggregation in response to collagen was reduced in most SPD pigs. Total platelet content of ADP, ATP, and serotonin was less than that of normal pigs. While the initial uptake of 14C-labeled serotonin into platelets was similar in SPD and normal pigs, retention of serotonin was reduced in platelets of SPD pigs. Transmission electron microscopy showed a large decrease of dense bodies in the platelets of SPD pigs. These findings support a diagnosis of SPD. Genetic analyses suggest an autosomal recessive mode of inheritance. A breeding program is under way to produce pigs affected only at the SPD gene, thus allowing further characterization of SPD and SPD-carrier pigs.

In 1967, a colony of swine affected by von Willebrand’s disease (vWD) was established at the Mayo Clinic research facility from a single boar and three unaffected females. Pigs homozygous at the Willebrand locus are characterized by trace levels of von Willebrand factor antigen (vWF:Ag) (>2 U/dL), low levels of factor VIII, bleeding time of 15 minutes or more, and a severe bleeding tendency. Heterozygous (vWD) pigs have low mean vWF:Ag and RCof values (26 and 30 U/dL, respectively), normal levels of factor VIII, and no bleeding tendency. After several generations, a new bleeding manifestation became apparent. These “variant” pigs were characterized by levels of vWF:Ag, RCof, and factor VIII, similar to those seen in vWD carriers, but had bleeding times of 15 minutes or more and a severe bleeding tendency. Epistaxis, dental bleeding, and parturition were potentially life-threatening occurrences in these “variant” pigs, as in homozygous vWD pigs. Genetic analysis suggests that a new bleeding disorder, like porcine vWD, is transmitted in an autosomal recessive manner.

To study this new bleeding disorder, pigs were obtained from the Mayo Clinic. Platelet aggregation in response to collagen was reduced in most SPD pigs. Total platelet content of ADP, ATP, and serotonin was less than that of normal pigs. While the initial uptake of 14C-labeled serotonin into platelets was similar in SPD and normal pigs, retention of serotonin was reduced in platelets of SPD pigs. Transmission electron microscopy showed a large decrease of dense bodies in the platelets of SPD pigs. These findings support a diagnosis of SPD. Genetic analyses suggest an autosomal recessive mode of inheritance. A breeding program is under way to produce pigs affected only at the SPD gene, thus allowing further characterization of SPD and SPD-carrier pigs.

MATERIALS AND METHODS

Preparation of platelet-rich plasma and gel-filtered platelets. Blood was collected in 0.1 volume of 3.8% trisodium citrate or porcine intestinal mucosal heparin (Organon, West Orange, NJ) to a final concentration of 5 U/mL. Platelet-rich plasma (PRP) was prepared by centrifuging blood at 120 × g for 20 minutes. PRP was assayed for platelet adenosine diphosphate (ADP), adenosine triphosphate (ATP), and serotonin was centrifuged for 4 minutes at 120 × g twice to ensure the absence of red blood cells.

Citrated PRP (9 mL) was gel-filtered on a 2.5- by 20-cm 2% gel. Components for high pressure liquid chromatography consisted of a Beckman 450 data system/controller and two 114 M pumps. Waters model 441 UV detector at 254 nm, and a 0.46- by 25-cm anion exchange column (Whatman Partisil 10 SAX). ADP was eluted in 0.1 mol/L potassium phosphate at pH 4.2; ATP was eluted in a linear 0.4 to 0.5 mol/L phosphate gradient (ambient temperature, flow rate 3 mL/min) at 0.05 absorbance unit full scale (AUFS). Run time was 42 minutes. Serotonin was eluted with 0.01 mol/L phosphate at pH 3.1 at 1 mL/min. The 450 controller plotting factors were set at 30,000 for the nucleotides and 200,000 for serotonin. Standards were obtained from Sigma (St. Louis, Mo.)

Uptake and retention of 14C-labeled serotonin. A modification of the method of Weiss et al was used. Five microliters of 14C-labeled serotonin creatinine sulfate (Amersham, 55 mCi/mmol, 8 μCi/mL) was incubated in 2.5 mL of citrated PRP (6.25 × 108 platelets) for 15 minutes at 37°C. Impiramine was then added to a final concentration of 10 μmol/L, and incubation was continued at 37°C. Aliquots were removed at intervals and centrifuged, and the radioactivity of the supernatant was determined in a liquid scintillation counter (Beckman LS7500).

Transmission electron microscopy. Blood samples extracted by venipuncture were mixed immediately with citrate-citric acid-dextrose, pH 6.5 (93 mmol/L sodium citrate, 70 mmol/L citric acid, and 140 mmol/L dextrose) in a ratio of 9 parts blood to 1 part anticoagulant. PRP was separated from whole blood by centrifugation at 100 × g.

Samples were combined with an equal volume of 0.1% glutaraldehyde in White’s saline, pH 7.3—a 10% solution of a 1:1 mixture of (1) 2.4 mol/L NaCl, 0.7 mol/L KCl, 46 mmol/L MgSO4, 64 mmol/L Ca(NO3)2, 4H2O, and (2) 0.13 mol/L NaHCO3, 8.4 mol/L Ca(NO3)2, 2.7 mmol/L KCl, 0.36 mmol/L NaH2PO4, 1 mmol/L MgCl2, 5 mol/L HEPES, 5 mmol/L glucose, 0.38% trisodium citrate, 0.35% bovine serum albumin, pH 7.35), and 4-mL fractions were collected. The platelets were pelleted and resuspended in normal Tyrode’s buffer (2 g/dL, Difco, Detroit, Mich) and homogenized intermittently in a Waring blender for 90 minutes at 0°C. The supernatant was sonicated for 3 seconds before use, and was either used undiluted or mixed with an equal quantity of buffer.

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mmol/L NaH₂PO₄·2H₂O, 3.8 mmol/L KH₂PO₄, and phenol red (0.1 g/L). After 15 minutes at 37 °C, the samples were sedimented to pellets, and the supernatant was discarded and replaced with 3% glutaraldehyde in the same buffer. Fixation was continued at 24 °C for 60 minutes. The cells were then washed in buffer and combined with 1% oxalic acid in barbitral acetate buffer (0.02 N HCl, a 20% stock solution containing 0.14 mol/L sodium barbitral and 0.145 mol/L sodium acetate, and a 6.8% solution of a stock solution containing 1.7 mol/L NaCl and 18 mmol/L CaCl₂). After exposure to the second fixative for 1 hour, the cells were dehydrated in a graded series of alcohol and embedded in an epoxy resin (Epon 812).

Contrast of thin sections cut from plastic blocks on an ultramicrotome was enhanced with uranyl acetate and lead citrate. Observations were made with an electron microscope (Philips 301).

Infusion studies. Two SPD swine were placed on their backs in wooden cradles and restrained by ropes; pigs lie quietly in this position. One 136-kg pig received 1,400 mL of normal pig cryoprecipitate prepared by the method of Slichter et al. The other SPD pig was infused with 410 mL of normal pig platelet concentrate prepared by plateletpheresis in a cell processor (Haemonetics model 30 blood cell processor). The total platelet content was 10.7 × 10¹¹, which was calculated to result in an increase of 3.0 × 10⁹ platelets/mL. Blood was removed at intervals for hemostatic tests, and car bleeding times were determined.

RESULTS

Aggregation studies. Collagen-induced aggregation in citrated PRP was absent or decreased in 10 of the 11 SPD pigs (Fig 1). The response to collagen of platelets from vWD and vWD-carrier pigs was varied (two of eight vWD pigs and two of six vWD-carrier pigs having absent or decreased collagen-induced aggregation). A likely explanation for this finding is that some vWD-carrier pigs were heterozygous at the SPD gene.

The response to collagen when resuspended in PPP from pigs with SPD was expressed as ΔT. SPD platelets were less responsive to collagen whether resuspended in normal, vWD, or autologous PPP. In contrast, normal platelets aggregated in response to collagen when resuspended in PPP from pigs with SPD.

ADP, ATP, and serotonin contents of platelets. The total content of adenine nucleotides, as well as serotonin, in SPD platelets was less than that of normal pigs (Fig 3). Mean values in SPD pigs studied were 31% for ADP, 34% for ATP, and 23% for serotonin of the mean values in normal pigs. To test whether the low values in SPD pigs were...
characteristic of swine in our closed herd, ADP and ATP contents were determined in three vWD pigs and serotonin content was determined in one vWD pig. Pigs with vWD whose platelets aggregated normally in response to collagen were used for these determinations. Mean values were ADP 109.8%, ATP 104.7%, and serotonin 91.8% of the mean values for unrelated normal pigs.

**Uptake and retention of \(^{14}\)C-labeled serotonin.** Uptake of \(^{14}\)C-labeled serotonin after 15 minutes was similar in the two groups of pigs (normal 76.2%, SPD 70.7%). At 15 minutes, imipramine was added to inhibit the reuptake of any serotonin released by the platelet. Release of \(^{14}\)C-labeled serotonin from SPD platelets was evident 15 minutes after the imipramine was added; and at 4 hours, SPD platelets had released approximately one third of the \(^{14}\)C-labeled serotonin originally taken up (Fig 4). Normal pig platelets retained the amine for 4 hours.

**Electron microscopic findings.** Thin sections of the platelet preparation from a normal and a SPD pig were examined by transmission electron microscopy. There were 57 dense granules in thin sections of 125 platelets in the normal pig and 15 dense bodies in the same number of cells from the SPD pig (Fig 5).

**Infusion studies.** Enough normal porcine cryoprecipitate was infused into a SPD pig to raise the levels of vWF:Ag from 26 to 97 U/dL and of RCof from 29 to 103 U/dL 30 minutes after infusion. The bleeding time remained abnormal (over 15 minutes) through 24 hours (Fig 6). Such an infusion corrects the bleeding time in vWD pigs. Another SPD pig received platelet concentrate from a normal pig, which raised its platelet concentration by a maximum of 2.1 x 10^10 platelets/mL 1 hour after infusion. Bleeding time was shortened and transiently corrected to normal (Fig 6). Increased collagen-induced platelet aggregation was apparent after platelet infusion (Fig 6). Before infusion, the response curve to collagen had a slope of 1.5 and a zero \(\Delta T\). (This can occur because SPD platelets undergo a transient change in shape, resulting in a measurable slope; however,

**DISCUSSION**

The pigs in this investigation that we identified as having SPD were all derived from a herd of vWD and vWD-carrier swine. Our studies have shown that the platelets of these SPD pigs have a decreased number of dense granules and decreased amounts of serotonin, ADP, and ATP. Furthermore, platelet retention of serotonin after the addition of imipramine was impaired.

Pigs with homozygous vWD are characterized in part by extremely low vWF:Ag and RCof values (less than 3 U/dL), bleeding times of more than 15 minutes, and a life-threatening bleeding diathesis. Pigs heterozygous for vWD have higher, but not normal, mean levels of vWD-Ag and RCof (26 and 30 U/dL, respectively), normal bleeding times, and no bleeding tendency. Pigs from our herd with SPD have vWF:Ag and RCof levels similar to those in pigs heterozygous for vWD but, in contrast, have prolonged bleeding times—often more than 15 minutes—and a life-threatening bleeding tendency.

The observation that the SPD pigs originated in a vWD herd prompted us to investigate the possibility that they were
affected by a variant form of vWD similar to that found in humans because they had long bleeding times and mean values of vWF:Ag and RCoF of 25 and 35 U/dL, respectively. Examination of the von Willebrand factor multimers, however, revealed no abnormality, and our experience with porcine vWD suggested that the plasma level of von Willebrand factor was not low enough to cause bleeding without some additional abnormality being present. This led us to investigate other causes of the prolonged bleeding time and to the discovery of platelet SPD.

Like vWD, SPD is inherited as a simple autosomal recessive locus segregating independently of the similarly autosomal recessive von Willebrand locus. The vWD pigs in our herd, therefore, are homozygous, heterozygous, or normal at the SPD locus. Those with homozygous SPD display impaired collagen-induced platelet aggregation; all others have normal collagen-induced aggregation and nucleotide content. The vWD carriers also are either heterozygous or normal at the SPD locus. The vWD carriers have normal bleeding times and therefore cannot be homozygous at the SPD locus. Pigs with prolonged bleeding times, low platelet nucleotide content, decreased collagen-induced aggregation, and mean levels of vWF:Ag and RCoF of 25 and 35 U/mL, respectively, are SPD pigs that also happen to be carriers of vWD. We have not been able to identify SPD pigs that definitely do not carry vWD. The lower values of vWF:Ag and RCoF in normals overlap the higher values in carriers.

Because our breeding program was focused on producing vWD and vWD-carrier pigs, it can be expected that most of the SPD pigs identified were carriers of vWD. In fact, pedigree analysis shows that all of the SPD pigs in this study may be carriers of vWD (eg, some SPD pigs were the offspring of one vWD pig, and the others were offspring of vWD carriers). We have initiated a backcross breeding program to produce pigs affected only at the vWD locus or the SPD locus. It will then be possible to determine if the decreased levels of vWF:Ag and RCoF are due to heterozygosity at the vWD locus or are linked to the expression of SPD.

Like platelets in the human disease, platelets in porcine SPD show abnormal aggregation with collagen. The secondary response of platelet aggregation to epinephrine and ADP is also abnormal in human SPD. Using our aggregation procedure, however, platelets of normal pigs do not aggregate in response to epinephrine, and there is only primary (reversible) aggregation with ADP. Thus, abnormal collagen-induced aggregation is the only detectable abnormality of porcine platelet aggregation that resembles SPD in humans.

Platelet storage pool deficiency in humans can be categorized on the basis of a decrease of substances stored in the dense granules (SPD or δ-SPD), rarely the α-granules (α-SPD or gray platelet syndrome), or both dense and α-granules (αδ-SPD). SPD involving dense granules often accompanies other clinical abnormalities, such as the Chédiak-Higashi syndrome, Hermansky-Pudlak syndrome, Wiskott-Aldrich syndrome, and thrombocytopenia absent radii syndrome.

SPD has been found in association with the Chédiak-Higashi syndrome in mice, cattle, and cats. Several pigment mutations in mice have been associated with SPD. Recently, it has been shown that SPD in fawn-hooded rats is a pleiotropic effect of the red-eyed dilution gene.

The only additional abnormality that we have thus far detected in our SPD pigs is reduced vWF:Ag and RCoF. We believe that the original breeding strategy would have produced many SPD pigs that were also carriers of vWD, so that pigs produced by the current breeding program and affected only at the SPD gene may have normal levels of vWF:Ag and RCoF. If this is true, these pigs may be an animal model for the idiopathic inherited SPD found in humans.

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