Molecular Genetic Analysis of Porcine von Willebrand Disease: Tight Linkage to the von Willebrand Factor Locus

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von Willebrand disease (vWD), one of the most common bleeding disorders in humans, is manifested as a quantitative or qualitative defect in von Willebrand factor (vWF), an adhesive glycoprotein (GP) with critical hemostatic functions. Except for the rare severely affected patient with a gene deletion as etiology of the disease, the molecular basis for vWD is not known. We studied the molecular basis for vWD in a breeding colony of pigs with a disease closely resembling the human disorder. The porcine vWF gene is similar in size and complexity to its human counterpart, and no gross gene deletion or rearrangement was evident as the pathogenesis of porcine vWD. A restriction fragment-length polymorphism (RFLP) within the porcine vWF gene was identified with the restriction endonuclease HindIII, and 22/35 members of the pedigree were analyzed for the polymorphic site. Linkage between the vWF locus and the vWD phenotype was established with a calculated LOD score of 5.3 (1/200,000 probability by chance alone), with no crossovers identified. These findings indicate that porcine vWD is due to a molecular defect within (or near) the vWF locus, most likely representing a point mutation or small insertion/deletion within the vWF gene.

PROBABLY the most common inherited bleeding disorder in humans is von Willebrand Disease (vWD), with prevalence estimated to be as high as 1% in some populations. The clinical severity and laboratory findings in vWD are heterogeneous and numerous subtypes and variants have been described, all characterized by a quantitative or qualitative abnormality in the von Willebrand factor (vWF) protein. vWF is generally transmitted in an autosomal dominant fashion. Rare patients have a severe form of the disease manifested clinically as a serious hemorrhagic diathesis with very low or undetectable levels of circulating vWF antigen (vWF:Ag).

vWF is an adhesive glycoprotein (GP) that mediates platelet adhesion at sites of vascular injury and serves as the major carrier in plasma of FVIII:C (antihemophilic factor). The basic subunit of vWF is a 220-kilodalton (Kd) protomer that undergoes complex posttranslational processing from its ~307-Kd propolypeptide, first into dimers and then larger multimers ranging up to 20 million daltons. vWF is only known to be synthesized in megakaryocytes and endothelial cells, the latter being the major source of plasma vWF. The full-length cDNA for human vWF has been isolated, and the gene has been localized as a single copy on chromosome 12p12--pter. Recently, a homologous sequence on human chromosome 22 has been identified which crosshybridizes with the midportion of the vWF cDNA.

When analyzed by Southern blotting, most patients with vWD have no readily detectable abnormalities in the vWF gene. Because of the complex nature of vWF multimer production and secretion, a defect at a number of steps, either at the vWF locus or perhaps in a variety of other genes involved in vWF processing, could result in clinically similar vWD phenotypes. Historically, attempts at genetic linkage analysis in human vWD have been limited to studies using HLA-type and a number of known polymorphisms for serum enzymes and RBC antigens. No convincing evidence for linkage to any of these markers has been obtained. Recently, restriction fragment-length polymorphisms (RFLPs) have been identified in our laboratory and by other investigators. A preliminary study using such RFLPs suggests linkage between the vWF locus and vWD in patients with the type IIa variant. No such studies have been reported in type I vWD, the most common form of the disease.

A porcine model for vWD has been identified and extensively studied. Affected pigs were originally described in 1941 and were subsequently inbred. A large colony of >700 pigs established at the Mayo Clinic in 1967 from one such inbred Poland China boar had abnormalities of primary hemostasis typical of the severe form of the disease in humans. Affected pigs with severe disease are relatively resistant to the development of atherosclerosis. Such severely affected homozygote pigs can be readily distinguished from obligate heterozygotes by clinical and laboratory parameters.

We report a molecular genetic analysis of porcine vWD. The porcine vWF gene is similar in size and complexity to the human gene, and no gross deletions or rearrangements are evident as the molecular basis for porcine vWD. An intragenic RFLP is identified with the restriction endonuclease HindIII using human full-length vWF cDNA as probe. This marker is tightly linked to the inheritance of vWD in a large porcine kindred, indicating that porcine vWD is caused by a molecular defect in (or near) the vWF gene.

MATERIALS AND METHODS

DNA extraction and Southern blotting. Approximately 25 to 50 milliliters of blood from human volunteers and normal and vWD pigs were obtained from peripheral veins and anticoagulated in tubes.
treated with 2.5 to 5 mL 3.8% sodium citrate. Porcine and human DNA was extracted from peripheral blood leukocytes by standard procedures, resuspended in 10 mmol/L Tris-HCl, pH 7.5, and 1 mmol/L EDTA (TE buffer) and quantitated by absorption spectrophotometry at 260 nm. DNA from the Chinese hamster ovary cell (CHO)-human somatic cell hybrid (Ade-I) containing only chromosome 22 was supplied by M. VanKeuren and D. Patterson. Approximately 5 to 10 µg DNA were digested with various restriction endonucleases (International Biotechnologies, New Haven, CT; Bethesda Research Laboratories, Gaithersburg, MD). The samples were size-fractionated by electrophoresis in 1% agarose gels in 1 x 0.04 mol/L Tris-acetate and 0.001 mol/L EDTA (TAE buffer), transferred to nylon membrane (Hybond-N, Amersham, Arlington Heights, IL) by Southern transfer, and then prehybridized for three hours at 68°C in 1 mol/L NaCl, 1% sodium dodecyl sulfate (SDS), and 10% dextran sulfate. Hybridization then proceeded for 18 hours at 68°C in the same solution with the addition of 2 mg/mL salmon sperm DNA and the radiolabeled probe. cDNA probes were labeled with 32PdCTP by hexamer priming directly in 1% low-melt agarose (Bethesda Research Laboratories) following restriction enzyme digestion and gel electrophoresis. Specific activity ranged from 1 x 10^6 to 1 x 10^7 cpm/µg DNA. Probes included full-length vWF cDNA, the EcoRI insert of clone pvWH33, and fragments of pvWH33 as shown in Fig 1B. Various wash conditions were empirically tested to maximize hybridization between the human vWF cDNA probe and homologous porcine sequences. Optimum results were obtained with a final wash stringency of 0.2 x 30 mmol/L NaCl, 3 mmol/L Na citrate (SCC), 1 mmol/L EDTA, 0.1% SDS, and 10 mmol/L PO4 at 42°C. The washed filters were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at -80°C with one intensifying screen, and the autoradiographs were developed after a 5- to 8-day exposure.

**Blot stripping and rehybridization.** Nylon membranes could be stripped and rehybridized approximately five to eight times by soaking in 0.4 mol/L NaOH for 30 minutes at 42°C, followed by a 30-minute wash at 42°C in 0.1 x SSC, 0.1% SDS, and 0.2 mol/L Tris-HCl, pH 7.5. Adequate stripping was then confirmed by overnight exposure to Kodak XAR-5 film at -80°C with one intensifying screen.

**Calculation of the log of the odds (LOD) score.** The LOD score was calculated using the MENDEL and LIPED computer programs. Allelic frequencies were estimated by random sampling of 15 normal pigs from three Minnesota farms.

**RESULTS**

**Porcine vWF gene.** Southern blot analysis of BalI-digested DNA from a human, a severely affected vWD pig, and an unrelated normal pig using full-length human vWF cDNA as probe is shown in Fig 2. A relatively high degree of homology between porcine and human vWF sequences is evident from the similar hybridization signals obtained at moderately high stringency. In addition, based on the number and size of bands, the human and porcine gene appear to be similar in size and complexity, with the human gene known to span ~175 kilobases (kb) and interrupted by a minimum of 20 introns. The patterns in DNA from normal and vWD pigs are identical. Similar identical pat-
terns were obtained with nine other restriction enzymes studied to date (EcoRI, BglII, BamHI, SacI, KpnI, RsaI, TaqI HindIII, and AwaI, data not shown). These findings indicate that no gross gene deletion or rearrangement has occurred within the vWF gene as the molecular basis for porcine vWD.

Identification of an RFLP in the porcine vWF gene. Southern blot analysis with the restriction endonuclease HindIII is shown in Fig 3A for eight members of the vWD pedigree. A difference in pattern between homozygous severe pigs (left four lanes) and heterozygote vWD pigs (right four lanes) is clearly evident (arrows). A similar analysis in four normal pigs, not from the vWD pedigree, is shown in Fig 3B. The two variant bands migrating at 4.1-kb and 3.8-kb appear to be allelic. Presence of the 3.8-kb band is denoted as plus(+) and the 4.1-kb allele is denoted as minus(−). In the normal pigs, heterozygote (+/−) as well as both homozygote genotypes (+/+, −/−) are readily observed, indicating that this is a frequent RFLP and that the + allele is neither the vWD molecular defect itself nor a private polymorphism restricted to the vWD pedigree. Fifteen normal pigs were evaluated for the presence of this RFLP. Allelic frequencies were 0.7 for the 4.1-kb allele and 0.3 for the 3.8-kb allele. The samples studied were obtained from three geographically adjacent farms, and inbreeding may have imparted a sampling bias. Failure to detect the hybridizing 300 basepair (bp) difference fragment in subjects with the 3.8-kb allele suggests that the polymorphic site is located within an intron.

Documentation of linkage in the vWD pedigree. The portion of the affected pedigree chosen for study is shown in Fig 4. The three generations shown contain 35 individuals, 17 of whom are phenotypic heterozygotes, and 18 of whom are severely affected vWD pigs. Of these, a total of 22 members were genotypically analyzed for the polymorphic site: 13 severely affected and 9 heterozygotes. In all cases, vWD heterozygotes were heterozygous for the HindIII RFLP (+/−), and severe vWD homozygotes were homozygous for the 3.8-kb band (+/+) (Figs 3A and 4). The 3.8-kb HindIII allele thus cosegregates with vWD in this pedigree with no recombinants. The maximum LOD score31 was calculated as 5.3 at a recombination fraction of Θ = 0.

Localization of the HindIII RFLP. Recently, Shelton-Inloes and colleagues18 described a sequence on human

Fig 3. Southern blot of HindIII RFLP using human full-length vWF cDNA as probe. (A) vWD pigs. Left four lanes: severely affected pigs revealing the 3.8-kb band only (left arrow). Right four lanes: heterozygote carriers who have both 3.8-kb and 4.1-kb alleles (right arrows). (B) Normal pigs. Two allelic variant bands representing the polymorphic site (arrows) at 4.1-kb and 3.8-kb are present in four normal unrelated pigs. The relative positions of HindIII-digested λ phage DNA fragments used as size markers are indicated to the left (23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb).

Fig 4. Family tree of porcine vWD pedigree. Twenty-two of 35 members were evaluated for the HindIII RFLP (denoted as the plus(+) or minus(−) allele. All severely affected individuals have only the 3.8-kb allele (+), whereas all heterozygotes have both the 3.8-kb and the 4.1-kb alleles (+/−). Squares and circles refer to males and females, respectively; half-shaded figures are phenotypic heterozygotes; and shaded figures are phenotypic severely affected pigs. Each member is identified by a number; Roman numbers (right) refer to the generation.
chromosome 22 that crosshybridizes with the midportion of the vWF cDNA, from approximate position 3,000 to 5,700 bp (Fig 1A). The precise function of this sequence, if any, is unknown. Thus, a given restriction fragment detected by full-length vWF cDNA could correspond to either the authentic vWF gene or to the chromosome 22 homologue. Figure 5A (left panel) shows a SacI digest of DNA from CHO, Ade-I and normal human, probed with C1, a fragment from the midportion of the human cDNA. The human lane contains two bands, one of which (arrow) hybridizes to both human and Ade-I, confirming the presence of homologous vWF sequences on human chromosome 22. The same blot was then stripped and reprobed with A3 (right panel), revealing only one band in the human lane that is not present in Ade-I, demonstrating that the region of the human vWF gene corresponding to A3 is present only in one copy on chromosome 12 and that the 5' border of the chromosome 22 sequence is distal to the first 2.3-kb of vWF cDNA. Detection of the vWF homologous chromosome 22 sequence by probe C1 (and also by probe A2 but not probe A1, not shown) localizes the 5' border of this sequence to between bp position 2,817 and 3,825 of the vWF cDNA. HindIII digests of porcine DNA were sequentially probed with multiple vWF cDNA fragments to sublocalize the region of human vWF cDNA corresponding to the segment of the porcine gene containing the HindIII RFLP. Probes A1, A2, and A3 (Fig 1B) continue to identify the HindIII polymorphic site, whereas probes B1, B2, B3, and C1 do not. Results with probes A2 and A3 are shown in Fig 5B (left two panels). BallI-digested DNA probed with A3 (Fig 5B, right panel) reveals only one hybridizing band (open arrow), confirming that this region of the vWF gene (containing the HindIII RFLP) is present as only a single copy in the porcine genome. Further work (Ginsburg, Bahou, Kelly, Harnden, unpublished observations) suggests that a vWF homologous sequence corresponding to the human chromosome 22 segment is absent in the pig and a number of other nonprimate species, implying that its appearance in humans represents a relatively recent evolutionary event.

DISCUSSION

Porcine vWD closely resembles the human disease by both laboratory and clinical parameters, and has served as an excellent model for studying both components of the vWF-FVIIIIC molecular complex.\textsuperscript{4,25,33} We showed that the porcine gene is similar in size and complexity to the human gene, which has been shown to span ~175 kb and to contain ~20 exons.\textsuperscript{32} The unusual size of the vWF gene might make it a particularly large target for the occurrence of random mutations, possibly contributing to the frequent occurrence of vWD in humans and identification of a similar disease in a number of animal species.\textsuperscript{34} The relatively high degree of coding sequence homology between the human and porcine genes, suggested by our Southern blot data (Figs 2 and 3), may indicate a strong evolutionary pressure for conservation of vWF structure across species. Such sequence homology has also been noted in several other mammalian species.\textsuperscript{32}

We confirmed the presence of a vWF homologous sequence on human chromosome 22 and mapped its 5' border as between bp 2,817 and 3,825 of the human vWF cDNA, further refining the data of Shelton-Inloes and colleagues.\textsuperscript{10} As shown in Fig 1A, the 3' border of this crosshybridizing sequence is somewhere upstream of cDNA residue 5,700.\textsuperscript{10} The porcine HindIII vWF RFLP is located upstream of this region, and additional Southern blot data confirms its presence within a single-copy region of the porcine vWF gene. In genetic linkage studies in humans, putative vWF RFLPs should similarly be investigated to distinguish their association with either the authentic vWF gene on chromosome 12 or its homologue on chromosome 22.\textsuperscript{14,15}

We successfully documented tight linkage between vWD
and the vWF gene in a large porcine vWD pedigree. The vWF molecule undergoes complex posttranslational processing, including dimer formation, glycosylation, multimerization, and cellular secretion through two distinct pathways. Each of these processing steps probably involves the products of many genetic loci. A defect in any one of these multiple steps could result in the vWF phenotype and could well result from defects at genetic loci distinct from the vWF gene itself. Such genotypic heterogeneity, i.e., causation of similar or identical phenotypes by mutations at different genetic loci, occurs in several human genetic disorders. The broad array of mucopolysaccharidoses that result from defects at multiple distinct loci involved in intracellular protein processing and trafficking was recently reviewed. Our highly significant LOD score strongly suggests that porcine vWF results from an abnormality within the vWF gene, although we cannot exclude the possibility of a defect in another gene in close proximity to the vWF locus. Given the normal gross gene structure in porcine vWD by Southern blot analysis, this defect most likely represents a point mutation or small insertion/deletion within the gene. Similar defects affecting all phases of gene processing and expression have been described in the thalassemias and a variety of other genetic diseases.

In addition to its application as a genetic linkage marker, this porcine vWF RFLP may also prove useful in bone marrow transplantation (BMT) studies of porcine vWD. To study the relative contribution of plasmatic and platelet vWF to vWF functional activity, we successfully performed a marrow transplant from a normal pig into a vWD animal, resulting in a chimeric subject with normal platelet vWF and very low plasma vWF. Additional BMT studies are in progress to elucidate this problem further. We previously described the application of RFLP analysis to study the origin of cell populations following BMT in humans. Similarly, analysis with the porcine vWF RFLP described here can now be used to demonstrate engraftment and to characterize the origin of cell populations in this animal model system.

In summary, we showed that the porcine vWF gene is highly homologous and is similar in size and complexity to its human counterpart. No gross gene deletion or rearrangement occurs in the vWF gene as an explanation for porcine vWD, a situation analogous to findings in most vWD cases in humans. We identified a frequent RFLP within the porcine vWF gene and established tight linkage to vWD in a large porcine kindred. A similar genetic approach to human vWD should eventually establish whether all or some vWF subtypes are similarly owing to defects within the vWF gene or defects at distinct distant loci.

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