Analysis of von Willebrand Factor mRNA From the Lung of Pigs With Severe von Willebrand Disease by Using a Human cDNA Probe

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To examine the control of porcine von Willebrand factor (vWF) biosynthesis we cloned human vWF complementary DNA (cDNA) and investigated the expression of the vWF gene in lungs from normal pigs and pigs with severe von Willebrand's disease (vWD). Recombinant clones spanning approximately 90% of human vWF cDNA were identified in a λ gt10 human lung cDNA library by screening with oligonucleotides. One clone spanning nucleotides 960 to 3,240 of human vWF cDNA was used to investigate the steady-state levels of vWF mRNA in lungs from normal pigs and from pigs phenotypically determined to be homozygous for vWD. This clone hybridized with genomic DNA from pig leukocytes when Southern blots were processed under very stringent conditions; therefore, human cDNA clones were considered valid probes to detect porcine mRNA. Northern blot analysis of total RNA from normal pigs and from pig umbilical vein endothelial cells identified the vWF mRNA as a molecular species of approximately 9.0 kilobases (kb). A very faint to undetectable band at 9.0 kb in total RNA from lungs of vWD pigs suggested a decreased rate of transcription of the vWF gene. Sucrose density gradient centrifugation of RNA from the vWD pigs confirmed by Northern analysis that the high-molecular weight fractions contained vWF mRNA and at the same size as normal pig mRNA. Dot blot hybridization analysis of vWF and actin mRNA processed under stringent conditions demonstrated that the relative ratio of vWF mRNA to actin mRNA in the vWD pigs varied from 21% to 41% of the ratio observed in normal pigs. Because the amount of vWF mRNA is not correlated to the amount of vWF activity or antigen in plasma of vWD pigs we conclude that posttranscriptional events are also probably involved in abnormal expression of vWF in these animals.

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expression of vWF in vWD pigs and that the defect in porcine vWD may not be solely in the control region of the gene.

MATERIALS AND METHODS

Materials. The cDNA probe for human cytoplasmic actin was kindly provided by Dr Elaine Fuchs of the University of Chicago.

Nucleic acid–grade phenol, cesium chloride, vanadyl ribonucleoside complexes, and RNA molecular weight (mol wt) markers (RNA ladder) were obtained from Bethesda Research Laboratories (Bethesda, MD). Nitrocellulose filters and the dot blot apparatus (Mini-fold) were purchased from Schleicher and Schuell, Inc (Keene, NH). Guanidinium isothiocyanate was from Fluka Chemical Corp (Buchs, Switzerland). Analytic-grade sucrose was obtained from Serva (Heidelberg, FRG). Polyallomer centrifuge tubes were from Kontron AG (Zurich). DNase I was obtained from Promega-Biotech (Madison, WI), and RNase A was from Sigma Chemical Co (St Louis). Other enzymes, transfer RNA, and salmon sperm DNA were purchased from Boehringer-Mannheim (Mannheim, FRG). Dextran sulphate and oligo(dT)-cellulose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Radioactive isotopes, nick-translation, and M13 sequencing kits were from Amersham Corp (Amersham, UK). All other reagents were from Sigma or Merck (Darmstadt, FRG).

Phenotypic diagnosis of vWD. A colony of pigs with vWD was established by artificial insemination with semen from pigs affected with homozygous vWD.14 Semen was obtained through the courtesy of Dr E.J.W. Bowie, Mayo Clinic, Rochester, MN. The animals were maintained on the experimental farm of the Institut National de la Recherche Agronomique (INRA; Jouy-en-Josas, France). All procedures on pigs were in accordance with INRA guidelines, and the pigs were killed by electrocution. The animals from the sixth generation of crossbreeding were used in this study. The phenotypic diagnosis for porcine vWD was based on assessing the bleeding time, plasma vWF Ag, and vWF activity (human platelet aggregating activity in the absence of ristocetin) as previously described.15 The three vWD pigs used in this study were females 6 to 7 months of age. They were the offspring from two different pairs of pigs who were phenotypically heterozygous for vWD (vWF Ag and activity, approximately 50% of normal values). The experimental pigs were deemed to be homozygous for vWD because they exhibit bleeding times longer than 15 minutes, no vWF activity in plasma, and levels of vWF Ag less than 0.01 U/mL (less than 1.0% of a normal porcine plasma pool). Previous results have shown that vWD pigs have levels of vWF Ag in platelets of approximately 0.3 U/million platelets compared with about 14 U/million platelets in the normal animals.15

Cloning of vWF cDNA. A cDNA library was constructed from mRNA isolated from adult human lung in the vector Agt10. Poly(A)⁺ RNA was isolated and cDNA synthesized by conventional methods using reverse transcriptase, Klenow polymerase, and S1 nuclease.20 The cDNA was size fractionated (0.5 kb), oligo dG-tailed, and annealed to a 3' end complementary to a partial vWF cDNA used in this study. The cDNA was size fractionated (>0.5 kb), oligo dG-nuclease.21 The cDNA was then ligated in EcoRI arms of Agt10 and plated on Escherichia coli, strain POP 101.22 Twelve probes of 21 nucleotides in length were synthesized by using an Applied Biosystems 380B synthesizer (Foster City, CA). The sequences of the oligonucleotides were based on the published sequences of vWF cDNA.6,7 The oligonucleotides were purified by high-performance liquid chromatography on a C₄ column. The probes were 5' end labeled by using (γ-³²P) adenosine triphosphate and T4 polynucleotide kinase.20 Approximately 1.3 × 10¹⁵ recombinant plaques were screened with these ³²P-labeled oligonucleotides. The sequences of the oligonucleotides that were used to identify the partial vWF cDNA used in this study correspond to the nucleotides 2781 to 2901 and 2924 to 2944 of the reported cDNA sequence.6 After plaque purification the cDNA inserts were excised with EcoRI and subcloned into the EcoRI site of pUC9 or m13mp18 for DNA sequence analysis performed by the dideoxy method.23 Isolation and characterization of the actin cDNA clone has previously been described.24

For hybridization studies the vWF cDNA insert was nick-translated to a specific activity of 5 to 14 × 10⁹ cpm/µg. For detection of actin mRNA or DNA the plasmid pBR322 containing the insert was used for nick-translation (5 × 10⁹ cpm/µg).

Isolation of RNA. For RNA isolation sterile disposable plastic-ware and, when needed, RNase-free glassware were used. Stock solutions and water were treated with 0.1% diethylpyrocarbamide (DEP) and autoclaved. Solutions for homogenization and ultracentrifugation were filtered (0.45 µm) immediately before use. Total RNA from lung tissue was isolated by modification of the guanidinium thiocyanate method of Chirgwin et al and Bahnak et al.25,26 Pieces of lung were collected from normal and vWD pigs within 30 minutes after the animals were killed and immediately frozen in liquid nitrogen or on dry ice. Lungs were pulverized on dry ice, and approximately 10 g was homogenized at room temperature in about 50 mL of 6 mol/L guanidinium thiocyanate, 25 mmol/L trisodium citrate, pH 7.0, 0.1 mol/L β-mercaptoethanol, and 0.5% Sarkosyl with a Polytron (Kinetica, Kriens-Luzern, Switzerland) at three-fourths speed for one to two minutes. The homogenate was passed through four layers of sterile gauze, and 1 g CsCl was added for every 2.5 mL of homogenate and allowed to dissolve. The homogenate was layered onto 3 mL of a CsCl cushion (5.7 mol/L CsCl, 0.1 mol/L EDTA, pH 7.5) in polyallomer centrifuge tubes and centrifuged at 20°C in a SW 41 Ti rotor (Beckman Instruments, Palo Alto, CA) at 30,000 rpm for 20 hours. After centrifugation the tubes were clamped with a hemostat below the labeled DNA and cut. The supernatants were removed, and the pellets were washed twice with ice-cold 70% ethanol and dissolved in 7.5 mol/L guanidinium HCl, 25 mmol/L trisodium citrate, pH 7.0, and 5 mmol/L diethiothreitol. To the dissolved RNA was added 0.025 vol of 1 mol/L acetic acid and 0.6 vol of ethanol, and the RNA was allowed to precipitate overnight at −20°C. The RNA was pelleted by centrifugation, washed once with 70% ethanol, and dissolved in sterile H₂O or 0.05% sodium dodecyl sulfite (SDS).

Sucrose density gradient centrifugation was used to select high mol wt RNA.27 The sucrose solutions (10% to 30%) were prepared in TE buffer (10 mmol/L Tris-HCl, pH 7.5, 0.1 mmol/L EDTA) and made 0.2% with DEP. The solutions were allowed to sit at room temperature for two to three hours and then were cooled to 4°C. The solutions were used on the same day to construct a 10% to 30% linear sucrose gradient in polyallomer centrifuge tubes. Total RNA was diluted in TE buffer to approximately 1 µg/µL, heated at 65°C for ten minutes, and cooled on ice and about 300 to 500 µg loaded onto a single gradient. Centrifugation was at 4°C for 16 hours at 36,000 rpm in a SW 41 Ti rotor. After centrifugation 0.5-mL fractions were taken from the top of the tube and 15 µL from each of the last eight fractions was electrophoresed in denaturing agarose gels as described later for Northern blots and stained with ethidium bromide to monitor the banding of the ribosomal RNA. The fractions that contained RNA with an S greater than 28 (usually the last 1 mL of the gradient) were used for further analysis. Polyadenylated mRNA was separated from other RNA species by binding to oligo(dT)-cellulose as described.28 RNA from cultured human umbilical vein endothelial cells (HUVEC), human fibroblasts, and U-937 cells29 was isolated by the hot phenol method in the presence of high concentrations of vanadyl ribonucleoside complexes (10 mmol/L).30,31

Dot blot analysis. RNA preparations were first incubated with RNase free DNase at 37°C for 45 minutes to eliminate the possibil-
vWF mRNA in Porcine vWD

The RNA samples were denatured in 70% formaldehyde and applied to nitrocellulose (0.45 μm) by using a dot blot apparatus as previously described. After blotting the nitrocellulose was air-dried and baked in vacuo for two hours at 70 to 80°C. Dot blots were prehybridized for at least four hours in a solution containing 40% deionized formamide, 0.1% SDS, 6 × SSEP (SSEP: 0.14 mol/L NaCl, 10 mmol/L NaH₂PO₄, pH 7.4, and 1.0 mmol/L EDTA), 2 mmol/L EDTA, 100 μg/mL sonicated salmon sperm DNA, 100 μg/mL yeast transfer RNA, and 5 × Denhardt’s solution. Hybridization was performed overnight at 42°C in the same solution as was used for prehybridization containing 1 × 10⁵ cpm/mL of nick-translated vWF cDNA or 0.5 × 10⁵ cpm/mL of actin cDNA. After hybridization the filters were quickly washed with 2 × SSEP with 0.1% SDS at room temperature and then twice for 30 minutes and 1 × SSEP and 0.1% SDS at 65°C and two 20-minute washes with 0.1 × SSEP and 0.1% SDS at 65°C. The nitrocellulose was air-dried and hybridization reactions detected by autoradiography using Kodak X-O AR film and X-Omatic cassettes with two intensifying screens (Eastman Kodak Co, Rochester, NY). The autoradiographs were quantitated by scanning with a densitometer and estimating the area under the curves gravimetrically.

Northern blot analysis. Northern blot analysis was essentially as described except the glyoxal denaturation of RNA was combined with formaldehyde–agarose gel electrophoresis. RNA samples were denatured with glyoxal and electrophoresed in 1.0% formaldehyde–agarose gels with buffer recirculation at 3 to 4 V/cm until the bromphenol blue dye ran 10 to 11 cm. The RNA ladder was used as mol wt markers. After electrophoresis the lane with the mol wt markers was removed for visualization with ethidium bromide, and the gel was washed in water for ten to 15 minutes at room temperature. The RNA was transferred to nitrocellulose presoaked in 20 × SSC (SSC: 0.15 mol/L NaCl and 0.015 mol/L trisodium citrate, pH 7.0) for 15 to 20 hours. After transfer the nitrocellulose was baked, prehybridized, and hybridized as described earlier for dot blots. The Northern blots were washed at low stringency conditions involving a five- to ten-minute wash in 2 × SSEP and 0.1% SDS at room temperature and two 20-minute washes in 2 × SSEP and 0.1% SDS at 50°C.

RESULTS

Fifteen cDNA clones showed positive hybridization signals after screening 1.3 × 10⁶ plaques of a human cDNA library with oligonucleotide probes. Seven overlapping clones spanned about 90% of vWF cDNA, and one clone was chosen for use in these studies. The cDNA was excised with the enzyme EcoRI and subcloned into pUC9. The recombinant clone (pWFIPC8) was 2,280 bp in length, and sequence analysis showed that it spanned nucleotides 960 to 3,240 of the published human cDNA sequence. This sequence includes 1,330 nucleotides that encode for the propeptide and 950 nucleotides that encode for the mature vWF. The human vWF cDNA and the actin cDNA probes were considered valid to detect porcine vWF mRNA and actin mRNA because they hybridized with restriction enzyme–digested genomic DNA from normal or vWD pig leukocytes under conditions of very high stringency (data not shown).

The results of Northern analysis of total RNA isolated from HUVEC, normal pig lung, cultured human fibroblasts, and poly(A)⁺ RNA from pig lung after sucrose density centrifugation are given in Fig 1. A mol wt species of 9.0 kb was found in both HUVEC and normal pig lung RNA preparations. Because of the low stringency washes of the Northern blots a 28S ribosomal mRNA was also present in total RNA. We used low-stringency washes for Northern analysis to avoid any loss of sensitivity resulting from possible base pair mismatches between the human and pig gene. High-stringency washes of Northern blots of human RNA eliminated the ribosomal binding. No other bands were detected in the pig RNA besides the 9.0-kb and the ribosomal bands.

Figure 2 (upper panel) shows Northern blot analysis of total RNA isolated from the lung of a normal pig, two vWD pigs, and the U-937 cell line. A band at 9.0 kb was not clearly discernable in mRNA from either vWD pigs when 10 μg of total mRNA was loaded on the gel. Figure 2 (lower panel) shows the results of Northern analysis of RNA from three vWD pigs after sucrose density gradient centrifugation. A band at 9.0 kb is identifiable in the vWD pigs; however, the quantity of the vWF mRNA appeared to vary significantly between the vWD pigs.

The Northern blots seemed to reflect the relative quantities of mRNA, but to avoid problems with the transfer of high-mol wt RNA we used dot blots hybridized in very stringent conditions to quantitate the relative amounts of vWF mRNA in normal and vWD pigs. Figure 3 shows an example of dot blots of total RNA from three vWD pigs, a normal pig, and cultured human fibroblasts hybridized with vWF (top) or actin (bottom) cDNA. The RNA samples were extensively digested with DNase before application, and RNase digestion of samples before blotting gave no appreciable signal. RNA samples from the lung of vWD pigs...
Fig 2. Northern blot analysis of total RNA from the lung of normal and vWD pigs and high-mol wt RNA from the lung of vWD pigs. Total and high-mol wt RNA from pig lungs were prepared, electrophoresed, and transferred to nitrocellulose as described in Materials and Methods and the legend to Fig 1. Upper panel: lanes 1 and 2, 5 and 10 μg of total RNA from normal pig lung; lanes 3 and 4, 5 and 10 μg of total RNA from vWD pig 1; lanes 5 and 6, 5 and 10 μg of total RNA from vWD pig 3; lane 7, 10 μg of total RNA from cultured U-937 cells. Autoradiography was for five days. Lower panel: lane 1, 4 μg of total RNA from normal pig lung; lanes 2 through 4, 5 μg of high-mol wt RNA from vWD pigs 1 through 3, respectively. Autoradiography was from one to five days.

The relative amounts of vWF mRNA in the lung of vWD pigs were calculated by constructing two identical dot blots using total RNA from a representative normal pig and three vWD pigs. These blots were first probed with the same vWF cDNA probe, and after decay of the radiolabeled probe the blots were reprobed with the same actin cDNA. Actin cDNA was used as a control because actin mRNA is abundant and easily detectable and there is no evidence that actin levels are modified in the vWD animals. Figure 3 (right) shows examples of the densitometric tracings corresponding to 4 μg total RNA blotted after probing with vWF (top) or actin (bottom) cDNA. After densitometric scanning of the blots a ratio of vWF mRNA to actin mRNA was calculated to normalize data for possible measurement or application differences in the RNA. The integrated areas under the curves of the densitometer scan were linear through the concentration range of RNA tested, and for quantitative analysis the tracings for the middle loading (4 μg RNA) were used. The ratio for the normal animal was arbitrarily set at 100%. Table 1 shows the average ratio of vWF mRNA to actin mRNA and relative normalized values for the two separate dot blots with the same RNA. All of the vWD pigs displayed significant amounts of vWF mRNA that varied from 21% to 41% of the ratio found in the normal animal.

Table 1. Ratio of vWF mRNA to Actin mRNA and Relative Percentage of vWF mRNA Found in Lungs From Normal and vWD Pigs

<table>
<thead>
<tr>
<th>VWF mRNA/Actin mRNA</th>
<th>Normal</th>
<th>vWD</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of normal</td>
<td>100</td>
<td>0.72</td>
<td>0.18</td>
<td>0.27</td>
<td>0.32</td>
<td>0.04</td>
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Discussion

Lung tissue was found to be a good source of RNA for cloning of human vWF cDNA and for investigating the steady-state levels of vWF mRNA from normal and vWD pigs by using human cDNA probes. The presence of endothelial cells and perhaps trapped megakaryocytes in the numerous capillaries of the lung made it possible to easily detect vWF mRNA by Northern hybridization analysis.
total RNA from normal pigs. The human cDNA probes hybridized with pig DNA and RNA when the blots were processed under conditions of high stringency, which suggests that porcine vWF is highly homologous to human vWF. Furthermore, the vWF mRNA from pig lung showed a band (9.0 kb) by Northern blotting the same size as RNA from HUVEC. This observation implies that the relationship between the propeptide and the mature vWF has been conserved in mammals and further supports the evidence that the propeptide has a functional role. Recent reports indicate that the propeptide may serve an important function for the correct formation of the large-mol wt multimers of vWF.4-11

All of the vWD pigs demonstrated the presence of vWF mRNA in lung tissue by Northern and dot blots, and the mRNA was the same size as that from the normal pig lung. To consistently detect vWF mRNA in vWD pigs it was necessary to enrich for vWF mRNA. We chose to take advantage of the size of vWF mRNA and to isolate high-mol wt RNA by using sucrose density gradient centrifugation instead of poly(A)+ selection of total mRNA because it gave better enrichment of vWF mRNA than oligo(dT)-cellulose chromatography. Difficulties in binding high-mol wt RNAs in good yields to oligo(dT)-cellulose have also been observed for other large mRNA species.12

The significant levels of vWF mRNA in the vWD pig do indicate that transcription of the vWF gene may not be the sole problem in this syndrome. There are several lines of indirect evidence to indicate that defects in translation or instability of transcripts may be involved in the final lack of expression of vWF in the vWD animals. If the defect was only in the control region of the gene affecting transcriptional rates, one would expect the mRNA that is present to be capable of producing a normal protein. The variability in the mRNA levels is not reflected in the variability of the phenotypic parameters for the pigs determined to be homozygous for vWD. The bleeding times were longer than 15 minutes, and there was no vWF activity in plasma. Although antigen levels can occasionally be detected, they are usually at the lower limits of the assay (less than 0.01 U/mL). In contrast, the relative mRNA levels in the vWD pigs ranged from 21% to 41% of those found in normal pig lungs.

Further evidence for posttranscriptional regulation of the expression of vWF in the vWD animals is suggested by the apparent identical size of the mRNA found in normal and vWD pigs. This is an important observation because it demonstrates that vWD pigs do not have a major deletion in the coding area of the gene that can be detected by Northern analysis. In this respect, severe vWD in pigs resembles the majority of the cases in humans where only two of 19 cases of patients with severe (type III) vWD had obvious gene deletions and these patients demonstrated alloantibodies to transfused vWF.13 All the other severe patients with vWD showed the same hybridization pattern as the normal controls after restriction enzyme digestion of genomic DNA. It is unknown, however, whether the transcripts that are present in the vWD pigs are being translated correctly or whether a mutation creating a new termination codon or other defects in the mRNA are affecting vWF synthesis or producing an unstable or incorrectly processed protein. Little is known of the trace amount of vWF that may be produced by vWD pigs and whether it has the characteristics of the normal protein. The significant amounts of vWF mRNA found in the lung of vWD pigs should facilitate future research on vWD in pigs including studies of translation that use in vitro techniques and direct cDNA cloning of the vWD mRNA. The latter possibility should allow for direct sequence comparison of the cDNA between normal and vWD pigs. Such studies could have important implications on the controls of synthesis of vWF and perhaps be directly related to certain forms of vWD in humans.

Finally, vWF mRNA from the lung of vWD pigs was very difficult to detect in Northern blots of total RNA, whereas dot blots always demonstrated easily detectable signals. This apparent paradox could be explained if more of the vWF mRNA was degraded in the vWD pigs. The degraded RNA could be more diffuse and not present in a single band in the Northern analysis, whereas partially degraded RNA could be detected on dot blots. There are, however, always technical problems when dealing with high-mol wt RNA that could account for these observations. Nevertheless, differential stabilities of mRNA are becoming more widely recognized and must always be considered as a potential mechanism of posttranscriptional control.14 Both the rate of transcription and the stability of transcripts could be investigated in vitro with cultured endothelial cells. These experiments would provide the information needed to determine the relative contributions of transcription and stability of transcripts to the lack of expression of vWF in the vWD pigs.

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