Molecular Studies of von Willebrand Disease: Reduced von Willebrand Factor Biosynthesis, Storage, and Release in Endothelial Cells Derived From Patients With Type I von Willebrand Disease

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Endothelial cells were cultured from the umbilical veins of two neonates with type I von Willebrand disease (vWD) and compared with cells cultured in parallel from normal control umbilical veins. In both cases, cultured vWD endothelial cells contained less messenger RNA (mRNA) encoding von Willebrand factor (vWF), and constitutively secreted two- to fourfold less vWF protein than their matched controls. Regulated secretion of stored vWF induced by thrombin or phorbol-12-myristate-13-acetate (PMA) was also diminished in vWD cells. Both the mRNA and protein produced by each of these type I vWD cells appeared to be of normal size. However, despite the diminished size of the vWF storage pool, electron microscopy of endothelial cells in situ showed normal appearing vWF storage organelles (Weibel-Palade bodies). These studies show that cultured umbilical vein endothelial cells can be used to explore the molecular defects in type I and perhaps other forms of vWD, and suggest that at least some forms of type I vWD are caused by diminished mRNA transcription or subsequent translation due to a defective vWF allele.

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VON WILLEBRAND disease (vWD) is a common inherited bleeding disorder characterized by decreased platelet adhesion to vascular subendothelium and mucosal bleeding. There are multiple subtypes of vWD that are presumed to arise from different qualitative or quantitative abnormalities in the von Willebrand factor (vWF) protein. One in the most common form of the disorder, type I vWD, patients have mild to moderate bleeding caused by reduced plasma vWF concentration and activity. They retain normal multimeric vWF structure and have variable intraplatelet stores of vWF. Although the clinical severity and laboratory abnormalities may vary widely among individual patients or even among affected members of the same family, type I vWD appears to be inherited as a single gene autosomal dominant trait. While it is most likely that the disorder is due to a defect in the vWF gene itself, extragenic factors including age, ABO blood group type, pregnancy, and hormonal manipulations can influence plasma vWF levels in normal individuals, and undoubtedly accounts for some of the heterogeneity seen among vWD patients. In addition, stress, exercise, or the infusion of pharmacologic agents such as the vasopressin analog, 1-deamino-8-D-arginine-vasopressin (DDAVP), temporarily elevates the plasma vWF, suggesting that there is a physiologic pool of vWF that can be rapidly mobilized. The good response to DDAVP observed in most type I vWD patients implies that this pool also exists in at least some subtypes of vWD.

The molecular genetic basis for most forms of vWD has not been elucidated, although there has been some recent progress in this area with the availability of full-length vWF cDNA probes. Ginsburg et al described missense mutations in two patients lacking the larger multimeric forms of vWF (type IIA vWD), and expressed recombinant vWF with appropriate mutations in heterologous cells. In addition, two groups have now reported deletions in the vWF gene in unrelated families with severe (type III) vWD. Finally, linkage between intragenic vWF restriction length polymorphisms (RFLP) and the inheritance of vWD has been established in both type IIA and type III pedigrees.

In view of the large size and complexity of the vWF gene, it may be difficult to detect many small deletions, rearrangements, or point mutations in the genomic DNA of vWD patients. One alternative strategy, which has been effective in many other diseases, is to analyze messenger RNA (mRNA) and protein metabolism in cells that express the protein of interest. Because vWF is only expressed in megakaryocytes and endothelial cells, obtaining tissue for such studies is problematic. Platelets contain traces of mRNA that can be amplified by polymerase chain reaction (PCR) but they do not synthesize appreciable quantities of protein. Their precursor, the megakaryocyte, is difficult both to isolate from bone marrow and to maintain in culture. Thus, at present, the only convenient source of cells in which to study vWF biosynthesis is the umbilical vein from affected offspring of vWD patients.

In this study we document a defect in vWF biosynthesis, storage, and secretion from umbilical vein endothelial cells (EC) isolated from the affected children of two patients with type I vWD. In each case, the vWD EC produced lower quantities of vWF mRNA and secreted less vWF protein than their normal counterparts. However, there were no abnormalities detectable in the vWF polypeptide, mRNA transcript size, or in the overall organization of the vWF gene. This reduction in vWF biosynthesis and secretion parallels the diminished plasma vWF levels in these families. Thus, vWD EC may prove to be a useful tool with which to analyze the pathogenesis of vWD.

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PATIENTS, MATERIALS, AND METHODS

Patients

**Family 1.** The mother is a 36-year-old woman with a history of recurrent epistaxis and abnormal bleeding after tonsillectomy and multiple dental extractions. Her mother also had a history of postoperative bleeding and was previously diagnosed as having vWD with vWF: Ag = 49%, ristocetin cofactor activity (RCO) = 25%. The biologic father of the child was unavailable for evaluation. The affected mother required amniocentesis for unrelated reasons. To establish the presence of the vWD genotype in the offspring, amniocyte DNA was subjected to restriction enzyme digestion along with leukocyte DNA from the mother and both of her parents. An informative intragenic SacI RFLP was detected in the maternal allele. The child was delivered vaginally at full term by elective Caesarean section.

**Family 2.** The mother is a 28-year-old woman with a history of bleeding after tonsillectomy at age 11 and easy bruising. Her mother also had a significant bleeding history but was never formally evaluated. There was no significant bleeding history in the father or in any members of his immediate family. The child was delivered at term by elective Caesarean section.

Clinical Laboratory Studies

Plasma vWF antigen was measured by the immunoelectrophoresis method of Laurell using polyclonal rabbit anti-human vWF antiserum. Ristocetin cofactor activity was measured using formalin-fixed platelets and the end-points determined in an aggregometer. Factor VIII activity was measured using a one-stage assay.

Cell Culture

Human umbilical vein endothelial cells were harvested by collagenase treatment of individual umbilical cord segments and subcultured in Medium 199 (Biofluids, Inc, Rockville, MD) containing 20% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 μg/mL porcine heparin (Sigma Chemicals, St Louis, MO), and 50 μg/mL endothelial cell growth factor (Collaborative Research, Lexington, MA) as previously described. Each of the two umbilical cords from patients with vWD was paired with a "control" cord and subjected to the same conditions of cell harvesting, primary culture, and subculture. Aliquots of EC at the third passage were frozen in culture medium containing 10% dimethyl sulfoxide and stored in liquid nitrogen for subsequent subculture. For experimentation, cells were plated onto dishes containing six 35-mm wells (Corning Glass Works, Corning, NY) that had been precoated with gelatin (Difco Laboratories, Inc, Detroit, MI).

Quantitation of vWF

Parallel cultures of vWD and normal EC were grown to confluence, washed four times with M199 supplemented with 0.1% gelatin (M199/G), and incubated for 30 minutes at 37°C with 1.0 mL M199/G, M199/G containing 1.0 U/mL human α-thrombin (2,160 NIH clotting units/mg, generously supplied by Dr John W. Fenton, New York State Department of Health, Albany), or M199/G containing 100 mmol/L phorbol-12-myristate-13-acetate (PMA). The amount of vWF released into conditioned medium was measured by an inhibition enzyme-linked immunosorbent assay (ELISA) using an avidin-biotin-peroxidase detection system as previously described. Significance of differences between means was analyzed by a Student's t test.

Radiolabeling, Immunoprecipitation, and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

To analyze both secreted and intracellular forms of vWF, vWD and normal EC were radiolabeled for 72 hours in complete EC growth medium supplemented with 0.5 mCi/mL L-[35S]cysteine. The cells were then harvested by brief treatment with trypsin, washed once, and lysed for 30 minutes at 4°C in a buffer containing 2% Nonidet P-40 (Sigma), 25 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 10 μg/mL each of aprotinin, leupeptin, and pepstatin. Radiolabeled vWF was isolated by sequential addition of rabbit anti-vWF antibody (Accurate Chemical & Scientific Corp, Westbury, NY) and protein A-Sepharose (Pierce Chemical Co, Rockford, IL). The beads were washed twice each in ice-cold buffers containing (1) 0.5 mol/L LiCl, 0.1 mol/L Tris-HCl pH 7.4, 5 mmol/L EDTA, 0.1 mmol/L PMSF, 0.1% Triton X-100 (Sigma); and (2) 0.02 mol/L NaCl, 0.05 mol/L Tris-HCl pH 7.4, 5 mmol/L EDTA, 0.1 mmol/L PMSF, 0.1% Triton X-100. PAGE in the presence of SDS was performed in slab gels containing 5% acrylamide using the buffer system of Laemmli.

Electron Microscopy of Endothelial Cells

Small segments (0.2 to 0.5-cm) of umbilical vein were isolated from freshly obtained normal and vWD cords, fixed in 2% paraformaldehyde/2.5% glutaraldehyde in cacodylate buffer, and embedded in LX112 (Ladd Research Industries, Burlington, VT) using standard techniques. Thin sections (70 nmol/L) were stained with uranyl acetate and lead citrate before electron microscopy.

Southern Blot Analysis

DNA was extracted from peripheral blood leukocytes by digestion with S但是用pase K, followed by sequential extractions with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation according to standard protocols. Amniocyte DNA from one of the potentially affected fetuses was extracted after the cells were established in culture. Ten micrograms of DNA was digested overnight with various restriction enzymes. The resulting DNA fragments were separated by electrophoresis on 0.8% agarose gels and transferred to Gene Screen Plus membranes (New England Nuclear Products) and hybridized with 32P-labeled cDNA probes. Final posthybridization washes were performed in 0.2 X standard saline citrate (SSC) at room temperature according to the manufacturer's specifications.

Preparation of vWF cDNA Probes

vWF cDNA probes were all digested with appropriate enzymes, and the desired inserts separated from vector sequences on low-melting point agarose gels. The cDNA probes were labeled with 32P-dCTP by the random hexanucleotide technique. The full-length, 8.3-kilobase (kb) cDNA was cloned into the EcoRI site of pUC19. A restriction map of full-length vWF cDNA has been published previously. Four smaller probes, designed to span the whole vWF cDNA, were then derived from the full-length cDNA clone: probe I was a 2.8-kb EcoRI-BamHI fragment; probe II was a 2.0-kb BamHI-KpnI fragment; probe III was a 2-kb KpnI-BglII fragment; probe IV was a 2-kb BglIII-EcoRI fragment.

Northern Blot and Dot Blot Analyses

Total cellular RNA was extracted from EC by the guanidine-HCl technique. Northern blot analyses, 10 to 15 μg aliquots of...
RNA were electrophoresed on 1% formaldehyde-agarose gels, transferred to Gene Screen Plus, and hybridized with 32P-labeled, full-length vWF cDNA probe. For quantitative analyses, additional aliquots of RNA were serially diluted, transferred to Gene Screen Plus, and hybridized with either a full-length vWF or a 2.1-kb fibronectin cDNA probe (a gift of Dr Thomas Maciag, American Red Cross, Rockville, MD).

RESULTS

Clinical Evaluation

As summarized in Table 1, each of the mothers and one of the two offspring had vWD documented by standard clinical tests. The second child had one set of laboratory values obtained shortly after delivery, when plasma vWF levels can be within the normal range in patients with vWD, and was unavailable for follow-up studies. However, the presence of the vWD genotype in this patient was documented by RFLP analysis (data not shown). As is true for the majority of patients with type I vWD, plasma vWF levels were within the normal range for both mothers at the time of delivery and then promptly fell in the peripartum period.

Southern Blot, Northern Blot, and Dot Analyses

Genomic DNA extracted from vWD and control leukocytes was digested with restriction endonucleases BamHI, EcoRI, and HindIII, and hybridized with a series of probes that span the entire full-length vWF cDNA (data not shown). No discernible deletions or rearrangements could be detected in the vWF gene with these techniques. These findings suggest that the molecular genetic defect(s) in these two patients are likely to be restricted to small deletions or point mutations, which may not be detected by Southern blot analysis.

Total cellular RNA was then extracted from both control and vWD EC and hybridized with the full-length vWF cDNA. As shown in Fig 1, serial dilutions of vWD EC RNA contained two- to fourfold less vWF mRNA than control EC, as measured by the intensity of hybridization with the vWF cDNA. In contrast, the quantity of fibronectin mRNA, another secreted adhesive glycoprotein of similar size, was identical in vWF and control EC. After electrophoretic separation and Northern blotting of RNA (Fig 2), the expected 8.3-kb mRNA transcript was detected in each of the patients. No abnormal vWF mRNA transcripts were detected in either vWD EC preparation by these analyses.

Table 1. Clinical Data

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Constitutive vWF Secretion

Cultured endothelial cells constitutively secrete newly synthesized vWF into the media in which they are maintained. To observe the possible effect of the vWD genotype on this secretion, similar numbers of control and vWD EC were subcultured and plated under identical conditions in adjacent culture wells. The growth curves of the vWD EC were indistinguishable from those of their normal counterparts, so that comparable numbers of cells were studied from each group. The rate of vWF secretion from the vWD EC cultures and two matched controls was measured by sampling replicate wells containing EC in serum-free culture media. As shown in Fig 3 (panel A), the constitutive rate of secretion for vWD1 was only 15% of the rate measured simultaneously in the matched control (2.84 ± 1.29 v 19.0 ± 4.7 ng/10^5/h; P = .02). Similarly, the constitutive rate of secretion for vWD2 (Fig 3, panel B) was 47.9% of its matched control (11.7 ± 3.6 v 24.5 ± 2.8 ng/10^5 cells/h; P = .06).

Regulated vWF Secretion

Endothelial cells store large multimeric forms of vWF in highly specialized storage organelles (Weibel-Palade bodies) for subsequent secretion. To determine whether production and mobilization of the vWF storage pool was also abnormal in type I vWD EC, confluent monolayers of the vWD EC and their matched controls were treated with 1 U/mL human thrombin for 30 minutes, and the quantity of vWF secreted was assayed at 10-minute intervals by sampling replicate wells. Constitutive vWF secretion was subtracted from the total vWF in the releasate at each time point. As shown in Fig 3 (panels C and D), stimulation with thrombin induced the release of only 11.7% as much vWF from EC storage pools in the vWD1 culture as from its matched control (3.35 v 28.6 ng/10^5 cells; P < .01), while the total amount of releasable vWF from the vWD2 culture was only 38.0% of that observed in its matched control (23.0 v 60.6 ng/10^5 cells; P = .01). The reduction in releasable vWF in each of the vWD cultures was confirmed in parallel experiments using PMA, a potent agonist of vWF secretion previously shown to elicit nearly quantitative release of EC Weibel-Palade bodies. Thirty-minute treatment with 100 nmol/L PMA induced the release of 14% (vWD1) and 78% (vWD2), as much vWF as the respective control cultures. Thus, the defect in the vWD EC likely resided principally in the size of the storage pools themselves rather than in defective signal transduction mechanisms.

Electron Microscopy of vWD Umbilical Veins

The finding of a diminished storage pool of vWF in vWD EC, as measured by agonist-stimulated secretion of vWF, raised the possibility that the defect might lie within the Weibel-Palade bodies, which might either be reduced in number or otherwise abnormal. To explore the latter possibility, a portion of the umbilical vein from the cord of one of the vWD patients (vWD2), along with a normal umbilical vein segment, were dissected free of surrounding tissue and
Fig 1. Quantitative analysis of RNA derived from vWD and normal EC. Total cellular RNA was prepared from vWD and normal EC and serially diluted onto Gene Screen Plus filters. Hybridization was performed with either a full-length vWF or a 2.1-kb fibronectin cDNA probe.

processed for transmission electron microscopy. As illustrated in Fig 4, an appreciable number of morphologically normal appearing Weibel-Palade bodies were detected in the vWD vessel segment.

vWF Biosynthesis in vWD EC

In light of the complexity of vWF biosynthesis, it is possible that the diminished storage of secretion observed in the vWD EC might be due to an inability of these cells to correctly process a normal vWF translation product, or alternatively, from the production of an abnormal vWF polypeptide that inhibits vWF multimer assembly. To assess the effect of the vWD genotype on vWF biosynthesis, radiolabeled vWF was isolated from the conditioned media and cell lysates (of control and vWD EC) with a polyclonal

Fig 2. Northern blot analysis of vWF RNA from vWD and normal EC. Total cellular EC RNA was extracted, electrophoresed on a 2% agarose formaldehyde gel, and transferred to Gene Screen Plus filters. The blot was hybridized with 32P-labeled full-length vWF cDNA. vWD1 and CON1, 25 µg total RNA/lane; vWD2 and CON2, 10 µg total RNA/lane.

Fig 3. Constitutive and thrombin-stimulated secretion of vWF from vWD and control EC. vWF secretion from confluent monolayers of vWD and control EC was assayed in replicate 35-mm diameter tissue culture wells over a period of 30 minutes in M199/gelatin (panels A and B), or M199/gelatin containing 1 U/mL α-thrombin (panels C and D). Data points represent the average of 3 to 6 wells. (○), CON1; (●), VWD1; (△), CON2, (△), VWD2.
rabbit anti-vWF antiserum and protein A-sepharose. When analyzed by SDS-PAGE under reducing conditions, both vWD and control EC secreted the fully processed vWF (Mr 220,000) polypeptide, as well as smaller quantities of the uncleaved pro-vWF (Mr 275,000) species usually detected in culture media (Fig 5, lanes A through D). Similarly, intracellular vWF, derived from both vWD and control EC, consisted of both the fully processed and the intracellular unprocessed (Mr 260,000) polypeptides (Fig 5, lanes E through H). There was no increase in the proportion of unprocessed pro-vWF immunoprecipitated from either vWD EC culture, nor were polypeptides of abnormal mobility detected in these gels.

**DISCUSSION**

In this study, human umbilical vein EC were obtained from two patients with type 1 vWD whose offspring had inherited the disorder. In each case, both constitutive and regulated secretion of vWF protein was diminished and this was accompanied by a two- to fourfold reduction in EC vWF mRNA. These results are in contrast to those of Levene et al. who reported a three- to 10-fold increase in vWF mRNA in one culture of type IIA vWD EC. However, as the vWF translation product of these cells appeared to be unusually sensitive to proteolysis, it may be hypothesized that these cells, unlike those described in this study, initially synthesize an increased quantity of protein that is subsequently degraded.

The finding of a diminished rate of basal vWF secretion in type 1 vWD EC is consistent with the previous study of Booyse et al. In this report we have extended this finding to two additional type I vWD cultures and have noted a parallel reduction in the releasable pool of vWF. Although regulated vWF secretion was diminished in our study, morphologically normal Weibel-Palade bodies were present in freshly pro-
ceased umbilical vein segments from the one affected umbilical cord available for study. Formal morphometric analysis was not performed, but the number of organelles observed in several fields was not appreciably different from that observed in control sections. The reason for this apparent discrepancy is not clear, but the possibility that there was an accelerated loss of Weibel-Palade bodies in the vWD cultures with successive passaging cannot be ruled out. Because the secretable pool of vWF was diminished in the EC derived from vWD patients, it is also possible that the quantity of vWF packaged in each Weibel-Palade body was reduced.

Recently, Wu et al. reported diminished mRNA levels in lung tissues derived from bleeder swine with severe, homozygous vWD. Despite the absence of plasma vWF in the bleeder swine, vWF mRNA was only modestly reduced to between 21% and 41% of the values obtained from normal porcine lungs. The investigators in that study concluded that "posttranscriptional events" caused the abnormal vWF expression in these animals, implying that the message they had detected may have been abnormal. Unfortunately, no further studies were performed to validate this hypothesis. In contrast, our study on human vascular EC has demonstrated a close correlation between endothelial cell vWF synthesis and vWF mRNA levels. We speculate that the reduced mRNA levels observed in each of the two vWD EC cultures are caused by either point mutations, or small deletions in one of the two vWF alleles that block vWF mRNA transcription or subsequent translation. Thus, the residual vWF produced by the EC is likely to be the product of the remaining, normally functioning vWF allele present in heterozygous patients. A similar conclusion was reached by Ginsburg et al. in a report of PCR amplified mRNA from platelets of one type I vWD patient. Whether these mutations are intronic, as in many of the \( \beta \)-thalassemias, or reside in the vWF coding sequences, is not known at this time.

There is increasing evidence that a number of extragenic factors can profoundly influence plasma vWF levels. These variables often confound the diagnosis of vWD and have frustrated previous attempts to understand the pathogenesis of this disorder. The finding that vWD EC continue to express the altered phenotype in culture is thus helpful, and suggests that these cells will provide a useful tool with which to study the molecular and cellular aspects of this common bleeding disorder.

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