Relationship Between Human Development and Disappearance of Unusually Large von Willebrand Factor Multimers From Plasma

By Julie A. Katz, Joel L. Moake, Patsy D. McPherson, Mark J. Weinstein, Kenneth J. Moise, Robert J. Carpenter, and Debra J. Sala

von Willebrand factor (vWF) multimers were examined in fetal, umbilical cord, and neonatal platelet-poor plasma (PPP) specimens. Sixty-five of 65 (100%) fetal PPP samples aged <35 weeks and seven of ten (70%) fetal samples aged >35 weeks had unusually large vWF (ULvWF) multimers. Thirty of 46 (65%) cord PPP samples from neonates ranging in gestational age from 34 to 41 weeks had ULvWF. There was no significant relationship between either gestational age at time of delivery or birth weight and likelihood of finding ULvWF multimers in cord PPP samples. No maternal PPP sample contained ULvWF multimers. Serial heelstick samples from 16 preterm and term neonates were analyzed for 8 weeks. ULvWF multimers disappeared from the PPP of ten of the neonates during this time. The PPP of four neonates had vWF patterns similar to those in normal adult PPP throughout the sampling period. The ULvWF multimeric forms of fetal and neonatal PPP samples were similar to those constitutively released from endothelial cells. They were not as slowly migrating in a very porous 0.5% agarose gel system as the ULvWF multimers released from Weibel-Palade bodies in response to the calcium ionophore A23187. A vWF protomer was present in 97% of fetal samples, 83% of cord blood specimens, and 11% of neonatal heelstick samples, but was not found in any maternal sample. These results indicate that control mechanisms operative in older children and adults to prevent circulation of ULvWF multimers and vWF protomeric forms are normally acquired late in uterine life or during the neonatal period. ULvWF multimers, which are normal components of fetal, most cord, and some neonatal plasma samples, may contribute to in utero and postnatal hemostasis.

THE MULTIMERS OF von Willebrand factor (vWF) are synthesized in endothelial cells and megakaryocytes, have molecular weights (mol wts) ranging into the millions of daltons, and are essential for normal hemostasis.1,2 vWF mediates adhesion of platelets to exposed subendothelium by promoting formation of platelet clumps at sites of vascular injury.

Unusually large vWF (ULvWF) multimers are vWF forms larger than those in normal plasma. They are present in endothelial cells and platelets, are secreted by human endothelial cells in culture, and can be extracted from subendothelial collagen.3,4 ULvWF multimers are ten to 20 times more active in inducing platelet agglutination than the somewhat smaller vWF multimers found in normal plasma,5 and large vWF forms secreted from the Weibel-Palade bodies of cultured human endothelial cells in response to calcium ionophore A23187 bind more avidly to the extracellular matrix of fibroblasts than do smaller vWF forms.6 They have been found in remission plasma of patients with the chronic relapsing type of thrombotic thrombocytopenic purpura (TTP).7

Weinstein et al8 recently studied fetal and neonatal plasma samples and reported that ULvWF multimers were present. We extended those initial observations by studying the vWF multimeric patterns in an expanded number of human fetal, umbilical cord, and neonatal platelet-poor plasma (PPP) samples in relationship to gestational age, birth weight, and postnatal development. We also analyzed neonatal PPP samples obtained serially to evaluate the process of change from plasma containing ULvWF multimers to plasma containing the somewhat smaller vWF multimeric forms characteristic of the PPP of normal children and adults.

MATERIALS AND METHODS

Aliquots of 75 fetal blood samples obtained by percutaneous transabdominal umbilical cord puncture9,8 were collected in 2-mL vacutainers containing EDTA. Indications for fetal blood sampling are shown in Table 1. Forty-six blood samples were collected during delivery from umbilical cords into a 2-mL tube containing 0.04 mL 7.5% EDTA solution. Fifteen paired maternal venous samples were obtained by a 21-gauge needle and placed in similar 2-mL EDTA tubes. Sixteen neonatal patients were followed for 8 weeks after initial sampling (except for one patient from whom PPP was also obtained in a 10-week period after the initial sample was obtained). Aliquots of 80 samples obtained by the heelstick method, using a 5-mm microlance1 were collected. Heel punctures were performed on the lateral or medial aspects of the plantar surface of the foot, and free-flowing blood was collected in a 0.75-mL microvette containing 1.5 mg EDTA solution per milliliter. All specimens were centrifuged at 3,000 rpm for ten minutes at 23°C. PPP samples were removed.

Table 1. Indications for Fetal Blood Sampling

<table>
<thead>
<tr>
<th>Fetal Sampling</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh alloimmunization</td>
<td>41</td>
</tr>
<tr>
<td>Chromosomal aberrations and/or fetal anomalies</td>
<td>25</td>
</tr>
<tr>
<td>Maternal ITP</td>
<td>6</td>
</tr>
<tr>
<td>Advanced maternal age</td>
<td>1</td>
</tr>
<tr>
<td>Positive maternal HIV titer</td>
<td>1</td>
</tr>
<tr>
<td>Decreased α-fetoprotein</td>
<td>1</td>
</tr>
<tr>
<td>Total samples</td>
<td>75</td>
</tr>
</tbody>
</table>

From the Departments of Pediatrics, Medicine, and Obstetrics and Gynecology, Baylor College of Medicine; the Biomedical Engineering Laboratory, Rice University; and the Department of Medicine, Boston University School of Medicine.

Submitted July 18, 1988; accepted January 10, 1989.

Supported by National Heart, Lung and Blood Institute Grants HL35387 and HL/8584. J.A.K. was supported in part by a Fellowship grant from the American Cancer Society.

Address reprint requests to Joel Moake, MD, Methodist Hospital, Hematology Section, Suite 930, 6565 Fannin, Houston, TX 77030.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc.

From www.bloodjournal.org by guest on November 10, 2017. For personal use only.
placed in plastic tubes, and frozen at −20°C. Any hemolysis was recorded.

Samples were thawed at 37°C, and vWF antigen was quantified by a rapid, sensitive enzyme-linked immunoassay (ELISA) method (Ramco Laboratories, Houston). Plots of vWF antigen level v versus dilutions of adult PPP (containing the range of vWF multimers found in normal plasma) or fetal PPP samples (containing ULvWF multimers, in addition to the vWF multimers found in adult PPP) produced parallel lines.

Both normal pooled PPP and patient PPP samples were diluted with veronal-buffered saline to 15% vWF antigen levels. Fifty microliters of the diluted control and patient samples, as well as 50 μL human umbilical vein endothelial cell supernatant,57 was placed into rocket tubes and denatured at 60°C for 20 minutes with 75 μL 0.02 mol/L Tris-HCl/2.5 mmol/L EDTA/8 mol/L urea/2% sodium dodecyl sulfate (SDS), pH 8.0. Fifteen microliters were applied to gel wells under a layer of running buffer, 0.04 mol/L Tris-HCl/0.02 mol/L sodium acetate/2 mmol/L EDTA/0.1% SDS, pH 7.4. Samples on 1% gels were run at 50 V for 60 minutes and then at 100 V until the tracking dye traveled 7 to 8 cm. The 0.5% gels were run at 40 V for 60 minutes and 85 V until the tracking dye traveled 7 to 8 cm. Temperature was maintained at 4°C.

vWF multimer patterns were analyzed by electrophoresis in SDS-agarose (0.5% or 1%) gel slabs (1.5 x 110 x 230 mm). The gels were overlaid with rabbit 125I-anti-human vWF IgG, and autoradiographs were produced at 24 and 48 hours.57,12

Human umbilical vein endothelial cell monolayers (6 to 8 x 10^5 cells) were prepared as described previously57 and either allowed to release vWF multimers at 37°C for 48 hours into serum-free medium 199 containing 5 μg/mL insulin (from bovine pancreas, Sigma, St Louis), 5 μg/mL human transferrin (Collaborative Research, Waltham, MA), 200 U/mL penicillin, 200 μg/mL streptomycin, 200 μg/mL neomycin, and 300 μg/mL L-glutamine (GIBCO Laboratories, Grand Island, NY) or induced to secrete vWF multimers into the same medium for ten minutes at 37°C with calcium ionophore A23187 (Sigma). The cell supernatant was then withdrawn for vWF quantification and multimeric analysis.

Approval for this experimental protocol was obtained from the institutional review boards of the Baylor College of Medicine, Texas Children’s Hospital and Harris County Hospital District, Houston.

RESULTS

vWF multimers were examined in fetal, cord, and neonatal PPP samples. Multimeric analysis of all 65 fetal samples <35 weeks and in seven of ten fetal samples >36 weeks showed the presence of ULvWF multimers (Fig 1). The mean vWF antigen level in these 75 PPP samples was

---

**Fig 1.** vWF multimeric patterns evaluated in 75 fetal and 46 cord PPP samples and grouped by gestational age.

---

**Fig 2.** SDS-1% agarose gel electrophoresis and autoradiographic analysis of vWF multimers. NP, normal pooled PPP; EC, endothelial cell supernatant; F1 through F6, fetal samples. M5, maternal sample obtained with the corresponding fetal sample. F5, Position of the unusually large vWF multimeric forms (in brackets). Position of the vWF protomer (arrow, bottom).
Fig 3. SDS-0.5% agarose gel electrophoresis and autoradiographic analysis of vWF multimers. (A) NP, normal pooled PPP; EC, endothelial cell supernatant containing the vWF forms secreted in response to stimulation by calcium ionophore A23187 for ten minutes; F-7, F-8, F-9, F-4, F-10, F-5, F-11, fetal PPP samples. Position of ULvWF multimeric forms of EC (larger bracket); multimeric forms of the fetal samples (smaller bracket). Position of the vWF protomer (arrow, bottom). (B) Comparison of NP, endothelial cell supernatant secreted in response to 1 μmol/L A23187 stimulation for 15 minutes (EC-S), and endothelial cell supernatant constitutively released during four days of endothelial cell incubation with medium 199 containing 20% FCS, penicillin-streptomycin-neomycin, and 1 mmol/L L-glutamine (EC-C).

111% (111 U/dL) (range 49% to 316%). This value may be an underestimate, because the small volumes of fetal blood obtained in some samples resulted in reduced ratios of blood to EDTA as compared with ratios of adult peripheral venous blood to EDTA used in the pooled PPP standards. SDS-1% agarose gel electrophoresis and autoradiography demonstrated that no qualitative differences existed in the vWF multimeric pattern of normal PPP separated from varying volumes of blood placed into 3-mL EDTA containers (not shown). Of 46 umbilical cord PPP samples studied (29 female and 17 male), 30 (65%) of the total samples had ULvWF (59% of the female and 76% of the male PPP samples) (Fig 1). The mean vWF antigen level was 134% (U/dL) with a range of 62% to 325%. These levels may be underestimates for the reason cited above for fetal samples. Seventeen cord plasma samples had mild hemolysis (30% of these samples had ULvWF multimers). There was no statistically significant relationship between either gestational age (Fig 1) or birth weight and the likelihood of finding ULvWF multimers in cord PPP. Twenty-six cord blood samples were evaluated for ULvWF multimers and grouped by weight. Sixty-two percent of these had ULvWF multimers in the following distribution: one of two at a birth weight of 1.5 to 2.0 kg; three of four at 2.0 to 2.5 kg; seven of nine at 2.5 to 3.0
cg; one of three at 3.0 to 3.5 kg; and six of eight at 3.5 to 4.0 kg. These differences were not statistically significant.

Autoradiographic analysis of 6 of the fetal samples is shown in Fig 2. A paired maternal PPP sample, M-5, is shown with the corresponding fetal sample, F-5. All fetal samples demonstrate the presence of both ULvWF multimers and a band that migrates with IgM (900,000 daltons) in 1.5% and 2% agarose gels (not shown). Under these electrophoretic conditions, this band might be assumed to be a tetramer. However, vWF multimers can have anomalous electrophoretic behavior, and other investigators using different gel systems have interpreted the band in this position as a dimer (the problem is reviewed in reference 13, p 143). Because of the intensity of this fastest migrating vWF band in endothelial cell lysates and supernatants, and because it is barely visible in autoradiograms of normal adult PPP, we call the band in this position a vWF “protomer.” Both ULvWF multimers and the vWF protomer are also found in cultured human umbilical vein endothelial cell supernatants but not in normal pooled PPP and maternal PPP. Ten fetal patients with Rh alloimmunization were sampled more than once between the gestational age of 20 and 36 weeks. Nine of

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gestational Age at Birth (wk)</th>
<th>Sex</th>
<th>Clinical status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>M</td>
<td>HMD, retinopathy of prematurity</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>F</td>
<td>HMD, stage 1 necrotizing enterocolitis, intraventricular hemorrhage, Staphylococcus aureus cellulitis</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>F</td>
<td>PDA, ileal perforation</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>M</td>
<td>Multiple congenital anomalies</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>F</td>
<td>Hemolytic anemia (etiology unknown), hyperbilirubinemia, apnea of prematurity, caffeine therapy</td>
</tr>
<tr>
<td>6</td>
<td>32</td>
<td>M</td>
<td>Hemolytic anemia (etiology unknown), hyperbilirubinemia</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>M</td>
<td>Infant of diabetic mother, HMD, group B streptococcal sepsis</td>
</tr>
<tr>
<td>8</td>
<td>23</td>
<td>M</td>
<td>HMD, BPD, PDA</td>
</tr>
<tr>
<td>9</td>
<td>30</td>
<td>M</td>
<td>HMD, laryngomalacia</td>
</tr>
<tr>
<td>10</td>
<td>23</td>
<td>F</td>
<td>HMD, BPD, enteroviral infection</td>
</tr>
<tr>
<td>11</td>
<td>27</td>
<td>M</td>
<td>PDA, group B streptococcal sepsis, staphylococcal scalded skin syndrome</td>
</tr>
<tr>
<td>12</td>
<td>25</td>
<td>F</td>
<td>HMD, BPD, ventricular septal defect</td>
</tr>
<tr>
<td>13</td>
<td>26</td>
<td>F</td>
<td>Maternal drug abuse, 20% placental abruption</td>
</tr>
<tr>
<td>14</td>
<td>25</td>
<td>F</td>
<td>Maternal systemic lupus erythematosus, mild HMD</td>
</tr>
<tr>
<td>15</td>
<td>28</td>
<td>F</td>
<td>HMD, pneumothorax</td>
</tr>
<tr>
<td>16</td>
<td>31</td>
<td>F</td>
<td>Mild HMD</td>
</tr>
</tbody>
</table>

NvWF, normal vWF multimeric pattern; BPD, bronchopulmonary dysplasia; PDA, patent ductus arteriosus; HMD, hyaline membrane disease.
these patients were transfused with either maternal or type-
compatible packed RBCs on several occasions. Twenty-five
of the 26 PPP samples analyzed had ULvWF multimers. The
PPP of one patient contained ULvWF at 30 and 32 weeks
but not at 36 weeks.

A very porous, SDS-0.5% agarose gel system (Fig 3A and
B) demonstrated that the ULvWF multimers of fetal sam-
ple were not so slowly migrating as the ULvWF multimers
secreted from the Weibel-Palade bodies of endothelial cells
in response to ten minutes of stimulation with calcium
ionophore A23187 (1 μmol/L).

Autoradiograms of two cord PPP samples are shown,
along with the corresponding maternal samples, in Fig 4.
Both ULvWF multimers and the vWF protomer were pres-
ent in the two cord samples but absent in the maternal
samples. No maternal PPP sample had ULvWF multimers.
The mean vWF antigen level in 15 maternal samples was
270% (U/dL), with a range of 126% to 523%.

Sixteen neonates were followed for 8 weeks. Patient data
for the early neonatal period and at the time of normalization
of the vWF multimeric patterns are summarized in Table 2.

No patient was ventilator dependent, and none was thrombo-
cytopenic during the study period. Each received oral multi-
vitamin and iron supplements. Other medications are noted.
The mean vWF antigen level was 222% (U/dL) (range 82% to
502%). (These values may be underestimates for the
reason previously stated.)

Serial analyses of the nine female and seven male neonatal
PPP samples are in Fig 5. In ten of 16 patients (five female
and five male), PPP samples that contained ULvWF mul-
timers early in the study period changed with time to PPP
that contained only the somewhat smaller vWF multimeric
forms characteristic of the plasma of normal children and
adults. Two patients were lost to follow-up, and four had
normal vWF multimeric patterns throughout the sampling
period. Neonatal vWF patterns on all PPP samples were
analyzed by cumulative frequency distribution (Fig 6). A
significant decrease in the percentage of PPP samples con-
taining ULvWF multimers was found at gestational ages
>40 weeks. An autoradiogram of seven of the neonatal
samples is shown in Fig 7A. Patient N-1 had ULvWF
multimers at 33 weeks but not at 40 weeks (Fig 7B). The
vWF protomer was present in 97% of fetal samples, 83% of umbilical vein blood specimens, and 11% of neonatal heel-stick samples, but in no maternal sample.

**DISCUSSION**

Factor VIII coagulant activity (VIII:C), vWF antigen and ristocetin cofactor activity (RCoF) have been evaluated previously by other investigators in both healthy and ill newborns. The VIII:C of neonatal plasma has been reported to be greater than, equal to, and less than adult values. Neonatal vWF antigen has been shown to be elevated as compared with that of adults, with the highest values in ill newborns. Ts’ao et al showed the RCoF activity of neonatal plasma to be greater than that of maternal plasma, although they reported lower than normal levels of VIII:C in the same infants. Their data suggested that vWF in infant plasma might be more functionally effective than vWF in adult plasma in inducing platelet agglutination. Initial observations extended considerably in this report, demonstrate that ULvWF multimers are present in almost all fetal PPP samples and are present in many umbilical cord and neonatal PPP specimens. These data contrast to plasma levels of other
coagulation factors (with the exceptions of factor V and fibrinogen), which are decreased in both preterm and term neonates as compared with adult levels.\textsuperscript{15-20}

ULvWF multimers were present in all 65 fetal plasma samples at gestational age <35 weeks. The consistent presence of ULvWF multimers in fetal PPP was confirmed by serial sampling in ten of the fetal patients. Seven of ten of the fetal plasma samples at gestational age >36 weeks had ULvWF multimers. One patient, sampled on three occasions, had ULvWF multimers at 30 and 32 weeks but not at 36 weeks.

At birth, 65% of 46 cord PPP samples had ULvWF multimers. Neither increasing gestational age nor increased weight at delivery was significantly related to the likelihood of finding ULvWF multimers in cord plasma. Furthermore, ULvWF multimers were not present in 15 maternal PPP samples obtained after delivery but were present in seven of the corresponding cord plasma samples. Serial neonatal heelstick plasma samples obtained in ten patients demonstrated that a transition from ULvWF multimers to the vWF multimeric forms characteristic of the plasma of normal children and adults occurred during the 31st to the 51st week of gestational age. Fifty-eight percent of neonatal patients born prematurely and studied before 40 weeks of gestational age had ULvWF multimers in their PPP as compared with only 18% of neonates whose gestational age was >40 weeks at the time of sampling. These data confirm that in fetal and early neonatal life ULvWF multimers are found physiologically in plasma and that ULvWF forms disappear as neonates age.

ULvWF multimers of two forms can be distinguished using very porous (0.5%) agarose SDS gel electrophoretic systems: (a) those constitutively released from endothelial cells, and (b) those released from Weibel-Palade bodies in response to agents that induce secretion of endothelial cell granule contents (eg, calcium ionophore A23187). These latter forms of ULvWF multimers are even more slowly migrating in 0.5% agarose than the constitutively secreted ULvWF forms. The ULvWF in fetal, cord, and neonatal PPP samples appear to be similar to the constitutively secreted ULvWF multimeric forms, although specific assays for the vWF propeptide (vWF antigen II) present in vWF forms constitutively secreted by endothelial cells\textsuperscript{21} have not been performed in patient samples. However, some ULvWF depolymerization, proteolysis, or other type of catabolism may occur, so that the electrophoretic migration of the ULvWF forms in fetal, cord, and neonatal plasma is slightly faster than the ULvWF multimers present in endothelial cell supernatant immediately after in vitro secretion.

Acquisition of vWF multimeric patterns lacking ULvWF multimers, characteristic of the PPP of normal children and adults, involves a maturation process that occurs progressively from late fetal to early neonatal life. This maturation process may involve functional changes in endothelial cell membranes or acquisition of an enzymatic processing activity, present in the plasma of normal adults, that converts ULvWF multimers to the somewhat smaller normal adult plasma vWF forms.\textsuperscript{3} ULvWF multimers, present in most fetal PPP samples and in many cord and neonatal PPP samples, are ten to 20 times more potent in promoting platelet aggregation in shear fields (in the absence of ristocetin or other exogenous chemical) than are the largest vWF multimers present in the plasma of normal children and adults. The relationship between the presence of ULvWF multimers in fetal and neonatal plasma and hemostasis was not addressed experimentally in this study. We speculate that ULvWF multimers, which have an augmented capacity to aggregate platelets under certain conditions, may serve as hemostatic compensation for the low level of vitamin K-dependent coagulation proteins present in utero.

REFERENCES

13. Lynch DC: Biosynthesis of von Willebrand factor, in Zimmer...


Relationship between human development and disappearance of unusually large von Willebrand factor multimers from plasma

JA Katz, JL Moake, PD McPherson, MJ Weinstein, KJ Moise, RJ Carpenter and DJ Sala