Evolution of Blood Coagulation Activators and Inhibitors in the Healthy Human Fetus

By P. Reverdieu-Moalic, B. Delahousse, G. Body, P. Bardos, J. Leroy, and Y. Gruel

Blood coagulation proteins were determined in 285 healthy fetuses from 19 to 38 weeks' gestation and compared with those of 60 normal full-term newborns and 40 adult controls. Prolongation of the coagulation screening tests, prothrombin time, activated partial prothrombin time, and thrombin clotting time, in fetuses throughout intrauterine life was explained by low levels of vitamin K-dependent factors (II, VII, IX, and X), contact factors (XI, XII, prekallikrein, and high-molecular-weight kininogen), factor V, factor VIII, and fibrinogen. Low levels of antithrombin III, heparin cofactor II, protein C and protein S, and tissue factor pathway inhibitor (TFPI) are clearly present at birth. In contrast, little is known about the coagulation system in the human fetus during intrauterine life. Some studies have shown small differences between the hemostatic system of premature infants after 30 weeks' gestation compared with full-term newborns. Reference values for most coagulation factors have been published for healthy infants who exhibit low levels of vitamin K-dependent factors (II, VII, IX, and X) at birth, as well as prolonged prothrombin time (PT) and activated partial prothrombin time (APTT), but in whom the equilibrium of coagulation activators/inhibitors is maintained. Thus, low levels of protein C (PC), protein S (PS), antithrombin III (ATIII), and tissue factor pathway inhibitor (TFPI) are clearly present at birth. However, only partial information, such as determinations of platelet antigens and glycoproteins, vitamin K-dependent factors, ATIII and heparin cofactor II (HCII), PS, and fibrinolytic proteins, has been reported on hemostasis from fetal samples before 30 weeks' gestation. Until now, only premature infants have been studied after 30 weeks, and this indirect approach has thus provided imprecise information about the real fetal hemostatic status.

Therefore, to enhance our knowledge of the coagulation system of the healthy human fetus, we conducted an extensive study of the most important coagulation activators and inhibitors from 19 weeks' gestation.

SUBJECTS AND METHODS

Subjects

Fetuses. Fetal blood was obtained from 285 fetuses between 19 and 38 weeks' gestation by direct puncture of the umbilical vein under high-resolution real-time ultrasound. Fetal blood sampling was performed in each case after obtaining informed consent for the antenatal diagnosis of congenital toxoplasmosis, rubella, or hemophilia. Only blood samples from fetuses diagnosed as normal were used for the study. Fetuses were not serially sampled. Since blood volume did not exceed 0.6 mL, not all assays for coagulation parameters could be performed on all fetus samples, but every assay was performed in at least 60 individuals. The absence of contamination by maternal blood in each fetal sample was demonstrated by analysis of red and white blood cell volume distribution curves on a Coulter S Plus II (Coultronics, Margency, France) and comparison to those from maternal blood. A Kleihauer test was also routinely performed.

Newborns. Whole blood from 60 newborns was collected directly from the umbilical vein immediately after delivery.

Normal healthy adults (adult controls). After obtaining informed consent, blood was collected from healthy subjects (20 males and 20 nonpregnant females not taking oral contraceptives) without previous history of hemorrhagic or thromboembolic disease (mean age, 35 years).

Samples

All samples were collected and mixed in 3.8% sodium citrate (1 part citrate to 9 parts blood). Within 30 minutes of sampling, platelet

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Submitted September 22, 1995; accepted April 2, 1996.

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poor plasma was obtained after centrifugation (3,000g for 15 minutes) at +10°C and kept frozen at −80°C.

**Methods**

Because the volume of fetal plasma was small (<250 μL), when possible, microtechniques\(^5\) were used for coagulation assays.

**Coagulation screening tests.** The coagulation screening tests\(^3\) consisted of PT using a rabbit-monkey thromboplastin for which International Sensitivity Index values were between 1.72 and 2.02 (Neoplastin; Diagnostica Stago, Asnières, France) and international normalized ratios were calculated. APTT testing was performed with APTT reagent from Organon Teknika (Fresnes, France) and results were expressed as seconds, and ratios were calculated with the mean adult clotting time. Thrombin clotting time (TCT) was performed with 4 NIH units of bovine thrombin diluted in a buffer without calcium (Thrombozyme; Diagnostica Stago).

**Assays of coagulation activators.** Coagulation factors were assayed by a one-stage coagulation time\(^3\) using deficient plasmas from Diagnostica Stago for factors II and V, from Immuno (Rungis, France) for factors VII, X, VIII, IX, XI, and XII, and from Sigma Diagnostics (St Louis, MO) for high-molecular-weight kininogen (HMWK) and prekallikrein (PK).\(^8\) Factor II antigen level was evaluated by Laurell immunoelectrophoresis\(^6\) using a rabbit polyclonal antibody (Diagnostica Stago). Proteins induced by vitamin K absence for factor II (PIVKA-II) and factor VII antigen were assayed by enzyme-linked immunosorbent assay (ELISA) (Asserachrom PIVKA-II and Asserachrom VII; Diagnostica Stago). Fibrinogen (factor I) was assayed by the Von Clauss method\(^4\) (Fibripreset; Diagnostica Stago) and by Laurell immunoelectrophoresis\(^6\) using a polyclonal antibody against fibrinogen (Anti–Fi; Diagnostica Stago). All coagulation assays were performed on a KC10 apparatus (Amelung, Maurepas, France).

**Assays of coagulation inhibitors.** ATIII and HCII antigen were determined with Laurell immunoelectrophoresis\(^6\) using polyclonal antibodies (Diagnostica Stago). PC\(^1\) and total PS\(^1\) levels were measured by ELISA (Asserachrom PC or PS; Diagnostica Stago). Free PS levels were evaluated by the same procedure after precipitation of C4b–BP–bound PS with polyethylene glycol as previously described.\(^9\) We then determined the ratio of free PS to total PS to study the plasma distribution of PS. PC activity was also estimated using a chromogenic assay (Staclot PC; Diagnostica Stago) on a Chromotimer (Behring, Rueil Malmaison, France). TFPI was assayed by ELISA (Imubind Total TFPI; American Diagnostica, Greenwich, CT).\(^7\)

**Statistical Analysis**

Results are expressed as the mean and range including 95% of the population. Differences between fetal, neonatal, and adult data were tested using Dunnett’s multiple comparison test, and P values less than .05 were considered significant.

**RESULTS**

**Coagulation screening tests (PT, APTT, and TCT) and fibrinogen levels.** PT slightly decreased during intrauterine life from 32.5 seconds to 22.6 seconds, but differences were statistically significant only between the last two groups (P < .01). PT in newborns remained significantly longer than in adults, with a mean value of 16.7 seconds (P < .05). APTT decreased gradually during gestation, with a ratio (calculated with the mean adult clotting time) of 5.0 (range, 2.5 to 8.2) at 19 to 23 weeks’ gestation and 3.1 (range, 2.3 to 4.2) at 30 to 38 weeks’ gestation. This ratio was still higher in newborns (1.1 to 1.8) than in adults (0.8 to 1.1). TCT was prolonged at 19 to 23 weeks, but decreased during intrauterine life to reach values at 30 to 38 weeks similar to those in newborns (P > .05) but always longer than in adults. In addition, fibrinogen levels were significantly lower in fetuses and newborns than in adults. Using an antigenic method, measurements of fibrinogen in samples obtained after 24 weeks’ gestation until birth always yielded higher values than those assayed by the Von Clauss method (Table 1).

**Vitamin K–dependent factors (II, VII, IX, and X).** Values for vitamin K–dependent factors are presented in Table 1. Levels of factors IX and X remained almost unmodified between 19 and 38 weeks, whereas a regular increase in factor VII levels was observed. Factor II increased significantly at the end of the third trimester. A notable increase in levels of factors II, IX, and X was recorded at birth, but with mean values (31.8% for factor IX and 43.5% for factor II) that always remained lower than in adults (Fig 1). Factor VII appeared to be the highest of the vitamin K–dependent factors in fetuses, and levels ranged from 20% in midpregnancy to 60% in the last 2 months, with identical values found when assayed by an immunologic method (data not shown). This is in contrast to factor IX levels, which remained at less than 15% during intrauterine life and were 32% at birth. No significant difference was obtained for factor II levels when assayed with an immunologic method (Fig 2). Moreover, PIVKA-II antigen levels (ELISA) were lower in fetuses than in newborns and adults, whatever the gestational age. This also supports the absence of non–γ-carboxylated factor II molecules in fetal plasma.

**Factors V and VIII.** Levels of factors V and VIII were higher than levels of vitamin K–dependent factors and remained stable at approximately 35% between 19 and 30 weeks’ pregnancy. They then increased slightly and reached adult levels at birth. As in adults, a wide variability was observed in neonates for both factors V and VIII, with values ranging from 50% to 140% for factor V and from 38% to 150% for factor VIII (Table 1 and Fig 3).

**Contact factors XI, XII, PK, and HMWK.** Levels of factor XI, PK, and HMWK remained at less than 30% in most fetuses during intrauterine life. On the other hand, factor XII regularly increased during gestation, with a mean value of 70% at birth (Table 1 and Fig 4).

**Coagulation inhibitors: ATIII, HCII, PC, PS, C4b-BP, and TFPI.** ATIII, HCII, PC, and total PS levels regularly increased in fetuses, but remained significantly lower than in newborns. PC was particularly low, with values that did not exceed 16% in fetuses between 30 and 38 weeks’ gestation. Neonates still had markedly low levels of PC and PS as compared with ATIII and HCII, which reached values higher than 50%. In addition, as in adults, no significant difference between immunologic and functional PC levels could be found in fetuses or newborns. Free PS levels were also lower in fetuses and newborns, with a ratio of free PS to total PS that was significantly higher than in adults. These results were well correlated with C4b-BP levels, which varied from 1.8% to 9.3% in fetuses and reached 18.6% at
Table 1. Coagulation Screening Tests and Coagulation Factor Levels in Fetuses, Full-Term Newborns, and Adults

<table>
<thead>
<tr>
<th>Parameter</th>
<th>19-23 (n = 20)</th>
<th>24-29 (n = 22)</th>
<th>30-38 (n = 22)</th>
<th>Newborns (n = 60)</th>
<th>Adults (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>32.5 (19-45)</td>
<td>32.2 (19-44)*</td>
<td>22.6 (16-30)†</td>
<td>16.7 (12.0-23.5)*</td>
<td>13.5 (11.4-14.0)</td>
</tr>
<tr>
<td>PT (INR)</td>
<td>6.4 (1.7-11.1)</td>
<td>6.2 (2.1-10.6)†</td>
<td>3.0 (1.5-5.0)*</td>
<td>1.7 (0.9-2.7)*</td>
<td>1.1 (0.8-1.2)</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>186.8 (83-250)</td>
<td>154.0 (87-210)†</td>
<td>104.8 (76-128)†</td>
<td>44.3 (35-52)*</td>
<td>33.0 (25-39)</td>
</tr>
<tr>
<td>TCT (s)</td>
<td>34.2 (24-44)*</td>
<td>26.2 (24-28)</td>
<td>21.4 (17.0-23.3)</td>
<td>20.4 (15.2-25.0)†</td>
<td>14.0 (12-16)</td>
</tr>
<tr>
<td>PT (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor I</td>
<td>16.9 (10-24)</td>
<td>19.9 (11-30)*</td>
<td>27.9 (15-50)†</td>
<td>43.5 (27-84)†</td>
<td>98.7 (70-125)</td>
</tr>
<tr>
<td>Factor II</td>
<td>27.4 (17-37)</td>
<td>33.8 (18-48)*</td>
<td>45.9 (31-62)</td>
<td>52.5 (28-78)†</td>
<td>101.3 (68-130)</td>
</tr>
<tr>
<td>Factor III</td>
<td>10.1 (6-14)</td>
<td>9.9 (5-15)</td>
<td>12.3 (5-24)†</td>
<td>31.8 (15-50)†</td>
<td>104.8 (70-124)</td>
</tr>
<tr>
<td>Factor IV</td>
<td>20.5 (14-29)</td>
<td>24.9 (16-35)</td>
<td>28.0 (16-36)†</td>
<td>39.6 (21-65)†</td>
<td>99.2 (75-125)</td>
</tr>
<tr>
<td>Factor V</td>
<td>32.1 (21-44)</td>
<td>36.8 (25-50)</td>
<td>48.9 (23-70)†</td>
<td>89.9 (50-140)</td>
<td>99.8 (65-140)</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>34.5 (18-50)</td>
<td>35.5 (20-52)</td>
<td>50.1 (27-78)†</td>
<td>94.3 (38-150)†</td>
<td>101.8 (65-170)</td>
</tr>
<tr>
<td>Factor IX</td>
<td>13.2 (8-19)</td>
<td>12.1 (6-22)</td>
<td>14.8 (6-26)†</td>
<td>37.2 (13-62)†</td>
<td>100.2 (70-135)</td>
</tr>
<tr>
<td>Factor X</td>
<td>14.9 (6-25)</td>
<td>22.7 (6-40)</td>
<td>25.8 (11-50)†</td>
<td>69.8 (25-105)†</td>
<td>101.4 (65-144)</td>
</tr>
<tr>
<td>HMWK (%)</td>
<td>15.4 (10-22)</td>
<td>19.3 (10-26)</td>
<td>23.6 (12-34)†</td>
<td>38.9 (28-53)†</td>
<td>98.8 (68-135)</td>
</tr>
</tbody>
</table>

Values are the mean, followed in parentheses by the lower and upper boundaries including 95% of the population.
Abbreviations: Ag, antigenic value; c, coagulant activity.
* P < .05.
† P < .01.

DISCUSSION

Knowledge of the development of hemostasis in the healthy human fetus would contribute to further understanding of hemostatic disorders in premature infants and in newborns. However, as shown by the report of the Scientific and Standardization Subcommittee on Neonatal Hemostasis, data concerning fetal coagulation and fibrinolysis are still not completely established. Previous studies have reported variable results on fetal blood samples obtained through fetoscopy or after therapeutic abortions. Moreover, studies on premature infants are difficult to interpret because many
such infants are unhealthy and develop hemorrhagic or thrombotic complications, and thus the morbidity is higher before 30 weeks’ gestational age. Mammalian models such as bovine,\textsuperscript{41} porcine,\textsuperscript{42} or ovine models\textsuperscript{43,44} have also been used to study hemostatic development in the fetus and newborn. The ovine model is of particular interest, since strong similarities have been found for the development of blood coagulation and fibrinolysis in lamb and human fetuses.\textsuperscript{45} However, these animal models are unable to provide enough relevant information to shed light on clinical problems in humans, and thus the establishment of all possible coagulation parameters in fetuses is still a necessity.

The most recent and safe technique to obtain pure human fetal blood is direct puncture of the umbilical vein under ultrasound guidance,\textsuperscript{25} and this has been used to diagnose a wide variety of hematologic\textsuperscript{26,27,46} and nonhematologic\textsuperscript{47-49} disorders. Using this sampling method, we evaluated 25 blood coagulation parameters, activators, and inhibitors in healthy human fetuses between 19 and 38 weeks’ gestation and in normal full-term newborns. Samples from 285 fetuses were necessary to establish relevant data, and 20 fetuses from each group were tested for these 25 parameters.

In 19- to 29-week-old fetuses, PT (international normalized ratio > 6) and APTT (mean ratio, 5) were greatly prolonged, which can be clearly related to the low levels of all coagulation factors, ie, vitamin K–dependent proteins, contact factors, factors V and VIII, and fibrinogen. All coagulation inhibitors, ie, ATIII, HCII, PC, PS, and TFPI, were also greatly reduced, and we can thus assume that at this age a well-balanced hemostatic equilibrium is maintained. Between 30 and 38 weeks’ gestation, a significant shortening of PT and APTT was recorded. At the same time, most coagulation factors increased slightly (up to 25% to 30%), whereas factors V, VII, and VIII were between 45% and 50% of adult values. All coagulation inhibitors remained at levels near or less than 20%, except ATIII, which reached a mean value of 37% at the end of pregnancy.

When compared with previous studies performed in human fetuses, our investigation provides further understanding of the fetal coagulation system. Forestier et al\textsuperscript{30} assayed only vitamin K–dependent proteins in 19- to 27-week-old fetuses and obtained results similar to ours. The low levels of factors II, VII, X, and IX, PC, and PS in fetuses until birth cannot be explained by vitamin K deficiency during intrauterine life, since similar results were found for factor II, factor VII, and PC with immunologic and functional assays. Moreover, according to others,\textsuperscript{30,51} the low or undetectable levels of PIVKA-II antigen in the fetus also support the fact that the vitamin K, content in fetal hepatic microsomes is apparently sufficient to ensure complete γ-carboxylation of synthesized molecules. However, these results concerning vitamin K–dependent factors do not exclude the possibility that fetal proteins exist. For example, increased glycosylation of fetal ovine PC has recently been demonstrated,\textsuperscript{52} but if this modification is present in humans, it apparently does not influence the functional activity of the protein, which was equal to the antigenic level. This resembles fetal features described for human fibrinogen, but in this case there were clear-cut differences between levels obtained by Von Claus and immunologic assays. As others have suggested,\textsuperscript{53-55} it appears that the ability of fibrinogen to polymerize is decreased in both the human fetus and newborn compared with the adult.

To our knowledge, this is the first report of procoagulant and anticoagulant activities during the last 2 months of intrauterine life for healthy fetuses. At this age (30 to 38 weeks), in comparison to younger fetuses, levels of coagulation activators and inhibitors were higher but remained significantly lower than in newborns. Andrew et al\textsuperscript{19} published reference values for coagulation parameters in healthy premature infants of 30 to 36 weeks’ gestation, and reported consistently higher levels of vitamin K–dependent proteins, fibrinogen, factors V and VIII, and contact factors compared with those we found in fetuses in the same intrauterine period. Moreover, they also showed slight differences between the coagulation systems of the healthy premature infant and the
Table 2. Blood Coagulation Inhibitors in Fetuses, Full-Term Newborns, and Adults

<table>
<thead>
<tr>
<th>Parameter (n = 20)</th>
<th>Fetuses (weeks' gestation)</th>
<th>19-23 (n = 20)</th>
<th>24-29 (n = 22)</th>
<th>30-38 (n = 22)</th>
<th>Newborns (n = 60)</th>
<th>Adults (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATIII (%)</td>
<td></td>
<td>20.2 (12-31)*</td>
<td>30.0 (20-39)</td>
<td>37.1 (24-55)†</td>
<td>59.4 (42-100)t</td>
<td>99.8 (65-130)</td>
</tr>
<tr>
<td>HCl (%)</td>
<td></td>
<td>10.3 (6-16)</td>
<td>12.9 (5-20)</td>
<td>21.1 (11-33)†</td>
<td>52.1 (9-99)t</td>
<td>101.4 (70-128)</td>
</tr>
<tr>
<td>TFPI (ng/mL)†</td>
<td></td>
<td>21.0 (16-29)</td>
<td>20.6 (13-33.2)</td>
<td>20.7 (10-40.4)†</td>
<td>38.1 (22-55.8)t</td>
<td>73.0 (50.9-90.1)</td>
</tr>
<tr>
<td>PC Ag (%)</td>
<td></td>
<td>9.5 (6-14)</td>
<td>12.1 (8-16)</td>
<td>15.9 (8-30)†</td>
<td>32.5 (21-47)t</td>
<td>100.8 (68-125)</td>
</tr>
<tr>
<td>PC Act (%)</td>
<td></td>
<td>9.6 (7-13)</td>
<td>10.4 (8-13)</td>
<td>14.1 (8-18)†</td>
<td>28.2 (14-42)†</td>
<td>98.8 (68-129)</td>
</tr>
<tr>
<td>Total PS (%)</td>
<td></td>
<td>15.1 (11-21)</td>
<td>17.4 (14-25)</td>
<td>21.0 (15-30)†</td>
<td>38.5 (22-55)†</td>
<td>99.6 (72-118)</td>
</tr>
<tr>
<td>Free PS (%)</td>
<td></td>
<td>21.7 (13-32)</td>
<td>27.9 (19-40)</td>
<td>27.1 (18-40)†</td>
<td>49.3 (33-67)†</td>
<td>98.7 (72-128)</td>
</tr>
<tr>
<td>Ratio of free PS to total PS</td>
<td></td>
<td>0.82 (0.75-0.92)</td>
<td>0.83 (0.75-0.95)</td>
<td>0.79 (0.70-0.89)t</td>
<td>0.64 (0.59-0.98)†</td>
<td>0.41 (0.38-0.43)</td>
</tr>
<tr>
<td>C4b-BP (%)</td>
<td></td>
<td>1.8 (6-12)</td>
<td>6.1 (0-12.5)</td>
<td>9.3 (5-14)</td>
<td>18.6 (3-40)†</td>
<td>100.3 (70-124)</td>
</tr>
</tbody>
</table>

Values are the mean, followed in parentheses by the lower and upper boundaries including 95% of the population.

Abbreviations: Ag, antigen; Act, activity.

* P < .05.
† P < .01.
‡ Twenty samples were assayed for each group, but only 10 for 19- to 23-week-old fetuses.

full-term newborn. In contrast, we observed considerable differences (P < .001) in the majority of blood coagulation factors, including inhibitors, between the oldest fetuses that we studied (30 to 38 weeks) and the newborns. However, among the oldest fetuses (>34 weeks) some exhibited levels that were close or identical to neonate levels, which indicates significant hepatic maturation at this stage of development. Our data thus support the fact that during the last month of intrauterine life and in the first hours following delivery, dynamic changes occur in the composition of the circulating blood, particularly the coagulation system. The fetal hemostatic mechanisms are obviously immature, but the birth process itself alters a number of coagulation parameters. The precise mechanisms or signals that initiate these modifications have not been fully described. The role of corticosteroid burst during the 48 hours before onset of labor has been evoked in the ovine model. Kisker et al. reported that betamethasone and cortisol injections in pregnant ewes were associated with increased levels of fetal ovine factors II, V, VII, IX, and X. The stress associated with delivery might also contribute to the maturation of the coagulation system, as suggested by Johnson et al., who reported significantly higher levels of von Willebrand factor in infants delivered vaginally compared with infants delivered by cesarean section. Our data thus provide reference values that might help in understanding the coagulation status of premature infants delivered by cesarean section, especially the least mature.

This study clearly supports the idea that fetal hemostasis is a dynamic system that gradually evolves by stages toward the adult state but always maintains equilibrium between activators and inhibitors throughout intrauterine life until birth.

ACKNOWLEDGMENT
We are grateful to Prof J. Lansac, Prof G. Body, and Dr A. Fignon from the maternity ward of Bretonneau hospital, Tours, who provided the blood samples. We also particularly thank M.C. Bernard and E. Rideau for excellent technical assistance and D. Raine for editorial assistance.

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COAGULATION IN THE HUMAN FETUS


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