Lower Maternal PLAC1 mRNA in Pregnancies Complicated with Vaginal Bleeding (Threatened Abortion <20 Weeks) and a Surviving Fetus, Antonio Farina,1,2,3* Nicola Rizzo,2 Manuela Concu,1 Irina Banzola,1 Akihiko Sekizawa,3 Silvia Grotti,27 and Paolo Carinci1 (1 Department of Histology, Embryology and Applied Biology and 2 Division of Frenatal Medicine University of Bologna, Bologna, Italy; 3 Department of Obstetrics and Gynecology, Showa University School of Medicine, Tokyo, Japan; * address correspondence to this author at: Via Belmeloro 8, 40126 Bologna Italy; fax 39-51-2094110, e-mail antonio.farina@unibo.it)

Trophoblastic cells of the placenta enter the maternal circulation and can be isolated there along with their specific mRNAs. Increased concentrations of mRNA for corticotropin-releasing hormone, which is synthesized in the placenta, are seen in the blood of patients affected by preeclampsia (3), and panels of mRNAs (4) can be used to test for a disease of interest. The mRNA concentrations in blood may vary with intracellular expression and with the number of trophoblastic cells per unit of blood expressing that specific mRNA. We hypothesize that pregnancies affected by a threatened abortion, but with a viable embryo, exhibit lower concentrations of mRNA for the placenta-expressed gene PLAC1 because of possible underlying delayed or abnormal placentation growth at the time of maternal blood collection.

We studied 10 pregnant women admitted for a threatened abortion at <20 weeks of gestation (threatened abortion group). The study was approved by our local ethics committee, and all patients gave informed consent. All fetuses with abnormal karyotypes and pregnant women with preeclampsia; pre- or postterm delivery; uterine pathology such as myomas or malformations; placenta abruptio; loss to follow-up; and a vaginal or cervical lesion visualized on clinical examination that could explain the vaginal bleeding were excluded from the study. We also excluded those women who had vaginal bleeding and a subsequent miscarriage because villus destruction, apoptosis, and embryo death could increase the number of circulating placental cells and nucleic acids in the maternal blood, as could abnormal fetal karyotype, fetal malformation, and abnormal maternal immune response. Placental abruption observed later in the pregnancy was also excluded as a possible source of feto-maternal hemorrhage. During hospitalization, all women were maintained at complete bed rest, and routine ultrasound examinations and endocrine (B-hCG) evaluation were carried out. We also studied 66 normal pregnancies as controls. All blood tests were performed 1–2 days after the first occurrence of bleeding.

Peripheral blood samples (500 μL) were collected in tubes containing EDTA and processed within 1 h. We added an equal volume of phosphate-buffered saline (PBS) and a double volume of Lysis Reagent (Applied Biosystems) to a final volume of 2 mL. The Lysis Reagent is part of the Starter Kit protocol and is needed to lyse the sample and to protect the free nucleic acids from degradation.

The samples were stored at −20 °C until further processing. We extracted total RNA with the reagents and disposable Starter Kit for the ABI PRISM 6100 Nucleic Acid Prep Station (Applied Biosystems), with a final volume of 100 μL. We included an additional step with Absolute RNA Wash Solution, which is designed to remove contaminating DNA and PCR-inhibitory substances from purified RNA. This solution is able to directly remove background DNA in situ from immobilized RNA on the purification tray during the standard protocol developed by Applied Biosystems. After a brief incubation, subsequent wash steps remove the reagent.

Reverse transcription was performed by use of the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer’s protocol. The whole sample of total RNA (50 μL) from peripheral blood was reverse-transcribed in a final volume of 100 μL, containing 10× reverse transcription buffer, 25× dioxynucleotide triphosphates, 10× reverse transcription random primers, and 50 U/μL MultiScribe reverse transcriptase. Incubation in a GenAmp PCR System 9600 thermal cycler was performed in two steps: 10 min at 25 °C, followed by 120 min at 37 °C.

Quantitative real-time PCR analysis was performed by use of a PE Applied Biosystems 5700 Sequence Detection System (Applied Biosystems).

To determine the amount of cDNA, we used the PLAC1 and hPL loci. Every sample was tripled tested for each gene in different tubes. The hPL locus was used as a positive control. The primer and probe combinations for PLAC1 (5) and hPL (6) are listed in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue1/. Calibration curves for PLAC1 quantifications used 1×104 to 1×107 copies of single-stranded synthetic DNA oligonucleotides (Proligo, CELBIO) specifying the PLAC1 amplicon. Concentrations were expressed as copies/mL of cDNA. The sequence of the synthetic DNA oligonucleotide for PLAC1 calibration was 5'-GATCGCTTCACC-TCTGCCGTTTTTCAGCAGGTTCAAGAAGATGCAATG-GACGTGCTGCTGCCCATAGACTGCTG-3'.

A calibration curve for hPL quantification was prepared as described above, with the sequence of the synthetic DNA oligonucleotide being 5'-TGGGACAGCCTCTA-GATTTGATTTCTGTGGCTTTTCCCTCATGTGGAGGTGTGGGAATTAGAGTCGAGCAGGAAGGTTCTCGGAGTGCATGC-3' (6). For the PCR analysis, 50 μL of reaction volume contained 17.5 μL of cDNA. The reaction mixture contained the amplification primer (400 nM for PLAC1 and 300 nM for hPL), the dual-labeled probe (100 nM for both genes), and the necessary components provided in the TaqMan Universal PCR Master Mix (Applied Biosystems). This corresponded to 1.25 U of AmpliTaq Gold DNA Polymerase; 0.5 U of AmplErase Uracil N-Glycosylase; 200 μM each of dATP, dCTP and dGTP; 400 μM dUTP; 10× TaqMan Buffer A; and 25 mM MgCl2. Buffer A contains Passive Reference I for signal normalization in all TaqMan
reactions. Each sample was analyzed in triplicate, and the means were used for calculations.

The thermal profiles were obtained by use of a 2-min incubation at 50 °C, followed by an initial 10-min denaturation step at 95 °C and by 40 cycles of 1 min each at 56 °C plus 20 s at 95 °C.

The clinical variables and PLAC1 mRNA data are summarized in Table 1 of the online Data Supplement. The data stratified according to estimated amount of bleeding and gestational age at the time of blood collection are shown in Table 1.

Median (range) PLAC1 mRNA values were 356 (2.42–16 237) copies/mL for the controls and 42 (10.72–281.78) copies/mL for the cases. Five cases with a median gestational age of 65 (49–70) days had much lower PLAC1 values than 23 controls with a median gestational age of 73 (49–75) days (P < 0.002). On the other hand, 5 affected cases with a median gestational age of 121 (105–126) days had a median PLAC1 value very similar to that observed for 43 controls [86 (78–91) days; Fig. 1]. This result could be consistent with the role of PLAC1 in early trophoblast development.

PLAC1 mRNA displayed a wider distribution in controls than in cases. We also stratified the series according to fetus gender because PLAC1 gene maps on X chromosome (region Xq26) (5, 7). We found no statistical difference for male and female fetuses, even when females showed slightly higher mRNA expression (Table 2 of the online Data Supplement).

As shown in Table 1, vaginal bleeding and/or number of previous bleeding episodes do not correlate with PLAC1 concentration. This could be related to the fact that the mRNA concentration is a real-time marker for cellular damage, as has been reported previously for fetal DNA.

When vaginal bleeding occurs, some kind of vascular disruption of the maternal-fetal interface resulting from abnormal or delayed placental differentiation may be hypothesized. The vascular injury has been reported to be so minimal that it cannot be detected by Doppler ultrasound (8), but such an event affects the maternal serum concentration of proteins produced by the placenta and the fetus (9, 10). Numerous factors, among them decidualized endometrium and the trophoblast itself, have been implicated in the regulation of extravillous trophoblast maturation. Subsequent development of the placenta depends on the proliferation, migration, and invasion of fetal trophoblast cells into the maternal uterus, by means of a dynamic change in gene expression (11). Interestingly, it is likely that the genes involved in preeclampsia belong to those important for implantation and maintenance of pregnancy, Specific gene products, as well as PLAC1, can provide a useful model to investigate whether some abnormal placental developments can be related to lower mRNA expression. It must be stressed, however, that vaginal bleeding may indicate an underlying placental dysfunction (which might subsequently lead to miscarriage, preeclampsia, fetal growth restriction, or preterm delivery) that manifests later throughout the pregnancy and is associated with higher fetal cells and/or free nucleic acids passage into the maternal circulation, a condition that might affect mRNA quantification. We

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>GA, days</th>
<th>Bleeding at the time of blood collection</th>
<th>Previous bleeding episodes, n</th>
<th>Subsequent bleeding episodes, n</th>
<th>Outcome and diagnosis of associated diseases</th>
<th>% of the median PLAC1 mRNA concentration of control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td>49</td>
<td>+ +</td>
<td>0</td>
<td>1 (16 weeks later)</td>
<td>Marginal placenta previa at 23 weeks</td>
<td>2.19</td>
</tr>
<tr>
<td>DM</td>
<td>49</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>Normal</td>
<td>18.9</td>
</tr>
<tr>
<td>VS</td>
<td>60</td>
<td>+ +</td>
<td>0</td>
<td>0</td>
<td>Normal</td>
<td>2.17</td>
</tr>
<tr>
<td>FS</td>
<td>65</td>
<td>+</td>
<td>1</td>
<td>0</td>
<td>Osteogenesis imperfecta at 21 weeks</td>
<td>5.18</td>
</tr>
<tr>
<td>CV</td>
<td>67</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>Normal</td>
<td>6.62</td>
</tr>
<tr>
<td>FP</td>
<td>70</td>
<td>+ +</td>
<td>0</td>
<td>0</td>
<td>Normal</td>
<td>3.57</td>
</tr>
<tr>
<td>DN</td>
<td>105</td>
<td>+</td>
<td>1</td>
<td>1 (1 week later)</td>
<td>Normal</td>
<td>145.5</td>
</tr>
<tr>
<td>GC</td>
<td>106</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>Normal</td>
<td>157.9</td>
</tr>
<tr>
<td>VE</td>
<td>121</td>
<td>+</td>
<td>1</td>
<td>0</td>
<td>Normal</td>
<td>70.33</td>
</tr>
<tr>
<td>CM</td>
<td>126</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>Normal</td>
<td>128.01</td>
</tr>
</tbody>
</table>

*ID, identifier; GA, gestational age.

+++, similar to mensis; +, spotting only.
reduced the risk of this bias by enrolling only those women whose pregnancies had a favorable outcome and who had no clinical complications reasonably able to affect mRNA expression. We included, however, in our series one case of marginal placenta previa not detectable at the time of blood collection (7 weeks gestation) because we believe that such evidence reinforces the role of the PLAC1 gene in proper placentation and its possible ability to detect, in advance, placental dysfunctions. In fact, even if not completely understood, abnormal trophoblastic infiltration (12) has been proposed as a mechanism for abnormal placement of the placenta. Plac1, a placenta-specific gene of the mouse, should facilitate trophoblastic interactions peculiar to the placenta-uterus interface (13). More recently, Fant et al. (5) examined the cellular location of PLAC1 (the human homolog of Plac1), and confirmed its placental expression throughout human pregnancy.

Our data are the first clinical evidence of a correlation between mRNA for a trophoblast-specific gene and vaginal bleeding. This evidence is in agreement with all reports describing how such pregnancies are at higher risk of adverse obstetric outcomes, including preeclampsia, intrauterine growth restriction, preterm delivery, or placental abruption, all associated with abnormal placenta that could start at the early stages of pregnancy.

In conclusion, the concentration of mRNA for the PLAC1 gene in maternal blood is lower in cases affected by early vaginal bleeding before 10 weeks of gestation with a viable embryo but not at 15–18 weeks. Such results are indicative of a role of PLAC1 in the regulation of a normal fetal-maternal interface during the early stages of human fetal development.

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References

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