ELECTRONIC LETTER

Measurement of mRNA of trophoblast-specific genes in cellular and plasma components of maternal blood

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Background: Placental mRNA in maternal plasma is suitable for quantitative analysis regardless of fetal gender and genetic polymorphism status.

Methods: We obtained 155 blood samples from pregnant women to compare human placental lactogen (hPL) and β -subunit of human chorionic gonadotropin (β hCG) mRNA and protein levels between the cellular and plasma components of maternal blood. To assess clearance of hPL mRNA expression, we obtained blood samples from nine women immediately before and after delivery by caesarean section. mRNA was extracted from the cellular and plasma components of all samples, and hPL and β hCG mRNA expression was analysed by reverse transcription-PCR assay.

Results: The concentration of β hCG mRNA in the cellular component positively correlated with the plasma concentration of β hCG protein and β hCG mRNA ($p=0.001$ for both). The concentration of hPL protein in the plasma correlated with the hPL mRNA concentration of the cellular component ($p<0.05$). For both hPL and β hCG, the mRNA concentration of the cellular component was greater than that of the plasma component (22.9-fold higher for hPL and 4.3-fold higher for β hCG). The half life of hPL mRNA clearance was significantly longer for the cellular fraction (mean half life=203.8 min, range 150–3465 min) than for the plasma fraction (mean half life=32.2 min, range 15–385 min) ($p=0.008$).

Conclusion: The present findings indicate that the concentration of hPL and β hCG mRNA is significantly higher in the cellular component of maternal blood samples than in the plasma component. Cellular mRNA in maternal blood is useful for non-invasive evaluation of placental function.

Fetal DNA in maternal plasma¹ is clinically valuable for non-invasive prenatal determination of fetal gender² and rhesus D status,³ and for the diagnosis of many fetal disorders, including single-gene disorders such as achondroplasia.⁴ In addition, reports indicate that abnormal concentrations of fetal DNA in maternal plasma are associated with many pathological conditions, including preeclampsia,⁵ fetal chromosomal aneuploidies,⁶ placenta accreta,⁷ and hyperemesis gravidarum.⁸ In most studies of fetal DNA in maternal plasma, Y chromosome-specific sequences are used as fetal markers in women carrying a male fetus. The use of Y chromosome-specific sequences ensures that fetal DNA is not confused with maternal DNA but also greatly limits the clinical usefulness of this promising method.

Lo *et al* have demonstrated that human placental lactogen (hPL) mRNA and β -subunit of human chorionic gonadotropin (β hCG) mRNA are detectable in maternal plasma.⁹ Because hPL and β hCG are only expressed by placental trophoblasts, any hPL or β hCG mRNA detected in maternal plasma is derived from these trophoblasts. Because plasma

RNA is coated to form small particles,⁹ it is relatively stable in the plasma and can be quantified by TaqMan RT-PCR assay. Studies have shown that preeclampsia can affect release of placental mRNA. Lo *et al* reported that the concentration of corticotropin-releasing hormone mRNA in plasma from pregnant women was almost 10 times higher for subjects with preeclampsia than for matched controls.^{10–11} Unlike fetal DNA in maternal plasma, placental mRNA in maternal plasma is suitable for quantitative analysis regardless of fetal gender and genetic polymorphism status.

Recently, we reported that mRNA of a placenta-specific gene, placental factor-1 (PLAC-1), was detectable in whole blood from pregnant women.¹² Because levels of PLAC-1 mRNA were higher in whole blood than in plasma, we concluded that some PLAC-1 mRNA in whole blood originates from the cellular component. Consequently, in the present study, we compared levels of placenta-specific gene mRNA between the cellular and plasma components of maternal blood.

METHODS

Subjects and blood sampling

Peripheral blood samples were collected from pregnant women who visited the Department of Obstetrics and Gynecology of Showa University Hospital, Tokyo, Japan, between April and September 2005. For negative controls, peripheral blood samples were obtained from eight non-pregnant women. All subjects gave informed consent, and the study was approved by the Research Ethics Committee of Showa University Hospital. For comparison of hPL and β hCG mRNA and protein levels between the cellular and plasma components of maternal blood, we obtained 155 maternal blood samples from pregnant women at 7–40 weeks' gestation. For assessment of clearance of mRNA expression, we obtained samples from nine women who delivered by caesarean section, before delivery and 15, 30, 60, 120 min, and 24 h after delivery. None of the nine women who delivered by caesarean section had any complications. The blood samples used to obtain plasma were 7 ml samples collected in EDTA-containing tubes. The blood samples used to obtain the cellular component were 2.5 ml samples collected in PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland).

Processing of blood samples and measurement of protein concentrations

Plasma harvesting was performed immediately after the blood arrived at the laboratory (within 3 h of being drawn). To obtain plasma, we centrifuged the 7 ml blood samples at 3000 rpm for 10 min at 4°C. The plasma was then carefully transferred into polypropylene tubes and stored at –20°C until used for RNA extraction. To obtain the cellular

Abbreviations: β hCG, β -subunit of human chorionic gonadotropin; hPL, human placental lactogen; PLAC-1, placental factor-1

component, each 2.5 ml blood sample in a PAXgene blood RNA tube was kept for 3 h at room temperature according to the manufacturer's protocol and then stored at -20°C .

Concentrations of hPL and βhCG protein in the plasma fractions were measured by radio-immunoassay and latex agglutination immunoassay, respectively.

RNA extraction

RNA was extracted from plasma using a method based on a previous report.⁹ Briefly, 1.6 ml of plasma was mixed with 2 ml of Trizol LS (Invitrogen, Carlsbad, CA) and 0.4 ml of chloroform. This mixture was centrifuged at 12 000 rpm for 15 min at 4°C , and the aqueous layer was transferred into new tubes. Then 1 vol of 70% ethanol was added to 1 vol of the aqueous layer. This mixture was applied to an RNeasy minicolumn (Qiagen, Hilden, Germany) and processed according to the manufacturer's instructions. Total RNA was eluted with 30 μl of RNase-free water and stored at -80°C .

Cellular component samples were centrifuged twice at 4000 g for 10 min at room temperature to remove the entire supernatant and any mRNA present in residual plasma. The pellet was then washed, resuspended, and incubated in optimised buffers containing proteinase K to digest proteins. A second centrifugation was performed to remove residual cell debris, and the resulting supernatant was transferred to a fresh microcentrifuge tube. We added 100% ethanol to the supernatant to adjust binding conditions, and the resulting lysate was applied to a PAXgene spin column (PreAnalytiX); RNA selectively binds to the silica gel membrane of the spin column. After the column was washed three times, pure RNA was eluted in 80 μl of RNase-free water.

Real-time quantitative reverse transcription-PCR

Reverse transcription of the mRNA was performed using an Omniscript RT Kit (Qiagen). Real-time quantitative PCR was then performed using a QuantiTect Probe PCR Kit (Qiagen). The hPL and βhCG primers and probe previously developed by Lo *et al*⁹ were used for the RT-PCR. RT-PCR was performed according to the manufacturer's instructions in a reaction volume of 25 μl . Multiple negative-control water blanks were included in every PCR. Thermal cycling of the PCR was as follows: initial denaturation at 95°C for 15 min; 40 cycles of denaturation at 94°C for 15 s and annealing at 60°C for 1 min.

Statistical analysis

Descriptive statistical analysis was performed using non-parametric routine tests. Data were expressed as median and interquartile range. $\log_{10}$ linear regression was used to evaluate association between variables and the gestational age at the time of blood sampling. A Spearman's r matrix of correlation was used to evaluate correlation among levels of cellular mRNA, plasma mRNA, and plasma protein. In order to estimate the apparent half life of hPL and βhCG mRNA clearance, we plotted the $\log_{10}$ of the peak hPL and βhCG mRNA concentrations to the first non-zero trough concentration. The R^2 values and slopes ($-k$) were calculated by linear regression. The apparent half lives of hPL and βhCG

mRNA clearance were then calculated. The half life was then computed using the following equation: half life = $0.693/k$.

RESULTS

Concentration of βhCG and hPL mRNA and protein

hPL and βhCG mRNA was detected in every sample obtained from pregnant women, but not in those from non-pregnant women. Table 1 shows the estimated median mRNA concentrations of plasma and cellular components, and plasma protein concentrations of βhCG and hPL.

All six $\log_{10}$ linear regressions yielded a significant p value, with R^2 ranging from 0.067 to 0.814 (fig 1A–F), as reported in the Appendix. Gestational age was most closely associated with hPL and βhCG protein levels, followed by plasma and cellular mRNA levels (see Appendix). Average hPL mRNA levels were 22.9-fold higher for the cellular component than for plasma, and average βhCG mRNA levels were 4.3-fold higher for the cellular component than for plasma. The matrix of correlations (table 2) showed that gestational age more closely correlated with βhCG ($p < 0.001$ for cellular mRNA, plasma mRNA, and protein) than with hPL (p values ranging from 0.244 to < 0.001). There was no significant correlation between cellular and plasma hPL mRNA.

Clearance of mRNA expression

In predelivery blood samples, the median hPL mRNA levels were 9584 copies/ml for plasma (range 3708–32 684) and 65 254 copies/ml for the cellular component (range 37 139–346 289). After delivery, plasma hPL mRNA cleared rapidly. Median apparent estimated clearance was 32.2 min for plasma (range 15–385; $R^2 = 0.84$ (range 0.44–1)) and 203.8 min for the cellular component (range 150–3465; $R^2 = 0.52$ (range 0.20–0.95)). In seven out of the nine women who delivered by caesarean section, plasma hPL mRNA fell below the minimum detectable level within 2 h postpartum, and no hPL mRNA was detected in plasma from blood samples obtained 24 h after delivery (fig 2). On the other hand, although we detected hPL mRNA in the cellular components of all nine women who delivered by caesarean section, the hPL mRNA level increased temporarily 15 min after delivery in eight of the nine women, and decreased slowly thereafter. For all nine women who delivered by caesarean section, hPL mRNA was detectable in the cellular component 24 h after delivery.

DISCUSSION

In the present study, we quantified concentrations of hPL and βhCG mRNA in the cellular and plasma components of maternal blood. In the cellular component, we found that the hPL mRNA concentration increased as pregnancy progressed, and that the βhCG mRNA concentration peaked around 10 weeks of gestation and then decreased gradually. Furthermore, for both hPL and βhCG , we observed significant correlations between plasma mRNA and plasma protein, as previously reported by Lo *et al*.⁹ Moreover, the βhCG mRNA concentration of the cellular fraction of maternal blood significantly correlated with plasma βhCG mRNA and plasma βhCG protein, and the hPL mRNA concentration of the cellular fraction correlated with plasma hPL protein. Because both hPL and βhCG are trophoblast-specific, these findings

Table 1 Changes in hPL and βhCG mRNA concentrations of plasma and cellular components

Trimester	Cases (n)	Cellular hPL	Cellular βhCG	Plasma hPL	Plasma βhCG
First	27	41 440 (22 880–67 840)	2850.83 (731.71–7351.04)	750 (416.66–1250)	334.58 (172.39–611.14)
Second	53	53 760 (20 480–99 120)	33.53 (0–723.93)	1770.83 (963.54–2932.29)	61.14 (22.91–116.82)
Third	75	61 760 (36 960–109 760)	3.63 (0–208.08)	3697.91 (2354.16–6260.41)	41.97 (16.66–78.02)

Data (copies/ml) are expressed as median (interquartile range).

Figure 1 Scatter plot of the available variables versus gestational age expressed in weeks. mRNA values are expressed in copies/ml.

might indicate that a certain number of trophoblasts circulate in maternal blood. If these trophoblasts represent placental function and placental gene expression, pathophysiological changes inside the placenta can be evaluated by analysing mRNA levels found in the cellular and plasma components of maternal blood. Thus, analysis of mRNA in maternal blood samples may be a useful method of non-invasive profiling of placental gene expression. Although cellular mRNA expression of β hCG was not higher than that of plasma at the second and third trimesters, the β hCG expression was approximately 100 times higher at the first trimester than at the second and third trimesters. The low β hCG expression of the cellular component may reflect the functional decrease in the trophoblasts released from the placental villi. Thus, we thought that levels of placenta-specific gene mRNA in maternal blood were higher in the cellular component than in the plasma. It might be feasible to measure placenta-specific gene mRNA earlier in gestation.

To recover the cellular component of maternal blood, we used a PAXgene Blood RNA System which preserves the

stability of cellular mRNA. Although the plasma mRNA level gradually decreased during 3 month storage at -20°C (data not shown), the system keeps the mRNA level relatively stable for at least 20 months when samples are stored at -20°C , and allows for easy transportation. To the best of our knowledge, this system provides the best preservation of cellular mRNA during storage of blood samples. Given the greater ease of preserving cellular mRNA, compared to plasma mRNA, cellular mRNA is easier to use for evaluation of placental function.

In the present study, we found that levels of hPL and β hCG mRNA were much higher in the cellular component than in the plasma (22.9-fold higher for hPL and 4.3-fold higher for β hCG). Because the concentration of hPL protein increases with the growth of the placenta as pregnancy progresses, the concentration of hPL may be associated with the size of the placenta or the number of trophoblasts. On the other hand, β hCG concentration peaked around 10 weeks of gestation, suggesting that β hCG levels are associated with changes in trophoblast function during gestation. Such functional differences between hPL and β hCG may be reflected by differences between hPL and β hCG mRNA expression in the cellular component. The present matrix of correlations (table 2) showed that gestational age more closely correlated with β hCG ($p < 0.001$ for cellular and plasma mRNA and protein) than with hPL (p values ranged from 0.244 to < 0.001). Although we found no significant correlation between cellular and plasma hPL mRNA, there was significant correlation between hPL protein and hPL mRNA concentration in the cellular fraction. This finding may suggest that cellular mRNA is more useful for evaluation of placental function than plasma component mRNA. However, because of the higher concentration of mRNA in the cellular fraction and the greater stability of cellular mRNA, cellular mRNA is easier to use than plasma mRNA for the evaluation of placental function.

Table 2 Matrix of correlation (Spearman's r) for hPL and β hCG (number of cases = 155)

	Cellular hPL	Plasma hPL	hPL
Cellular hPL	1.000	0.094	0.168*
Plasma hPL	0.094	1.000	0.705**
hPL	0.168*	0.705**	1.000
	Cellular β hCG1	Plasma β hCG2	β hCG3
Cellular β hCG1	1.000	0.584**	0.676**
Plasma β hCG2	0.584**	1.000	0.648**
β hCG3	0.676**	0.648**	1.000

*Correlation is significant, $p < 0.05$ (two-tailed); **correlation is significant, $p < 0.01$ (two-tailed).

Figure 2 Clearance (min) for cellular (dotted line) and plasma (solid line) components.

In the present analysis of hPL mRNA clearance, we found that the half life of hPL mRNA after delivery was 32.2 min for plasma mRNA (range 15–385) and 203.8 min for cellular mRNA (range 150–3465). Clearance of cellular mRNA had a more gradual curve than clearance of plasma mRNA. The half life of hPL mRNA was significantly longer for the cellular component than for plasma ($p = 0.08$). We speculate that the plasma mRNA does not come from the cellular component of maternal blood and that the clearance of mRNA in the plasma is more efficient than that in the cellular component. Furthermore, we observed that the concentration of hPL mRNA peaked at 15 min after caesarean section, suggesting that there is an influx of trophoblasts into the maternal circulation at the time of placenta removal.

In the present study, we could not determine the source of the cellular mRNA. However, when we extracted mRNA from maternal blood, we discarded the supernatant of the blood samples, thus preventing contamination of plasma mRNA. The concentration of cellular hPL mRNA was lower before caesarean section than 15 min after caesarean section. The half life of hPL mRNA after delivery was much longer in the cellular component than in plasma. Furthermore, many studies indicate that trophoblasts circulate in maternal blood.^{13–14} The present findings also indicate that trophoblasts are present in maternal blood, and that hPL mRNA in the cellular component of maternal blood might be produced by trophoblasts in maternal blood. Furthermore, the higher levels of hPL and β hCG mRNA that we detected in the cellular component than in the plasma component of maternal blood suggest that maternal blood contains many trophoblast cells.

In this study, we studied hPL and β hCG mRNA because we want to assess whether the placental function can be evaluated through analysis of the cellular component of maternal blood. Since many genes including those whose antibodies are not commercially available, can be detected by this method, this technique has an advantage compared with conventional protein measurements.

In conclusion, this is the first report to indicate that placental gene expression can be evaluated by measuring

mRNA levels in the cellular component of maternal blood, and that the concentration of placental gene mRNA is significantly higher in the cellular component than in plasma. We conclude that cellular mRNA in maternal blood is useful for non-invasive evaluation of placental function. Evaluation of pathophysiological changes in the placenta by evaluating cellular mRNA may allow prediction of pregnancy complications.

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APPENDIX

Output of $\log_{10}$ -linear equations for variables versus gestational age

Variable	R ²	F value	p value	Intercept	Slope
Cellular hPL	0.067	6.35	0.014	4.4870	0.0104
Cellular β hCG	0.534	101.00	0.000	2.5528	0.0343
Plasma hPL	0.814	384.90	0.000	-0.7054	0.0482
Plasma β hCG	0.201	22.07	0.000	3.5174	-0.0438
hPL	0.271	32.74	0.000	2.6811	-0.0354
β HCG	0.432	66.92	0.000	1.9177	-0.0307

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