were also observed in plasminogen activator inhibitor-1, tissue-type plasminogen activator, corticotrophin-releasing hormone, selectin P and placenta-specific 1 mRNA (Purwosunu *et al.*, 2007a,b). Therefore, evaluation of placental mRNA levels in maternal plasma may allow for the indirect monitoring of the placental function.

That the expression of placenta-specific genes, such as hPL and βhCG, is detectable in the cellular component of maternal blood (Okazaki *et al.*, 2006) suggests that some trophoblasts circulate in the blood of normal pregnant women. Furthermore, the mRNA concentrations of hPL and βhCG correlate with the protein assay. We have reported that the cellular mRNA concentration of hPL during the third trimester was 16.7 times greater, and that of βhCG during the first trimester was 8.5 times greater, than those of the plasma component of maternal blood (Okazaki *et al.*, 2006). These results suggest that analysis of the cellular component of maternal blood may be ideal for evaluating the placental function. We reported the up-regulated mRNA expression of pregnancy-specific β1-glycoprotein 1 (PSBG) and trophoblast glycoprotein (TPBG) in the cellular component of blood from patients affected with PE, and a direct correlation between the PSBG expression levels and the clinical severity of PE (Okazaki *et al.*, 2007). As oxidative stress may play an important role in the pathogenesis of PE, in this study we assessed the mRNA expression levels of anti-oxidant enzymes in the cellular component of blood from women with PE.

MATERIALS AND METHODS

Subjects

We included pre-eclamptic and normal pregnant women who visited the Department of Obstetrics and Gynecology, University of Indonesia at Cipto Mangunkusumo National Hospital. Subjects were recruited between December 2005 and February 2006. A total of 48 singleton pregnancies were included, with 24 in each group of PE and control. All women provided their written informed consent to participate in the study, and the protocol was approved by the Research Ethics Committees of both University of Indonesia and Showa University. We defined mild and severe PE, as well as hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome, as described elsewhere (Purwosunu *et al.*, 2007b). The severities of hypertension and proteinuria were defined by highest level of hypertension during hospital stay and urinary protein excretion in a 24-hour urine specimen, respectively. The control group included pregnant women with no pre-existing medical diseases or prenatal complications.

Processing of blood samples

The blood samples (2.5 mL) were collected in PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and kept at room temperature for 3 h, and then were stored at −20 °C until they were transported to Japan. A molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo, Japan. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) were performed according to protocols described elsewhere (Okazaki *et al.*, 2006). In brief, the blood samples were centrifuged twice at 4000 g for 10 min at room temperature; the entire supernatant and any mRNA present in the residual plasma were removed. The pellet was then washed, resuspended and incubated in optimized buffer solution containing proteinase K to digest protein. A second round of centrifugation was performed to remove any residual cell debris, and the resulting supernatant was then transferred to a fresh microcentrifuge tube. We added 100% ethanol to the supernatant to adjust the binding conditions, and the resultant lysate was then applied to a PAXgene spin column (PreAnalytiX); thus resulting in the selective binding of RNA to the silica-gel membrane of the spin column. After the column was washed three times, pure RNA was then eluted in 80 μL of RNase-free water.

Real-time quantitative RT-PCR

The mRNA was reverse transcripted using an Omniscript RT Kit (Qiagen). Real-time quantitative PCR was then performed using a QuantiTect Probe PCR Kit (Qiagen), according to the manufacturer's instructions. The cDNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (QuantiTect Probe PCR kit, Qiagen) using a 2-μL aliquot of cDNA and the kit's components in a reaction volume of 20 μL. TaqMan PCR analyses for HO-1, HO-2, SOD, GPx and CAT were performed using predeveloped and commercially available primers and probe sets (Cat# Hs00157965_m1 for HO-1, Cat# Hs00157965_m1 for HO-2, Cat# Hs00166575_m1 for SOD, Cat#Hs00829989_gH for GPx and Cat# Hs00156308_m1 for CAT: Applied Biosystems, Foster City, CA). As an initial step, we verified that each PCR assay was specific to mRNA and not to genomic DNA. The amplification data were collected and analyzed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analyzed in duplicate, and multiple negative water blanks were included in every analysis. The following thermal profile was used: 15-min denaturation at 95 °C and 15-s annealing at 94 °C, followed by 1-min extension at 60 °C. The quantification of the gene expression was performed with investigators blinded to sample background. The calibration curves for HO-1, HO-2, SOD, GPx and CAT mRNA quantification were made using placental mRNA obtained from normal pregnancies undergoing elective cesarean delivery, with calculations performed as previously described (Farina *et al.*, 2006) and they were expressed as relative concentrations (RCs).

Table 1—Demographics and clinical variables

	Control ($n = 24$)	Pre-eclampsia ($n = 24$)	p value
Maternal age (year)	27 (18–42)	27 (18–37)	ns
BMI	22.5 (20–28)	23.5 (16–32)	ns
Gestational age at blood sampling (week)	38 (35–40)	39 (36–41)	ns
Systolic blood pressure (mmHg)	115 (100–125)	160 (130–200)	—
Diastolic blood pressure (mmHg)	77.5 (70–85)	100 (90–140)	—
Proteinuria (g/day)	0 (0–0.1)	5.1 (1.5–8)	—
Birth weight (g)	3075 (2500–4200)	2875 (2000–3500)	0.03
Primiparae (%)	45.8	41.7	ns

BMI, body mass index; ns, not significant.

Statistical analysis

The distribution of the demographic characteristics and mRNA concentrations were expressed as the median and min-max. Mann-Whitney U-test was used for univariate comparison of the variables. A weighted (for the number of cases) log-linear regression analysis was performed in women with PE and controls by plotting the actual mRNA values against the systolic and diastolic blood pressures, as well as for absolute proteinuria. For all the analyses, a p value <0.05 was considered to be significant.

RESULTS

Among the 24 cases of the PE group, 11 (45.8%) were mild PE, 7 (29.2%) were severe PE and 6 (25%) were HELLP syndrome. As shown in Table 1, there was no difference in maternal age, gestational age, percentage of primipara and body mass index between the PE and control groups.

In Table 2, we show the RC of each mRNA in the cellular component of maternal blood. All of the mRNA species showed a statistically different distribution in PE versus controls. In addition, the values were a function of the severity of the disease as shown in Figure 1. The expression levels of HO-1 and HO-2 were inversely related to the clinical severity of pre-eclampsia. In the PE group, HO-2 correlated with systolic blood pressure ($p = 0.022$), and HO-1, HO-2 and CAT with proteinuria ($p = 0.001$, $p < 0.001$, $p = 0.026$), respectively (Figure 2). The statistical data are presented in Table 3.

Table 2—Median values (min-max) of the different mRNA species in the cellular blood component in the control and pre-eclampsia groups

	Control ($n = 24$)	Pre-eclampsia ($n = 24$)	p value*
Log HO-1	9.87 (8.61–10.53)	9.13 (5.42–10.19)	<0.001
Log HO-2	7.05 (3.19–7.47)	6.81 (4.73–7.34)	0.019
Log SOD	5.91 (4.95–6.44)	5.40 (3.90–6.23)	0.001
Log GPx	7.56 (7.03–8.109	6.90 (4.54–7.52)	<0.001
Log CAT	7.38 (4.39–7.77)	7.07 (4.90–7.63)	0.006

* Mann-Whitney U-test.

Table 3—Significant correlations between mRNA species in the cellular blood component and systolic blood pressure and proteinuria

	Systolic blood pressure		Proteinuria	
Variable	Slope	p value	Slope	p value
HO-1	−0.035	0.013	−0.521	<0.001
HO-2	−0.024	<0.001	−0.286	<0.001

DISCUSSION

Recently, the manifestation of clinical symptoms of PE, including endothelial dysfunction, hypertension and proteinuria, is reported to be mediated by high circulating concentrations of anti-angiogenic factors such as soluble FLT-1 (sFLT-1) (Levine *et al.*, 2004) and soluble Eng (sEng) (Venkatesha *et al.*, 2006). The protein concentration of sFLT-1 starts rising at least 5–6 weeks before the onset of symptoms, and the sEng is elevated in the serum of preeclamptic women 8–12 weeks before the clinical onset (Levine *et al.*, 2006). Furthermore, although the administration of sFLT-1 to pregnant rats induced the clinical manifestations of PE (Maynard *et al.*, 2003), the simultaneous adenoviral administration of sFLT-1 and sEng induced symptoms of severe proteinuria, severe hypertension and HELLP syndrome (Venkatesha *et al.*, 2006). These observations suggest that excess circulating sFLT-1 and sEng contribute to the clinical manifestation of PE.

Although the molecular mechanism regulating the release of these anti-angiogenic factors is unknown, it is suggested that hypoxia or oxidative stress of trophoblasts is associated with the production of anti-angiogenic factors. Li *et al.* reported the up-regulation of sFLT-1 to be associated with increased oxidative stress as a consequence of hypoxia in placental trophoblasts (Li *et al.*, 2005). HO-1 is known to have anti-oxidant, anti-inflammatory and cytoprotective functions. HO-1 is an oxygen sensor and its expression is inducible under hypoxic conditions (De Marco and Caniggia, 2002). The up-regulation of HO-1 is considered to be a marker of increased oxidative stress in cultured trophoblast cells (Li *et al.*, 2005). Previous

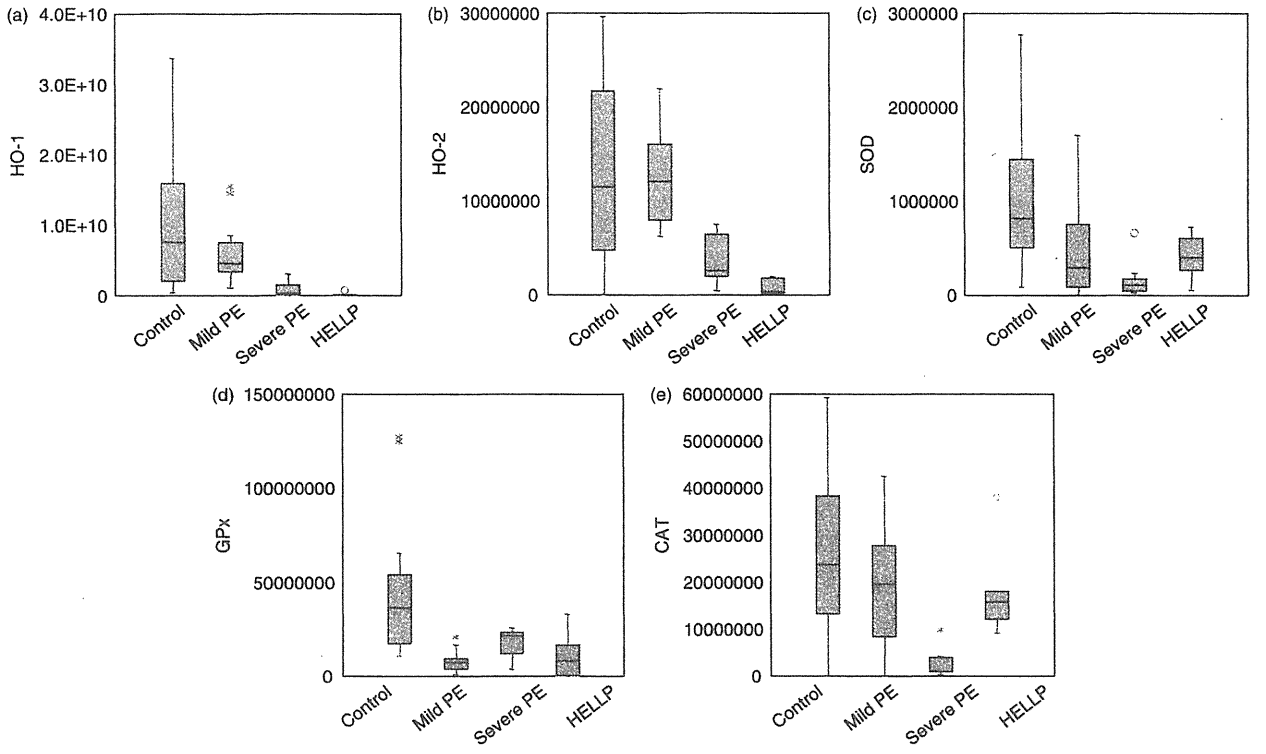

Figure 1—Box and whiskers plot of the distribution of HO-1, HO-2, SOD, GPx and CAT mRNA levels in the controls and women with pre-eclampsia, stratified according to the severity of pre-eclampsia [mild, severe preeclampsia, and hemolysis, elevated liver enzyme, low platelets (HELLP) syndrome]. Unit of each mRNA level is relative concentration per milliliter of maternal plasma. The medians are indicated by a line inside each box, the 75th and 25th percentiles by the box limits; the upper and lower bars represent the 10th and 90th percentiles, respectively. Small star (*) indicates above 90th or 10th percentile samples

data showed that low HO-1 levels in the placenta resulted in an abortion (Zenclussen *et al.*, 2005), and that the up-regulation of HO-1 by adenoviral administration worked protectively during pregnancy (Zenclussen *et al.*, 2006). PE has been reported to be associated with diminished placental HO levels (Ahmed *et al.*, 2000). Furthermore, in the previous study, we obtained tissue samples of villous trophoblasts at the time of fetal karyotype analysis through chorionic villous sampling (CVS), and assessed the mRNA expression of anti-oxidant genes. The results revealed that the expression levels of HO-1 and SOD were significantly lower in the CVS tissues from the pregnant women that developed PE later in gestation (Farina *et al.*, 2008). Although Li *et al.* reported the sFLT-1 production to increase in trophoblast cells when cultured under hypoxic conditions, the increased sFLT-1 production positively correlated with the increased lipid peroxide production, thus indicating that hypoxia promotes both the sFLT-1 and lipid peroxide production in placental trophoblasts (Li *et al.*, 2005). Moreover, the adenoviral overexpression of HO-1 inhibited the sEng release in placental villous explants, while also inhibiting the sFLT-1 production in endothelial cells (Cudmore *et al.*, 2007).

In the present study, we revealed that the mRNA expressions of HO-1, HO-2, SOD, GPx and CAT decreased in the cellular component of blood from patients with PE, and that the mRNA expressions of HO-1 and HO-2 negatively correlated with the clinical severity of PE. Furthermore, the HO-1 level correlated with the severity of proteinuria, while the HO-2 level correlated with that of proteinuria and the systolic blood pressure, thereby providing continuing evidence that PE may be associated with excess oxidative stress, which cannot be neutralized by the HO activity, thus leading to the increased release of anti-angiogenic factors in the placenta.

In conclusion, we herein demonstrated that the mRNA concentrations of HO-1, HO-2, SOD, GPx and CAT significantly decreased in the cellular component of the maternal blood of pregnant women with PE. The values of HO-1 and HO-2 correlated with the severity of PE. These findings indicate that anti-oxidant enzymes play important roles in PE. As a result, the alterations of the placental function can be evaluated by a cellular RNA analysis of maternal blood, and this approach may offer an alternative way for evaluating anti-oxidant factors involved in the pathogenesis of PE. A prospective study examining the expression of various placental genes might therefore be worthwhile to further evaluate the possible mechanisms underlying pathological pregnancies.

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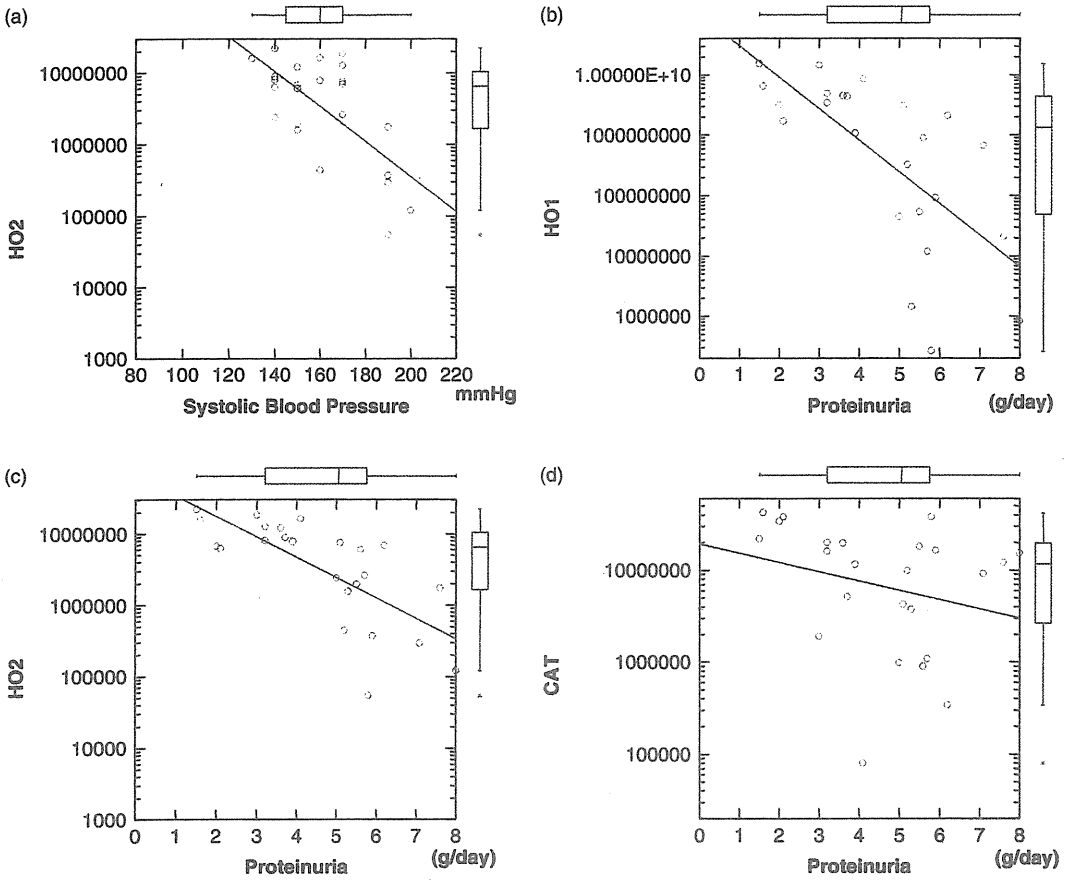

Figure 2—Correlation between the mRNA levels and systolic blood pressure and proteinuria in the subgroup of patients with pre-eclampsia. Only the significant correlations are reported. A unit of each mRNA level is relative concentration per milliliter of maternal plasma

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Expression of Angiogenesis-Related Genes in the Cellular Component of the Blood of Preeclamptic Women

Yuditiya Purwosunu, MD, Akihiko Sekizawa, MD, Shiho Yoshimura, MD, Antonio Farina, MD, Noroyono Wibowo, MD, Masamitsu Nakamura, MD, Hanako Shimizu, MD, and Takashi Okai, MD

The purpose of this study is to assess the changes in the expression of angiogenesis-related genes in the cellular component of the blood from preeclamptic patients. Blood samples were obtained from the preeclampsia (PE) and control participants. Cellular RNA was analyzed by reverse transcription polymerase chain reaction (PCR) to identify any angiogenesis-related genes and thereby assess the mRNA expression among women with PE and controls during weeks 35 to 41 of gestation. Significant differences were observed between PE and controls in all of the angiogenesis-related genes examined. In PE, for the increased expression of transforming growth factor-β1 (TGF-β1), endoglin and fms-like tyrosine kinase-1 (Flt-1); and the reduced expression of vascular endothelial growth factor (VEGF), placental growth factor (PlGF). fms-Like tyrosine kinase-1 and endoglin significantly correlated with the systolic pressure, while VEGF, Flt-1, and endoglin all correlated with proteinuria. An altered expression of angiogenesis-related genes was demonstrated in the cellular component of blood from preeclamptic patients. These findings indicate that this approach may offer an alternative way for evaluating the pathogenesis of PE.

KEY WORDS: Preeclampsia, cellular RNA, maternal blood, Flt-1, endoglin.

INTRODUCTION

Despite intensive research, preeclampsia (PE) continues to be a leading cause of fetomaternal mortality and morbidity in the developed and developing world. The current consensus is that the disorder is initiated by a placental defect, which triggers a cascade of events leading to maternal syndrome. These events include dysregulated placental angiogenesis, thus resulting in a release of antiangiogenic factors that might induce systemic endothelial dysfunction.[1,2] Among the angiogenesis-related genes produced by trophoblasts, altered concentrations of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), VEGF receptor-1 (VEGFR-1) known as fms-like tyrosine kinase-1 (Flt-1), transforming growth factor-β1 (TGF-β1), and endoglin (ENG) are associated with the preeclamptic outcome.[3-5]

Vascular endothelial growth factor is crucial to normal fetal development[6] and it is induced by hypoxia. Vascular endothelial growth factor acts through 2 receptors, VEGFR-1 and VEGFR-2, also known as Flt-1 and the kinase domain region (Flk/KDR), respectively. fms-Like tyrosine kinase-1 is expressed by trophoblast cells and is thought to play an essential physiological role during pregnancy.[7] A soluble form of Flt-1 (sFlt-1) is formed by alternative splicing and binding with VEGF. Vascular endothelial growth factor causes vasodilatation through the production of nitric oxide and prostacyclin.[8-10] Because sFlt-1 combines with VEGF and PlGF and the

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serum level of sFlt-1 increases in pregnant women who develop PE,[4] the free serum PlGF and free serum VEGF thus decline prior to the development of PE.[4] The circulating protein levels of sFlt-1 correlate with the severity of PE before the onset of hypertension and proteinuria.[4] Patients receiving VEGF-signaling antagonists for the treatment of cancer develop hypertension and proteinuria.[11] Moreover, the administration of exogenous sFlt-1 to pregnant rats produces a syndrome resembling PE, including hypertension and proteinuria.[12] These findings suggest that excess circulating sFlt-1 may thus play a causal role in PE.

Endoglin is a cell-surface coreceptor for TGF-β1 and TGF-β3. Elevated expression of ENG on the cell membranes of the vascular endothelium and syncytiotrophoblasts modulates the activity of TGF-β1 and TGF-β3.[13,14] Endoglin transcription is inducible by hypoxia.[15] Endoglin knockout mice die at mid-gestation due to defective angiogenesis and abnormal cardiovascular development.[16] Endoglin also regulates the endothelial nitric oxide synthase activity and local vascular tone.[17] Venkatesha et al[5] reported the placenta to be a major source of soluble ENG (sENG) during pregnancy and that placental ENG is upregulated in PE releasing sENG into the maternal circulation, which correlates with the severity of PE. In addition, the administration of sENG to pregnant rats produces hypertension and modest proteinuria. Furthermore, it is interesting to note that the coadministration of sENG and sFlt-1 results in severe PE, including HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome.[3] Therefore, it is thought that sENG and sFlt-1, which are derived from the placenta, play critical roles in the pathogenesis of PE.

Cell-free mRNA derived from the placenta circulates in the plasma of pregnant women, and the discovery of circulating fetal/placental RNA in maternal plasma has enabled the development of several promising approaches for noninvasive evaluation of placental function. A previous report showed that VEGF, Flt-1, and ENG mRNAs are detectable in the plasma of pregnant women in the third trimester, and that these levels increase in pregnancies complicated by PE, in comparison to normal pregnancies. Furthermore, mRNA concentrations of VEGF, Flt-1, and ENG correlate with the severity of PE.[18] These changes are observed in other genes, such as plasminogen activator inhibitor-1, tissue-type plasminogen activator, corticotrophin-releasing hormone, selectin P, and placenta-specific 1 mRNA.[19,20] An evaluation of the placental mRNA levels in maternal plasma thus may allow for the indirect monitoring of the placental function. A molecular-level analysis of cell-free mRNA in maternal plasma may therefore prove to be useful for monitoring the placental function.

The expression of placenta-specific genes, such as human placental lactogen (hPL) and human chorionic gonadotropin beta (β-hCG),[21] is detected in the cellular component of the blood from pregnant women, thus indicating that some trophoblasts or placental debris circulate in the blood of normal pregnant women. Furthermore, the cellular mRNA concentrations of hPL and β-hCG correlate with the plasma protein levels. The cellular mRNA concentration of hPL during the third trimester was 16.7 times greater, and that of β-hCG during the first trimester was 8.5 times greater, than that of maternal plasma.[21] Based on these results, an analysis of the cellular component of maternal blood may therefore be ideal for evaluating alterations of placental function. Another study reported upregulated mRNA expression of pregnancy-specific β1-glycoprotein (PSBG) and trophoblast glycoprotein in the cellular component of blood from patients affected with PE and a direct correlation between PSBG expression levels and the clinical severity of PE.[22] These findings are thus considered to provide further insight into identification of pathological pregnancies through mRNA analysis in the cellular component of maternal blood. Therefore, this study attempted to assess the changes in the mRNA expression levels of angiogenesis-related genes in women with PE.

MATERIALS AND METHODS

Participants

This study was performed as part of a series along with previously reported studies.[19,20] The study population included preeclamptic and normal pregnant women who were treated at the Department of Obstetrics and Gynecology, University of Indonesia, Cipto Mangunkusumo National Hospital. The participants were recruited between December 2005 and February 2006. All women gave their informed consent to participate in the study, which was approved by the Research Ethics Committee.

A total of 48 singleton pregnancies were observed with 24 in each group of PE and control participants. In the PE group, we excluded preeclamptic patients of superimposed type, such as chronic hypertension or proteinuria. Mild and severe PE as well as HELLP syndrome were defined as described in previous reports.[19,23] Fetal growth restriction (FGR) was defined as birth weight

2.0 SD below the mean expected weight for the gestational age. The control group included pregnant women with no pre-existing medical diseases or antenatal complications.

Processing of Blood Samples

The blood samples (2.5 mL) were collected in PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and kept at room temperature for 3 hours and then stored at −20°C until transported to Japan. The molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo. RNA extraction and RT-PCR were performed according to protocols described elsewhere.[21] Briefly, the blood samples were centrifuged twice at 4000*g* for 10 minutes at room temperature, and the entire supernatant and any mRNA present in the residual plasma were removed. The pellet was then washed, resuspended, and incubated in optimized buffer solution containing proteinase K to digest protein. A second round of centrifugation was performed to remove any residual cell debris, and the resulting supernatant was transferred to a fresh microcentrifuge tube. Next 100% ethanol was added to the supernatant to adjust the binding conditions, and the resultant lysate was then applied to a PAXgene spin column (PreAnalytiX), thus resulting in the selective binding of RNA to the silica-gel membrane of the spin column. After the column was washed 3 times, pure RNA was eluted in 80 μL of RNase-free water.

Real-Time Quantitative RT-PCR

Reverse transcription (RT) of the mRNA was performed using an Omniscript RT Kit (Qiagen, Hiden, Germany). Real-time quantitative PCR was then performed using a QuantiTect Probe PCR Kit (Qiagen). Reverse transcription was performed according to the manufacturer's instructions. Complementary DNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (QuantiTect Probe PCR kit; Qiagen) using a 2-μL aliquot of cDNA and the kit's components in a reaction volume of 20 μL. TaqMan PCR analyses for PlGF, VEGF, Flt-1, TGF-β1, and endoglin were performed using predeveloped and commercially available primers and probe sets (Cat # Hs00182176_m1 for PlGF, Cat # Hs00900054_m1 for VEGF, Cat # Hs01052936_m1 for Flt-1, Hs00171257_m1 for TGF-β1, Cat # Hs00923997_g1 for ENG: Applied Biosystems, Foster City, CA). Each PCR assay was verified to be specific to mRNA and not to genomic DNA. The amplification data were collected and analyzed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analyzed in duplicate, and multiple negative water blanks were included in every analysis. The following thermal profile was used: 15-minute denaturation at 95°C and 15-second annealing at 94°C, followed by 1-minute extension at 60°C. Quantification of the gene expression was performed with investigators blinded to the sample background. Calibration curves for PlGF, VEGF, TGF-β1, and ENG mRNA quantification were made using placental mRNA obtained from a normal pregnancy with elective cesarean delivery, with calculations performed as previously described[24] and expressed as relative concentrations (RC). The amounts of Flt-1 mRNA were expressed in term of copies/mL. Plasmid DNA for calibration curves was prepared as previously described to quantify these mRNA concentrations.[19]

Statistical Analysis

The distribution of demographic characteristics and mRNA concentrations were expressed as the median and minimum-maximum. The Mann-Whitney *U* test was used for univariate comparison of the available variables. A weighted (for number of cases) log-linear regression analysis was performed in women with PE and controls by plotting the actual mRNA values against the systolic and diastolic blood pressure, as well as absolute proteinuria. For all of the analyses, a P value <.05 was considered significant.

RESULTS

A total of 48 pregnant women with PE (n = 24) or normal pregnancies (n = 24) were assessed in this study. In the analysis of demographic variables, the median (minimum-maximum) ages of pregnant women for control and PE group were 27.0 (18-42) and 27.0 (18-37) years, respectively. The gestational ages at blood drawing were 38 (35-40) and 39 (36-41) weeks, body mass index of pregnant women were 22.5 (20-58) and 23.5 (16-32), and the percentage of nulliparae were 45.8% and 41.7%, respectively. Therefore, no significant differences between groups were determined. At the time of blood drawing, the systolic and diastolic blood pressures of the control group were 115 mm Hg (100-125) and 77.5 mm Hg (70-85), and those of the PE group were 160 mm Hg (130-200) and 100 mm Hg (90-140), respectively. The amount of proteinuria in the control and PE groups were 0.2 g/d (0.1-0.4) and

Table 1. Median Values (Minimum-Maximum) of the Different mRNA Species in the Control and Preeclampsia Groups

	Control	Preeclampsia	*P* Value[a]
Case #	24	24	-
GA (week)	38 (35-40)	39 (36-41)	NS
PlGF	4892 (526-17 194)	626 (0-2942)	<.001
VEGF	4 930 245 (1 271 006-10 667 872)	1 094 860 (9111–6 005 707)	<.001
Flt-1	3064 (51-19 626)	22 435 (3945-57 759)	<.001
TGF-β1	6 638 002 (178 632-33 179 507)	29 445 121(11 392 034–56 181 272)	<.001
Endoglin	402 530 (9130-1 102 747)	1 337 860 (647 661–2 214 079)	<.001

Abbreviations: Flt-1, fms-like tyrosine kinase-1; GA, gestational week at blood drawing; PlGF, placental growth factor; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor.
[a] Mann-Whitney *U* test.

5.0 g/d (1.5-8.0), respectively. The median birth weight of newborns were 3075 g (2500-4200) for the control and 2875 g (2000-3500) for the PE group, and the birth weight in the PE group was significantly lower than the control group ($P = .03$).

The quantitative results of each cellular RNA species are shown in Table 1. All mRNA species studied showed a statistically different distribution on PE versus controls. The levels of Flt-1, TGF-β1, and ENG in the PE group were higher than the control group, whereas the VEGF and PlGF levels were lower in the PE group.

The distributions of mRNA levels for the controls and patients with different severities of PE and manifestations of HELLP syndrome are shown in Figure 1. According to the clinical severity, the expression levels of TGF-β1, ENG, and Flt-1 apparently increased, and the levels of PlGF and VEGF decreased. Figure 2 shows the correlations between the mRNA levels and the actual severity of hypertension or proteinuria. In the PE group, Flt-1, ENG, and VEGF correlated with systolic pressure ($P < .001$, $R^2 = .558$; $P < .001$, $R^2 = .631$; $P < .001$ and a $R^2 = .30$), and Flt-1, ENG, and VEGF correlated with proteinuria ($P < .001$, $R^2 = .545$; $P < .001$, $R^2 = .671$; $P < .001$, $R^2 = .41$). Furthermore, all the 3 markers were correlated among them with a Spearman coefficient of −0.774 for VEGF versus ENG ($P < .01$), −0.711 for VEGF versus fms-Like tyrosine kinase-1 ($P < .01$) and 0.880 for Flt-1 versus ENG ($P < .01$), respectively (Figure 3).

COMMENTS

The present report confirmed that the mRNA expression of TGF-β1, Flt-1, and ENG increased in the cellular component of blood from women with PE, whereas VEGF and PlGF expressions decreased, thereby providing continuing evidence for the association of angiogenesis-related genes with this disorder. Whereas the Flt-1 and ENG expression increases in the plasma of the blood from preeclamptic women,[18] the concentrations of Flt-1 and ENG in the cellular mRNA also increased. Plasma RNA expression of Flt-1 and ENG were closely correlated with the clinical severity of PE, and the cellular RNA expression of Flt-1 and ENG was also correlated with severity of systolic blood pressure and proteinuria. These facts indicate that cellular RNA also reflects the functional alterations of the placenta as shown previously in the plasma mRNA, and that Flt-1 and ENG play a critical role in the pathogenesis of PE. Preeclampsia patients showed increased mRNA expression of TGF-β1, of which ENG is a coreceptor. Because the increased production of ENG may be greater than the production of TGF-β1 in the trophoblasts of preeclamptic patients,[5] the amount of free TGF-β1 therefore decreased and this decrease may be associated with the pathogenesis of PE.

Although the cellular PlGF expression decreased in the preeclamptic patients, this decrease was consistent with previous reports of serum protein in preeclamptic patients.[25] The cellular VEGF expression was decreased in preeclamptic patient, and the level was gradually decreased according to the severity of PE. This finding was not consistent with that of a previous plasma RNA analysis. Hypoxia is known to upregulate VEGF production.[26] Increased VEGF is thought to originate from the placenta and is upregulated in response to local ischemia due to chronic underperfusion and/or infarction of an area of the placenta in PE. In vitro and in vivo studies suggest that the circulating levels of free VEGF decrease during active PE due to binding of VEGF and sFlt-1.[4,27] However, early reports present conflicting results regarding VEGF concentrations in PE. Although the elevated cell-free RNA concentration of VEGF

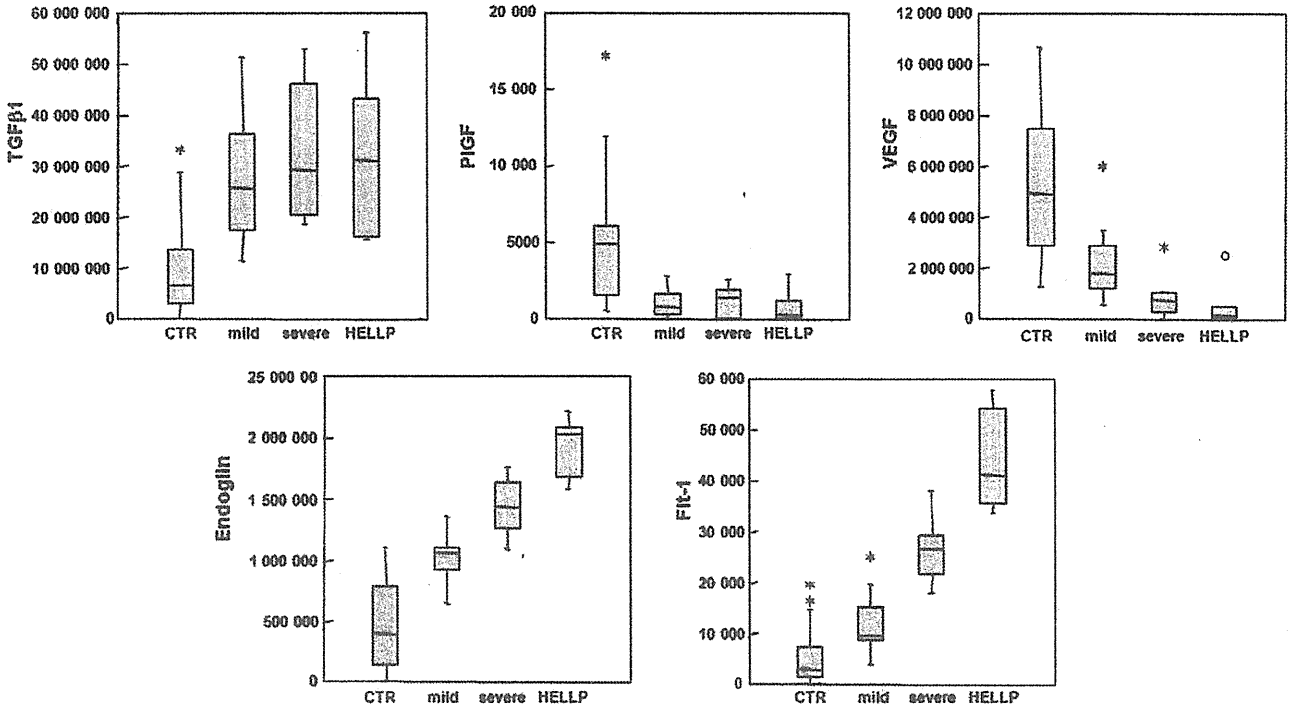

Figure 1. Box and whiskers plot of the distribution of PlGF, TGF-β1, VEGF, Flt-1, and ENG mRNA levels in controls and women with preeclampsia, stratified in accordance with the severity of preeclampsia (mild, severe preeclampsia, and hemolysis, elevated liver enzyme, low platelets [HELLP] syndrome). The medians are indicated by a line inside each box, the 75th and 25th percentiles by the box limits; the upper and lower bars represent the 10th and 90th percentiles, respectively. Asterisks (*) indicate each sample above 90th or below 10th percentile. The units of gene expression are relative concentrations except for Flt-1 (copies per milliliter). CTR indicates control; ENG = endoglin; Flt-1 = fms-like tyrosine kinase-1; HELLP = hemolysis, elevated liver enzymes, and low platelets syndrome; mild = mild preeclampsia; PlGF= placental growth factor; severe = severe preeclampsia; VEGF = vascular endothelial growth factor.

correlates with the severity of PE in a previous study, the cellular VEGF concentration decreased and this finding correlated with the disease severity. The half-life of plasma VEGF expression is 108 minutes, and the majority of the expression is in the placenta.[18] Although trophoblasts, syncytial knots, and placental debris clearly circulate in the maternal blood,[21] the cellular component of maternal blood may include endothelial cells as well as trophoblasts. This hypothesis may affect the difference in VEGF expression between cellular and plasma RNA. Another hypothesis is the decreased viability of circulating trophoblasts. In the villi of preeclamptic placenta, damaged and apoptotic trophoblasts may be released from the surface of the villi, while the VEGF expression of the trophoblasts would be decreased.

A new aspect of the current study is the use of cellular mRNA analysis in maternal blood. The advantage of cellular RNA in the evaluation of placental function is a higher mRNA concentration in comparison to the plasma RNA. The median RNA concentrations (minimum-maximum) of ENG, VEGF, and Flt-1 in the plasma of normal pregnant women are 888.1 RC/mL (121.6-1314), 43.2 RC/mL (4.9-64.9), and 106.4 copies/mL (0.0-181.2), respectively.[18] In the cellular RNA, those concentrations were 402 530 RC/mL (9130-1 102 747), 4 930 245 RC/mL (1 271 006-10 667 872), and 3064 copies/mL (51-19 626), respectively. Therefore, the values of cellular RNA are much higher than those of plasma, as previously reported in the mRNA analysis of hPL and β-hCG.[21] Furthermore, the cellular RNA is much more stable and can be easily stored at −20°C for a long time. To study the mechanism of PE in a prospective study, it will be able to apply this tool, which permits noninvasive assessment during early gestation. In this context, the present data suggest that analysis of the cellular component of maternal blood may be more suitable as a tool for evaluating alterations in placental function than that of maternal plasma. It would therefore be interesting to analyze the levels of cellular mRNA of angiogenesis-related genes in early pregnancies with subsequent PE,

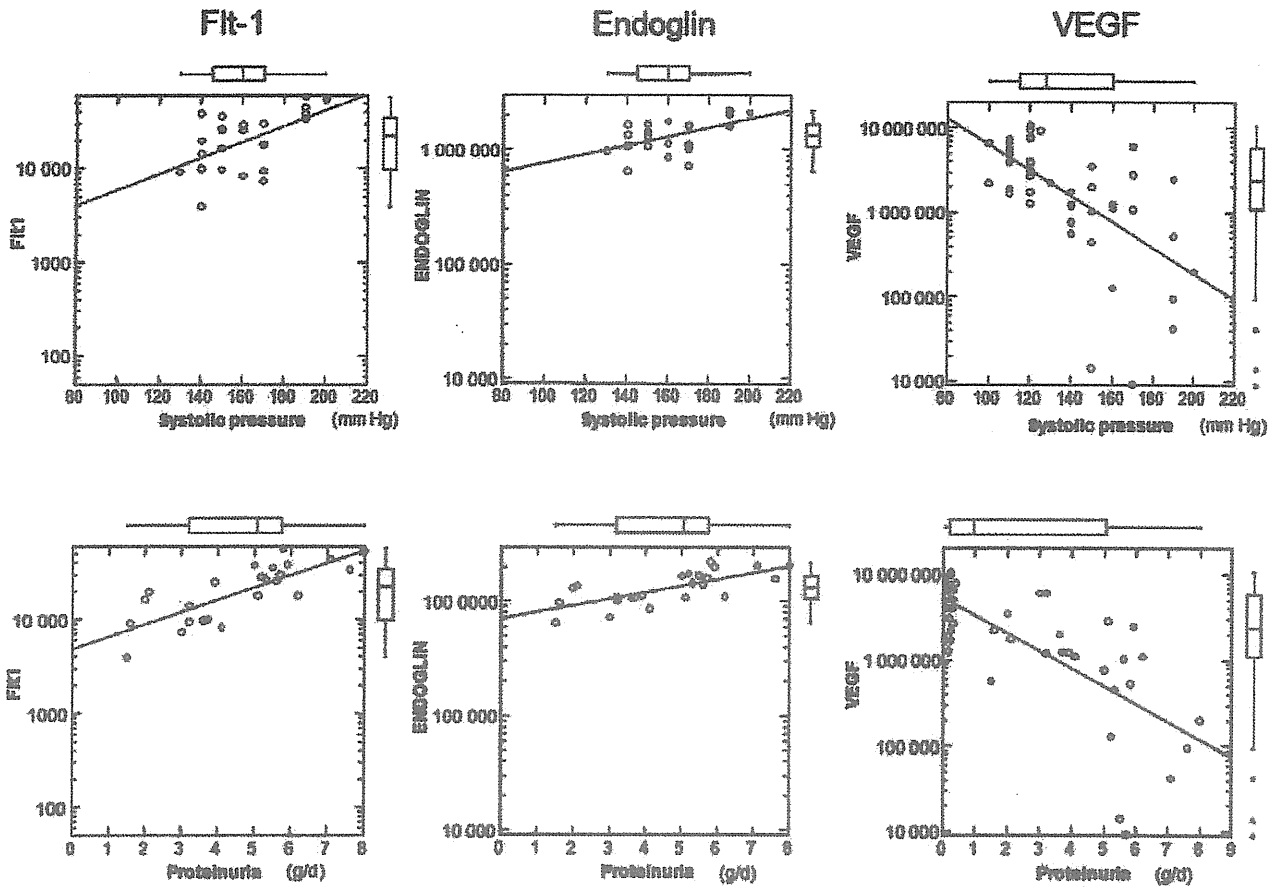

Figure 2. Correlation between cellular mRNA levels and systolic blood pressure, diastolic blood pressure, and proteinuria in the subgroup of patients with preeclampsia. Only the significant correlations are reported. The units of endoglin and VEGF expression are relative concentrations. The unit of Flt-1 is copies per milliliter. Flt-1 indicates Flt-1, fms-like tyrosine kinase-1; mRNA = messenger RNA; VEGF = vascular endothelial growth factor.

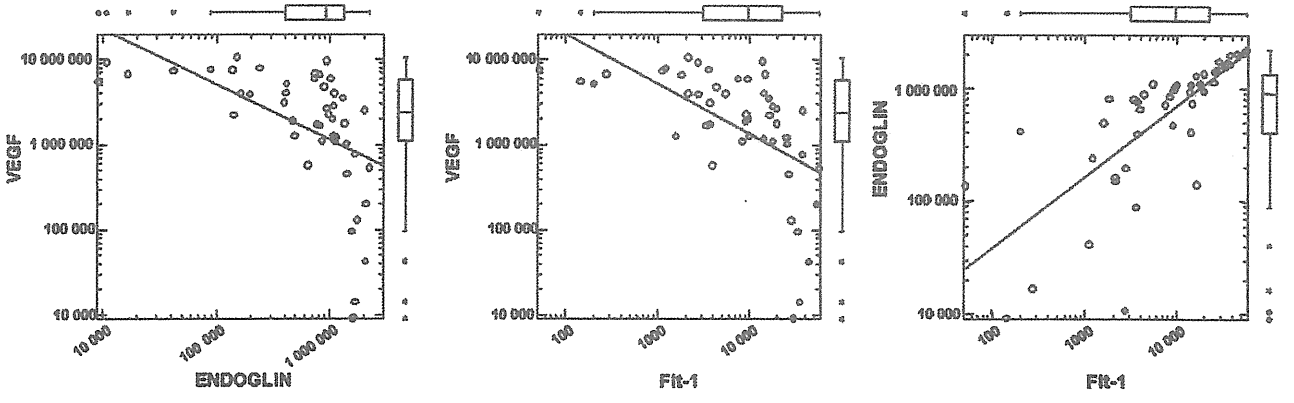

Figure 3. Correlation among cellular mRNA levels in maternal blood. Only the significant correlations are reported. The units of endoglin and VEGF expression are relative concentrations. The unit of Flt-1 is copies per milliliter. Flt-1 indicates Flt-1, fms-like tyrosine kinase-1; mRNA = messenger RNA; VEGF = vascular endothelial growth factor.

because developments in early placentation may heavily influence the subsequent health of the pregnancy.

In conclusion, significantly increased mRNA concentrations of Flt-1, ENG, and TGF-β1 and decreased concentrations of PlGF and VEGF were observed in the cellular component of maternal blood from preeclamptic patients. The values were correlated with the severity of PE. These facts provide further insight into dysregulated angiogenesis-related genes in PE through cellular mRNA measurement in maternal blood. A prospective study examining the expression of various placental genes might therefore be worthwhile to further evaluate the possible mechanisms underlying pathological pregnancies.

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PP13 mRNA Expression in Trophoblasts From Preeclamptic Placentas

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To assess the role of placental protein 13 (PP13) in the pathogenesis of preeclampsia, the messenger RNA expression of PP13 was investigated in the trophoblasts from first and third trimester placenta. First, syncytiotrophoblasts and extravillous trophoblasts were separated from frozen section of third trimester placenta obtained from pregnant women with and without preeclampsia by laser microdissection. The PP13 levels of syncytiotrophoblasts were significantly higher than those of extravillous trophoblasts. The expression levels in syncytiotrophoblasts from the preeclampsia were significantly lower than those from normal. Next, the PP13 expression was assessed in trophoblasts from residual samples of chorionic villus sampling at 11 weeks of gestation, who subsequently developed preeclampsia. The levels in the preeclampsia group were significantly lower than those of normal cases. These findings indicate that an alteration in the PP13-messenger RNA expression in the trophoblasts may be associated with the pathogenesis of preeclampsia.

KEY WORDS: Trophoblast, PP13, mRNA expression, preeclampsia.

INTRODUCTION

Preeclampsia is a leading cause of maternal and perinatal mortality and morbidity.[1] Preeclampsia is a multisystem disorder unique to human pregnancy and its clinical features are well recognized, characteristically manifesting in the second to third trimester. The underlying pathology is associated with a failure of trophoblastic invasion of the maternal arteries during early gestation.[2] Because preeclampsia has a long preclinical phase before clinically manifesting in later gestation, clinical prediction is thought to be possible and the clinical prediction could offer an early opportunity for intervention.

A variety of methods have been reported as potential early markers for preeclampsia. Potential early pregnancy markers under investigation include Doppler ultrasound of the uterine arteries in the first trimester,[3] decreased pregnancy-associated plasma protein-A (PAPP-A)[4] and a pentraxin (PTX3).[5] Melchiorre et al assessed uterine artery Doppler finding at 11-14 weeks. In the receiver-operating characteristics curves for the prediction of preterm preeclampsia, area under the curve was 0.76 (95% CI, 0.66-0.86).[6] Dugoff et al reported that adjusted odds ratio of low PAPP-A (<5th percentile) for the prediction of preeclampsia was 1.54 (95% CI, 1.16-2.03).[4] However, none of these alone have sufficient clinical discrimination to be used in clinical practice. Recently, Chafetz et al reported the maternal serum levels of placental protein 13 (PP13) at 9-12 weeks of gestation to be a promising screening tool for prediction with high sensitivity.[7,8] They suggested that the pathophysiological

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alteration of PP13 expression starts before 10 weeks of gestation and that these changes play a crucial role in the development of preeclampsia.

Placental protein 13 is a 32-kd dimer protein and is thought to be involved in implantation and maternal artery remodeling.[9-11] In the present study, to assess the role of PP13 in the pathogenesis of preeclampsia, the expression of PP13 messenger RNA (mRNA) was investigated in the syncytiotrophoblasts and extravillous trophoblasts (EVTs) from the placenta of preeclamptic women. Furthermore, the expression of PP13 mRNA was investigated in first trimester trophoblasts from residual samples of chorionic villus sampling (CVS) obtained for fetal karyotyping, who subsequently developed preeclampsia.

MATERIALS AND METHODS

In the first part of the study, mRNA expression of PP13 was quantified in both syncytiotrophoblasts and EVTs from the placenta. In brief, just after a caesarean section, placenta samples were obtained from pregnant women with and without preeclampsia (n = 5 for each group). Tissue sections (5 × 5 × 5 mm) were cut off from the maternal side of the cotyledons and frozen sections were made and stored at −80°C until use.[12]

For the separation of each cell type, a laser microdissection system (PALM Microbeam: PALM Microlaser Technologies GmbH, Bernried, Germany) was used. Slides were prepared on which a 4-μm thin LM (laser microdissection) Film (PALM Microlaser Technologies GmbH) was fixed. Tissue specimens were cut into 10-μm thick sections and placed on the slide. The sections were then fixed with 100% methanol. After toluidine blue staining, syncytiotrophoblasts and EVTs were separated by laser microdissection.[12] Although the number of syncytial knots in the villi increased in cases with preeclampsia, we retrieved syncytiotrophoblasts from the surface of the villi, not including the part of knots. Extravillous trophoblasts were separated from decidua of placental basal plate. In each case, approximately 500 cells were retrieved for mRNA analyses. RNA was separated using conventional methods.[12] This study was approved by the Ethic Committee of Showa University and written informed consent was obtained from each pregnant woman.

In the second part of the study, the expression of PP13 mRNA was assessed in first trimester trophoblasts. Residual CVS tissues were obtained from pregnant women who underwent CVS for an assessment of the fetal karyotype. All women gave their informed consent to participate in the study, which was approved by the Institutional Review Board of University of Bologna. Out of the 95 participants initially enrolled while under care at the Division of Prenatal Medicine at the University of Bologna, Bologna, Italy, 5 cases developed preeclampsia (cases) during later geststion. In the cases, 2 cases developed severe preeclampsia and were early onset type. Cesarean section was performed in 3 cases. Each case was randomly matched with 5 controls for gestational age at the time of CVS and fetal gender. We therefore enrolled 5 women who developed preeclampsia and 25 controls with a normal course of pregnancy. All the female participants were older than 35 years. Any pregnancies with major fetal defects, such as congenital heart diseases and aneuploidies, and preexisting complications, such as hypertension and diabetes, were excluded. Gestational age was calculated by ultrasonographic measurements at 11 weeks' gestation.

Preeclampsia was defined as gestational hypertension (systolic pressure >140 mm Hg or diastolic blood pressure >90 mm Hg on ≥2 occasions after gestational week 20) with proteinuria (>0.3 g/d). Severe preeclampsia was defined by the presence of ≥1 of the following: (1) severe gestational hypertension (systolic pressure >160 mm Hg or diastolic blood pressure >110 mm Hg on ≥2 occasions after gestational week 20) or (2) severe proteinuria (≥5 g protein in a 24-hour urine specimen).[13]

Villous samples were centrifuged at 1500*g* for 5 minutes at 4°C, resuspended in 1 mL of phosphate buffered solution (PBS) and then centrifuged at 1500*g* for 5 minutes at 4°C. Each villous sample was mixed with 0.8 mL of TRIzol Reagent (Invitrogen, Carlsbad, Calif) and lysed by repetitive pipeting. For the RNA extraction, 0.16 mL of chloroform was added to the sample; after vigorous shaking, the sample was incubated at 4°C for 15 minutes and centrifuged at 10 000*g* for 15 minutes at 4°C; following centrifugation, the aqueous phase was transferred to a fresh microtube. Precipitation of total RNA from the aqueous phase was obtained by mixing with 0.4 mL of isopropyl alcohol, incubation at 4°C for 15 minutes, and centrifugation at 10 000*g* for 15 minutes at 4°C. The RNA pellet was washed once with 0.8 mL of 75% ethanol and briefly air-dried. Finally, the total RNA was dissolved in 20 μL of RNase-free water and transferred to Japan under −20°C and then stored at −80°C.

Real-time Quantitative Reverse Transcription-polymerase Chain Reaction

A molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School

Table 1. Demographic Characteristics in a Study of the Third Trimester Trophoblasts

	Control Group	Preeclampsia Group	*P* Value
Maternal age (years)	35 (26-38)	35 (28-43)	ns
Gestational age (week)	37 (37-38)	30 (27-33)	$P = .007$
Birth weight (g)	2740 (3018-2506)	963 (725-1490)	$P = .009$
Systolic blood pressure (mm Hg)	120 (116-123)	165 (157-182)	-
Diastolic blood pressure (mm Hg)	70 (68-73)	97 (88-114)	-
Proteinuria (g/d)	0	2.5 (0.4-3)	-
HELLP syndrome (# cases)	0	2	ns

Abbreviations: HELLP, hemolytic anemia, elevated liver enzymes and low platelets, ns, not significant.

of Medicine, Tokyo. RNA was reverse-transcribed using an Omniscript RT kit (Qiagen, Hilden, Germany). cDNA products were amplified by real-time quantitative polymerase chain reaction (PCR) according to the manufacturer's instructions (QuantiTect Probe PCR kit; Qiagen) using a 2-μL aliquot of cDNA and the kit components in a reaction volume of 20 μL. TaqMan PCR analyses for PP13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed using predeveloped and commercially available primers and probe sets (Cat # Hs00747811_m1 for PP13 and Cat# 4333764F for GAPDH; Applied Biosystems, Foster City, Calif). Initially, each PCR assay was verified to be specific to mRNA and not to genomic DNA. The amplification data were collected and analyzed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analyzed in duplicate and multiple negative water blanks were included in every analysis. The thermal profile used was as follows: 15 minutes of denaturation at 95°C, followed by 15 seconds of annealing at 94°C and 1 minute of extension at 60°C. The quantification of gene expression was performed with investigators blinded to the outcome of pregnancy. The amounts of mRNA samples were expressed as copies/μL. To quantify these mRNA concentrations, plasmid DNA was prepared for calibration curves as previously described.[14] Because the initial volumes of the tissue specimens could not be quantified, the level gene expression was expressed as a ratio to the GAPDH expression.

Statistical Analysis

In the first arm of the study, descriptive statistics was performed by routine analysis. The median mRNA values of PP13 for each subgroup were calculated and analyzed using the Kruskal-Wallis test and relative post hoc test for paired comparisons between the groups.

In the second arm of the study, the data were matched for gestational age and fetal gender in a 1:5 case-control design. The median mRNA concentrations of each available marker of PP13 in cases and controls were calculated. A rank analysis and a nonparametric analysis (Mann-Whitney *U* test) were used to detect any differences between the cases and controls.

Differences were considered to be significant for a *P* value <.05.

RESULTS

Demographic characteristics of pregnant women subjected in a microdissection analysis were shown in Table 1. Although no differences were observed in the maternal age between groups, significant differences were noted in the gestational age at the time of blood drawing and birth weight. First, we compared mRNA levels of PP13/GAPDH between syncytiotrophoblasts and EVTs. The levels in syncytiotrophoblasts were significantly higher than those of EVTs in both normal and preeclamptic placenta. Figure 1 shows the box-plot for the generated subgroups of patients. The Kruskal-Wallis test yielded a *P* value = .003. Paired comparisons between the subgroups were performed by an adequate post hoc test. Syncytiotrophoblasts in the controls differed statistically from all the other groups (preeclampsia group and EVT in controls, *P* value < .05). The expression levels in syncytiotrophoblasts from the preeclamptic placenta were significantly lower than those from the normal placenta. No further differences have been found between the other subgroups.

In the CVS samples, the median (min–max) gestational age at the time of CVS was 82 (81-84) days or 11 weeks 5 days for both cases and controls. The maternal age was 36 (35-38) for both cases and controls. In cases and controls, the percentage of primiparae was 36 and 38, the week at delivery was 38 (35-41) and 39 (36-41),

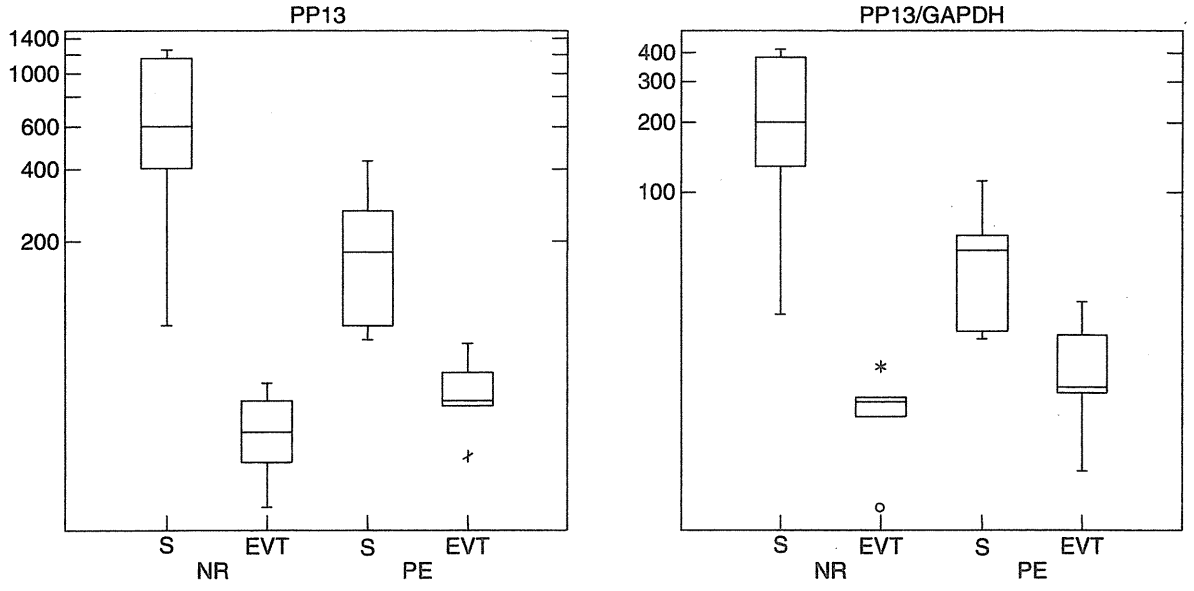

Figure 1. Box and whiskers plot of PP13 and PP13/GAPDH in the syncytiotrophoblasts and EVTs from pregnant women with and without preeclampsia. The box represents the interquartile range, which contains the 50% of value. The whiskers are lines that extend from the box to the highest and lowest value (1.5 times the interquartile distance), excluding outlines. A line across the box indicates the median. S = syncytiotrophoblasts; EVT = extravillous trophoblasts; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; NR = normal control; PE = preeclampsia; PP13 = placental protein 13.

the fetal weights were 2850 g (2000-3510 g) and 3100 g (2560-4180 g), respectively. No statistical differences were found between cases and control. The PP13 levels were lower in the CVS samples than those in microdissected samples (P value = .008, Mann-Whitney U test). The median (min-max) PP13/GAPDH levels in the group who subsequently develop preeclampsia and the controls were 1.636 (0.80-11.5) $\times$ 10^{-4} and 7.653 (1.1-49.4) $\times$ 10^{-4}, respectively, and the PP13/GAPDH levels in the preeclampsia group were significantly lower than those of normal cases. The mean observed rank of 1.8 in the preeclampsia group was significantly lower than expected (3.84; P value = .016, Mann-Whitney U test). Out of 6 possible rank values for each set, 3 cases had a value of 1 and 2 cases a value of 3 (Figure 2).

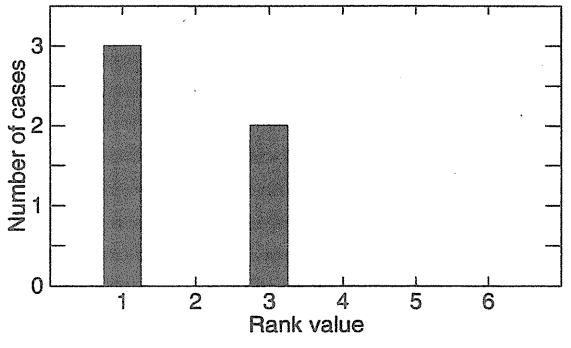

Figure 2. Results of a rank-sum analysis of the CVS samples. The number of cases of preeclampsia with each rank value is represented by the bar graph. CVS = chorionic villus sampling.

DISCUSSION

Placental protein 13 is one of the more than 50 known proteins synthesized by the placenta. Although the function of PP13 is not clear, it is reported that PP13 is associated with implantation and maternal artery remodeling, and that PP13 prevents erythrocyte adhesion in the area with reduced blood flow such as intervillous space.[9-11] Furthermore, PP13 is suggested to have special immune functions at the feto-maternal interface.[9-11] Placental protein 13 is known to be placenta-specific. An immunohistochemical study indicated that PP13 is localized in the brush border membrane of syncytiotrophoblasts.[11] However, the function of PP13 in normal pregnancy and its role in the pathogenesis of preeclampsia has not yet been elucidated. The present study quantified the expression of PP13 mRNA by both syncytiotrophoblasts and EVTs in

normal and preeclamptic placentas by real-time reverse transcription (RT)-PCR assay. The results showed that PP13 is predominantly expressed in the syncytiotrophoblasts in comparison to EVTs and that the expression of syncytiotrophoblasts in the preeclamptic placenta was significantly lower than those in the normal placenta. Burger et al reported that serum PP13 levels in patients with preeclampsia during the second and third trimester were significantly higher than in normal serum.[9-11] However, a previous study revealed that mRNA expression of PP13 was decreased in the cellular components of blood from pregnant women with preeclampsia, in comparison to normal blood (data not shown). The present results proved the cell-level expression of PP13 in the syncytiotrophoblasts from preeclamptic patients to be lower than those from normal pregnancies.

The second part of the study demonstrated mRNA expression of PP13 to be detectable in the CVS tissues at 11 weeks and that the PP13 mRNA levels in CVS tissues from pregnant women who were destined to develop preeclampsia were significantly lower than those from pregnant women with a normal course of pregnancy. This result indicates that mRNA expression in CVS tissues in the trophoblasts from pregnant women who will develop preeclampsia at later gestation had already been altered before the CVS procedures, although a higher risk of preeclampsia has been associated with the CVS procedure.[15]

Lower PP13 protein levels were reported in the serum samples of pregnant women at 9 to 12 weeks, who subsequently developed preeclampsia during later gestation, in comparison to those of normal pregnant women.[7] The decrease in the serum PP13 level could be affected by the decrease in the PP13-mRNA level in the trophoblasts from early gestation. The decreased expression of PP13 in the trophoblasts may lead to the development of preeclampsia. An impaired invasion of trophoblasts is well known to be associated with the pathophysiological changes of preeclampsia. The oxygen tension of the intervillous space dramatically increase between 10 and 12 weeks through endothelial remodeling of the spiral arteries in normal pregnancy.[16] However, in cases where the tension does not sufficiently increase, hypoxia and subsequent oxidative stress of villous trophoblasts occur, leading to the development of preeclampsia. It is particularly worth noting that PP13 expression is affected at this early stage of gestation. This finding indicates that the alterations associated with the pathogenesis of preeclampsia in the villous trophoblasts have already started at this gestational age. It is suggested that the decrease of PP13 may be results from hypoxia of the intervillous space and that it may be the result of factors which are more directly associated with the etiology of preeclampsia, such as impaired invasion of trophoblasts. Further research to resolve the mechanism regulating PP13 expression in the trophoblasts during early gestation may elucidate the pathogenesis of preeclampsia.

In conclusion, we found a decrease in the PP13 mRNA expression to occur in the trophoblasts obtained from women in their 11th week of pregnancy who develop preeclampsia at a later gestation. This finding indicates that an alteration in the PP13 mRNA expression in the trophoblasts may be associated with in the pathogenesis of preeclampsia.

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