厚生労働科学研究費（子ども家庭総合研究事業）
分担研究報告書

母体血漿遺伝子を用いた妊娠高血圧症候群の予知

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研究要旨

妊娠高血圧症候群の発症に、妊娠初期の絨毛の病態生理学的な変化が関与していることを直接確認した。さらに、母体血漿中 cell-free RNA を用いて胎盤における遺伝子発現のモニターが可能であること、妊娠 15-20 週の妊婦血漿中の抗血管増殖因子である FLT-1 や Endoglin の遺伝子発現の測定で、妊娠高血圧症候群の発症予知が高い精度で可能なことを示した。

A. 目的及び背景

妊娠高血圧症候群は、妊娠初期の絨毛細胞の母体脱落膜への侵入障害と関連している。正常妊娠では、絨毛が脱落膜のラセン動脈の血管内皮を置換することで血管抵抗が低下し、絨毛間腔に多量の母体血が流入する。しかし、絨毛細胞の侵入障害が起こると絨毛間腔の酸素分圧は十分に上昇せず、また変動も大きいため、絨毛は低酸素負荷及び酸化ストレスを受ける。この環境で絨毛細胞は抗血管増殖因子の産生を増加し、それらが母体血中を循環し血管内皮障害の原因になると考えられる。そこで今回、このような絨毛の病態変化を母体血漿中に循環する cell-free RNA で評価可能であることを確認するため、我々は、妊娠高血圧症候群を発症した症例の cell-free RNA を用いた遺伝子発現変化を検討した。vascular endothelial growth factor (VEGF), VEGF-receptor 1 (VEGFR1, FLT-1), Endoglin (ENG), placenta-specific 1 (Plac 1), selectin P, plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator (tPA), Corticotrophin releasing hormone (CRH) の遺伝子を標的に、母体血漿中から抽出した RNA を逆転写し、real-time PCR 法でそれらの遺伝子発現量を定量し、それらの定量値とその患者の臨床症状を比較した。その結果、妊娠高血圧症候群の患者血漿中でこれら８種類全ての遺伝子発現が、正常に妊娠経過している妊婦血漿に比較し、有意に高値を示した。また、妊娠高血圧症候群の重症化に伴いそれぞれの発現量が増強し、HELLP 症候群ではさらに全ての遺伝子が高発現であった。蛋白尿、高血圧の個々の重症度との相関を検討したところ、それらの重症化に伴い、有意に全ての遺伝子発現量が増加することが分った(1-3)。さらに、多変量解析の結果、これら遺伝子の中で PAI-1、tPA と蛋白尿の程度の間に特に強い相関が確認されたことから、PAI-1、tPA と

妊娠高血圧症候群の発症には強い関連があると推察された(1-3)。そこで、どの時期からこの変化を検出できるかを確認する目的で研究1を、また、遺伝子発現変化がどの時期の絨毛細胞で検出できるかを明らかにすることを目的に研究2を行った。

さらに、近年、妊娠初期の母体血中でplacental protein 13 (PP13)蛋白を測定し、将来妊娠高血圧症候群を発症する症例のPP13濃度が低値を示し、妊娠高血圧症候群の発症予知への応用が期待されている。そこで、研究3として、PP13遺伝子発現を、将来妊娠高血圧症候群を発症する症例、及び発症後の症例の絨毛で定量し、その動態について検討した。

B. 対象・方法

研究1：妊娠15週から20週の臨床症状がない時期に妊婦末梢血を採取し(n=683)、その後に妊娠高血圧症候群を発症した症例としなかった症例で、上記の遺伝子発現量を定量し、発症予知の可能性を検討した。この研究は、インドネシア大学で倫理委員会承認の下、患者から同意を得て血液を採取した。血液から血漿成分を分離後、凍結保存し、日本に輸送後、昭和大学でヒトゲノム倫理委員会の承諾の下、RNA解析を行った。

研究2：母体の年齢が35歳以上であることを理由に、絨毛採取を行った妊婦を対象に、余剰絨毛を凍結保存した(n=90)。この研究のサンプルは、ボローニャ大学病院で倫理委員会承諾の下、患者の同意を得て採取した。サンプルからRNAを抽出後、日本に凍結下で輸送し、昭和大学倫理委員会の承認の下、遺伝子発現解析を行った。標的とした遺伝子は、血管増殖因子関連遺伝子であるVEGF、FLT-1、PlGF、transforming growth factor-β1 (TGF-β1)、ENGと、抗酸化因子であるheme oxygenase-1 (HO-1)、superoxide dismutase (SOD)である。

研究3：妊娠高血圧症候群を発症し帝王切開を行った妊娠後期の胎盤（n=5）と合併症なく選択的に帝王切開を行った症例（n=5）の胎盤の凍結切片から、Laser Microdissection法で絨毛のS細胞とExtravillous trophoblasts (EVT)を500細胞ずつ各症例採取し、PP13遺伝子発現量を比較した。さらに、研究2で用いたCVS組織でもPP13発現量の定量を行った(6)。

（倫理的な配慮）

本研究は、すべて昭和大学倫理委員会の承諾の下、文書による承諾を得て行っている。また、他施設との共同研究においても当該施設の倫理委員会の承諾を得て行っており、その検体分析に関しても昭和大学倫理委員会の承諾を得ている。

C. 研究結果

研究1：妊娠15-20週の合併症のない683人の妊婦から血液を採取し、妊娠高血圧症候群をその後に発症した症例(n=62)と合併症なく正常に妊娠経過した症例のうち、統計学的に1:5マッチでランダムに選んだ310例で遺伝子発現量を定量した。8種類の遺伝子発現の中で、CRH遺伝子は発現が検出できない症例が多くあったことから除外し、7種類の遺伝子について検討した。その結果、その後に妊娠高血圧症候群を発症した症例(n=62)でPAI-1、tPAは、それぞれ8.9倍、8.0倍と増加していた。さらに、全ての遺伝子でこの時期に既に有意に遺伝

子発現量が増加していた（図1）。そこで、ROC curve を用いて、それら7種類の遺伝子発現量による妊娠高血圧症候群の発症予知の可能性について検討した（図2）。その結果、単一マーカーとしては、FLT-1 が、次いで、ENG が優れた妊娠高血圧症候群の予知マーカーであることが分かった。さらに、単一の遺伝子による予知精度に比較し、7種類全てを組み合わせることで、その精度が最大限に高まることが分かり、その組み合わせで妊娠高血圧症候群の 84%が、疑陽性率 5%で予知できることが分かった(4)。

研究2：絨毛検査で 90 例の余剰絨毛を採取した。その内、5 例がその後に妊娠高血圧症候群を発症した。5 例のうち 2 例は早発型の重症妊娠高血圧腎症であり、帝王切開で分娩した。3 例は軽症妊娠高血圧症候群であった（図3）。

統計学的に 1:5 マッチの分析を行うため、この5例の妊娠高血圧症候群症例と25例のランダムに選んだ正常コントロールを対象に検討を行った。対象患者の背景においては、平均絨毛採取施行時期、日齢 82 日（妊娠11週5日）を含め、両群に差はなかった。余剰絨毛組織から RNA を抽出し、逆転写して cDNA を作成した。

その後、VEGF、FLT-1、PlGF、TGF-β1、ENG、HO-1、SOD の遺伝子発現量を同様に real-time PCR で測定したところ、表1に示すように、将来、妊娠高血圧症候群を発症した症例で VEGF、FLT-1、TGF-β1、ENG の遺伝子発現が有意に高値を、さらに、PlGF、HO-1、SOD の遺伝子発現は有意に低値を示した[$p<0.05$, *Rank analysis and nonparametric analysis (Mann-Whitney U test)*] (5)。

研究3

妊娠高血圧症候群発症後の胎盤の PP13 発現は S 細胞優位であった。また、正常の絨毛 S 細胞に比較し、妊娠高血圧症候群では、発現が低下していた。次に、CVS で採取した妊娠 11 週の絨毛では、その後に妊娠高血圧症候群を発症する症例の PP13 発現が、既に低値を示すことがわかった (6)。

図3．将来妊娠高血圧症候群を発症する症例の絨毛細胞での遺伝子発現の検討(方法)

表1．将来妊娠高血圧症候群を発症する症例の絨毛細胞における遺伝子発現の変化

mRNA species	Mean rank in PIH (n=5)	Mean rank in controls (n=25)	p-value	Expression in PIH cases vs controls
FLT-1	5.60	3.08	0.045	↑
Endoglin	5.80	3.04	<0.001	↑
VEGF	5.40	3.12	0.009	↑
PlGF	1.60	3.88	0.031	↓
TGF-β1	5.60	3.08	0.009	↑
HO-1	2.00	3.80	0.049	↓
SOD	2.60	3.68	0.041	↓

図1．妊娠 15-20 週の臨床症状のない妊婦の血漿中 cell-free RNA 濃度(文献 4 より)
妊娠高血圧症候群の症例数：mild PE 26 例、severe PE 24 例、HELLP 12 例；Control　310 例
* p<0.05, ** p<0.01:　Scheffe post hoc test

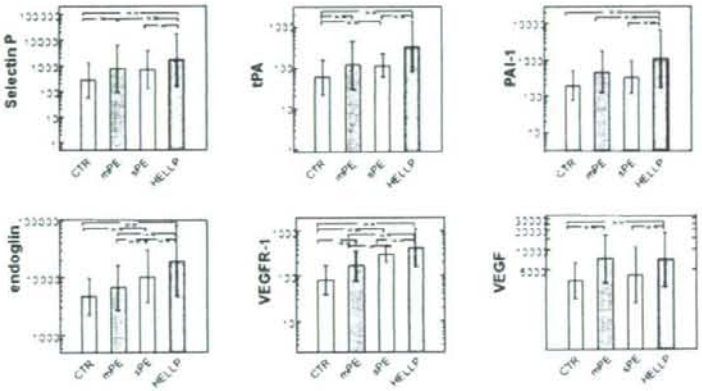
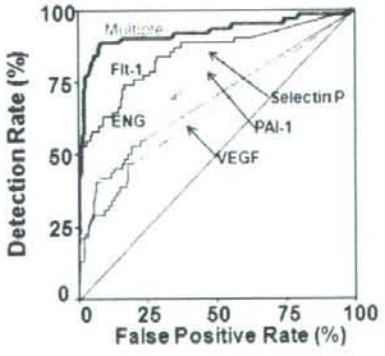


図2．ROC カーブを用いた母体血 cell-free RNA からの妊娠高血圧症候群の発症予知



	Area (AUC)		95% Confidence Interval		
			Lower Bound	Upper Bound	
VEGFR1	.846	.000	.793	.899	69.0%
Endoglin	.756	.000	.693	.820	43.5%
PAI-1	.732	.000	.669	.795	29.0%
Selectin P	.727	.000	.662	.792	24.2%
tPA	.686	.000	.624	.748	33.9%
VEGF	.651	.000	.588	.714	29.0%
PLAC-1	.645	.000	.581	.709	17.7%
Multiple	.927	.000	.877	.976	84.0%

D．結論

妊娠 15-20 週の合併症のない妊婦から血液を採取し、妊娠高血圧症候群をその後に発症する症例で、臨床症状のない妊娠中期にこれらの 7 種類の遺伝子発現に変化がみられるかを検討した結果、この時期に既にすべての遺伝子の発現量が有意に増加していた。このことは、これらの遺伝子が、妊娠高血圧症候群の病態形成に深く関与していることを示すものであり、さらに、これらのマーカーとなる遺伝子の組み合わせで、その後の妊娠高血圧症候群の発症予知が可

能であることを示す。ROC curve を用いた検討で、単一マーカーとしては、FLT-1 が、次いで、ENG が優れた妊娠高血圧症候群の予知マーカーであることが分かった。さらに、7 種類全てを組み合わせることで、その精度が最大化できることが分かり、その組み合わせで妊娠高血圧症候群の 84%が、疑陽性率 5%で予知できることが分かった(5)。この診断感度は、従来、妊娠高血圧症候群の予知に用いられている子宮動脈などの超音波血流計測、FLT-1 や ENG などの生化学的な評価などの過去の報告に比較して優れている。

次に、絨毛におけるこれらの発現変化がどの時期から起こっているかを明らかにするため、妊娠 11 週の絨毛組織の遺伝子発現を検討した。その結果、FLT-1, ENG, VEGF の遺伝子発現量は妊娠 11 週の段階で既に有意に上昇しており、PlGF、PP13 の遺伝子発現は有意に低下していることを見出した(5-6)。前述したようにその後に妊娠高血圧症候群を発症する母体の血清蛋白濃度では soluble FLT-1 蛋白濃度が発症の約 5 週前に、soluble ENG 蛋白濃度が 2-3 ヶ月前に上昇することが報告されているが、我々の結果はそれよりもはるか前の妊娠 11 週の段階でこれらの遺伝子発現が絨毛細胞局所で既に発現変化していることを確認し、妊娠初期の胎盤形成過程での異常が妊娠高血圧症候群の病態形成の基になるとする考え方を直接裏付ける結果である。さらに、この結果は、soluble FLT-1 や soluble ENG が妊娠高血圧症候群の病態形成に深くかかわっていることを示すとともに、cell-free RNA 解析で、もっと早い時期から胎盤内で起こる機能的な変化を捉えることが可能なことを示唆している。

このように、母体血漿中 cell-free RNA を分析することで、いままで“Black Box”であった胎盤の機能的な変化が real-time にモニターできる。この方法は、妊娠高血圧症候群の発症予知のみではなく、妊娠高血圧症候群やその他の妊娠合併症の病態形成メカニズムの研究にも利用できると考えられる。さらに、妊娠高血圧症候群などの新しい治療法や予防法の開発にも寄与するものと期待される。

〈来年度の課題〉

上記研究 1 は、インドネシア大学との共同研究で、妊娠高血圧症候群の発症率の高いインドネシアでの結果である。平成 20 年度以降、日本人の妊娠初期から中期の血液をサンプリングしており、日本人のサンプルでも同様の結果が導き出せるかを検討していきたい。同時に、母体の細胞成分由来の遺伝子発現(cellular RNA)でも胎盤の機能変化が評価できることを確認しており、そのサンプルも同時に採取しているので、それを用いた予知の可能性についても検討する予定であり、cell-free RNA と cellular RNA のどちらが優れたマーカーになるかについての比較も行いたい。

G. 研究発表

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H. 知的財産権の出願・登録状況

予定なし

Reproductive Sciences OnlineFirst, published on December 15, 2008 as doi:10.1177/1933719108328615

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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Reproductive Sciences Vol. 000 No. 00 Month 2009 1-6
DOI. 10.1177/1933719108328615

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),

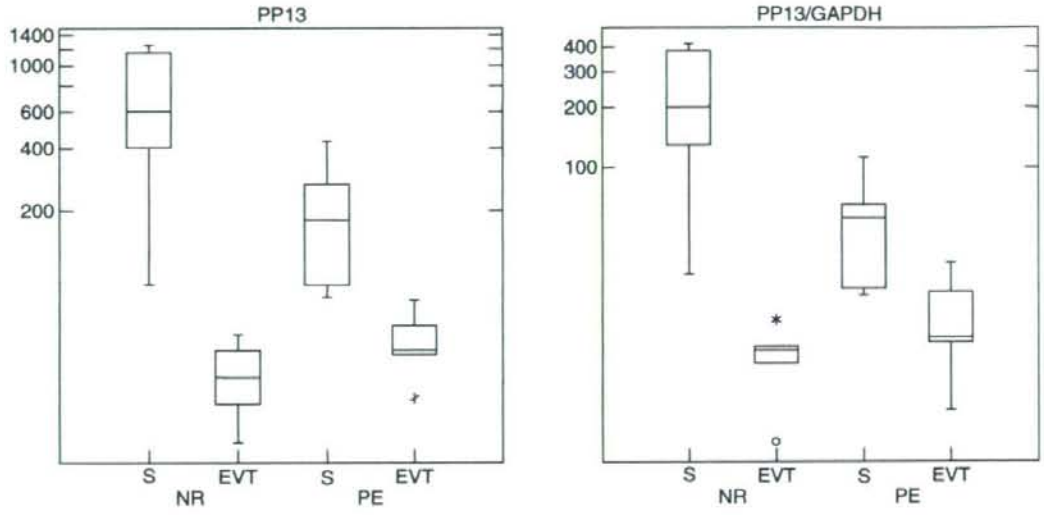

Figure 1. Box and whiskers plot of PP13 and PP13/GAPDH in the syncytiotrophoblasts and EVTs from pregnant women with and without preeclampsia. The box represents the interquartile range, which contains the 50% of value. The whiskers are lines that extend from the box to the highest and lowest value (1.5 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).

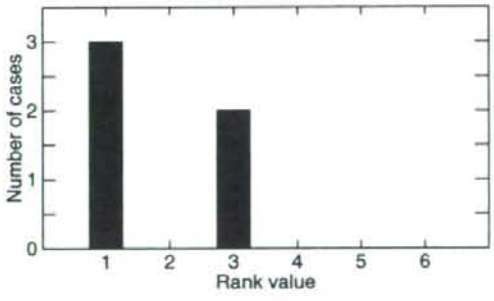

Figure 2. Results of a rank-sum analysis of the CVS samples. The number of cases of preeclampsia with each rank value is represented by the bar graph. CVS = chorionic villus sampling.

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.

ACKNOWLEDGMENT

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (Nos. 20591930 and 20791169); a Grant for Child Health and Development (20C-1) from ministry of Health, Labour and Welfare of Japan; Health and Labour Sciences Research Grants from Ministry of Health, Labour and Welfare of Japan; and also in part by Progetto Pluriennale E.F. 2003, Università di Bologna and ex 60% (AF).

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PRENATAL DIAGNOSIS
Prenat Diagn 2008; **28**: 956–961.
Published online 15 September 2008 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/pd.2109

Gene expression in chorionic villous samples at 11 weeks' gestation from women destined to develop preeclampsia

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Objective To evaluate the direct alterations in mRNA expression among chorionic villous samples from 11 weeks' pregnant women who would develop preeclampsia (PE) later in the pregnancy.

Method Case-control study encompassing five women destined to develop PE [cases matched 1:5 for gestational age (GA) with 25 controls]. We quantified mRNA expression on tissue samples from chorionic villous sampling (CVS) of normal and PE patients. We then assessed mRNA expressions of vascular endothelial growth factor (VEGFA), VEGFA receptor 1 (Flt-1), endoglin (Eng), placental growth factor (PlGF), transforming growth factor-β1 (TGF-β1), heme oxygenase-1 (HO-1) and superoxide dismutase (SOD). Data were analyzed by nonparametric rank analysis.

Results For all the mRNA species considered in this study, all the mean observed ranks in the PE group were significantly altered compared to the rank expectation among controls. mRNA for Eng and TGF-β1 were the markers with the highest degree of aberration in PE, in respect to controls. The results are consistent with those already reported for the corresponding circulating proteins. mRNA for HO-1 and SOD were instead associated with the lowest aberration.

Conclusion It is assumed that the pathogenesis of PE is associated with pathophysiological alterations to trophoblasts in early gestation. Our study has directly proved that gene expressions relating to angiogenesis or oxidative stress are altered in the first trimester trophoblasts that go on to develop PE later. These results would put the basis for a possible screening method for PE by using residual CVS. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS: mRNA; preeclampsia; real-time PCR; chorionic villous samples

INTRODUCTION

Preeclampsia (PE) complicates 5–8% of all pregnancies (De Groot *et al.*, 1999) and remains one of the main causes of maternal and fetal mortality and morbidity (Walker, 2000). PE is a multisystem disorder unique to human pregnancy and its clinical features are well recognized, characteristically manifesting in the second to third trimester. However, the underlying and major changes in the pathology arise in the earlier stages of pregnancy owing to a failure of trophoblast cell invasion associated with increased apoptotic and necrotic indices manifesting in the second to third trimester. The earlier the gestational age (GA) at diagnosis, the higher the risk of maternal death (MacKay *et al.*, 2001). In fact, the risk of maternal death is fourfold higher if PE is diagnosed before gestational week 32 compared to after this GA. Given the morbidity associated with PE and the long preclinical phase before it manifests clinically in later gestation, an enormous variety of biomolecules have been studied to detect evidence of alteration in the maternal circulation during early pregnancy, prior to the presentation of clinical symptoms (Myatt and Miodovnik, 1999).

It has recently been reported that increases in antiangiogenic factors such as vascular endothelial growth factor receptor-1 [VEGFR-1, also known as fms-like tyrosine kinase-1 (Flt-1)] and endoglin (Eng) play important roles in the development of PE and that the protein levels of maternal serum increase long before the onset of the clinical manifestation (Levine *et al.*, 2006). Also, vascular endothelial growth factor (VEGFA), as well as placental growth factor (PlGF) have been extensively proposed as predictive markers for PE long before the clinical onset as reviewed by Lyell *et al.* (2003). Transforming growth factor-β1 (TGF-β1) was also associated with the prediction of PE but with discordant results, being reported both higher or lower in PE cases (Enquobahrie *et al.*, 2005; Lim *et al.*, 2008). Finally, *in vitro* study has revealed that these gene expressions in trophoblasts are up-regulated by a decrease in heme oxygenase-1 (HO-1), which works protectively against

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Received: 19 May 2008
Revised: 15 July 2008
Accepted: 4 August 2008
Published online: 15 September 2008

oxidative stress (Cudmore *et al.*, 2007) while superoxide dismutase (SOD) is increased in trophoblast cells under hypoxia condition (Li *et al.*, 2005). Thus, it is speculated that angiogenesis and oxidative stress play important roles in the pathogenesis of PE.

However, for a variety of reasons, numerous aspects of normal and pathological pregnancies, particularly with regard to PE, remain difficult to study and are thus poorly understood. For example, a direct evidence of *in vivo* alteration of gene expressions in the trophoblast from patients destined to develop PE later has not been reported. Thus in the present study, to test for direct alterations in gene expression among trophoblasts that would later develop PE, we prospectively collected tissue samples of villous trophoblasts at the time of fetal karyotype analysis through chorionic villous sampling (CVS). mRNA expressions of factors that are reported to play roles in the development of PE were assessed and compared with the clinical outcomes.

MATERIALS AND METHOD

As many as thirty pregnant women in care at the Division of Prenatal Medicine at the University of Bologna, Bologna, Italy, were enrolled in the present prospective case-control study. All the women were more than 35 years old at delivery and bearing a single male fetus. Pregnancies with major fetal defects (like congenital heart diseases and aneuploidies) were excluded. CVS was performed for assessment of fetal karyotype. Residual CVS from five women with a diagnosis of PE (performed at the third trimester of pregnancy) were matched with 25 controls (1 : 5 ratio) for GA and fetal gender. GA was calculated by ultrasonograph measurements at 11 weeks' gestation. All women gave informed consent to participate in the study, which was approved by the Institutional Review Board.

PE was defined as gestational hypertension (systolic pressure >140 mmHg or diastolic blood pressure >90 mmHg on ≥2 occasions after gestational week 20) with proteinuria (>0.3 g/day). Severe PE was defined by the presence of ≥1 of the following: (1) severe gestational hypertension (systolic pressure >160 mmHg or diastolic blood pressure >110 mmHg on ≥2 occasions after gestational week 20); or (2) severe proteinuria (≥5 g protein in a 24-h urine specimen) (ACOG practice bulletin, 2002).

Tissues and RNA preparation

Villous samples were centrifuged at 1500 g for 5 min at 4 °C, resuspended in 1 mL of phosphate buffered solution (PBS) and then centrifuged at 1500 g for 5 min at 4 °C. Each villous sample was mixed with 0.8 mL of Trizol Reagent (Invitrogen, Carlsbad, CA) and lysated by repetitive pipetting. 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 min and centrifuged at 12 000 rpm for 15 min 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, incubated at 4 °C for 15 min and centrifuged at 12 000 rpm for 15 min at 4 °C. The RNA pellet was washed once in 0.8 mL of 75% ethanol and briefly air-dried. Finally, the total RNA was dissolved in 20 μL of RNase-free water and stored at −80 °C.

Real-time quantitative reverse transcription-PCR

RNA samples were transferred to Japan at under −20 °C. Molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo. RNA was reverse-transcripted by 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 VEGFA, Flt-1, Eng, PlGF, TGF-β1, HO-1, SOD, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed using predeveloped and commercially available primers and probe sets (Cat # Hs00900054_m1 for VEGFA, Cat # Hs01052936_m1 for Flt-1, Cat # Hs00923997_g1 for Eng, Cat # Hs00182176_m1 for PlGF, Cat # Hs0000171257_m1 for TGFβ1, Cat# Hs00157965_m1 for HO-1, Cat# Hs00166575_m1 for SOD, and Cat# 4 333 764F for GAPDH; Applied Biosystems, Foster City, CA). As an initial step, we verified that each PCR assay was specific to mRNA and not to genomic DNA. 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 min of denaturation at 95 °C, followed by 15 s of annealing at 94 °C and 1 min of extension at 60 °C. Quantification of gene expression was performed with investigators blinded to the outcome of pregnancy. Amounts of mRNA samples were expressed in term of copies per milliliter. To quantify these mRNA concentrations, we prepared plasmid DNA for calibration curves as previously described (Purwosunu *et al.*, 2007). Owing to the fact that initial volumes of tissues could not be measured, the level of each gene expression was expressed as a ratio to the GAPDH expression.

Statistical analysis

Data were matched for GA in a 1 : 5 case-control study design. Median mRNA concentrations of each available marker of Flt-1, Eng, VEGFA, PlGF, TGF-β1, HO-1, and SOD in cases and controls were calculated. Rank and nonparametric analysis (Mann–Whitney U test or Fisher exact test) were used to detect differences between cases and controls. Differences were considered significant for a p-value <0.05.

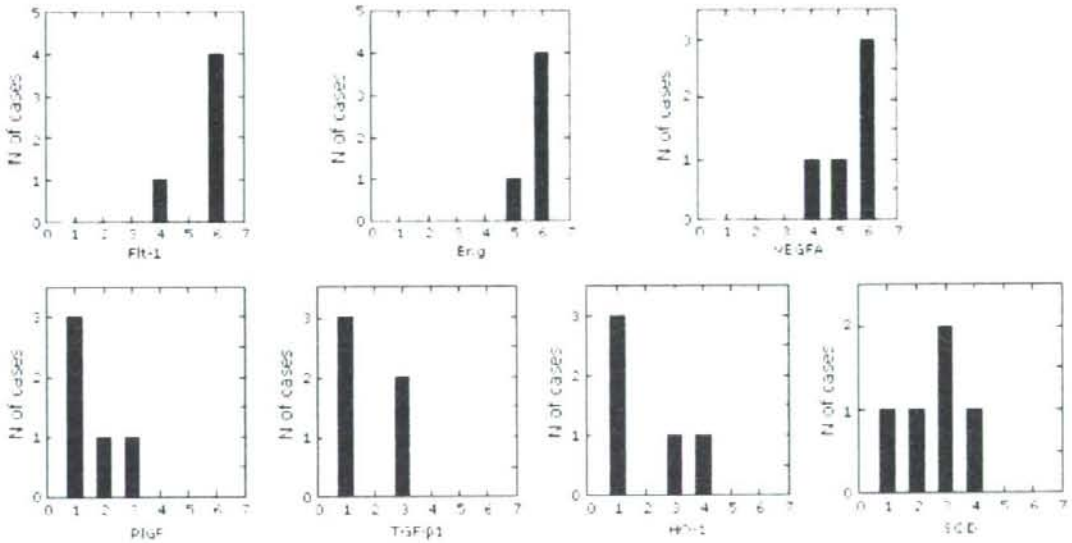

Figure 1—Results of rank-sum analysis of the CVS samples. The number of PE cases with each numerical rank is represented by the bar graph

RESULTS

Table 1 and 2 reports the clinical information available for the data set. Table 3 reports the median values (min–max) for the mRNA species considered in the study. Quantitative amplification data were examined using matched rank-sum analysis. The ranks of the mean mRNA levels from each PE sample were found to be significantly higher or lower than the expected rank for the controls. The mean observed ranks for PE cases and controls are reported in Table 4 and Figure 1, showing a statistically different distribution between the cases and controls. As shown, Eng was the marker with the highest difference, followed by TGF-β1 Eng. HO-1 and SOD were the markers with the lowest level of aberration in PE.

DISCUSSION

Trophoblast differentiation and, specifically, pseudovasculogenesis during early pregnancy are crucial for normal placentation and a well-functioning maternal–fetal interface (Red-Horse *et al.*, 2004). In PE, trophoblasts fail to mimic a vascular adhesion phenotype, causing impaired invasion of the spiral arteries (Zhou *et al.*, 1997). PlGF and VEGFA, which share close amino acid homology, are potent angiogenic agents. PlGF and VEGFA are inhibited by the soluble form of Flt-1 (sFlt-1), an endogenously secreted form of Flt-1 produced by alternative splicing (Kendall and Thomas, 1993). Soluble Flt-1 prevents interactions of PlGF and VEGFA with membrane-bound, functional Flt-1 in vascular tissues and thereby leads to endothelial dysfunction (Maynard *et al.*, 2005). Serum concentrations of sFlt-1 are increased in PE and correlate with the severity of the disease (Chaiworapongsa *et al.*, 2004). As sFlt-1 combines with P1GF and VEGFA, free serum PlGF and free serum VEGFA decline before the development of PE (Levine *et al.*, 2006). It is interesting to note that in a rat model, over-expression of Flt-1 has been shown to trigger a PE-like phenotype (Maynard *et al.*, 2005). Furthermore, cancer patients who were treated with an anti-VEGFA antibody showed hypertension and proteinuria (Yang *et al.*, 2003). Taken together, these data suggest that elevated sFlt-1 and, subsequently, lowered VEGFA and PlGF levels may play a pathogenic role in the development of PE.

Recently, sEng was identified as a possible factor causing the development of PE (Venkatesha *et al.*,

Table 1—Demographic and clinical characteristics of the patients. Data are expressed as median (min–max) or percentage

Variable	PE $n = 5$	Controls $n = 25$	p-value[a]
Gestational age (days) at the time of CVS	82 (81–84)	82(81–84)	ns
Maternal age (year)	36 (35–38)	36 (35–38)	ns
% of primiparae	36	38	ns
Week at delivery	38 (35–41)	39 (36–41)	ns
Neonatal weight (gr)	2850 (2000–3510)	3100 (2560–4180)	ns

[a] Mann–Whitney U test or Fisher exact test.

Prenat Diagn 2008; **28**: 956–961.
DOI: 10.1002/pd

Table 2—Clinical data available for the PE cases

Case ID	Week of PE insurgence	PE degree	Second trimester doppler of uterine arteries (mRI[a])	Type of delivery
SB	29	Severe	0.68, no incisure	Cesarean section
IR	31	Severe	0.50, monolateral incisure	Cesarean section
DM	33	Mild	0.38, no incisure	Cesarean section
AF	32	Mild	0.44, no incisure	Vaginal
MC	36	Mild	0.46, no incisure	Vaginal

[a] mRI, Mean Resistance Index.

Table 3—Median (Min–Max) mRNA relative concentrations for cases and controls

mRNA species	PE $n = 5$	Controls $n = 25$	p-value[a]
Flt-1	84 249 (15 383.10–182 662.80)	21 710 (1056.70–86 196.00)	0.038
Eng	164 208 (123 381–2 865 316)	4150 (209.20–443 766.10)	<0.001
VEGFA	17.3 (9.30–87.80)	6.45 (1.70–50.50)	0.016
PlGF	1.57 (0.60–12.90)	12.50 (0.90–187.60)	0.014
TGF-β1	876 (206.8–7543.10)	143 (16.80–1347.40)	0.008
HO-1	0.50 (0.07–3.15)	2 (0.16–55.91)	0.047
SOD	166 (8.40–345.00)	237 (13.20–1460.90)	0.041

The gene expression is normalized for GAPDH.
[a] Mann–Whitney U test.

2006). Eng or CD105, a cell-surface co-receptor for TGF-β1 and TGF-β3 isoforms, is highly expressed in endothelial cells and syncytiotrophoblasts and modulates actions of TGF-β1 and TGF-β3. Serum levels of placenta-derived sEng have been shown to be elevated in PE. Furthermore, over-expression of sEng in pregnant rats has been associated with increased vascular permeability and hypertension. These symptoms worsened dramatically in cases where both Eng and Flt-1 were overexpressed, resulting in a severe preeclamptic phenotype with proteinuria and features of hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome. Caniggia *et al.* (1997) have previously reported that overproduction of Eng would cause inhibition of trophoblast differentiation and invasion. Yinon *et al.* (2008) speculate that in pathological hypoxic conditions, Eng is inappropriately up-regulated, leading to abnormal trophoblast invasion and complications such as PE. Furthermore, excessive production of Eng would lead to increased sEng in the maternal circulation, which in turn may cause endothelial dysfunction resulting in PE (Venkatesha *et al.*, 2006). Previously, we also have demonstrated increased VEGFA, Flt-1, and Eng mRNA concentrations in plasma from women with PE at term of pregnancy. The main origin of such mRNA is from the placenta, in view of the rapid decrease in these mRNA levels after delivery (Purwosunu *et al.*, 2008). Thus, it is speculated that antiangiogenic factors may play a very important role in the pathogenesis of developing PE.

It is proposed that oxidative stress and inflammation associated with pregnancy may be controlled by vascular protective factors and that the lack of such compensatory systems leads to PE (Ahmed *et al.*, 2000). HO-1 has been implicated in several physiological functions throughout the body including control of vascular tone and regulation of the inflammatory and apoptotic

Table 4—Mean ranks for mRNA species in PE cases and controls

mRNA species	Mean rank in PE ($n = 5$)	Mean rank in controls ($n = 25$)	p-value[a]	Expression in PE cases vs. controls
Flt-1	5.60	3.08	0.045	↑
Eng	5.80	3.04	<0.001	↑
VEGFA	5.40	3.12	0.010	↑
PlGF	1.60	3.88	0.031	↓
TGF-β1	5.60	3.08	0.009	↑
HO-1	2.00	3.80	0.049	↓
SOD	2.60	3.68	0.041	↓

[a] Rank-sum test.

cascades. HO-1 is an antiinflammatory and provides a defense against oxidant injury. Administration of an HO-1 antagonist to pregnant rats resulted in fetal resorption (Alexandreanu and Lawson, 2002), and adenoviral overexpression of HO-1 rescues pregnancy in abortion-prone mice (Zenclussen *et al.*, 2004). The activity of HO-1 isolated from chorionic villi is reported to decrease under hypoxic conditions (Appleton *et al.*, 2002). A reduced expression of HO-1 protein in placenta from preeclamptic pregnancy has been reported. Recently, Cudmore *et al.* have reported an association between HO-1 activity and antiangiogenic factors; inhibition of HO-1 activity in placental villous explants stimulates the release of sFlt-1 and sEng (Cudmore *et al.*, 2007). It is suggested that the loss of HO-1 activity may be central to the pathogenesis of PE.

Nevertheless, whether such a phenomenon occurs *in vivo* in PE placenta at early gestation remains unknown. In the present study, we evaluated these mRNA expressions in villous tissue samples, in cases

where the pregnant women subsequently developed PE. We showed that Flt-1, Eng, VEGFA, and TGF-β1 mRNA levels were significantly higher in CVS at 11 weeks' gestation in women destined to develop PE, whereas, PlGF, SOD, and HO-1 mRNA levels were significantly lower. The fact that mRNA expressions of protective factors against oxidative stress were decreased and that those of antiangiogenic factors were increased was concordant with a previous *in vitro* study (Maynard *et al.*, 2003). Our findings have directly shown that these mRNA expressions were already altered in the trophoblasts at 11 weeks, and that placental alteration at first trimester may trigger the onset of PE long before the clinical manifestation of PE. To the best of our knowledge, this is a first report of mRNA expression analysis in the trophoblasts *in vivo* from cases where the outcome of pregnancy could be evaluated.

Several studies report that the diminishing protein levels of PlGF early in pregnancy may reflect either less placental production of PlGF or increased binding to local circulating and membrane bound receptors, but the mechanisms remain unclear (Maynard *et al.*, 2005). There are some reports that the protein level of plasma PlGF in early gestation was not significantly different and was later decreased in pregnant women who subsequently developed PE (Livingston *et al.*, 2001). There are also reports that the serum protein level of VEGFA was increased (Hunter *et al.*, 2000) or decreased (Polliotti *et al.*, 2003) in early gestation. No consensus has been reached. In this study, the mRNA expression levels of angiogenic factors of PlGF and VEGFA were decreased and increased, respectively. We then directly proved that the local expression of PlGF was decreased and that of VEGFA was increased in the villous trophoblast *in vivo*.

In conclusion, we quantified mRNA expressions of genes associated with angiogenic factors and oxidative stress in the early villous trophoblasts, in cases where the pregnant women would develop PE later in the pregnancy. In such women, it emerges that the gene expression of antiangiogenic factors and oxidative stress related genes is already altered in the villous trophoblasts, and that Eng TGF-β1 and HO-1 seem to be critical factors for the pathogenesis of developing PE at least in tissue samples. Given that the women who have CVS for fetal karyotype are at higher risk for PE, mRNA dosage can be useful for a possible detection of higher risk patients.

ACKNOWLEDGEMENTS

This study was supported in part by a Progetto Pluriennale E.F. 2003, Università di Bologna and ex 60% AF; in part also by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (Nos. 20591930, and 20591308).

Health and Labour Sciences Research Grants from Ministry of Health, Labour and Welfare, Japan.

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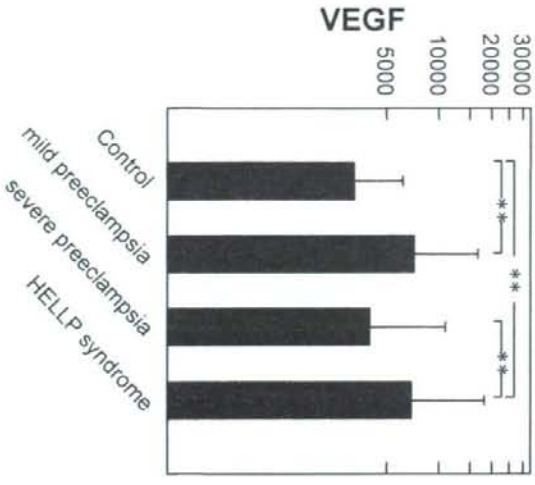
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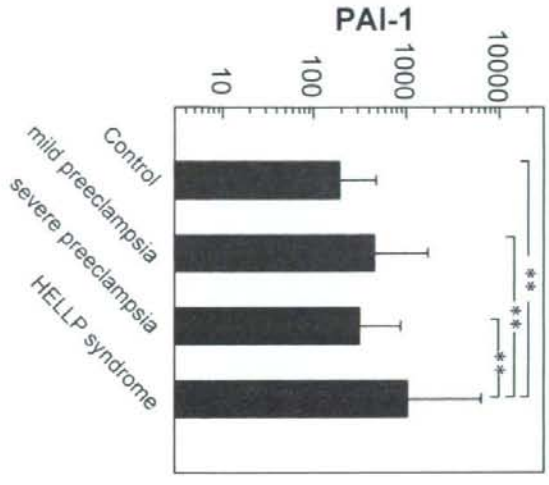
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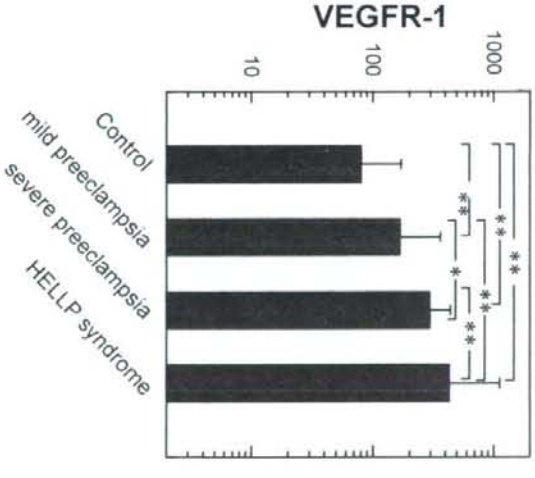
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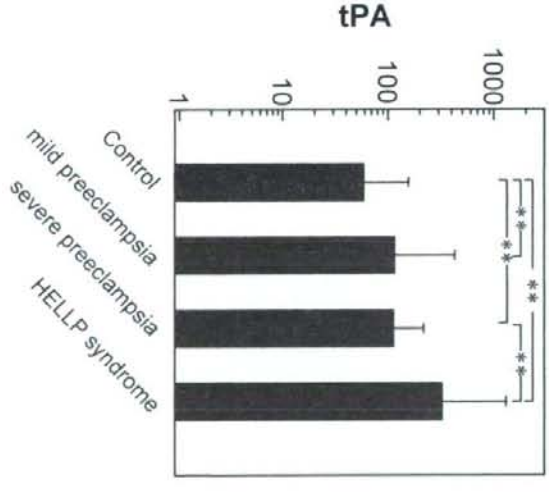
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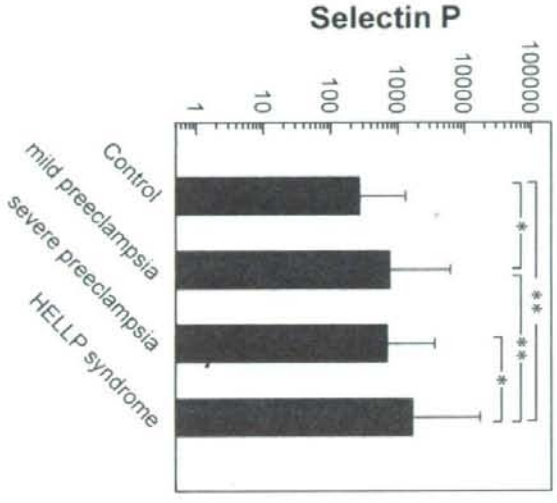
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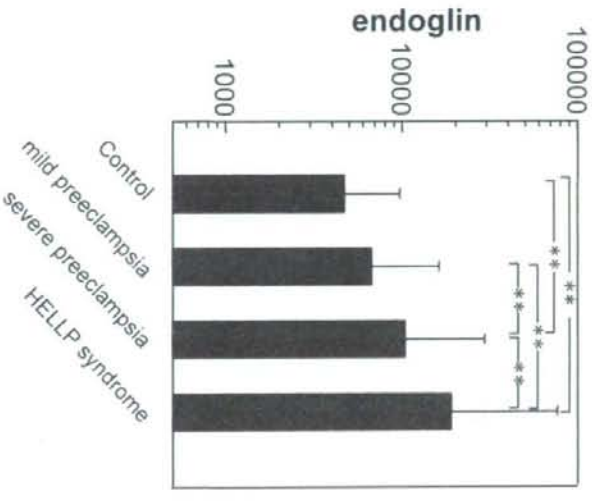
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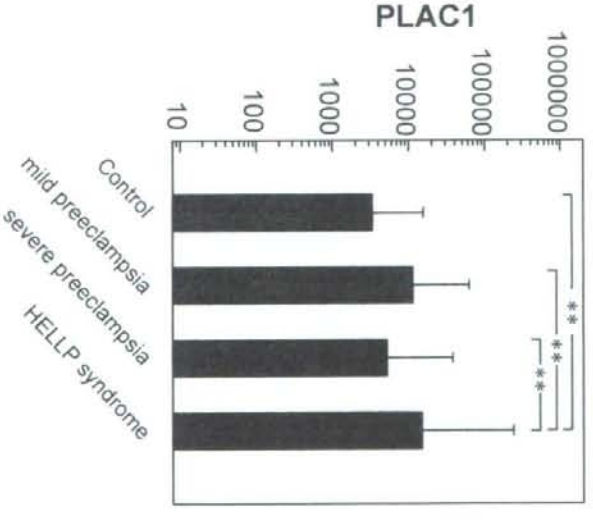
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