

Implication of Umbilical Cord in Preeclampsia

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Key Words

Preeclampsia · Umbilical cord · Placenta · Angiogenesis

Abstract

Objectives: To investigate the role of biochemical changes in the umbilical cord and placenta in developing preeclampsia (PE). **Subjects and Methods:** Thirty women with PE and 15 healthy pregnant women as controls were enrolled in this study. Vascular endothelial growth factor (VEGF), soluble vascular endothelial growth factor receptor 1 (sVEGFR-1), platelet-derived endothelial cell growth factor (PD-ECGF), neutrophil elastase and nitric oxide (NO) were measured. **Results:** Both serum (maternal and fetal) and tissue (placenta and umbilical cord) levels of VEGF, sVEGFR-1, PD-ECGF and neutrophil elastase were significantly increased, whereas NO was significantly decreased (except placental tissue showed no changes) in preeclamptic patients. The cord serum level of PD-ECGF was significantly higher in severe PE compared to mild PE and normal pregnant women. The placental and cord tissue levels of PD-ECGF and neutrophil elastase were significantly higher in severe PE, while the cord tissue level of NO was significantly lower in severe PE. **Conclusion:** Our data showed that umbilical cord vessels and stroma can serve as an additional source of vasoactive and angiogenic substances that contribute to the biochemical changes occurring in PE.

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Introduction

Preeclampsia (PE) is a multisystem disorder that remains a major cause of maternal and fetal morbidity and death. The Egyptian National Maternal Mortality Study [1] revealed that hypertensive disorders in pregnancy were the second cause of maternal mortality and represent 13% of direct obstetric death. To date, no treatment had been found to prevent the development of PE. Oxidative stress has been increasingly postulated as a major contributor to endothelial dysfunction in PE [2].

Angiogenesis is considered as key in clarifying the pathogenesis of PE. Many angiogenic factors have been shown to be expressed in the placenta as exemplified by vascular endothelial growth factor (VEGF), placental growth factor, soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) and platelet-derived endothelial cell growth factor (PD-ECGF) [3, 4]. The expression and actual role of these markers in maternal and fetal blood remain conflicting [5]. The placental PD-ECGF contains 5 extra amino acids compared to PD-ECGF purified from human platelets but both have angiogenic and mitogenic activity [3]. sVEGFR-1 is essential for the normal development and function of the placenta [6]. Many investigators [5, 6] reported sVEGFR-1 as a possible PE factor. Increased neutrophils have been demonstrated in women with PE, and neutrophil activation liberates neutrophil

elastase [7]. Hence, in this study, we assessed these biochemical markers in the umbilical cord and placenta of preeclamptic patients, and evaluated their impact on the mother and fetus.

Subjects and Methods

The study included 45 pregnant women, admitted to the inpatient and labor ward in the Women and Health Reproductive Hospital, Assiut University Hospital. The patients and controls were matched by age and gestational age. The studied group included 30 women with PE; 15 had mild and the remaining 15 had severe PE. The control group consisted of 15 healthy pregnant women in labor with blood pressure less than 140/90 mm Hg. Women with chronic hypertension, diabetes mellitus, renal and liver diseases or smokers were excluded. Demographic data and routine clinical investigations were recorded for each participant. Ethical approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine. Informed consent was obtained from all patients.

Bioassays

A sample of 5 ml of maternal blood was withdrawn from both preeclamptic and control women on admission. A similar amount of blood was taken from the umbilical cord after delivery. Serum was separated by centrifugation at 300 g and immediately stored at -70°C till the time of assay. Placental and cord tissue samples (2 g of each) were randomly taken immediately after labor from both preeclamptic and control women. The tissue samples were washed repeatedly with cold saline solution and then homogenized by a tissue homogenizer (IKA ULTRA-TURRAX[®] T25, Werke Staufen, Germany) in 4 volumes of cold saline solution (w/v). The supernatant was collected after centrifugation (4,000 g, 20 min, 4°C) and the total protein concentration was determined using total protein kit. The sample was stored frozen at -70°C till the time of assay. Quantitative determination of serum VEGF was performed using a competitive enzyme immunoassay kit supplied by Accucyte[®] Sciences, USA. Accucyte human VEGF is designed to measure the 'total' (bound + free) amount of VEGF in the samples. This assay system is not hindered by autoantibodies, soluble receptors, or binding protein that can interfere with most commercial sandwich assays. Quantitative determination of serum sVEGFR-1 was performed using a colorimetric sandwich ELISA kit, supplied by R&D Systems, USA.

PD-ECGF was measured as thymidine phosphorylase activity by a spectrophotometer using the Scocca method [8]. Briefly, 1 g of the crystalline thymidine (T-9250) powder was supplied by the Sigma Chemical Company, USA. A sample of 290.64 mg was dissolved in 100 ml of 0.14 M potassium phosphate buffer, pH 7.4, to obtain 12 mM thymidine solution. Samples of 70 μl serum or tissue extract were added to 630 μl buffered thymidine solution (pH 7.4). The reaction mixture was incubated at 37°C . Aliquots of 200 μl were taken at 0 and after 20 min and added to 1.7 ml of ice-cooled 0.01 N NaOH. Free thymine base was produced as a result of the enzyme catalytic reaction. This method depends on the measurement of the increase in optical density at 300 nm. The extinction coefficient was 3.7. One unit of the enzyme corresponded to the amount of the enzyme that produces 1 nmol/ml of thymine base per hour [8].

The neutrophil elastase activity was determined by its amidolytic effect on the substrate N-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide. The rate at which *p*-nitroaniline was released was measured photometrically at 405 nm after stopping the reaction with acetic acid. Briefly, 10 mg of elastase substrate was reconstituted with 7 ml of dimethyl sulfoxide. One volume of this stock solution was diluted with 3 volumes of distilled water before use. Fifty microliters of Tris-HCl buffer, pH 8.3, was added to the sample tube and incubated at 37°C for 3 min, and then 50 μl of serum or tissue extract was added. After being mixed and incubated at 37°C for 2–3 min, 50 μl of elastase substrate in dimethyl sulfoxide (37°C) was added. After that, mixed and incubated at 37°C for 3 min, 50 μl of 20% acetic acid was added. A blank tube was prepared as a sample tube except that no incubation at all and elastase substrate was added after acetic acid. The absorbance of the sample was read against the sample blank at 405 nm. The elastase activity of the sample was calculated from the formula: elastase activity (μl) = absorbance \times 138 [9, 10].

Serum levels of nitric oxide (NO) were determined as total nitrite concentration after reduction of nitrate to nitrite using cadmium and reaction with the Griess reagent then measured at 550 nm [11].

Statistical Analysis

Data were statistically analyzed using SPSS version 12 for Windows and expressed as mean \pm SE. Statistical analysis was performed using the Mann-Whitney U test and correlations by Pearson's test. A *p* value of <0.05 was considered statistically significant.

Results

The birth weight, placental weight and placental thickness were significantly lower in women who had PE. On the other hand, blood pressure, either systolic or diastolic, and body mass index were significantly higher in preeclamptic patient as shown in table 1.

Platelet count ($\times 10^9/\text{l}$) was significantly lower in preeclamptic patients (mild PE: 197.8 ± 3.31 , severe PE: 171.9 ± 3.47) than in controls (303.7 ± 6.1) and the difference was statistically significant ($p < 0.001$). Hemoglobin concentration was significantly decreased in PE patients compared to the normal healthy controls (mild PE: 10.33 ± 0.11 mg/dl, severe PE: 9.72 ± 0.13 mg/dl, controls: 12.98 ± 0.07 mg/dl; $p < 0.001$). Although the levels of some routine laboratory parameters such as urea, creatinine, alanine transaminase, and aspartate transaminase were higher in women with PE than the normal group, these values were within the normal range. Also, there was no significant difference between mild and severe PE considering these parameters (data not shown).

Serum VEGF, sVEGFR-1, PD-ECGF, and neutrophil elastase were increased significantly while NO was decreased significantly in all preeclamptic patients com-

Table 1. The demographic data of the studied patients with mild and severe PE compared to the normal pregnant women

Demographic data	Preeclampsia			p values	
	control (n = 15), I	mPE (n = 15), II	sPE (n = 15), III	I vs. II	I vs. III
Maternal age, years	26.40 ± 0.49	29.53 ± 0.51	30.87 ± 0.37	NS	NS
Gestational age, weeks	38.40 ± 0.09	37.13 ± 0.14	37.27 ± 0.11	NS	NS
Parity	1.80 ± 0.15	2.00 ± 0.20	2.46 ± 0.14	NS	NS
Birth weight, g	3,053 ± 21.36	2,597 ± 42.66	2,330 ± 46.25	<0.05	<0.01
Placental weight, g	593.3 ± 6.28	453.3 ± 8.58	436.7 ± 6.60	<0.01	<0.001
Placental thickness, cm	4.96 ± 0.03	3.53 ± 0.05	3.30 ± 0.06	<0.001	<0.001
BMI, kg/m ²	27.53 ± 0.20	31.55 ± 0.29	31.92 ± 0.34	<0.01	<0.01
BP, mm Hg					
SBP	122.7 ± 0.53	158.3 ± 0.68	184.0 ± 0.93	<0.001	<0.001
DBP	75.33 ± 0.42	99.33 ± 0.30	117.0 ± 0.79	<0.001	<0.001

mPE = Mild preeclampsia; sPE = severe preeclampsia; BMI = body mass index; BP = blood pressure; SBP = systolic blood pressure; DBP = diastolic blood pressure; NS = non significant. No significant difference was found between mild (II) and severe (III) PE except in systolic blood pressure (p < 0.001) and diastolic blood pressure (p < 0.001).

Table 2. The serum levels in maternal and fetal circulations of the angiogenic indices in all cases of PE (mild and severe) compared to the normal pregnant women

Angiogenic parameters	Control (n = 15)	Preeclampsia (n = 30)	p values
Maternal circulation			
VEGF, ng/ml	3.83 ± 0.11	5.46 ± 0.09	<0.01
sVEGFR-1, pg/ml	428.9 ± 10.47	1,123.5 ± 8.54	<0.001
NO, nmol/ml	35.40 ± 0.22	32.78 ± 0.22	<0.05
PD-ECGF, µg/ml	18.41 ± 0.34	28.41 ± 0.47	<0.001
Elastase, µg/l	57.42 ± 1.99	89.36 ± 1.96	<0.01
Fetal circulation			
VEGF, ng/ml	1.78 ± 0.05	2.8 ± 0.07	<0.01
sVEGFR-1, pg/ml	379 ± 9.89	733.5 ± 15.07	<0.001
NO, nmol/ml	32.92 ± 0.20	29.26 ± 0.28	<0.01
PD-ECGF, µg/ml	37.99 ± 0.36	52.83 ± 0.60	<0.001
Elastase, µg/l	51.86 ± 1.39	75.48 ± 1.57	<0.01

Table 3. The placental and cord tissue levels of angiogenic indices in all cases of PE (mild and severe) compared to the normal pregnant women

Angiogenic parameters	Control (n = 15)	Preeclampsia (n = 30)	p values
Placental tissue			
VEGF, ng/mg protein	1.25 ± 0.02	3.34 ± 0.01	<0.05
sVEGFR-1, pg/mg protein	217.4 ± 8.30	523.2 ± 9.40	<0.05
NO, nmol/mg protein	0.53 ± 0.01	0.47 ± 0.01	NS
PD-ECGF, µg/mg protein	10.12 ± 0.19	17.15 ± 0.21	<0.001
Elastase, µg/g protein	4.19 ± 0.22	7.93 ± 0.24	<0.01
Cord tissue			
VEGF, ng/mg protein	0.52 ± 0.01	1.4 ± 0.02	<0.05
sVEGFR-1, pg/mg protein	123.4 ± 4.50	326.4 ± 8.60	<0.05
NO, nmol/mg protein	1.61 ± 0.02	1.30 ± 0.03	<0.05
PD-ECGF, µg/mg protein	7.08 ± 0.22	9.89 ± 0.27	<0.05
Elastase, µg/g protein	3.89 ± 0.17	6.24 ± 0.20	<0.05

pared to the normal pregnant women, as shown in table 2. Neutrophil elastase and PD-ECGF were significantly increased in the placental and cord tissue, whereas NO was significantly decreased in cord tissue only (table 3). No significant difference between mild and severe PE was detected in these parameters, except that the cord serum (fetal circulations) level of PD-ECGF was significantly higher in patients with severe PE compared to mild PE and normal pregnant women (table 4).

There was a strong correlation between PD-ECGF and VEGF (r = 0.7, p = 0.0001), and sVEGFR-1 (r = 0.8, p = 0.0001), and an inverse correlation between placental thickness (r = -0.3, p = 0.01) and placental weight (r = -0.5, p = 0.001). Regarding clinical data, the fetal weight was negatively correlated with cord serum level of sVEGFR-1 (r = -0.4, p = 0.005), PD-ECGF (r = -0.35, p = 0.01), and neutrophil elastase (r = -0.3, p = 0.03), whereas NO was positively correlated with birth weight

Table 4. The cord tissue levels of NO, PD-ECGF and neutrophil elastase and cord serum PD-ECGF in mild and severe PE compared to the normal pregnant women

Demographic data	Preeclampsia			p values		
	control (n = 15), I	mPE (n = 15), II	sPE (n = 15), III	I vs. II	I vs. III	II vs. III
NO, nmol/mg protein	1.61 ± 0.02	1.36 ± 0.03	1.24 ± 0.04	NS	<0.05	NS
PD-ECGF, µg/mg protein	7.08 ± 0.22	8.64 ± 0.23	11.14 ± 0.28	NS	<0.05	NS
Elastase, µg/g protein	3.89 ± 0.17	5.93 ± 0.20	6.54 ± 0.20	NS	<0.05	NS
Serum PD-ECGF, µg/ml	37.99 ± 5.53	49.57 ± 9.90	56.09 ± 7.16	<0.001	<0.001	<0.05

mPE = Mild preeclampsia; sPE = severe preeclampsia.

($r = 0.36$, $p = 0.01$). However, no relationship was detected between cord serum VEGF and fetal weight ($r = -0.2$, $p = 0.08$), whereas the blood pressure of the mother, either systolic or diastolic, was correlated with sVEGFR-1 ($r = 0.8$), PD-ECGF ($r = 0.7$), neutrophil elastase ($r = 0.6$) and VEGF ($r = 0.5$; $p < 0.5$ in all parameters).

Discussion

Our result confirmed the previous report [12] of the rise in VEGF and its receptor in maternal and fetal circulation in PE. However, our result was not similar to previous reports [13, 14] in which VEGF levels did not differ significantly between preeclamptic women and the controls and the mean maternal serum VEGF levels were significantly lower in preeclamptic women when compared to the normal healthy controls [14]. Regarding the difference in VEGF and VEGFR-1 between mild and severe PE, we did not detect any such difference between mild and severe PE as previously reported. This may be because our cases of mild PE represented a subset of women who were on the higher spectrum of the disease.

In this study, there is a positive correlation between total VEGF and sVEGFR-1 in the maternal circulation ($r = 0.6$, $p = 0.0001$). sVEGFR-1 was used as a sensitive and specific biomarker for the diagnosis of early onset (≤ 34 weeks) of PE [6]. Elevated sVEGFR-1 early in gestation may signal an antiangiogenic imbalance due to its strong antagonistic activity. It is bound to all isoforms of free VEGF and placental growth factor with high affinity, and so reduces their levels below a critical threshold required for the maintenance of the established vasculature. As a result, placental angiogenesis is decreased, and placental underperfusion occurs, generating a hypoxic envi-

ronment and stimulating additional sVEGFR-1 release, which further aggravate the situation [15].

Moreover, this placental hypoxia increases the PD-ECGF expression rate [16] as there is a tendency for PD-ECGF and VEGF to be co-expressed [17]. This hypothesis could be the explanation for the positive correlations between PD-ECGF and VEGF in both the maternal and cord serum of women with PE ($r = 0.8$, $p < 0.001$ and $r = 0.6$, $p < 0.001$, respectively), as the expression of VEGF is also upregulated by hypoxia. Cord serum level in this study showed a strong correlation between PD-ECGF and sVEGFR-1 ($r = 0.8$, $p = 0.0001$). Either systolic or diastolic blood pressure ($r = 0.7$, $p = 0.0001$) was inversely correlated with placental thickness ($r = -0.3$, $p = 0.01$), placental weight ($r = -0.5$, $p = 0.001$) and fetal weight ($r = -0.35$, $p = 0.01$). Platelet count was significantly lower in preeclamptic patients when controls were compared to mild or severe PE. This may be due to the release of PD-ECGF by lysis of platelets. The decreased platelet counts in PE led to a reduced number of placental microparticles derived from various cells forming procoagulant vesicles [18]. The thymidine phosphorylase, PD-ECGF, and over-expression increased the rate of production of the highly reducing sugar 2-deoxy-D-ribose-1-phosphate which generated oxygen radicals and induced cellular oxidative stress. This in turn consumed the antioxidants including NO in the reaction involving these reactive oxygen species [19].

Furthermore, the decreased placental angiogenesis and placental hypoxic environment in PE produced inflammatory responses. These inflammations increased neutrophil activation and liberation of neutrophil elastase which hindered the angiogenic balance. Neutrophil elastase was reported to be involved in the generation and processing of a number of antiangiogenic factors from their precursors, including the potent angiogenesis in-

hibitors, endostatin from collagen XVIII and angiostatin from plasminogen [20]. Neutrophil elastase was also found to cleave angiogenin, a protein with potent angiogenic activity. Elastase-cleaved angiogenin has lost its ability to undergo nuclear translocation in endothelial cells, a process essential for angiogenic activity [21]. The maternal plasma levels of neutrophil elastase may serve as a possible cell-free marker to quantify neutrophil activation as demonstrated in women with PE. The neutrophil elastase levels were elevated in both early- and late-onset forms of PE [7, 22]. Neutrophils, but not lymphocytes or monocytes, infiltrate maternal systemic vasculature causing the majority of vascular cell dysfunction in women with PE [23].

Evidence suggested that clinical manifestations of PE were caused by endothelial dysfunction. NO, which is synthesized from L-arginine in endothelial cells by the endothelial nitric oxide synthase, provided a tonic dilator

tone and regulated the adhesion of white blood cells and platelet aggregation. Alterations in the L-arginine-NO pathway had been associated with the development of PE. Various studies report decreased, increased or unchanged levels of nitrite and nitrate. NO level was decreased due to increased degradation by superoxide in states of infection and inflammation [24]. Novel insights into the role of the placenta in the development of PE had been provided [25]. Herein, we provide novel insights into the role of umbilical cord in the development of PE.

Conclusion

Our data showed that the umbilical cord vessels and stroma can serve as an additional source of vasoactive and angiogenic substances that contribute to the biochemical changes occurring in PE.

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