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ORIGINAL ARTICLE

The relationship between circulating endothelin-1, soluble fms-like tyrosine kinase-1 and soluble endoglin in preeclampsia

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Placental overproduction of anti-angiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) has a key role in the development of preeclampsia (PE). Circulating endothelin-1 (ET-1) levels are also elevated in PE. In this study, we investigated the correlation between ET-1 and sFlt-1, placental growth factor (PlGF), sEng levels during uncomplicated normotensive pregnancy and PE. A total of 218 pregnant primigravid women were enrolled: 110 with PE and 108 uncomplicated normotensive pregnancies. PE was defined as new onset of elevated blood pressure (BP) $>140/90$ mm Hg and $\geqslant 2+$ proteinuria on two occasions after 20 weeks of gestation in previously normotensive pregnant women. Circulating ET-1, sFlt-1, sEng and PlGF levels were estimated using enzyme immunoassays, and correlation between variables was ascertained. Women with PE showed higher levels of sFlt-1 (41.5 ± 15.7 vs 6.15 ± 3.4 ng ml^{-1}, $P<0.001$), sEng (84.9 ± 38.8 vs 13.2 ± 6.3 ng ml^{-1}, $P<0.001$), ET-1 (1.52 ± 0.55 vs 0.88 ± 0.35 pg ml^{-1}, $P<0.001$) and sFlt-1:PlGF ratio (591.1 ± 468.4 vs 18.3 ± 2.1, $P<0.001$); and lower levels of PlGF (96.3 ± 47.2 vs 497.6 ± 328.2pg ml^{-1}, $P<0.001$). BP levels showed an independent relationship with sFlt-1:PlGF ratio in normotensive pregnant women and with sFlt-1:PlGF ratio and ET-1 in PE. sFlt-1 and sFlt-1:PlGF ratio correlated with proteinuria. ET-1 correlated significantly with sFlt-1, sEng and sFlt-1:PlGF ratio in PE. Our results show an association between elevation of sFlt-1 and sEng and ET-1 in the maternal circulation in PE, and strengthen the possibility that ET-1 may be a mediator in genesis of PE syndrome secondary to anti-angiogenic factors released by the placenta.
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Introduction

Preeclampsia (PE), a multifactorial hypertensive syndrome of late pregnancy of unknown etiology, is an important cause of maternal and fetal morbidity and mortality.[1,2] The key pathophysiological processes are believed to be initiated by reduced placental perfusion secondary to inadequate trophoblast invasion.[3,4] Placental response to ischemia is manifested by overproduction of anti-angiogenic peptides, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng),[5] resulting in a clinical syndrome characterized by widespread systemic endothelial dysfunction.

Both sFlt-1 (a splice variant of fms-like tyrosine kinase-1) and sEng (a truncated form of endoglin) are thought to act by binding with their ligands, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF); and transforming growth factor-β, respectively, in circulation, thereby preventing the binding of these pro-angiogenic molecules to their native endothelial cell-surface receptors. Experimental studies confirmed the development of hypertension, proteinuria and histological lesions of PE in various organs in pregnant rats on infusion of recombinant adenovirus encoding sFlt-1 and sEng1.[6,7]

Elevations in circulating sFlt-1 and sEng and reduction in PlGF levels antedate the appearance of PE.[8–11] This suggests that perturbation of other pathways may be necessary for the development of clinical manifestations. Endothelin-1 (ET-1) is a

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potent vasoconstrictive peptide secreted by endothelial and vascular smooth muscle cells. We[12] and others[13–16] have shown elevated ET-1 levels in PE. It is possible that endothelial dysfunction induced by the elevated sFLt-1 and sEng levels results in overproduction of ET-1, leading to hypertension and proteinuria.

In this study, we evaluated sFlt-1, sEng, PlGF and ET-1 levels in normotensive and preeclamptic pregnancies to investigate the relationship, if any, between these markers and ET-1.

Subjects and methods

Subjects were recruited from the Antenatal Clinic and Wards of the Department of Obstetrics and Gynecology, Postgraduate Institute of Medical Education and Research, Chandigarh, a large tertiary care hospital in north India. The study protocol was approved by the Institute Ethics Committee. A total of 218 pregnant women were enrolled in this case–control study: 108 normotensive pregnant women (N) and 110 with PE. PE was defined as maternal systolic blood pressure (BP) $\geqslant$140 mm Hg and/or diastolic BP $\geqslant$90 mm Hg on two occasions separated by at least 6 h and proteinuria $>$300 mg per day or 2+ on dipstick after 20th week of gestation in previously normotensive pregnant women. Subjects were excluded if they had one or more of the following: hypertension before 20 weeks of gestation, diabetes, asthma, heart disease, kidney disease, haematological disorder, autoimmune disease, urinary tract infection, current or past history of smoking, twin pregnancy, molar pregnancy and eclampsia. Mean arterial pressure ((2 $\times$ diastolic BP + systolic BP)/3) was calculated for all subjects.

After an informed consent, 4 ml venous blood was collected in vacutainers; plasma and serum were separated by centrifugation and stored in sterile cryovials at $-80\,^{\circ}$C until analysis. For serum separation, blood samples were allowed to clot for 30 min prior to centrifugation.

sFlt-1, sEng, PlGF and ET-1 levels were determined in duplicate using commercial ELISA kits as per manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Intra- and inter-assay coefficients of variation were 3.2 and 7.4% for sFlt1, 3.0 and 6.5% for sEng, 5.4 and 11.2% for PlGF and 4.43 and 5.57% for ET-1, respectively. The sFlt-1:PlGF ratio was calculated for all subjects.

The average of duplicate assays represented the value of individual samples in the statistical analysis. All values are expressed as mean $\pm$ s.d. and analysed using SPSS v 13.0 (Chicago, IL, USA) software. One-factor *post hoc* analysis of variance with Bonferroni correction was used to compare the means of clinical characteristics. Inter-group comparison of continuous data was done using Mann–Whitney *U*-test and correlation between two variables was ascertained using Spearman's rank correlation coefficient. Stepwise multiple regression analysis was done to investigate the effect of the various measured molecules with the mean arterial pressure. A double-sided *P*-value of <0.05 was considered significant.

Results

Clinical characteristics (Table 1) showed no significant differences in the mean age of the participants, but the mean gestational age at delivery was significantly lower in PE. As per the grouping criteria, the systolic BP and diastolic BP were significantly higher in PE group with PE patients exhibiting proteinuria. In the normotensive group, there were two small for gestational age deliveries, whereas there were 6 (5%) fresh stillbirths and 24 (22%) small for gestational age deliveries in the PE group. The BPs normalized following delivery in all PE subjects at 14.5 $\pm$ 4.2 days after delivery.

Figure 1 shows the circulating ET-1, sFlt-1, sEng and PlGF values and the sFlt-1:PlGF in the two study groups. Compared with the normotensive pregnant women, the circulating levels of sFlt-1 (41.5 $\pm$ 15.8 vs 6.11 $\pm$ 3.3 ng ml^{-1}, $P<0.001$), sEng (86.8 $\pm$ 38.3 vs 13.4 $\pm$ 6.1 ng ml^{-1}, $P<0.001$) and ET-1 (1.52 $\pm$ 0.55 vs 0.88 $\pm$ 0.35 pg ml^{-1}, $P<0.001$) were significantly elevated in PE patients. In six PE subjects, the sFlt-1 levels were greater than the upper detection limit of the kit even after diluting the samples twice the recommended dilution. In contrast, a significant decrease in the mean PlGF levels (96.1 $\pm$ 46.4 vs 508.2 $\pm$ 332.3 pg ml^{-1}, $P<0.001$) was observed in this group. The sFlt-1:PlGF ratio was greater in PE when compared with normotensive group (591.1 $\pm$ 468.4 vs 19.4 $\pm$ 17.9, $P<0.001$).

ET-1, sFlt-1, sEng and sFlt-1:PlGF ratio showed a significant positive association with MAP in PE group, whereas PlGF levels showed a negative association (Table 2). In the normotensive group, the MAP correlated positively with sFlt-1:PlGF ratio and negatively with PlGF, but not with ET-1, sFlt-1

Table 1 Demographic characteristics of study subjects

Parameters	*Preeclampsia (PE)*	*Normotensive (N)*	P-*value*
Number of subjects	110	108	—
Mean age (years)	25.9 $\pm$ 3.9	26.2 $\pm$ 3.9	0.596
Mean gestational age at delivery (weeks)	33.1 $\pm$ 2.6	38.9 $\pm$ 2.73	$<$0.0001
Systolic blood pressure (mm Hg)	153.4 $\pm$ 10.7	116.7 $\pm$ 4.7	$<$0.0001
Diastolic blood pressure (mm Hg)	101.9 $\pm$ 8.1	76.3 $\pm$ 5.1	$<$0.0001
Mean arterial pressure (mm Hg)	119.1 $\pm$ 8.0	89.6 $\pm$ 4.3	$<$0.0001
Proteinuria (g per day)	3.05 $\pm$ 0.78	Nil	$<$0.0001

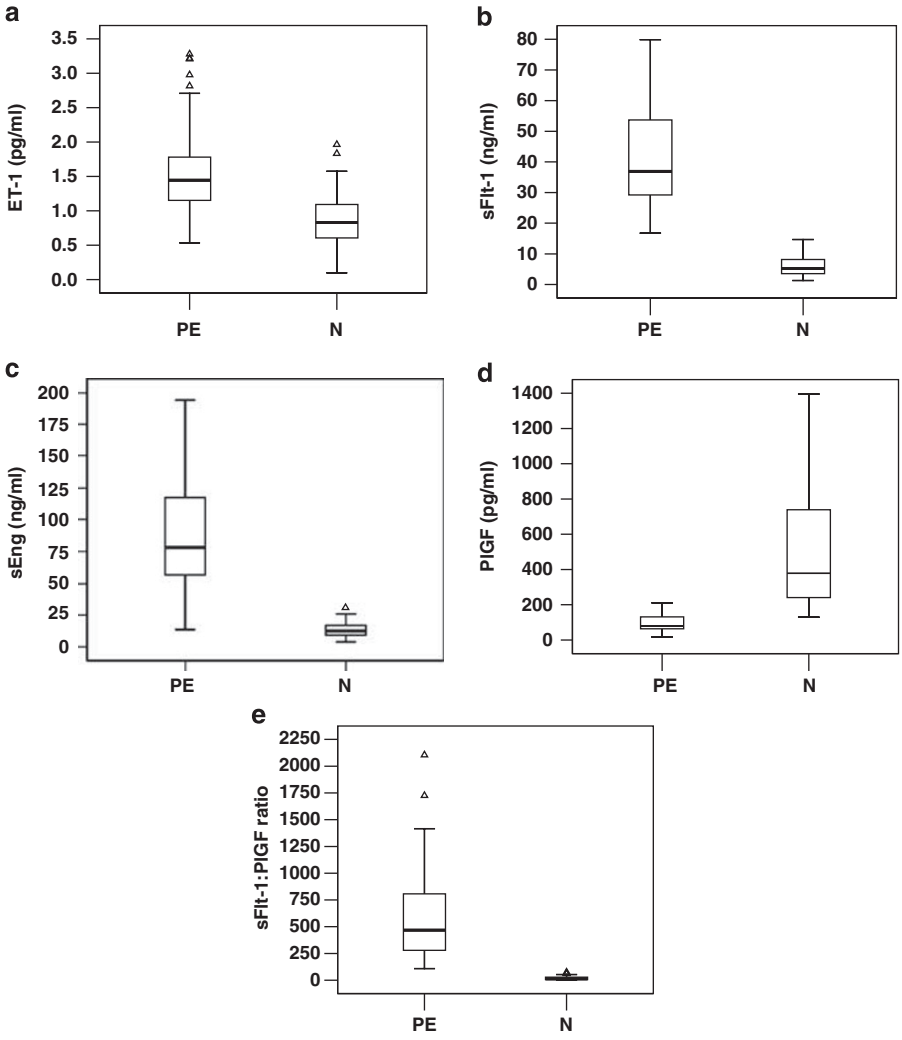

Figure 1 Circulating (**a**) ET-1, (**b**) sFlt-1, (**c**) sEng and (**d**) PlGF levels and (**e**) sFlt-1:PlGF ratio in the two groups. The ET-1, sFLt-1, sEng and sFlt-1:PlGF ratio are increased and the PlGF levels decreased in the PE group compared with normotensive women. The lower and upper bars represent the 10th and 90th centiles, respectively, and the interquartile range is indicated by the box; the median value being the horizontal line in the box.

Table 2 Relationship of ET-1, sFlt-1, sEng and PlGF levels and sFlt-1:PlGF ratio with mean arterial pressure in PE and N group

	PE		*N*	
	Spearman's coefficient (ρ)	P-*value*	*Spearman's coefficient* (ρ)	P-*value*
ET-1	0.3	0.002	0.03	0.7
sFlt-1	0.24	0.01	0.11	0.28
sEng	0.25	0.01	0.11	0.16
PlGF	−0.21	0.04	−0.26	0.02
sFlt-1:PlGF ratio	0.28	0.006	0.34	0.004

Abbreviations: ET-1, endothelin-1; PE, preeclampsia; PlGF, placental growth factor; N, normotensive; sEng, soluble endoglin; sFlt-1, soluble fms-like tyrosine kinase-1.

and sEng. On multiple regression analysis, ET-1 as well as sFlt-1:PlGF ratio were independently correlated with MAP in the PE group ($P=0.01$ and 0.03, respectively), whereas only sFlt-1:PlGF ratio showed an independent association with MAP ($P=0.006$) in N group.

The degree of proteinuria correlated with sFlt-1 levels ($\rho=0.2$, $P=0.046$) and sFlt-1:PlGF ratio ($\rho=0.25$, $P=0.015$). The correlation with sEng, PlGF and ET-1 was just short of reaching statistical significance ($P=0.051$, 0.052 and 0.054, respectively).

A significant correlation between sFlt-1 and sEng levels was noted in both PE and N groups ($\rho=0.38$, $P=0.0001$ and $\rho=0.42$, $P<0.0001$, respectively), whereas PlGF correlated negatively with sFlt-1 in PE as well as N groups ($\rho=-0.24$, $P=0.18$ and $\rho=0.27$, $P=0.19$, respectively).

ET-1 levels showed a significant correlation with sFlt-1, sEng and sFlt-1:PlGF ratio in PE, but not in the N group (Table 3). No statistically significant correlation was observed between PlGF and ET-1 levels in either group.

Table 3 Relationship of ET-1 levels with sFlt-1, sEng and PlGF levels and sFlt-1:PlGF ratio in the PE and N groups

	PE		*N*	
	Spearman's coefficient (ρ)	P-*value*	*Spearman's coefficient (ρ)*	P-*value*
sFlt-1	0.285	0.003	0.033	0.756
sEng	0.276	0.006	0.086	0.423
PlGF	−0.050	0.630	0.048	0.689
sFlt-1:PlGF ratio	0.231	0.025	−0.029	0.811

Abbreviations: ET-1, endothelin-1; PE, preeclampsia; PlGF, placental growth factor; N, normotensive; sEng, soluble endoglin; sFlt-1, soluble fms-like tyrosine kinase-1.

Discussion

In this study, for the first time, we show a relationship of circulating ET-1 levels with sFlt-1, sEng and sFlt-1:PlGF ratio in women with PE. This study also validates our previous finding that ET-1 levels are elevated, and correlate with BP in women with PE.[12] Consistent with other published reports, we confirmed the changes in circulating sFlt-1, sEng, PlGF levels and sFlt-1:PlGF ratio in PE in our cohort of Indian subjects.

Significant progress has been made in the understanding of the pathogenesis of PE in the last decade. The key breakthrough came in a series of studies by Karumanchi and co-workers. They showed abnormally increased production of sFlt-1 and sEng, two powerful anti-angiogenic molecules by preeclamptic placentae, and went on to confirm the significance of these findings in animal models.[6,7] These findings were validated in a cohort of pregnant women, where the levels correlated with disease severity and even antedated the clinical manifestations.[8,9]

sFlt-1 and sEng are believed to exert their pathogenic effects by limiting the availability of their pro-angiogenic ligands, viz. VEGF, PlGF and transforming growth factor-β to their native cell-surface binding partners on the endothelium.[17] This 'angiogenic imbalance' is believed to induce endothelial dysfunction, systemic vasoconstriction, hypertension and proteinuria.

The exact mechanism by which this imbalance increases arterial pressure or induces proteinuria has remained unclear. It is likely that the imbalance leads to perturbations of other pathways that result in the PE phenotype. The endothelium responds to changes in its milieu by altering the synthesis and/or release of vasoactive peptides, such as ET-1 and nitric oxide.[18,19] Pregnant rats developed hypertension and increased circulating ET-1 levels when subjected to chronic reductions in uteroplacental perfusion pressure.[20] This hypertension was abolished by selective blockade of the ET-1 type A receptor. Exposure of human umbilical vein endothelial cell cultures to sera collected from reductions in uteroplacental perfusion pressure was followed by an increase in the ET-1 concentration in the media, whereas no such increase was noted when cells were exposed to sera from normal pregnant rats, suggesting that a substance present in the PE sera induced ET-1 synthesis.[21] In other studies, hypertension produced by placental ischemia in pregnant rats has been shown to be associated with increased sFlt-1 and sEng levels.[22,23]

In this study, ET-1 levels showed a significant correlation with sFlt-1, sEng and sFlt-1:PlGF ratio in PE, but not in the N group. The sFlt-1:PlGF ratio, a measure of angiogenic balance, independently correlated with the BP in both normotensive and preeclamptic pregnancies, whereas ET-1 showed an independent association with MAP only in PE. Past studies have shown that the sFlt-1:PlGF ratio goes up near term even in normal pregnancy, and it is at this time that a slight rise in BP from the mid-pregnancy levels (within the physiological range) occurs. Our finding confirms this relationship, and suggests that this alteration is a physiological phenomenon, probably required to shut off angiogenesis as pregnancy nears term. However, a more substantial alteration in the angiogenic balance in PE probably leads to endothelial dysfunction, and the consequent elevation in ET-1 leads to a rise in BP beyond the normal limits.

A direct link between angiogenic factors and ET-1 was shown recently.[24] VEGF blockade induced ET-1 release from cultured glomerular endothelial cells, and the conditioned medium thus obtained triggered nephrin loss from podocytes. Conditioned medium obtained from cells incubated with PE sera induced a similar effect, which could be reversed by ET-1 receptor antagonist. Indeed, VEGF blockade by anti-VEGF antibodies and sFlt-1 induces proteinuria.[25] sFlt-1 infusion in pregnant rats was shown to increase arterial pressure and preproendothelin mRNA expression in renal cortices.[26] ET-1 A receptor blockade completely abolished this BP response to sFlt-1. In another study, recombinant sFlt-1 infusion in pregnant rats was followed by increased placental and vascular superoxide production and decreased vasorelaxation to acetylecholine and sodium nitroprusside.[27] In addition, decrease in nitric oxide (a potent vasodilator) formation has been shown to be associated with the increased levels of both sFlt-1 and sEng in women with PE.[28]

Our findings show an association between the ET-1 and various anti-angiogenic proteins in maternal circulation of preeclamptic women, and suggest a link between these pathways in the genesis of clinical syndrome of PE. It seems likely that the raised sFlt-1 and sEng cause release of ET-1 by the endothelial cells, either through a direct effect on the endothelial cells or through reduced availability of VEGF, which in turn causes hypertension and could contribute to proteinuria by modulating nephrin expression. Previously, we have shown that placental ET-1 synthesis is downregulated in PE,

suggesting that the circulating ET-1 is of maternal origin.[29] In fact it is possible that the local vascular concentrations of ET-1 were even higher, since its secretion is polarized, and the circulating levels are usually lower. In a recent study, Wang *et al*.[30] showed increased levels of ET-1 and sFLt-1 in the amniotic fluid obtained at 16–19 weeks of gestation in women who later developed PE, and noted a positive correlation between the two.

Some limitations of this study must be acknowledged. Whereas we have shown a statistical relationship between ET-1 and anti-angiogenic factors, causality has not been established. Longitudinal studies are required to establish the temporal profile of elevation in the anti-angiogenic molecules and ET-1. Interventional studies using agents that block the actions of sFlt-1 and/or ET-1 in women with PE will provide the final answer to these questions. It is likely that other effector molecules are involved in the genesis of the full clinical syndrome. ET-1 correlated well with BP elevation, but not with proteinuria, whereas the anti-angiogenic factor elevation correlated with both.

In conclusion, we show a relationship between ET-1, a potent vasoconstrictor peptide and the anti-angiogenic sFlt-1 and sEng, suggesting a possible link between these pathways in PE. This interaction might contribute to the development of hypertension and proteinuria. Prospective longitudinal studies are required to further elucidate the relationship between these systems.

What is known about this topic

- Overproduction of anti-angiogenic factors, viz. soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) is a key event in the development of preeclampsia (PE).
- The exact mechanism of development of the clinical syndrome, however, is not known.
- There is a need to identify mediators, if any, that might explain the clinical features.

What this study adds

- This case–control study examined the relationship between levels of endothelin-1 (ET-1), a potent vasoconstrictor peptide and sFlt-1, sEng and placental growth factor in maternal circulation in normotensive pregnant women and those with preeclampsia.
- There was a significant correlation between the levels of ET-1, sFlt-1 and sEng in women with PE; the levels correlated with severity of hypertension and proteinuria.
- This study suggests the possibility of an interaction between these pathways in the development of hypertension and proteinuria in PE.

Conflict of interest

The authors declare no conflict of interest.

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