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Promoter region polymorphisms in the transforming growth factor beta-1 (TGFβ1) gene and serum TGFβ1 concentration in preeclamptic and control Iranian women

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ABSTRACT

Preeclampsia (PE) is a pregnancy associated disorder characterized by hypertension and proteinuria, which causes neonatal and maternal morbidity and mortality. The Th1/Th2 cytokine paradigm of the immune adaptation in pregnancy is now expanded to include Th1/Th2/Th17 and regulatory T (Treg) cells. Among cytokines, TGFβ1 has properties that justify evaluation of its role in PE etiopathology. In this investigation the polymorphisms of the TGFβ1 gene at promoter region, positions −800G→A and −509C→T, were studied in 142 PE and 140 normal pregnant female subjects using PCR-RFLP. Additionally, serum TGFβ1 was determined by ELISA. At position −800G→A genotypes and allele frequencies showed no significant differences between PE patients (GG 73.9%; GA 21.1%; AA 4.93%) and normal control (GG 70%; GA 28.6%; AA 1.4%) women. However the AA genotype at this position was more frequent in PE patients than in the control group. At −509C→T position, genotypes and allele frequencies showed no significant differences between PE patients and control individuals. The CC genotype at −509C→T position was more prevalent in PE patients than in the control group. The mean serum TGFβ1 level was significantly higher (62.14 ng/ml) in PE patients compared with pregnant and non-pregnant control groups (and 47.01 and 40.68 ng/ml, respectively). In conclusion, the promoter region polymorphisms of TGFβ1 may not be associated with PE, but serum levels of this cytokine may contribute to the etiopathology of PE.

1. Introduction

Preeclampsia (PE) is pregnancy associated disorder with symptoms that include hypertension and proteinuria. It is responsible for substantial neonatal and maternal morbidity and mortality (Sibai et al., 2005). It occurs in 3–5% of pregnancies and is a major cause of maternal mortality in developed countries (Sibai et al., 2005; Khan et al., 2006). The common hypothesis about the etiology of PE has been focused on deviation of immune responses and a Type 1/Type 2 cytokine disequilibrium (Wilczyski et al., 2003; Matthiesena et al., 2005). Recent data show that the Th1/Th2 paradigm is now insufficient to explain immunology of normal pregnancy, and this paradigm has been expanded to include Th1/Th2/Th17 and regulatory T (Treg) cells (Saito et al., 2010). It is believed that delicate

Abbreviations: PE, pre-eclampsia; RSA, recurrent spontaneous abortion; RFLP, restriction fragment length polymorphism.

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and complex balances between various cytokines are required to establish and maintain a successful pregnancy (Saito, 2000; Raghupathy, 2001).

TGFβ1 is a 25-kDa homodimeric cytokine secreted in a latent form (Khalil, 1999) by several cell types including peripheral blood mononuclear cells, endothelial cells, platelets, Treg cells and also renal cells. TGFβ1 is multifunctional cytokine present in semen and expressed in several pregnancy associated tissues, and it plays an important role in the physiology of normal pregnancy (Bowen et al., 2002). TGFβ1 biological actions are mediated in an autocrine or paracrine manner. This cytokine supports type 2 immune responses and also shifts immune responses toward the Th3 type (Raghupathy, 2001). In the placenta, both mRNA and protein of TGFβ1 appear to be present throughout gestation.

TGFβ1 has been implicated in many hypertensive disorders. It may be an important mediator of angiotensin II-induced hypertensive damage (Border and Noble, 1998). Data from TGFβ1 transgenic mice have demonstrated that high circulating levels can mediate renal fibrosis and progressive loss of renal function (Border and Noble, 1998). It seems that TGFβ1 may contribute to PE onset with shallow placentation and may also be involved in the pathology of PE by affecting both hypertension and renal cell function in an endocrine manner (Border and Noble, 1998).

The human TGFβ1 gene is located at chromosome 19q13 and contains seven exons (Clark and Coker, 1998). Several polymorphisms of TGFβ1 have been reported (Cambien et al., 1996). Three polymorphisms have been located in the TGFβ1 promoter region at positions −988, −800, −509 (Cambien et al., 1996). It has been reported that the −509C→T polymorphism is significantly associated with higher (Grainger et al., 1999) and the −800G→A substitution with lower production of total TGFβ1 in the circulation (Syrris et al., 1998).

Serum level and cytokine gene polymorphisms have previously been studied by several investigators in PE patients (Hennessy et al., 2002; Muy-Rivera et al., 2004; Enquobahrie et al., 2005). In this investigation, serum level and promoter region polymorphisms in the TGFβ1 gene (position −800G→A and −509C→T) were studied in Iranian women with preeclampsia and normal matched controls.

2. Materials and methods

2.1. Subjects

The study group consisted of 142 women (aged 17–38 years, mean 28 years) with preeclampsia and no history of kidney disorders and hypertension before pregnancy. Patients with anatomical, hormonal, chromosomal, infections and autoimmune disorders were excluded from the study. All subjects attended the Department of Obstetrics and Gynecology of Shahid Beheshti University of Medical Sciences Tehran-Iran. The diagnosis of preeclampsia was made by a gynecologist after clinical and paraclinical examination of cases. From 142 selected PE women; 112 suffered moderate (mild) and 30 suffered severe preeclampsia. The patients with blood pressure (BP) of more than 140/90 after 20 weeks of pregnancy or proteinuria of more than 300 mg in 24 h urine collected were classified as preeclamptic. The severe form of preeclampsia was defined on the basis of symptoms of BP more than 160/110, proteinurea >300 mg in 24 h urine, with headache, oliguria, visual disturbances, upper abdominal pain, high creatinine and liver enzymes and thrombocytopenia.

The control group consisted of 140 ethnically matched women; 100 pregnant of whom were in the third trimester of pregnancy and 40 non-pregnant women (aged 16–50 years, mean 35) with normal blood pressure (<140/90) and no proteinurea. The non pregnant women had at least one previous pregnancy without PE. The gestational age of PE patients and control pregnant women were 32–37 and 30–35 weeks, respectively. The women that entered into this study did not have any IUGR or systemic disorders. None of the patients had received any medication before blood sampling. Twenty PE patients and six control women had a family history of preeclampsia. All case and control individuals in this study were non-smokers and all had normal BMI. The patient and control individuals participated in this study after informed consent.

2.2. DNA extraction and TGFβ1 genotyping

Peripheral venous blood was collected in EDTA-coated tubes. DNA was extracted from whole blood using the salting-out method (Miller et al., 1988). Specific oligonucleotide primers were used as previously described (Luedecking et al., 2000; Cotton et al., 2002). The following primers (MOLBIOL, Germany) were used for amplification of TGFβ1 polymorphisms. Position −800 forward primer: 5′-tgg ggc cga ccg cta tcg, and reverse primer: 5′-gcc acc cca tac att tac. For the position −509, forward primer: 5′-cag taa atg tat ggg gtc gca g, and the reverse primer sequence: 5′-ggt gtc agt ggg agg agg g. Polymerase chain reaction (PCR) amplification of each polymorphism was performed in a total reaction volume of 20 μl with 300 ng DNA as template. Genotyping was performed by restriction fragment length polymorphism (RFLP) analysis. The following restriction enzymes (Fermantas, Lithuania) were used for the digestion of amplified PCR products. For digestion of PCR products contain position −800, NmuCI and, for the PCR products containing position −509, Eco81 I were applied. The digestion conditions were in accordance with the manufacturer's procedures.

2.3. Measurement of TGFβ1 protein level

Peripheral venous blood was obtained from the preeclamptic and control women that were in same gestational age (third trimester). The sera were isolated and stored at −70 °C until assay for TGFβ1. The biologically active TGFβ1 protein concentration was determined using a solid-phase TGFβ1-specific sandwich ELISA (Bendermed system, USA) as described by the manufacturer's protocol. Prior to analysis sera were transiently by acidification to activate latent TGFβ1, and then tested at a 1:12 dilution.

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Table 1
Distribution of TGFβ1 genotype and alleles in PE patients and control groups.

TGFβ1 genotype and allele	Preeclampsia ($n = 142$)	Control ($n = 140$) (pregnant and non-pregnant)	P-value*
Genotype: −800 (G→A)			
GG	105 (73.9%)	98 (70%)	
GA	30 (21.1%)	40 (28.6%)	
AA	7 (4.9%)	2 (1.4%)	0.06
Allele			
G	0.845	0.843	
A	0.155	0.157	0.1
Genotype: −509(C→T)			
CC	40 (28.2%)	35 (25%)	
CT	59 (41.5%)	62 (44.3%)	
TT	43 (30.3%)	43 (30.7%)	0.82
Allele			
C	0.49	0.471	
T	0.51	0.529	0.77

* Statisticial test: χ^2.

2.4. Statistical analysis

Allele frequencies for each polymorphic site were calculated by the allele counting method. Differences in the genotype and allele frequencies between patients and controls were tested by χ^2 analysis. Differences in serum TGFβ1 concentration between the PE patients and normal control group were determined by student's t-test. Serum TGFβ1 levels in PE patients and control groups in relation to different genotypes were analyzed by ANOVA test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Position −800G→A

In this investigation, the change at position −800G→A of the TGFβ1 gene was studied using PCR-RFLP in 142 cases of preeclampsia and 140 normal pregnant women. Results indicated that 105 (73.9%) of the PE cases, and 98 (70%) of the normal subjects, were homozygote GG at this position. In addition 30 (21.1%) of PE cases and 40 (28.6%) of normal subjects were heterozygote AG, respectively. Seven (4.9%) of the PE cases and two of the normal subjects were found to be homozygote AA (Table 1). There was no statistically significant difference in genotype or allele frequency distributions between PE patients and controls at this position ($P > 0.05$).

3.2. Position −509C→T

In addition, the genotype at position −509C→T of TGFβ1 gene was evaluated in the same study groups. The results indicated that 40 (28.2%) of PE cases, and 35 (25%) of normal subjects, were homozygote CC at this position. In addition, 59 (41.5%) of the PE cases and 62 (44.3%) of normal subjects were heterozygote CT. Homozygote TT was found in 43 (30.3%) of PE cases and 43 (30.7%) of normal subjects (Table 1). Statistical analysis of genotype distribution and allele frequency at this position showed no significant difference between PE cases and normal controls ($P = 0.8$).

3.3. Measurement of serum TGFβ1 concentration

The biologically active TGFβ1 protein concentration was determined using a solid-phase TGFβ1-specific sandwich ELISA. A TGFβ1 standard curve was constructed using 200, 100, 50, 25, and 12.5 ng/ml of recombinant human TGFβ1 protein.

Mean serum levels of TGFβ1 in PE patients were 62.14 ng/ml, compared with 47.01 and 40.68 ng/ml in pregnant and non pregnant control women, respectively. As summarized in Table 2, the mean serum TGFβ1 level was significantly higher in preeclamptic patients in comparison with both of the two control groups ($P = 0.0001$; Table 2). Serum TGFβ1 levels were not significantly different between pregnant and non pregnant control individuals ($P = 0.2$).

The serum TGFβ1 levels in relation to different genotypes in PE, pregnant control and non pregnant control women are summarized in Table 3. In all groups, the serum TGFβ1 level was not statistically different between the three genotypes at either position. At the −800 position, individuals with GG genotype, and at −509 position individuals with CC genotype, showed high TGFβ1 serum levels (Table 3).

4. Discussion

Cytokines have important roles in endometrial receptivity, implantation, placental development and fetal growth. Their actions include establishment and control of maternal immune system balance during gestation, and this has been extensively studied (Bowen et al., 2002). Some published data indicate that maternal Th2 type cytokines

Table 2
Serum TGFβ1 levels in PE patients and control groups.

	Serum TGFβ1 mean (range) (ng/ml)	P-value*
Pre-eclampsia (PE), $n = 140$	62.14 (22.19–152.13)	
Pregnant control, $n = 100$	47.05 (31.48–142.25)	0.0001
Non-pregnant control, $n = 40$	40.68 (15.92–137.26)	0.0001

* Statisticial test: student's t-test.

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Table 3
Serum TGFβ1 (ng/ml) levels in PE patients and control groups in relation to different genotypes.

SNP position	Genotype	Preeclampsia PE (n = 142)	Pregnant control (n = 100)	Non-pregnant control (n = 40)	P-value*
−800	GG	62.80 (105)	49.57 (71)	44.41 (27)	0.146
	GA	57.75 (30)	41.86 (28)	35.17 (12)	0.176
	AA**	71 (7)	14 (1)	6 (1)	
P-value		0.578	0.532	0.548	
−509	CC	71.37 (40)	47.08 (14)	45.5 (18)	0.131
	CT	55.41 (59)	51.07 (50)	31.17 (12)	0.121
	TT	62.79 (43)	45.22 (33)	43.4 (10)	0.217
P-value		0.521	0.627	0.519	

* Statisticial test: ANOVA.
** AA genotype omitted from statistical analysis because of small sample size.

are associated with successful pregnancy outcome while abnormally elevated maternal Th1 type cytokines are incompatible with normal pregnancy outcome (Wilczyski et al., 2003; Borzychowski et al., 2005; Matthiesena et al., 2005). Recent reports indicate that the Th1/Th2 paradigm is now insufficient to explain immunology of normal pregnancy. The Th1/Th2 paradigm now incorporates Th1/Th2/Th17 and regulatory T (Treg) cells (Saito et al., 2010).

In this study we analyzed polymorphisms of the TGFβ1 promoter region at −800G→A and −509C→T positions in 142 Iranian women with PE and 140 matched control individuals (100 pregnant and 40 nonpregnant) and we also measured serum levels of this cytokine in the study groups.

Serum TGFβ1 levels in PE patients have been previously studied but the data are not conclusive. In agreement with other reports (Enquobahrie et al., 2005; Muy-Rivera et al., 2004) our results showed statistically significant differences in mean TGFβ1 serum level (P = 0.001) between PE and control groups. While in contrast to our findings, Hennessy et al. (2002) have been reported no significant differences in serum TGFβ1 between PE and control individuals. Also in a recent comprehensive article; Szarka et al. (2010) reported no significant differences in serum TGFβ1 levels between PE patients, healthy non-pregnant and healthy pregnant women. This controversy may be attributed to the different racial and genetic background of the different cohorts, as well as the small sample size for PE patients in these studies.

There are many parameters (e.g. BMI, smoking, gestational age at sampling, medication, other reproductive disorders, etc.), which could affect TGFβ1 serum levels in PE patients and pregnant control women. These parameters should be considered when designing a study. As indicated in Section 2 of this article both PE patients and control pregnant women were in the third trimester of gestational age at sampling and none of the studied cases were smokers or were overweight. Also none of patients had been received any medication before blood sampling. The setting of this study decreases the likelihood of an effect of the abovementioned variables on serum TGFβ1 levels.

Genotype distribution and allele frequencies at position −800G→A SNP were not significantly different between PE and control groups. The −800G→A substitutions is thought to disrupt a consensus half-site for binding of the nuclear transcription factor CRE-binding protein, consequently contributing to a lower production of total TGFβ1 in the circulation (Syrris et al., 1998). Our findings showed that mean TGFβ1 serum level in persons with the GG genotype (PE = 62.08, pregnant control = 49.5 and non pregnant control = 44.4 ng/ml) is higher than individuals with GA genotypes. Our findings thus confirm the results of Syrris et al. (1998).

Genotype distribution and allele frequencies at position −509C→T polymorphisms were not significantly different between PE and control groups. It has been reported that the −509C→T polymorphism is significantly associated with a high plasma concentration of TGFβ1 (Grainger et al., 1999). Our findings showed that at position −509, the mean TGFβ1 serum level in PE women with a CC genotype was higher than individuals with either CT or TT genotypes, but these differences were statistically not significant. Also, PE patients with a CC genotype had higher TGFβ1 serum levels than both control groups (pregnant and non-pregnant individuals) with the same genotype. These data do not support previously published result by Syrris et al. (1998). These differences may be attributable to the differences in race and genetic background and the different sample sizes in the two studies.

TGFβ1 gene polymorphisms (at positions +10 and +25) in PE patients have been studied by Daher et al. (2006) and no associations were found. We are also analyzing polymorphisms of the coding region of TGFβ1 in Iranian women with PE (data not shown). Recently TGFβ1 gene polymorphisms have been studied in RSA patients and no associations were found (Amani et al., 2004, 2005).

TGFβ1 gene polymorphisms in other immunologic diseases such as systemic lupus erythematosis (Wang et al., 2007), psoriasis vulgaris (Baran et al., 2007), systemic sclerosis (Ohtsuka et al., 2002), rheumatoid arthritis (Sugiura et al., 2002), Crohn's disease (Schulte et al., 2001) and asthma (Pulleyn et al., 2001) have been previously reported by other investigators.

Investigation of other cytokine gene polymorphisms in PE patients has resulted in controversial data. The TNFα −308G→A polymorphism was associated with PE complicated by severe fetal growth restriction (Molvarec et al., 2008). Also, an association between TNFα gene variant at position −850C→T and PE has been investigated by several researchers and no association have been found (Stonek

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et al., 2008; Vural et al., 2010). Association of interleukin-10 gene polymorphism at position −1082C→T with PE were studied by Vural et al. (2010) and Stonek et al. (2008), but the results were controversial. There is no association between IL-6 polymorphism at 174G→C position and PE, as shown by Vural et al. (2010) and Stonek et al. (2008).

Promoter region polymorphisms of TGFβ1 gene may have no effect on cytokine production during pregnancy, but evaluation of organ-specific expression and serum levels of this cytokine may be important in diseases of pregnancy.

These data suggest that TGFβ1 is associated with the etiopathology of PE and high serum TGFβ1 levels are likely to be due to increased production of this cytokine. An elevated serum TGFβ1 level could contribute to the clinical signs of PE in several ways:

1. TGFβ1 has dose dependent growth stimulatory and inhibitory effects and can contribute to shallow placentation with growth inhibition (Moustakas et al., 2002).
2. TGFβ1 is involved in elevated hypertension in PE by stimulation of renin release from jaxtaglomerolar cells (Antonipillai et al., 1993) and angiotensin II expression (Border and Noble, 1998). Also this cytokine causes endothelial dysfunction by stimulating vasoactive peptide endothelin-1 production. A positive association between angiotensin converting enzyme and angiotensin gene polymorphisms with PE and lack of association between PE and endothelin-1 type A receptor gene polymorphism (−231G→A) has been reported (Serrano et al., 2006; Lisi et al., 2007; Miskovi et al., 2008).
3. Elevated TGFβ1 serum level can cause glomerolar cell dysfunction and proteinurea. Also TGFβ1 stimulates extracellular matrix (ECM) production and inhibits ECM degradation, which results in ECM deposition in kidney cells and subsequently glomerular cell dysfunction (Docherty et al., 2002).

However, TGF-β1 has potential beneficial effects. This cytokine has powerful immunoregulatory properties. TGFβ1 is known to promote human Treg cell generation and to inhibit Th17 cell differentiation (Saito et al., 2010). Furthermore, elevated levels of its soluble co-receptor, endoglin, have been previously reported in preeclampsia. Soluble endoglin impairs binding of TGFβ1 to its receptors and prevents downstream signaling, including effects on activation of eNOS and vasodilation, suggesting that this anti-angiogenic protein leads to dysregulated TGFβ signaling in the vasculature (Venkatesha et al., 2006).

In conclusion, TGFβ1 genetic variants at two promoter region (positions: −800 and −509) may not be associated with PE, but elevated expression and production of TGFβ1 during pregnancy may affect susceptibility to PE. Future investigations are needed to clarify the role of this important multifunctional cytokine in PE.

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