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Evidence of sexual dimorphism in the placental function with severe preeclampsia

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ABSTRACT

Preeclampsia (PE) affects 5–8% of pregnancies and is responsible for 18% of maternal deaths in the US, and for long-term complications in mother and child. PE is an inflammatory state and may influence placental function in a sex-specific manner. We determined if there is a sexual dimorphism in the placental inflammatory and apoptotic responses in preeclamptic pregnancies. Placentas were collected from normotensive and preeclamptic pregnancies with either male or female fetuses (MPE and FPE respectively) after c-section at term with no labor. Expression patterns of markers of inflammation measured by ELISA, as well as hypoxia, apoptosis and angiogenesis markers measured by Western blotting were determined in the placenta. Consistent with previous studies, an increase in inflammation, hypoxia, and apoptotic cell death was observed in PE compared to normotensive pregnancies. Levels of TNFα, IL-6 and IL-8, and HIF-1α were significantly greater, whereas the angiogenic marker VEGF was significantly reduced in MPE vs. FPE. Sexual dimorphism was also observed in the activation of cell death: the number of TUNEL-positive cells, and the expression pro-apoptotic markers PUMA and Bax being higher in MPE vs. FPE. We also found an increase in the levels of protein and DNA-binding activity of NFκB p65 in MPE vs. FPE. In summary, we show here that in preeclamptic pregnancies the placentas of males were associated with significantly higher expression of inflammatory, hypoxia and apoptotic molecules but reduced expression of a pro-angiogenic marker compared to placentas of female fetuses. We propose that the transcription factor NFκB p65 might, at least partially, be involved in sexual dimorphism during PE.

1. Introduction

The syndrome of preeclampsia (PE) is defined by hypertension and significant proteinuria developed at or after 20 weeks of gestation in previously normotensive women and which resolves postpartum [1]. It is a multisystem disorder, which complicates 5–8% of pregnancies in the United States [1]. The incidence of PE has increased over past decades in association with increasing maternal age, and the incidence of diabetes, obesity and multiple births [2]. Several mechanisms have been suggested to play a role in the etiopathogenesis of PE including an abnormal immune response, defective placentation, relative placental hypoxia or ischemia and oxidative/nitrative stress [3]. This leads to an exaggerated maternal inflammatory response [4] and generalized maternal endothelial cell activation, the causes of which are still uncertain but thought to be triggered by angiogenic or other factors released from the placenta [5].

Sexual dimorphism is now increasingly recognized as a factor in placental function and placental disorders. Microarray analysis has shown distinct sexual dimorphism in gene expression in the human placenta, in particular immune genes were expressed at higher level in female placenta compared to male [6]. Gene expression in the placenta also responds to maternal inflammatory status in a sex-dependent manner [7]. Expression of 59 genes were changed in the placenta of women with asthma vs. no asthma with a female fetus compared to only 6 genes changed in those with asthma but a male fetus [8]. Some of these genes were associated with growth, inflammatory and immune pathways. Changes in diet provide distinctive signature of sexually dimorphic genes in placenta with expression generally higher in female than male placentas [9]. The male placenta has higher TLR4 expression and a greater production of TNFα in response to LPS than the female placenta, which may underlie the propensity to preterm birth in males [10].

Abbreviations: BMI, body mass index; TNFα, tumor necrosis factor alpha; IL-8, interleukin-8; IL-6, interleukin 6; PE, preeclampsia; HIF-1α, hypoxia inducible factor-1alpha; NFκB, nuclear factor kappa B; VEGF, vascular endothelial growth factor; PUMA, p53 upregulated modulator of apoptosis.

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A male fetus is more at a risk of poor outcome than the female fetus in association with complications such as placental insufficiency, preeclampsia, infection, IUGR and preterm delivery [11]. A Norwegian population-based study of 1.7 million singleton births has clearly identified that preterm delivery and perinatal mortality and morbidity are dominated by the male sex [12]. While some reports suggest that preeclampsia is more prevalent with male fetuses, the Norwegian study shows an increased incidence of PE at <37 weeks gestation with a female fetus [13], which may reflect the fact that male fetuses are delivered earlier due to other problems and may not therefore stay *in utero* to allow the mother to develop PE [14]. The male fetus is also associated with more vasoconstricted state in the maternal microcirculation and greater endothelial dysfunction of preeclamptic women compared to those with female fetus [15]. Despite all these data, the mechanism responsible for a sexual dimorphic effect in preeclampsia remains unknown. The aim of present study was to determine if there was a sexual dimorphism in the placenta from pregnancies complicated by PE in expression of markers for inflammation, hypoxia, apoptosis and angiogenesis.

2. Materials and methods

2.1. Ethical approval and study participants

Placentas were collected from the Labor and Delivery Unit at University Hospital under a protocol approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio with informed consent from patients. Placentas were collected immediately following delivery by c-section in the absence of labor from normotensive (CTRL, $n = 10$, 5 male/5 female) and severe preeclamptic pregnancies (PE, $n = 10$, 5 male/5 female). Women with chronic hypertension, diabetes, renal disease, multifetal gestations or any other medical complication together with smokers were also excluded from the study. Severe preeclampsia was defined as hypertension (systolic blood pressure >160 mmHg and/or diastolic blood pressure >110 mmHg on two occasions 2–240 h apart), and proteinuria (≥2 protein on dipstick) occurring after 20 weeks of gestation in a previously normotensive woman [16].

2.2. Materials

TUNEL assay kit was obtained from Roche Diagnostics, EMSA kit from Pierce, Thermo Scientific. Antibodies were purchased from BD Biosciences (HIF-1α), Santa Cruz (VEGF), Cell Signaling (NFκB p65, caspase-3, caspase-9, Bcl-2, p53, PUMA and BAX), and Sigma (β-actin). ELISA kits for TNFα, IL-6 and IL-8 were purchased from R&D Systems.

2.3. Tissue processing and sampling

A random sampling technique was used to collect tissue from 5 sites around the circumference of the placenta at least one inch from the periphery [17] Villous tissue was dissected out from beneath the chorionic plate, avoiding the basal plate, flash frozen and stored at −80 °C. Tissues were homogenized by bead beater (Biospec Products, USA) in lysis buffer as described [18]. Total protein in the homogenates was estimated using Bradford's reagent (BioRad).

2.4. ELISA

Fresh placental villous tissue homogenates were used to measure the levels of TNFα, IL-6 and IL-8 by ELISA by the manufacturer's protocol (R&D Systems).

2.5. TUNEL assay

Frozen sections from CTRL and PE placentas were analyzed by terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) according to the manufacturer's protocol. Nuclei were counterstained by DAPI (Invitrogen). Image acquisition was performed by fluorescence microscope (Nikon).

2.6. Western blotting

Proteins were separated on 4–20% gradient precast gels (Bio-Rad), transferred onto nitrocellulose membranes and blocked with 5% nonfat milk in 0.1% Tween, 20 mM Tris (pH7.5)-buffered saline (TTBS) (w/v) for 1 h. Blots were probed with primary antibody in 1% nonfat milk powder/TTBS overnight at 4 °C and were detected using HRP-conjugated secondary antibody in 5% nonfat milk/TTBS for 1 h. Products were visualized by chemiluminescent HRP substrate (Millipore). Band intensity was measured in a G:Box using Gene Snap and Gene Tools software (Syngene).

2.7. EMSA

For high salt extraction of nuclear proteins, frozen tissue (~50–100 mg) was homogenized in 1 ml of low salt buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, and 1 mM dithiothreitol) containing 0.1% NP-40 (Sigma) and protease inhibitors (Roche), followed by centrifugation at 16,000 × *g* for 5 min at 4 °C. The pellet was resuspended in 50 μl of high salt buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, and 0.2 mM EDTA) and further incubated on ice for 30 min for high salt extraction. The samples were centrifuged at 16,000 × *g* for 30 min at 4 °C, and the supernatant were transferred to a pre-chilled tube. Binding of p65 to consensus DNA sequence was performed using the chemiluminescent nucleic acid detection module according to the manufacturer's protocol (Thermo Scientific). 5′-Biotin labeled consensus sequence (sense: 5′-AGTTGAGGGGACTTTCCCAGGC-3' and antisense: 5′-GCCTGGGAAAGTCCCCTCAACT-3') was obtained from the nucleic acid core facility at UTHSCSA.

3. Statistical analysis

Data are reported as mean ± SEM. Comparisons between two groups were performed with Student's *t*-test. One-way Analyses of Variance (ANOVA) with Tukey's post hoc test were used where appropriate. $P < 0.05$ was considered as significant.

4. Results

4.1. Clinical data

The clinical and demographic characteristics of the patients involved in the present study are listed in Table 1. Systolic and diastolic blood pressures were significantly greater in the preeclamptic group than the controls. No difference in BMI, maternal age and gestational age were found between normotensive controls and preeclamptics with a female fetus. Among the groups with a male fetus, the gestational age of preeclamptics was slightly but significantly smaller than of normotensive pregnancies with no difference in other characteristics. Birth weight percentile did not differ between the groups. Placental weight is not routinely measured at University Hospital and was therefore not available.

4.2. Cytokine and chemokine production in preeclamptic placentas

Release of inflammatory cytokines by the placenta in response to hypoxia/ischemia may lead to increased levels of them in maternal circulation and endothelial dysfunction during preeclampsia [19]. Among the pro-inflammatory cytokines, placental TNFα and IL-6, as well as the chemokine IL-8, were previously reported to be elevated during preeclampsia [20]. As shown in Fig. 1, we found significantly higher levels of TNFα, IL-8 and IL-6 in both male and female placentas of preeclamptic women compared to normotensive controls. While there was no difference between male and female placentas in normotensive women, in preeclampsia, the male PE placenta had significantly higher levels of TNFα, IL-8 and IL-6 compared to female PE placenta.

4.3. Expression of markers for angiogenesis and hypoxia

It is well-known that preeclampsia is associated with altered production of angiogenic peptides caused by relative hypoxia in the placental tissue [21]. We measured expression of VEGF protein as a marker for angiogenesis and HIF-1α as a hypoxia marker. We found significantly reduced expression of VEGF in male preeclampsia (MPE) compared to the male CTRL (Fig. 2) but with no differences seen in female placentas. Previously we have already shown an increase in HIF1α levels in preeclamptic placentas [18]. In this