BBA Clinical 6 (2016) 25–30

Contents lists available at ScienceDirect

BBA Clinical

journal homepage: www.elsevier.com/locate/bbaclin

Apoptotic and stress signaling markers are augmented in preeclamptic placenta and umbilical cord

Syeda H. Afroze [a], Ram R. Kalagiri [b], Michelle Reyes [c], Jacqueline D. Zimmerman [c], Madhava R. Beeram [b], Nathan Drever [c], David C. Zawieja [a], Thomas J. Kuehl [b,c], Mohammad N. Uddin [b,c,d,*]

[a] Department of Medical Physiology, Texas A&M Health Science Center College of Medicine, Temple, TX, USA
[b] Department of Pediatrics, Baylor Scott & White Health, Texas A&M Health Science Center College of Medicine, Temple, TX, USA
[c] Department of Obstetrics and Gynecology, Baylor Scott & White Health, Texas A&M Health Science Center College of Medicine, Temple, TX, USA
[d] Department of Internal Medicine, Baylor Scott & White Health, Texas A&M Health Science Center College of Medicine, Temple, TX, USA

ARTICLE INFO

Article history:
Received 1 February 2016
Received in revised form 10 May 2016
Accepted 23 May 2016
Available online 25 May 2016

Keywords:
Apoptotic
Stress
Placenta
Pregnancy
Preeclampsia

ABSTRACT

Objective: Preeclampsia (preE) has a significant link to alterations of placental function leading to stress and apoptotic signaling, which pass the placental barrier and leave persistent defect in the circulation of the offspring. We assessed apoptotic signaling in placentas and umbilical cords from patients with and without preE.
Methods: We collected placental and cord tissues from 27 normal pregnant (NP) women and 20 preE consenting patients after delivery in an IRB approved prospective study. p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation, pro-apoptotic Bcl-2-associated X (Bax), anti-apoptotic Bcl-2, caspase-9, and pro-inflammatory cyclooxygenase-2 (Cox-2) were evaluated by western blot and immunohistochemistry. Comparisons were performed using Student's *t*-test.
Results: p38 phosphorylation (Placenta: 1.5 fold, Cord: 1.7 fold), ratio of Bax/Bcl-2 (Placenta: 1.7 fold, Cord: 2.2 fold), caspase-9 (Placenta: 1.5 fold, Cord: 1.8 fold) and Cox-2 (Placenta: 2.5 fold, Cord: 2.3 fold) were up-regulated ($p < 0.05$) in preE compared to NP patients. Average hospital stays for preE babies were longer than NP babies. No complications were reported for NP babies; however, all of preE babies had multiple complications.
Conclusions: Apoptotic and stress signaling are augmented in preE placenta and cord tissue that alter the intrauterine environment and activates the detrimental signaling that is transported to fetus.

1. Introduction

Preeclampsia (preE), is a clinical syndrome characterized by a systolic blood pressure (BP) of ≥140 mm Hg or a diastolic BP of ≥90 mm Hg accompanied by proteinuria of ≥0.3 g in a 24-hour urine sample that begins after 20 weeks gestation [1–4]. Its prevalence rate is 3 to 8% worldwide and it is one of the leading causes of maternal and fetal morbidity and mortality [1–4]. The mechanism of preE is extensively being studied; however the reason why preE occurs is elusive. Several theories point to abnormal placentation caused by hypoxic insults and oxidative stress leading to placental apoptosis is being pursued [1–4]. The placenta in turn releases vasculotoxic substances which passes the placental barrier and goes to the circulation of the offspring that may predispose to a pathological response to the fetus, upon birth or later in life [1–4].

Theories proposed to cause preeclampsia include angiogenic imbalance [2,5,6], autoantibodies to angiotensin type 1 (AT_1) receptor [7,8], cardiotonic steroids [9–11], genetic predisposition [3] and immunological factors [12]. The angiogenic imbalance is the effect of vascular endothelial growth factor (VEGF) on the proliferation and survival of endothelial cells. It has a vasodilator effect on the systemic vessels that increases vascular permeability [2,3,5,12]. However, it was found that there is an increased sensitivity to elevated levels of circulating angiotensin receptor AT_1 activating antibody that may lead to preE [7,8]. The cardiotonic steroids particularly Marinobufagenin (MBG) from the bufodienolide group has the ability to inhibit the Na^+/K^+ ATPase, increase plasma MBG promotes volume expansion and hypertension [3,9–11]. MBG has also been shown to alter the proliferation and migration of specialized cytotrophoblasts (CTBs) in the placenta thereby decreases the perfusion of the feto-maternal unit resulting in oxidative stress and endothelial dysfunction [9–11,13]. MBG plays a major role in the pathogenesis of preE including activation of apoptotic signaling and alteration of endothelial cell growth [3,11,14].

preE can occur through two connected pathways placental trophoblast dysfunction and endothelial dysfunction within the maternal

* Corresponding author at: Scott & White Hospital (Building 1) Room 352, 2401 South 31st Street, Temple, TX 76508, USA.
E-mail address: MNUDDIN@sw.org (M.N. Uddin).

http://dx.doi.org/10.1016/j.bbacli.2016.05.003

systemic vasculature. The formation of various toxic compounds within the placenta such as vasoconstricting agents and altered cytokines can cause greater oxidative stress leading to endothelial dysfunction [15]. This refers to the fact that several endothelial cells did not show the proper response to specific stimuli in preE women. One study by Gant et al. found vascular resistance produced in response to increases in levels of angiotensin II was lost in preE patients [16,17]. Possible culprit factors for endothelial dysfunction include Platelet-activating factor and P-selectin, which when unregulated favored increased platelet activity and endothelial retraction [18,19]. Once a preE pregnancy is terminated however, disturbances in maternal circulation dissolve rapidly due to elimination of these placental factors [19]. Indeed, when endothelial dysfunction is combined with pre-existing conditions such as vascular, renal, and metabolic diseases and other genetic factors, there is a much greater risk for developing preE. While placental pathophysiology is not the primary pathway for developing preE, it is an important contributor in the development of the disorder during pregnancy.

In this prospective study, we assessed the apoptotic and stress signaling proteins in the placenta and umbilical cord of normal pregnant and preE patients The outcomes of the pregnancy were also been followed. The purpose of the study is to correlate the presence of stress and apoptotic signaling markers in the placenta and umbilical cord and their relationship to the outcome in the offspring.

2. Methods and materials

2.1. Human subjects

In this study, we recruited 20 pregnant women who present with preeclampsia defined as BP ≥ 140/90 with proteinuria of >300 mg of protein in a 24 hours urine sample and 27 pregnant women with uncomplicated pregnancy as control from the department of Obstetrics and Gynecology at Baylor Scott & White Hospital in Temple Texas. The hospital's Institutional Review Board approved the study and informed consent was granted by the subjects. The clinical status and assessments at the time of admission for maternal symptoms and maternal age were taken. The infants' weight, length, gestational age, length of hospitalization and associated morbidities were recorded. Samples of placenta and umbilical cord were collected from the recruited subjects after delivery.

The p38 MAPK phosphorylation was evaluated by Western blot. Apoptotic markers; Bcl-2 associated X protein (Bax), pro-apoptotic Bcl-2 protein, caspase-9 and pro-inflammatory protein cyclo-oxygenase-2 (Cox-2) expression were assayed both by Western blot and immunohistochemistry.

2.2. Western blot analysis for p38 MAPK, Bax, Bcl-2, caspase-9 and Cox-2 proteins

Placenta and umbilical cord were homogenized with a cell lysis buffer (Cell Signaling Technology) containing 0.1 M Tris at pH 7.4, 50 M NaCl, 0.5 M EDTA at pH 8, Igepal and water and protease inhibitor cocktail. Protein concentrations were determined by BCA reagent (Pierce, Rockford, IL). An equal amount of protein in each sample was separated using NuPage Novex 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk probed with anti-p38 MAPK, Bax, Bcl-2, caspase-9 and Cox-2 antibodies. After incubation with the corresponding secondary antibody, proteins were visualized with chemiluminescence detection system (Pierce). The intensity of the bands was determined using ImageQuant LAS 4000 (GE Healthcare Life Sciences). The expression of p38 MAPK, Bax, Bcl-2, caspase-9 and Cox-2 was quantified by densitometry analysis using Image J software where the target protein is normalized to a structural protein (β-actin) to control between groups and ensures correction for the amount of total protein on the membrane. The phospho-p38 was normalized to total p38.

2.3. Immunohistochemistry (IHC) of placenta and umbilical cord samples from preE and NP patients

Placenta and umbilical cord samples were frozen in Optimal Cutting Temperature (OCT) compound and cut on the Cryostat as 20 micron thick slices. Tissue slices were put on positively charged slides. Slides were incubated at 37 °C for 15 min and washed in PBS (phosphate buffered saline) for 5 min. Slides were places in 0.01% hydrogen peroxide for 20 min and washed in PBS for 5 min. A hydrophobic pen was used to circle tissue sections and 5% goat serum was added to the circled tissue sections for 2 h. Slides were placed in a humidified box and the Anti-Bax, Bcl-2, caspase-9 and Cox-2 antibodies (Abcam) were added in 1% goat serum. Negative control samples were placed in the humidified box without the antibody and only 1% goat serum. The humidified box was placed in the refrigerator overnight. Slides were washed in PBS three times for 30 min. The secondary antibody was added in 1% goat serum to slides for 2 h. Then slides were again washed in PBS three times for 30 min. Diaminobenzidine was added to the slides for less than 30 s then washed in water. Slides were then dipped 10 times in 50% alcohol, 70% alcohol, 90% alcohol, 95% alcohol, and xylene consecutively. Finally, dibutyl phthalate xylene mountant was added to the slides and a coverslip was placed over the tissue.

2.4. Statistical methods

Heterogeneity of variance of patient characteristics and assay values were examined with Levene's test. For those measurements with heterogeneous variance, Mann-Whitney *U* tests were used for comparisons of the two patient groups. Otherwise, Student's *t*-tests were used to compare groups. Data from the NP patients were compared to preE patients using Student's *t*-test. A p-value of less than 0.05 was considered significant.

3. Results

3.1. Human data

There was no significant difference between the normal pregnant and preE patients in terms of maternal age, maternal height and mean gestational age at birth. As expected, pregnant patients with symptoms of preE differed from those with normal pregnancies in variables related to these symptoms. The mean systolic BP for the preE patients (166 ± 11 mm Hg) differed from the normal patients (122 ± 10 mm Hg) respectively ($p < 0.0001$). The mean diastolic BP differed were 93 ± 10 and 74 ± 9 mm Hg, respectively (p 0.0001). The mean urinary protein levels differed was 1974 ± 1149 mg/24 h in the preE group and 149.0 ± 56.6 mg/24 h in the NP group ($p = 0.0001$). We did not find any difference in body mass index (BMI) between NP (30.6 ± 6.9 (27)) and preE women (34.2 ± 7.8 (20)), $p = 0.095$. Patients with NP were about 4 weeks further along in pregnancy than those with preE pregnancies, but the range of gestations for both groups was 28 to 39 weeks of gestation (Table 1).

We divided the preE subjects into early (before 34 weeks) and late preE (after 34 weeks) groups and compared their outcomes. The placental thickness in early preE subjects was 25 mm compared to 32 mm in late preE ($p = 0.05$) and placental volume in early preE 296 cm^3 compared to 393 cm^3 ($p = 0.0498$). Gestational age at delivery in early preE is 32.4 weeks vs 36.8 weeks in late preE ($p = 0.011$). About 56% of the infants (5 out of 9) who are born to early preE are small for gestational age (SGA) and 30% of the infants (3 out of 10) who are born to late preE are SGA (Fig. 1). Qualitative demographic variables are shown in Table 2.

3.2. WB data for p38 MAPK, Bax, Bcl-2, caspase-9 and Cox-2 proteins

The p38 MAPK phosphorylation in both the placenta and umbilical cord was upregulated in preE patients compared to control (*$p < 0.05$)