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Preeclampsia serum induces human glomerular vascular endothelial cell hyperpermeability via the HMGB1-Caveolin-1 pathway

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ABSTRACT

To explore new ideas about the pathogeny of preeclampsia (PE) proteinuria, this study focused on whether severe PE serum (PES) could induce high-molecular-weight protein (HMWP) hyperpermeability in glomerular endothelial cells (GEC) via the HMGB1-Caveolin-1 (CAV-1) pathway. Normal pregnancy serum (NPS) and severe PES were used to treat primary human GEC monolayer for 24 h. The CAV-1 inhibitor methyl-beta-cyclodextrin (MBCD), the HMGB1 inhibitor glycyrrhizicacid (GA), recombinant HMGB1 (rHMGB1) were also used to treat GEC monolayer that were stimulated by NPS or severe PES. The dynamic permeability of GEC to HMWP was detected by Evans blue-labeled BSA and CAV-1 expression in GEC was analyzed by immunofluorescence staining and Western blotting. We detected HMGB1 expression in placenta and serum in normal pregnancy and severe PE. The results showed that severe PES significantly promoted GEC hyperpermeability and CAV-1 expression. By inhibiting CAV-1 expression, MBCD reversed severe PES-induced GEC monolayer permeability. HMGB1 expression in PE placenta and serum was significantly increased. Compared with that in normal placenta, HMGB1expression was increased in the cytoplasm of syncytiotrophoblast cells in PE placenta. GA decreased the severe PES-induced hyperpermeability and CAV-1 expression in GEC. rHMGB1 induced high expression levels of CAV-1 and HMWP hyperpermeability in GEC. In conclusion, HMGB1 is increased in severe PE patients and induces the expression of CAV-1 in GEC. High expression of CAV-1 in GEC can promote HMWP hyperpermeability, which may contribute to the development of PE proteinuria.

1. Introduction

Preeclampsia (PE) is a common hypertensive disorder in pregnancy that is characterized by hypertension, proteinuria and other systemic disturbances at or after 20 weeks of gestation. Increased proteinuria can lead to serious hypoproteinemia, a possible risk factor for failing expectant management of early-onset PE. However, the underlying mechanism of proteinuria remains unknown. Disease symptoms generally abate following delivery, suggesting that the placenta plays a central role in PE (Wang et al., 2014). Placental implantation in PE features abnormal trophoblastic invasion of uterine vessels (Jiang et al., 2012). Abnormal trophoblastic invasion can result in placenta hypoxia and anoxia stress and is associated with an increased inflammatory state. HMGB1 can be released into maternal circulation when the placenta becomes hypoxic in PE (Holmlund et al., 2007; Riley and Nelson, 2009).

HMGB1 is a nuclear protein that acts as an architectural chromatin-binding factor. HMGB1 is involved in the maintenance of nucleosome structure and the regulation of gene transcription (Muller et al., 2001; Thomas, 2001). It can be released into the extracellular milieu from immune and nonimmune cells in response to various stimuli, including hypoxia and ischemia (Hamada et al., 2008). Our main concern was whether HMGB1 enhanced the permeability of human glomerular endothelial cells (GEC) to high-molecular-weight protein (HMWP).

GEC, which are located inside the capillary walls of the glomerulus, play vital roles in albumin permeability. Caveolae are a type of plasma membrane invaginations with a diameter of 50–100 nm. Caveolae are abundant in endothelial cells of intramuscular capillaries with

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approximately 73 caveolae per μm^2 of endothelium (Gabella and Blundell, 1978). Caveolin-1(CAV-1), a 21-22KD protein, is a structural protein responsible for maintaining the Ω shape of caveolae. CAV-1 is involved in cholesterol trafficking and homeostasis (Bosch et al., 2011). However, whether CAV-1 regulates the barrier function of GEC to HMWP in PE needs further investigation.

Thus, our study was designed to answer the following questions: such as whether hypoxic placenta trophoblast cells produce the danger signal HMGB1; whether HMGB1 in PE leads to hyperpermeability of GEC monolayer; and whether CAV-1 expression is related to the GEC monolayer permeability.

2. Materials and methods

2.1. Patient selection

This study was approved by the Institutional Review Board of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. All participants provided informed consents and permissions to collect placenta and blood. PE was defined following the guidelines of the American College of Obstetricians and Gynecologists (Roberts et al., 2013). Maternal maximum systolic blood pressure of 160 mmHg and/or diastolic blood pressure of 110 mmHg on admission was defined as severe PE. Twenty women with severe PE at 25–32 weeks of gestation and twenty women with uncomplicated pregnancies at the same period were enrolled in this study. None of the patients had a medical history of liver or kidney disease, diabetes, primary hypertension, or other cardiovascular diseases.

2.2. Sample collection

Twenty milliliters of peripheral blood were collected from each patient and stored separately. The serum from severe PE patients were obtained after diagnosis but before receiving treatment. The serum from normal pregnancy patients obtained during routine prenatal examinations served as control. Maternal venous serum was frozen at -80℃ until analysis. Placental biopsies were collected from the maternal aspect of the placenta within 15 min after delivery. Five small separate biopsies were taken from the placental center and each quadrant to avoid sampling bias. All samples included full-thickness placental blocks.

2.3. Culture of human GEC

Human GEC were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). These cells were isolated from human kidneys and they can be characterized by immunofluorescence with antibodies specific to vWF and CD31. GEC were seeded into flasks and dishes and were cultivated with endothelial cell medium (ECM, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS), 1% endothelial cell growth supplement, and 1% penicillin/streptomycin solution.

2.4. Cytotoxicity assays

The cytotoxicity of methyl-beta-cyclodextrin (MBCD, Sigma) and glycyrrhizic acid (GA, Sigma) was assessed using CCK-8 assays (DojinDo). Briefly, GEC ($1*10^4$ cells/well) were seeded in a 96-well flat-bottom plate, cultured for 48 h, and then placed in serum-starved conditions for an additional 8 h. Subsequently, cells were incubated with 100 μl of ECM with MBCD (1, 2, 3, 4, 5, 6, and 7 mM) or GA (1, 2, 3, 4, 5, 6, and 7 mM) for 24 h. Moreover, GEC were incubated with serum of normal pregnancy patients in a concentration gradient (1%, 5%, 10%, 15%, 20%, and 25%) for 24 h. Five wells per group were tested at each concentration on the gradient. After 10 μl of CCK-8 solution was added to each well, cells were incubated at 37℃ for 2 h, and the absorbance was measured at 450 nm using a microplate reader.

2.5. CAV-1 gene silencing in GEC by small-interfering RNA (siRNA)

GEC ($2*10^5$ cells/well) were plated into a six-well culture plate. At 60–70% confluence, GEC were transfected with Opti-MEM with RNAi-MAX (Invitrogen) and CAV-1-siRNA (Gene Pharma, China) for a period of 24 h. After changing the ECM, the cells were cultured for another 24 h, and then, GEC monolayer permeability was measured. The sequences of the CAV-1 siRNA and CAV-1 primers are listed in the supplementary table.

2.6. Permeability of GEC

Permeability was quantified by spectrophotometric measurement of the flux of Evans blue-bound bovine serum albumin (BSA) across functional GEC monolayer using a Transwell (Corning) model as previously described (Lee et al., 2012). Briefly, GEC were plated ($5*10^4$cells/well) in 4-μm pore size and 6.5-mm diameter Transwells for 3 days to obtain a confluent monolayer. The confluent monolayer were incubated with the collected PE serum (PES), normal pregnancy serum (NPS), and serum pretreated with MBCD, GA or rHMGB1(1 μg/ml, Chimerigen Laboratories) for 24 h (Zhang et al., 2011; Jiang et al., 2014). Wells with NPS alone were used as controls. Inserts were washed with PBS before adding 100 μl of 0.67 mg/ml Evans blue (Sigma) diluted in growth medium containing 4% BSA. Fresh growth medium was added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue-BSA solution. After 20 min, the OD at 650 nm was measured in the lower chamber.

2.7. Immunofluorescence analyses

Immunofluorescence analysis was performed as previously described (Zhang et al., 2003). GEC were seeded on cover slips in 6-well plates and cultured to 70–80% confluence and then incubated with PES, NPS and/or MBCD for 24 h. The cells were fixed in 4% paraformaldehyde for 15 min. After being blocked with 5% BSA for 30 min, the cells were probed with anti-CAV-1 antibodies (1:200, CST) overnight at 4 ℃, and then incubated with secondary antibodies (1:3000, Jackson). To visualize the nucleus, the cells were stained with DAPI (Sigma). Fluorescence images were observed under a laser-scaning confocal microscope (Zeiss). GEC at passage 5 were labeled with vWF antibodies (1:200, Abcam) to evaluate whether the GEC at passage 5 still had stable endothelial features.

2.8. Immunohistochemical analyses

Immunohistochemical analysis was performed as previously described (Lv et al., 2016). Paraffin-embedded placental tissue sections were deparaffinized in xylene and hydrated with ethanol followed by microwave treatment in antigen unmasking solution for 10 min. After treatment with 3% hydrogen peroxide for 15 min and blocking with 10% BSA for 30 min, the tissue sections were probed with anti-HMGB1 antibodies (1:400, Abcam) followed by incubation with the biotinylated secondary antibodies (1:3000, Jackson). The images were captured using a Nikon fluorescence microscope. Scoring was based on the percentage of positive cytoplasm staining: 0–10% scored 0; 10–35% scored 1; 36–70% scored 2; and more than 70% scored 3. We used the final score to determine a high or low expression as follows: a score of 0 or 1 indicated low expression, and a score of 2 or 3 indicated high expression. The scoring was performed by two senior pathologists in a blinded manner.

2.9. Enzyme-linked immunosorbent assay

The serum collected from the above mentioned 20 normal pregnancy and 20 PE patients were used to determine HMGB1 levels with an ELISA kit (IBL) according to the manufacturer's instructions.