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The defect of both angiogenesis and lymphangiogenesis is involved in preeclampsia

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ABSTRACT

Introduction: Pre-eclampsia (PE) is characterized by failed remodeling of maternal vessels perfusing the placenta. Blood vessels and lymphatic system are involved in vessel remodeling and flow homeostasis in the uterus during pregnancy. This study aims to investigate the involvement of angiogenesis and lymphangiogenesis in PE.

Methods: Placental and decidual tissues were obtained from pregnancies with PE ($n = 90$), including PE cases with decidual vasculopathy (DV) ($n = 52$) and without DV ($n = 38$), and healthy pregnancies (control, $n = 20$). The clinical characteristics of these groups were analyzed. The expression levels of VEGF1, CD34, PROX-1, VEGFR3, and CD31 in the placenta and decidua were detected through immunohistochemistry, reverse-transcription polymerase chain reaction, and Western blot.

Results: The lymphangiogenic markers PROX-1 and VEGFR3 were negatively expressed in the placenta but positively expressed in the decidua. The expression levels of the angiogenic markers VEGF1 and CD34 and the panendothelial marker CD31 were significantly lower in the placenta and decidua of the PE group than in those of the control group. The expression levels of VEGF1, CD34, and CD31 were significantly lower in the placenta and decidua with DV than in those without DV. Furthermore, the expression trends of PROX-1 and VEGFR3 was similar to those of VEGF1, CD34, and CD31 among the groups.

Conclusions: Lymphangiogenesis occurred in the decidua but not in the placenta. Impaired angiogenesis and lymphangiogenesis were associated with PE, particularly in the presence of DV.

1. Introduction

Pre-eclampsia (PE) affects approximately 2.5%–3% of all pregnancies and is a leading cause of maternal morbidity and mortality in developing countries [1]. Although the exact pathogenesis of PE remains unclear, the placenta and decidua may play important roles in this condition. A defective placenta during remodeling of spiral arteries can release many factors, resulting in systemic endothelial dysfunction and PE [2]. Moreover, the spiral arteries of patients with PE (approximately 40%–60%) undergo pathological changes but are unaffected by physiological remodeling during pregnancy. These changes occur in the decidua, an important fetal–maternal interface, and referred as decidual vasculopathy (DV) or acute atherosis [3]. Several studies suggested that DV is related to poor clinical outcomes and increased placental pathology [4,5]. Hence, the dysfunctional uteroplacental blood flow is assumed as the common pathway in the early preclinical stages that lead to PE [2].

Maternal uterine blood vessels undergo dramatic vascular remodeling to accommodate increased uteroplacental blood flow during pregnancy. An adequate uteroplacental blood flow is essential for normal placental perfusion and critical to fetal health,

Abbreviations: BMI, body mass index; PE, preeclampsia; DV, decidual vasculopathy; RT-PCR, reverse-transcription polymerase chain reaction; pre-BMI, pre-pregnancy body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Pr/Cr, urine protein-to-creatinine ratio; LDH, lactate dehydrogenase; AST, aspartate transaminase; ALT, alanine transaminase; PLT, platelet count; VEGF, vascular endothelial growth factor; PROX-1, prospero-related homeobox gene-1; VEGFR3, vascular endothelial growth factor receptor 3.

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survival, and successful pregnancy [6]. In addition to uterine blood vessels, the uterine lymphatic vessel system significantly changes in the vascular remodeling process during pregnancy [7,8]. To date, studies on placental lymphatic development have been limited and have provided significantly different or even opposing conclusions [9–12]. Nevertheless, lymphatic vessels have been recently identified in the decidua [8,12,13].

Blood vessels and lymphatic system are important in vessel remodeling and fluid homeostasis in the uterus during pregnancy. Thus, this study aims to investigate the involvement of angiogenesis and lymphangiogenesis in PE.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Institutional Review Board of the Guangdong Women and Children Hospital.

2.2. Tissue sample collection

Pregnancies with PE and healthy pregnancies (control) were admitted for delivery at the Guangdong Women and Children Hospital on 2011 to 2013. Women who delivered through the caesarean route with complete pregnancy data and single pregnancy were included in this study. Caesarean section was performed in the pre-eclamptic patients because vaginal delivery was unsuitable considering the disease progression and/or unfavorable cervical ripening. The initial available total population of women with PE was 121. A total of 90 cases with PE were finally enrolled in this study after applying the exclusion criteria, including incomplete data ($n = 8$), multiple pregnancy ($n = 7$), and unavailability of placental samples for histological reanalysis ($n = 16$). Control subjects included healthy normotensive women who had no antenatal medical or obstetric complications, presented normal fetal growth at ≥37 weeks of gestation, and underwent caesarean section between 37 and 42 weeks of gestation because of the following indications: breech presentation, transverse presentation, fetal macrosomia, cephalopelvic disproportion, or psychological reasons. The initial number of women in the control group was 45. A total of 20 women were set as the controls after applying the exclusion criteria, including incomplete data ($n = 13$), multiple pregnancy ($n = 3$), and unavailability of placental samples for histological reanalysis ($n = 9$).

Placental and decidual tissues were collected as previously described by Staff et al. [14] and Harsem et al. [15]. Decidua basalis is the area between the placenta and the mother that is poorly studied in PE [41]. Thus, the deciduas used in this study were mainly obtained from the placental bed, i.e., decidua basalis. The collected samples were divided into two parts: the first portion was fixed in 10% formaldehyde solution and paraffin-embedded for immunohistochemical staining, whereas the remaining portion was immersed into liquid nitrogen for further study. All sections were counterstained with hematoxylin and observed under a bright-field microscope. DV was defined as vascular fibrinoid necrosis and lipid-filled foam cells in the vascular wall of spiral arteries in the decidua basalis or parietalis [16,17]. Hence, pregnancies with PE were further divided into two subgroups: without DV ($n = 38$) and with DV ($n = 52$).

PE was defined as gestational hypertension with proteinuria (with blood pressure values ≥140/90 mm Hg on two measurements at least 6 h apart and 24 h urinary protein ≥300 mg or urine dipstick protein ≥ +) after the 20th week of pregnancy of previously normotensive and non-proteinuric women [18].

2.3. Immunohistochemistry analysis

In brief, 5 μm sections of formalin-fixed and paraffin-embedded tissues were cut on silanized glass slides, deparaffinized in xylene, rehydrated in graded ethanol concentrations (100%, 95%, 70%, and 50%), and submerged in phosphate-buffered saline. The sections were blocked using endogenous peroxidase with 3% hydrogen peroxide solution for 15 min and then placed in an autoclave with 0.01 mol/L sodium citrate solution at 121 °C for 3 min to remove antigens. The sections were incubated with the primary antibodies of vascular endothelial growth factor 1 (VEGF1, 1:100), CD34 (1:100), prospero-related homeobox gene-1 (PROX-1, 1:100), VEGF receptor 3 (VEGFR3, 1:50), and CD31 (1:200) overnight at 4 °C. The sections were further incubated with biotin-labeled secondary antibody at room temperature for 1 h. Negative controls were obtained by replacing the primary antibodies with phosphate-buffered saline. Diaminobenzidine tetrahydrochloride was used as a chromogen. All sections were counterstained with hematoxylin and then observed under a bright-field microscope. The antibodies and their companies of origin were as follows: VEGF1 (rabbit polyclonal antibody, PA513297) from Pierce (Rockford, IL, USA), CD31 (mouse monoclonal, clone JC70A) and CD34 (mouse monoclonal, clone M7165) from Dako Laboratories (Carpinteria, CA, USA), VEGFR3 (rabbit polyclonal antibody) from Reliatech GmbH (Braunschweig, Germany), and Prox-1 (rabbit polyclonal antibody) from Novus Biologicals Inc. (Littleton, Co., USA).

The expression of different proteins was determined by semi-quantitatively assessing the percentage of stained cells and the staining intensity using a previously described method with minor modifications [19]. The percentage of positive cells was rated as follows: 2 points, 11%–50% positive tumor cells; 3 points, 51%–80% positive cells; and 4 points, >81% positive cells. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. The obtained points for the expression and percentage of positive cells were added. The specimens were categorized according to their overall score into four groups: negative (−), 0 to 1 point, ≤ 10% positively stained cells, regardless of the intensity; weak expression (+), 2 to 3 points; moderate expression (++), 4 to 5 points; and strong expression (+++), 6 to 7 points. A blinded analysis was performed.

Table 1
Primers used for RT-PCR.

Primer	Sequence	Product size (bp)
VEGF1	F: GCCTTGCTGCTCTACCTCC R: GCAGTAGCTGCGCTGATAGA	120
CD34	F: ACCCTGATTGCACTGGTCAC R: GTCTTCGCCCAGCCTTTCTC	114
PROX-1	F: GAGATGTGCGAGCTAGACCC R: GAGGCAGACTGGTCAGAGGA	467
VEGFR3	F: AGTCACACGTCATCGACACC R: GCGTCCTTCCTCTCACGAAC	300
CD31	F: AGACGTGCAGTACACGGAAG R: AGTATCTGCTTTCCACGGCA	128
β-actin	F: GGGGAGCTGTCACATCCAG R: GAAGCATTTGCGGTGGACG	192

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from treated and untreated cultures using TRIzol reagent according to the manufacturer's protocol (Invitrogen). First-strand cDNA was synthesized using a PrimeScript RT-PCR reagent kit (Takara, China) according to the manufacturer's instructions. The specific primers used for RT-PCR are shown in Table 1. β-actin was used as the reference control gene to normalize the expression of the target genes. The PCR products were separated through electrophoresis on 1.5% agarose gels and then visualized with ethidium bromide under UV light. Images were analyzed with the GEL DOC 2000 system (Bio-Rad, USA). The relative expression level (%) was calculated by dividing the gene band density by the β-actin band density.

2.5. Western blot analysis

In brief, samples were prepared in a homogenizer with lysis buffer (Tris HCL, pH 8, 20 mM; NaCl, 137 mM; EDTA, 2 mM; glycerol, 10%; and Nonidet P-40, 1%) and protease inhibitor cocktail (Thermo Scientific). The homogenates were centrifuged at 14,000 *g* for 10 min at 4 °C. The supernatant containing the target protein was used for Western blot analysis. Protein concentrations were determined using the BCA protein assay (Thermo Scientific). Equal quantities of protein (40 μg) were subjected

Table 2
Clinical characteristics of the pregnant women included in the study.

Characteristics	DV (n = 52)	Non-DV (n = 38)	Controls (n = 20)	*P*
Maternal age (years)	33.2 ± 2.7	32.4 ± 2.8	31.6 ± 2.4	0.067
Pre-BMI (kg/m^2)	23.9 ± 1.1	23.8 ± 0.4	23.5 ± 0.7	0.166
Primigravid	35 (67.3%)	26 (68.4%)	15 (75%)	0.814
SBP (mmHg)	161.7 ± 8.5[a]	164.5 ± 8.7[a]	118.6 ± 6.8	0.000
DBP (mmHg)	111.2 ± 7.7[a,b]	106.1 ± 6.5[a]	69.8 ± 5.3	0.000
Delivery weeks	35.3 ± 0.9[a,b]	36.4 ± 0.4[a]	39.2 ± 0.3	0.000
Fetal weight (g)	2327.2 ± 132.2[a,b]	2547.5 ± 89.1[a]	3137.6 ± 95.7	0.000
APGAR 1 min	5 (3–8)[a]	6 (3–8)[a]	7 (5–9)	0.000
APGAR 5 min	8 (6–10)[a]	8 (5–10)[a]	10 (9–10)	0.000
Urine Pr/Cr (g/10 mmol)	2.41 ± 0.78[a]	2.30 ± 0.61[a]	0.09 ± 0.03	0.000
Use of antihypertensive	31 (59.6%)	20 (52.6%)	–	0.509
Use of magnesium sulfate	24 (46.2%)	13 (34.2%)	–	0.255
PLT (10^9/l)	123.8 ± 25.8[a]	137.4 ± 30.3[a]	186 ± 31	0.000
LDH (U/l)	374.5 ± 84.1[a]	401.0 ± 90.0[a]	176.2 ± 20.0	0.000
AST (U/l)	73.7 ± 30.3[a]	80.7 ± 26.8[a]	23.7 ± 8.3	0.000
ALT (U/l)	71.9 ± 28.9[a]	80.1 ± 28.4[a]	20.5 ± 7.6	0.000
Use of tobacco	9 (17.3%)	5 (13.2%)	2 (10%)	0.701

[a] *P* value < 0.05 VS controls.
[b] *P* value < 0.05 VS non-DV.