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Original article

Downregulation of indoleamine 2, 3-dioxygenase expression in the villous stromal endothelial cells of placentas with preeclampsia

Naoyuki Iwahashi*, Madoka Yamamoto, Sakiko Nanjo, Saori Toujima, Sawako Minami, Kazuhiko Ino

Department of Obstetrics and Gynecology, Wakayama Medical University, 811-1 Kimiidera, Wakayama,641-0012, Japan

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ABSTRACT

Introduction: Previous studies have shown that indoleamine 2, 3-dioxygenase (IDO), an immunosuppressive enzyme that converts tryptophan to kynurenine, is expressed in the placenta and might play a role in the maintenance of pregnancy, although its associations with the pathogeneses of preeclampsia (PE) and fetal growth restriction (FGR) remain unclear. The objective of this study was to investigate the differences in IDO expression among normal, PE, and FGR placentas, and the associations between IDO expression and clinical symptoms, or the expression of fms-like tyrosine kinase receptor-1 (Flt-1).
Methods: Immunohistochemical studies of IDO and Flt-1 expression were performed in human placentas that were complicated with FGR alone (n = 19), PE alone (n = 20), or both PE and FGR (n = 39), and gestational age-matched controls (n = 23).
Results: It was found that IDO was expressed on endothelial cells in the villous stroma, while Flt-1 was located on trophoblast cells. The IDO expression level of the PE alone group was significantly lower than those of the FGR alone and control groups. The IDO expression of the PE + FGR group was significantly lower than that of the FGR alone group. Lower IDO expression was significantly correlated with more severe maternal hypertension or proteinuria in PE patients, who exhibited higher Flt-1 expression. The late onset PE patients exhibited significantly lower IDO expression than the early onset PE patients.
Conclusion: This study demonstrated that the downregulation of IDO expression on the endothelial cells of the villous stroma was associated with PE, but not FGR, suggesting that IDO might be involved in the pathophysiology of PE.

1. Introduction

Preeclampsia (PE) is one of the most common and crucial complications of pregnancy and can cause maternal morbidity and mortality. It is characterized by the development of maternal hypertension and significant proteinuria preceded by endothelial cell activation and an inappropriate inflammatory response (MacKay et al., 2001). A considerable body of evidence indicates that PE has an immunological etiology (Kudo et al., 2003; Saito and Nakashima, 2014; Nishizawa et al., 2007). PE is frequently accompanied by fetal growth restriction (FGR). Placental ischemia is involved in the development of PE. It also causes fetal hypoxia and acidosis, and subsequently leads to FGR as well as adverse maternal and neonatal outcomes (Zamudio et al., 1995; Soothill et al., 1987). However, the pathogeneses of these severe complications are complex and not fully understood.

Recent studies have suggested that the breakdown of immune tolerance, hypoxia, oxidative stress, excessive inflammation, and increased production of anti-angiogenic factors are associated with the etiology of PE (Saito and Nakashima, 2014; Nishizawa et al., 2011). Fms-like-tyrosine-kinase receptor-1 (Flt-1), a vascular endothelial growth factor (VEGF) receptor, is expressed in the human placenta throughout gestation and contributes to the regulation of placental angiogenesis (Andraweera et al., 2012). In PE patients, its placental expression increases proportionally with the severity of the disease (Andraweera et al., 2012; Maynard et al., 2003; Gu et al., 2008; Munaut et al., 2012; Helske et al., 2001). In contrast, soluble Flt-1 (sFlt-1), a VEGF antagonist, is known to have potent antiangiogenic properties (Andraweera et al., 2012; Maynard et al., 2003). It has been reported that an imbalance between angiogenic and anti-angiogenic pathways in the placenta and increased secretion of sFlt-1 into the maternal plasma circulation might contribute to maternal endothelial dysfunction,

* Corresponding author.
E-mail addresses: naoyuki@wakayama-med.ac.jp (N. Iwahashi), madoka-y@wakayama-med.ac.jp (M. Yamamoto), sakiko-n@wakayama-med.ac.jp (S. Nanjo), toujima@wakayama-med.ac.jp (S. Toujima), awako@wakayama-med.ac.jp (S. Minami), kazuino@wakayama-med.ac.jp (K. Ino).

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resulting in the clinical symptoms of PE (Andraweera et al., 2012; Maynard et al., 2003; Gu et al., 2008; Munaut et al., 2012).

From the viewpoint of the immunological response network, pregnancy is a unique phenomenon as the fetus, which possesses paternal antigens, is not normally rejected by the maternal immune system. In recent years, it has been reported that indoleamine 2,3-dioxygenase (IDO), an immunosuppressive enzyme that mediates the conversion of tryptophan to kynurenine, might play a key role in placental development during normal pregnancy (Kudo et al., 2003; Munn et al., 1998; Mellor et al., 2001). In early pregnancy, IDO is mainly expressed on extravillous trophoblasts and might be involved in immune tolerance at the feto-maternal interface (Hönig et al., 2004). In term placentas, IDO is reported to be present in the endothelia of larger vessels in the terminal villi, although its role is still unclear (Blaschitz et al., 2011; Ligam et al., 2005). While some previous studies have examined IDO enzymatic activity in PE placentas (Nishizawa et al., 2007), little is known about human placental IDO expression and its functional role in pregnancies complicated by PE.

Here, we focused on the possible role of endothelial IDO in the villous stromal vessels of 3rd trimester placentas from patients with PE, and investigated IDO expression among placentas associated with FGR alone, PE alone, or both PE and FGR (PE + FGR). Furthermore, we analyzed the associations between IDO expression and the severity of clinical symptoms, or the onset of PE, and compared those with Flt-1 expression.

2. Materials and methods

2.1. Study subjects

This retrospective analysis included 101 females who delivered their babies between January 1, 2006, and December 31, 2010, at Wakayama Medical University Hospital. Human placenta samples were collected from 3rd trimester pregnancies that were complicated with FGR alone (n = 19), PE alone (n = 20), or PE + FGR (n = 39). PE was defined as when the patient's blood pressure (BP) was ≥140/90 mmHg after 20 weeks' gestation and they exhibited proteinuria (≥300 mg protein/24 h). Severe hypertension was defined as ≥160/110 mmHg, and severe proteinuria was defined as proteinuria ≥2000 mg protein/24 h. The eligibility of the PE cases was determined according to the diagnostic criteria of the International Society for the Study of Hypertension in Pregnancy. PE with an onset time of between 20 and 32 weeks' gestation was defined as early onset and that with an onset time of ≥32 weeks' gestation was defined as late onset. To remove the effects of infectious inflammation, women that had suffered both preterm premature rupture of membranes and breech presentation pregnancies and had been matched for maternal and gestational age were used as controls (n = 23). FGR was defined as a newborn weight of less than the 10th percentile for the infant's gestational age. None of the subjects had hemolysis, elevated liver enzyme levels, and low platelet (HELLP) syndrome. Patients with multiple pregnancies, fetal chromosomal abnormalities, or fetal anomalies were excluded from the study. All of the women delivered their babies by caesarean section prior to the onset of labor. Written informed consent was obtained from individual patients for the use of the placental specimens. This study was approved by the ethics committee of Wakayama Medical University.

2.2. Placental specimen collection

All placental specimens were obtained after caesarean sections. To avoid the effects of labor on placental gene expression, only specimens from women who had not undergone labor were included. A central area of chorionic tissue was dissected from the attachment site of the umbilical cord and placenta. After the tissues had been washed with saline to remove excess blood, they were immediately frozen in liquid nitrogen and stored at −80 °C until use. The remaining placental samples were fixed in 10% formaldehyde overnight and then embedded in paraffin. Sections (2 μm) were collected onto silane-coated slides and dried in a conventional oven at 60 °C for 24 h. Hematoxylin-eosin staining was then performed to enable histological examinations to be performed.

2.3. Western blot analysis

Placental samples were prepared in a homogenizer with lysis buffer (20 mM Tris HCL, pH 8; 137 mM NaCl; 2 mM ethylenediaminetetraacetic acid [EDTA]; 10% glycerol, and 1% Nonidet P-40) and protease inhibitor cocktail (Roche Life Science, Indianapolis). The homogenates were centrifuged at 15000*g* for 15 min at 4 °C. The resultant supernatant was used for the Western blot analysis. Protein concentrations were determined using the bicinchoninic acid protein assay (Thermo Scientific, Waltham). Equal quantities of protein (40 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica). The samples were incubated with mouse monoclonal anti-human IDO antibody (1:500) (Takikawa et al., 1988) or anti-β-actin antibody (1:1000) (Abcam, Cambridge), before being incubated with the secondary antibody (1:1000) (R&D Systems, Minneapolis). Immunoreactive proteins were detected using a LuminoGraph (ATTO, Tokyo, Japan). β-actin was used as an internal loading control.

2.4. Immunohistochemistry

For immunostaining, the samples were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. For antigen retrieval, the slides were boiled in 1 mM EDTA (pH 8.0) in a pressure cooker for 10 min. Mouse monoclonal anti-IDO antibody (Takikawa et al., 1988), mouse monoclonal anti-Flt-1 antibody (Abcam, Cambridge), and mouse monoclonal anti-CD-34 antibody (Seikagaku Corporation, Tokyo, Japan) were used. The signals were detected using a peroxidase-based method involving simple stain MAX-PO (Nichirei Biosciences Inc., Tokyo, Japan) and 3,3-diaminobenzidine as a substrate. Non-immune mouse IgG1 (Seikagaku corporation, Tokyo, Japan) was used as the negative control. Counterstaining was performed with Mayer's hematoxylin solution. The staining was graded by two observers that were not aware of the subjects' clinical data based on both the staining intensity and positivity as follows (Fig. 3): Grade 1, no cells were stained or the cells were weakly stained and scarcely positive; Grade 2, the cells were moderately stained and focally positive; Grade 3, the cells were strongly stained and diffusely positive.

2.5. Statistical analyses

Statistical analyses were performed using the JMP Pro statistical software version 12.1.0 for Windows (SAS Institute Inc., Cary). Statistical comparisons between the groups were performed using Spearman's rank correlation coefficient, the Kruskal–Wallis test, the Steel–Dwass test, or Mann–Whitney *U* test as appropriate. Differences were considered to be significant at $P < 0.05$. Data are reported as the mean ± SD for each group.