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Impaired Wnt5a signaling in extravillous trophoblasts: Relevance to poor placentation in early gestation and subsequent preeclampsia

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ABSTRACT

Background: Defective decidual endovascular trophoblast invasion and subsequent impaired spiral artery remodeling is highly associated with the pathogenesis of preeclampsia (PE). Since there are scant and conflicting data regarding the function of Wnt5a signaling in extravillous trophoblasts (EVT), the aim of this study was to investigate whethere impaired Wnt5a signaling affects the invasive and tube forming capabilities of EVT.

Methods: Expression levels of Wnt ligands were compared between first trimester chorionic villi of women who later developed PE and women with unaffected pregnancies using publicly available microarray data (GSE12767). Wnt5a expression was examined in placentas using quantitative RT-PCR, Western blot analysis and immunohistochemistry. The function of Wnt5a signaling in EVT was investigated in an immortalized first trimester EVT cell line, HTR-8/SVneo, using small-interfering RNAs, recombinant human Wnt5a (rhWnt5a), and inhibitors of JNK or PKC.

Results: Microarray data analysis of the first trimester placentas showed that, among Wnt ligands, *Wnt5a* expression was significantly lower in women who later developed PE. The mRNA and protein expression levels of Wnt5a were significantly decreased in PE placentas compared with normal term placentas. Wnt5a knockdown significantly suppressed invasion and tube formation of HTR-8/SVneo cells, while the addition of rhWnt5a augmented the cell migration, invasion, and tube formation. Repression of Wnt5a/PKC signaling in HTR-8/SVneo cells inhibited cell invasion, but did not alter cell tube formation. In contrast, inhibition of Wnt5a/JNK signaling attenuated rhWnt5a-induced invasion and tube formation capabilities.

Conclusions: These findings suggest that impaired Wnt5a signaling is associated with poor placentation and subsequent PE.

1. Introduction

Preeclampsia (PE) is estimated to complicate 2–8% of all pregnancies, and is a leading cause of maternal and neonatal morbidity and mortality [1]. It has been postulated that PE occurs in two stages, i.e. that poor placentation typified by insufficient remodeling of spiral arteries (first stage) results in the release of excessive amounts of placental materials that lead to an excessive maternal inflammatory response and endothelial dysfunction (second stage) [2]. Placentation is a highly complex, regulated process that is crucial for normal fetal growth and the maintenance of a healthy pregnancy. During normal placentation, extravillous trophoblasts (EVT) invade into the decidua and the inner one third of the myometrium [3,4]. A subset of EVT, i.e. endovascular trophoblasts, invade maternal vessels, disrupt the endothelium and the smooth muscle layer, and replace the vascular wall [3,4]. These conversions allow spiral arteries to become widely dilated independently of vasomotor control, thereby providing a sufficient blood supply in intervillous space to meet the requirements of the fetus. On the other hand, defective decidual endovascular trophoblast invasion and subsequent impaired spiral artery remodeling are thought to result in less dilated vessels and a lack of adequate placental perfusion, leading to the development of PE [3,4]. Although early placentation has long been the focus of intense research efforts, the mechanism of impaired spiral artery remodeling has not been fully elucidated.

Accumulating evidence suggests that Wingless (Wnt) signaling plays an important role in placental development and human trophoblast differentiation [5,6]. Wnt proteins comprise a family of 19 secreted

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glycoproteins that act as ligands via 12 putative cell-surface receptors and co-receptors [7]. Wnt signaling is a complex pathway that modulates a number of signal transduction pathways and regulates diverse biological functions in a highly cell- and tissue-dependent manner [7,8,9]. Wnt signaling is broadly divided into two main categories, i.e. beta-catenin-dependent and beta-catenin-independent signaling. The former signaling pathway regulates target gene expression via beta-catenin stabilization and its translocation to the nucleus, and is closely associated with cell proliferation, cell fate determination, and differentiation [7,9]. On the other hand, the latter includes activation of c-Jun NH2-terminal kinase (JNK) and protein kinase C (PKC), which regulate cell functions such as cell movement and polarity [7,8]. Of the 19 known Wnt ligands, 14 have been reported to be expressed in the first trimester placenta [10]. However, the exact role of Wnt ligands in early placentation, especially in spiral artery remodeling, remains controversial. We hypothesized that Wnt signaling is associated with poor placentation and subsequent PE. The aim of this study was therefore to explore the role and underlying mechanisms of Wnt signaling in impaired early placentation and PE.

2. Methods

2.1. Microarray data mining for Wnt ligands in early placentation

Publicly available gene expression data of chorionic villus sampling were obtained from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/gds/) as series matrix files. There was only one data set (GSE12767) that was composed of the first trimester chorionic villi of women who later developed PE (n = 4) and women with unaffected pregnancies (n = 8). Expression values of Wnt ligands were log2 transformed, and probe sets whose maximum expression value across samples was less than 5 were removed from further analysis. Data were normalized across all samples by subtracting the mean and then dividing by the standard deviation of the expression value, and those values were converted into a heat map using Python (https://www.python.org).

2.2. Placenta samples

Placental villous tissues of the first trimester (n = 4) were obtained from normal pregnancies following induced abortion (6, 7, 8, and 11 weeks of gestation). In addition, placentas were collected from normal term pregnancies (n = 10) and PE pregnancies (n = 12) for quantitative real-time PCR (Table 1). Furthermore, since we have used up placental specimens for quantitative real-time PCR, another sample set of placentas (normal control = 6, PE = 6, respectively, Table 1) were prepared for Western blot analysis of Wnt5a expressions. Placentas were obtained immediately after Cesarean section in the absence of labor at Kyoto University Hospital, Japan. Villous tissues were collected from the central part of the placenta, and were macroscopically free of infarction or calcification. After a brief rinse in saline solution, these tissues were stored in RNAlater (Ambion) at −80 °C. PE was defined as a new onset of hypertension and proteinuria after 20 weeks of gestation with systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on at least two occasions at least 6 h apart, and proteinuria ≥ 300 mg/24 h.

2.3. Cell culture, RNA interference, and reagents

An immortalized first trimester extravillous trophoblast cell line, HTR-8/SVneo, was kindly provided by Dr. C. Graham (Queen's University, Kingston, Canada). The HTR-8/SVneo cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. In order to examine the effect of Wnt5a on cell function, RNA interference was performed by transfecting cells with two different small-interfering RNAs (siRNAs) targeting human Wnt5A mRNA or control siRNA (QIAGEN), using the HiPerfect Transfection Reagent (QIAGEN). HTR-8/SVneo cells were treated in the absence or presence of 100 and 200 ng/ml human recombinant Wnt5a (rhWnt5a; R&D Systems). PKC inhibitor GF109203X (5 μM) and the JNK inhibitor SP600125 (5 μM). All were assayed at least in duplicate in each experiment, and each experiment was performed at least three times.

2.4. RNA extraction and quantitative real-time PCR

Total RNA extraction from placental tissues and extravillous trophoblast cells (HTR-8/SVneo cells) was performed using the RNeasy Mini kit (Qiagen) as previously described [11]. Of 19 Wnt ligands, the expression of eight representative ligands in placental villous tissues was examined. The forward and reverse primers used for cDNA amplification are shown in Supplementary Table 1. 18S ribosomal RNA (rRNA) was used as the internal standard (Ribosomal RNA Control Reagents, #4308329, Applied Biosystems). Quantitative real-time PCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo) on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Gene expression was estimated using the comparative crossing point method for relative quantification. All data were normalized using 18S rRNA as an internal control and expressed relative to controls.

2.5. Western blotting

Western blotting was performed as previously reported [11]. The sources of the antibodies and their concentrations were shown in Supplementary Table 2. Signals were detected by Pierce ECL Plus Western Blotting Substrate (#32132, Thermo Scientific) and visualized by the ChemiDoc system (BioRad). The band intensity of Wnt5a and Tubulin in each sample was quantified by Image J Imaging System Software Version 1.3 (National Institutes of Health).

2.6. Immunohistochemistry

Immunohistochemical staining was conducted by the streptavidin-biotin-peroxidase method as previously reported [12]. Briefly, sections were incubated with mouse monoclonal antibody against Wnt5a or cytokeratin 7 (Supplementary Table 2) overnight at 4 °C. Slides were washed and incubated with biotinylated rabbit anti-mouse IgG (Nichirei), followed by incubation with streptavidin-peroxidase complex solution for 30 min at room temperature. Peroxidase activity was visualized by treatment with diaminobenzidine. The nuclei were counterstained with Mayer's hematoxylin and sections were observed under a light microscope (Olympus).

2.7. Immunofluorescent staining

Human placentas were fixed in 10% formaldehyde. After sections (5 μm) were deparaffinized, antigen retrieval was performed by boiling in 10 mM sodium citrate buffer (pH 6.0). Sections were then pre-incubated with 10% normal goat serum (50062Z, Life Technologies) with 0.3% Triton X-100 for 30 min at room temperature. The sources of the antibodies and their concentrations were shown in Supplementary Table 2. Slides were mounted with Prolong Gold Antifade Reagent with 4′,6-Diamidino-2-Phenylindole (DAPI, #P36935, Molecular Probes). Images were taken by Leica TCS SP8 confocal microscopy (Leica Microsystemsy).

2.8. Comparison of Wnt5a expression in placenta using cDNA microarray gene expression data

In order to investigate whether the level of Wnt5a expression in the