Biomedicine & Pharmacotherapy 80 (2016) 102–108

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Leukemia inhibitory factor promote trophoblast invasion via urokinase-type plasminogen activator receptor in preeclampsia

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ARTICLE INFO

Article history:
Received 18 November 2015
Received in revised form 7 March 2016
Accepted 7 March 2016

Keywords:
LIF
uPAR
Preeclampsia
Trophoblast
Invasion
PI3K/Akt

ABSTRACT

Preeclampsia is a pregnancy-related syndrome which can cause perinatal mortality and morbidity. Inadequate invasion by trophoblast cells may lead to poor perfusion of the placenta, even result in preeclampsia. Understanding the molecular mechanisms underlying placentation facilitates the better intervention of preeclampsia. Urokinase-type plasminogen activator receptor (uPAR) is involved in the physiological and pathological processes. Leukemia inhibitory factor (LIF) is an important regulator in the establishment of pregnancy. However, the expression of uPAR in preeclamptic patients and its relationship with LIF remains unclear. In the current study, we found that the level of uPAR was relatively lower in the placentas from preeclamptic patients as compared with normal pregnant women. LIF promoted trophoblast cell outgrowth by upregulating uPAR in an explants culture, and LIF also enhanced migration and invasion potential through uPAR in trophoblast JAR and JEG-3 cell lines, and with increased gelatinolytic activities of matrix metalloproteinase 2 (MMP-2). The effect of LIF and uPAR on trophoblast migration and invasion was mediated by PI3K/AKT signaling pathway. Our data indicates the roles of LIF in promoting trophoblast migration and invasion through uPAR and suggest that abnormal expression of uPAR might be associated with the etiology of preeclampsia.

1. Introduction

Preeclampsia is a pregnancy-related syndrome which remains a major cause of maternal and fetal mortality and morbidity [1,2]. It affects approximately 2–8% of all pregnant women worldwide. During normal pregnant, trophoblast invades the uterine epithelium and spiral arteries to establish maternal-fetal circulation [3,4]. Poor trophoblast migration and invasion result in fetal shallow implantation, insufficient circulation and nutrient exchange between the mother and fetus, which has been observed in severe preeclampsia (PE) and fetal intrauterine growth restriction [5–8]. However, the molecular mechanisms underlying the preeclampsia remain unclear.

The urokinase-type plasminogen activator receptor (uPAR) is a highly glycosylated protein bound to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor [9]. uPAR is involved in many physiological and pathological processes including inflammation reaction, cancer metastasis, and embryo implantation [10–12]. It is expressed at all stages of decidual tissue development in mice [13]. Naruse et al. found that uPAR was expressed during early pregnancy in uterine natural killer cells, which regulates EVT invasion and spiral artery remodeling [14]. Our studies found that uPAR, which was expressed in normal trophoblast cells, influenced trophoblast differentiation and placenta development (unpublished data). However, the expression of uPAR on preeclampsia and the associated regulatory mechanisms is poorly understood.

Trophoblast migration and invasion are mediated by many molecules, such as hormones, growth factors and cytokines [15,16]. Leukemia inhibitory factor (LIF) is a pleiotropic cytokine in the interleukin 6 (IL-6) family. LIF plays an important role in the establishment of pregnancy by supporting decidual and placental differentiation, as well as embryo development. Both glandular and luminal epithelial cells express LIF, which reach the peaks during the secretory/postovulatory phases of the menstrual cycle and promotes uterine transformation to a receptive state. Additionally, LIF regulates trophoblast behavior by promoting proliferation, invasion and differentiation [17,18]. LIF-deficient

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http://dx.doi.org/10.1016/j.biopha.2016.03.005
0753-3322/

female mice show an inability of blastocysts to attach the uterus [19]. A reduced level of LIF had also been observed in infertile women with implantation failure [20]. Nevertheless, the mechanism of LIF regulation via uPAR in trophoblast migration and invasion is not clear.

In this study, we detected the expression of uPAR in the human placental villi from preeclamptic patients and in women with normal early pregnancies. We investigated whether LIF and uPAR mediated the invasion of trophoblast cells in explant culture and *in vitro* implantation model. Furthermore, the underlying signaling pathways were studied.

2. Materials and methods

2.1. Tissue samples

The protocols for human study were in accordance with the Institutional Review Board of Dalian Medical University. Samples were obtained from the first Affiliated Hospital of Dalian Medical University from 2014 to 2015. Human placental tissues from the first trimester (7–9w) were obtained from healthy women undergoing legal abortion for nonmedical reasons. Term placentas, including normal (n = 10) and preeclampsia (n = 8), were collected after cesarean birth. Preeclampsia was defined according to the definition in Williams Obstetrics (23rd edition). Briefly, patients had new onset of both hypertension (systolic/diastolic blood pressure $\geq$140/90 mmHg measured on two occasions of at least 4 h apart) and proteinuria ($\geq$300 mg per 24 h) after 20 weeks of gestation. For the normal pregnant group, women were excluded from obesity, chronic hypertension, gestational diabetes mellitus (GDM), or eclampsia. All the preeclampsia patients were aged 23–35, developed hypertension and proteinuria after gestational age of 32 weeks, and delivered at 36–40 weeks, which matched with normal controls.

2.2. Cell culture

The JAR and JEG-3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in DMEM/F2 (Invitrogen) supplemented with 10%FBS, 100 U/mL penicillin, and 100 lg/mL streptomycin at 37 °C under 5% CO_2 in humidified air according to standard procedures.

2.3. Immunohistochemistry

Tissue slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. Slides were blocked with 10% normal goat serum for 10 min, and incubated with anti-human uPAR antibody (1:50; Cell Signaling Technology, Beverly, MA, USA) and anti-human keratin 7 antibody (1:50, protein tech, USA) at 4 °C for 12 h. The slides were incubated with biotinylated secondary antibody at 37 °C for 15 min, and reacted with a streptavidin-peroxidase conjugate at 37 °C for 10 min, then used 3, 3′-diaminobenzidine as a chromogen substrate. Meyer's hematoxylin was used as a counterstained dye. A negative control was obtained by replacing the primary antibody with PBS. Images were captured with the Olympus fluorescence microscope (Olympus, Japan). The average optical density was used to score the staining strength of the slides, and analyzed by the Image J software.

2.4. Explant culture

The explant culture was performed as described previously. In brief, small pieces of tissues (2–3 mm) from tips of first trimester human placental villi (7 w) were dissected and explanted in Millicell-CM culture dish inserts (0.4 μm pore size, Millipore, Carrigtwohill, Co., Cork, Ireland) pre-coated with phenol red-free matrigel substrate (Becton Dickinson, Bedford, MA, USA). Inserts were placed into 24-well culture dishes (Costar, Cambridge, MA, USA). The explants were cultured in serum-free DMEM/F12 media with 100 IU/ml penicillin and 100 mg/ml streptomycin at 3%O_2/5% CO_2/92%N_2. Trophoblast cell sprouting and migration from the distal end of the villous tips were recorded for 48 h. To test the effect of uPAR on the trophoblast cell migration, siRNA specifically targeting uPAR (500 nM), an equal concentration of the control siRNA and LIF was introduced into wells of culture media.

2.5. Transfection of uPAR siRNA

Cells were plated into 60-mm culture dishes and then transfected with 40 nM of siRNA using lipofectamine 2000 following the manufacturer's instructions. All assays were performed 48 h after transfection. uPAR siRNA sequences were as follows: uPAR siRNA (559): sense 5′-GCCGUUACCUCGAAUGCAUTT-3′, antisense-5′ AUGCAUUCGAGGUAACGGCTT-3′.

2.6. Real-time PCR

Cells were treated with RNAiso Plus reagent (TaKara, Japan) for RNA extraction, and PrimeScript™ RT reagent Kit with gDNA Eraser kit (Takara, Japan) was used for synthesizing cDNA. SYBR® Premix Ex Taq™ (Takara, Japan) was used for quantitative Real-time PCR. Primers were as follows: uPAR: 5′- AGGACCCTGAGC-TATCGGACTG-3′ (forward), and 5′-TGCATTCGAGGTAACGGCTTC-3′ (reverse). GAPDH: 5′-GCACCGTCAAGGCTG AGAAC-3′ (forward), and 5′-TGGTGAAGACGCCAGTGGA-3′ (reverse). The reactions were performed with Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies, USA).

2.7. Western blot

To prepare whole-cell extracts, cells at 90% confluent were washed in PBS before incubation with RIPA lysis buffer. Protein concentration was determined with the Coomassie Protein Assay Reagent using bovine serum albumin as a standard. Cell lysates (40 μg) were separated by 12% SDS-PAGE min-gel and transferred onto nitrocellulose membranes, blocked with TTBS containing 5% fat-free dry milk. The membranes were incubated with the primary antibodies at 4 °C overnight. The primary antibodies used included anti-human uPAR antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA), anti-human *p*-PI3K, PI3K, *p*-PDK, PDK, *p*-Akt (468), *p*-Akt(308), Akt (Cell Signaling Technology, Beverly, MA, USA) antibody and anti-human GAPDH (1:2000; protein tech, USA). After incubation with a HRP-conjugated secondary antibody, immunoreactive proteins were visualized with Enhanced Chemiluminescence Plus kit (Thermo Scientific, Rockford, USA) detection system.

2.8. Matrigel cell invasion and transwell cell migration assay

For Matrigel cell invasion assay, transwell inserts (Costar, Cambridge, MA, USA) containing polycarbonate filters with 8 μm pores were precoated with 50 ml of 1 mg/ml Matrigel matrix. For cell migration assay, the inserts were not precoated with matrigel. 1.0×105 of cells in serum-free medium were plated in the upper chamber, whereas medium with 10% FBS was added to the lower chamber. After incubating for 24 h, the cells on the Matrigel side of the inserts were removed by cotton swab. The inserts were fixed in methanol and stained with Crystal Violet. The number of invaded or migrated cells attached to the other side of the insert was counted under a light microscope (Olympus IX51, Japan) in five