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Effects of CXCL3 on migration, invasion, proliferation and tube formation of trophoblast cells

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ABSTRACT

CXCL3 was reportedly associated with the invasion and metastasis of various malignancies, the role of CXCL3, however, in preeclampsia has not been fully discussed. We previously found placental CXCL3 level in severe preeclampsia was significantly lower than that in healthy pregnancy and exogenous recombinant human CXCL3 protein was able to promote trophoblasts' migration and proliferation. The current study, therefore, is further to investigate effects of endogenous CXCL3 on migration, invasion, proliferation, tube formation and apoptosis of trophoblasts.

Immunofluorescence staining demonstrated that CXCL3 localized in both trophoblasts of placenta and HTR-8/SVneo cells. Moreover, data showed that migration, invasion, proliferation and tube-formation capability of HTR-8/SVneo cells transfected with *siRNA-CXCL3* were suppressed by down-regulation of CXCL3, while those behaviors of HTR-8/SVneo cells transfected with *pEZ-CXCL3* were enhanced by upregulation of CXCL3. Nevertheless, the apoptosis of HTR-8/SVneo cells was not affected by neither siRNA nor overexpression plasmid. The result suggests that CXCL3 is involved in migration, invasion, proliferation and tubule formation of trophoblasts and may play a key role in the pathogenesis of preeclampsia.

1. Introduction

Preeclampsia is a multi-system dysfunction severe pregnancy related disease featured by hypertension and proteinuria and affects 3%–7% of all pregnancies [1], which is still one of prime reasons for maternal and perinatal mortality and morbidity worldwide. Exact pathophysiology of preeclampsia is still unknown, whereas abnormal placental development may point out the reasons why [2]. During early normal placentation, partial cytotrophoblasts detached from the villous become extravillous trophoblasts (EVTs) that subsequently generate both interstitial EVTs invading into the maternal decidua even superficial myometrium and endovascular EVTs remodeling uterine spiral arteries, resulting in transforming the high-resistance, low-capacity uterine spiral arteries into the high-capacity, low-resistance blood circulation beneficial to fetal development [3]. This process is controlled complicatedly by the interaction of chemokines, growth factors and transcription factors [4]. Recently, the two-stage model hypothesis puts the origin of clinical symptoms of preeclampsia down to the inadequate EVTs invasion and the consequent insufficient remodeling of spiral arteries with it, which further lead to generalized maternal endothelial dysfunction and inappropriate maternal inflammatory response, but the mechanism underlying this hypothesis is still unknown [5,6]. Yet studies have shown that abnormal immune cells and cytokine signaling arisen from excessively maternal immune and inflammatory responses are involve in these cascade amplification reactions and implicated in the migration, invasion and proliferation of trophoblasts [7,8]. In addition, chemokine exists extensively in the maternal-fetal interface and associates with the regulation of trophoblasts, which may play an inevitable key role in the immune mechanism of preeclampsia [9,10].

Chemokine is a bioactive protein superfamily with low molecular weight classified into four subgroups (CC or β, CXC or α, C and CX3C), which participates in various pathophysiological processes. CXCL3, encoded by the human GRO gene and also known as growth-related oncogene γ (GRO-γ), is a member of ELR + CXC group subdivided from CXC family [11]. Reportedly, it plays a crucial role in chemotactic process [12], vascularization [13], tumorigenesis [14], cell differentiation [15], and cell invasion and migration [16,17] by binding with CXCR1 and CXCR2 expressed in diverse cells like leukocytes [18],

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tumor cells [19], smooth muscle cells [20], decidual and villous cells [21] as well as human vascular endothelial cells [22]. In recent years, CXCL3 is widely attracted for the findings in oncology that it up-regulates in aggressive breast cancer [23] and prostate cancer [16] and enhances the metastasis of cholangiocarcinoma [24] and melanoma [25]. Moreover, CXCL3 also connects with the migration of such non-tumor cell as neuron precursor cells and airway smooth muscle cells [20,26].

Gestational trophoblast is known as the pseudo-tumor cell. Admittedly, analogical invasive properties exist between EVTs and malignant cells. Nevertheless, the role of CXCL3 in gestational trophoblasts remains unclear. Similarities between maternal-fetal interface and tumor microenvironment and the striking likeness between EVTs and cancer cells in their biological behavior [27,28] may be conducive to give us enlightenment upon the role of CXCL3 in the pathogenesis of preeclampsia. Our previous research has shown that placental expression of CXCL3 in severe preeclampsia was significantly decreased and exogenous CXCL3 was able to promote migration and proliferation of trophoblasts [29]. The role of endogenous CXCL3, however, in the pathogenesis of preeclampsia remains unclear up to now. Therefore, the present work is to explore effects of endogenous CXCL3 on migration, invasion, proliferation, tube formation and apoptosis of trophoblasts, which may make a certain contributions to reveal the pathogenesis of preeclampsia.

2. Methods and materials

2.1. Tissue sections, cell climbing slides and immunofluorescence staining

Placenta tissue was collected promptly from normal full-term pregnant women after elective cesarean delivery for such obstetrical factors as the presence of placenta previa and cephalopelvic disproportion as described previously [30]. Informed consent was obtained from all participants, and the research was approved by the ethics committee of West China Second University Hospital of Sichuan University.

Tissue blocks of 1.0 cm^3, avoiding vessels, calcification or infarction, were taken from the center of maternal side of placenta, which were washed with physiological saline, fixed in 4% formaldehyde solution for 48 h, then routinely embedded in paraffin and sectioned in 2 μm slices.

HTR-8/SVneo cell, the cell line of extravillous trophoblasts in early pregnancy, was kindly granted by Prof. Yali Hu of Drum Tower Hospital Affiliated to Medical College of Nanjing University (Nanjing, China) [31] who was provided by Dr Charles Graham from Queen's University (Canada), the establisher of the HTR-8/SVneo cells [32]. Furthermore, HTR-8/SVneo cell line in our study was identified by short tandem repeat (STR) genotyping, consistent with the STR database in ACTT, an authority of STR genotyping. HTR-8/SVneo cell line was plated on climbing slides placed in 6-well flat-bottomed plate and then washed with PBS, air-dried, and finally fixed with 4% formaldehyde after cell adhesion and expansion.

The placenta sections and HTR-8/SVneo cell climbing slides were immunolocalized with fluorescent secondary antibodies. Slides were deparaffinized, rehydrated and repaired the antigen, which were successively interacted with primary antibody, secondary antibody and 40, 6-diamidino-2-phenylindole (DAPI) at proper conditions. Then, signals were detected by confocal microscopy (Olympus FV1000, Japan). Mouse anti-human cytokeratin 7 (CK7) antibody (Abcam, U.K.), rabbit anti-human CXCL3 antibody (Absin, China), rabbit anti-human CXCR2 antibody (Bioss, China), isotype-matched control IgG (NSB), Alexa Fluor-488 goat anti-rabbit secondary antibody, Alexa Fluor-546 goat anti-mouse secondary antibody (ZSGB-BIO, China) and DAPI (ZSGB-BIO, China) were diluted at a ratio of 1:50, 1:100, 1:200, 1:100, 1:400, 1:300 and 1:200 respectively.

2.2. Cell culture and transfection of siRNA and plasmid

HTR-8/SVneo cell was cultured in RPMI-1640 medium (Hyclone, USA) with 10% fetal bovine serum (KangYuan, China) and incubated at 37 °C with 5% CO_2. Cells cultured in 6-well flat-bottomed plate and reached 70–80% convergence were transfected with either siRNA targeting CXCL3 (*siRNA-CXCL3*, GenePharma, China) to downregulate CXCL3 using Lipofectamine3000 siRNA transfection reagent (Invitrogen, U.S.A.) or plasmid targeting CXCL3 (*pEZ-CXCL3*, GenePharma, China) to upregulate CXCL3 applying the X-tremeGene HP DNA Transfection Reagent (Roche, Germany), according to the kit's operating manual. Negative control siRNA (*siRNA-NC*) and empty vector (*pEZ-Vec*) were provided as the control of *siRNA-CXCL3* and *pEZ-CXCL3* respectively. In addition, the untransfected HTR-8/SVneo cell was served as the blank control of the study. After incubating for 48 h, transfected cells were serum starved overnight and then used for migration, invasion, proliferation, tube formation and apoptosis experiments. Meanwhile, a portion of RNA lysates and protein lysates from different groups were collected to measure the transfection efficiency of knockdown and overexpression by QRT-PCR and Western blot.

2.3. Real time PCR

Extraction of cellular total RNA from RNA lysates was performed with Trizol reagent system (Bioteke, China) and quantified by absorbance at 260 nm. Synthesize cDNA from RNA and QRT-PCR were employed with Revert cDNA synthesis kit (GeneCopoeia, U.S.A, Lot: AORT-0020QP001) and SYBR Green PCR kit (GeneCopoeia, U.S.A, lot: AOPR-0200) respectively, according to the operational manual. Gene quantification was expressed with the relative CT value ($2^{-\triangle\triangle Ct}$), normalized with GAPDH levels, the loading control, in the same sample. The primer sequence of CXCL3 and GAPDH was designed, synthesized and validated (GeneCopoeia, U.S.A). The primers sequences were: *CXCL3* (Forward: *5′-CGC CCA AAC CGA AGT CAT-3′, reverse: 5′-GTG CTC CCC TTGTTC AGT ATC T-3′*); *GAPDH* (Forward: *5′-TTG GTA TCG TGG AAG GAC TCA-3′; Reverse: 5′-TGT CAT CAT ATT TGG CAG GTT-3′*).

2.4. Western blot

Cell total protein was extracted from protein lysates with the general protein extraction reagent (Bioteke corp. China) containing 1 mM PMSF and quantified by BAC reagent kit. Then, samples (30μg/15μl) were loaded, separated on 15% SDS-PAGE gels (Beyotime, China) and transferred to PVDF membranes (Millipore, Germany). Sequentially, PVDF membranes were blocked with 5% skim milk in TBST (Tris-buffered saline (TBS) and Tween-20) for 1 h at room temperature, washed with TBST for three times, incubated at 4 °C overnight with primary antibodies specific to CXCL3 and β-actin. After washing, membranes were further to incubate with secondary antibody for 1 h at room temperature, washed with TBST, dried with neutral absorbent paper and finally scanned by ChemiDoc MP imaging system (Biorad, U.S.A.). Rabbit anti-human CXCL3 antibody (Absin, China), rabbit *anti*-β-actin antibody (CST, U.S.A.) and goat anti-rabbit IgG (CST, U.S.A.) were diluted with blocking buffer at a ratio of 1:100, 1:1000 and 1:2000 respectively. β-actin was used as loading control.

2.5. Cell migration and invasion assays

Corning Transwell permeable supports (Corning, U.S.A) were applied to detect cell migration and cell invasion. In cell migration assay, 5×10^4 transfected cells suspended in 0.2 ml FBS-free RPMI-1640 medium were seeded into the upper compartment, the bottom of which is a microporous membrane with 8.0 μm pore size, the lower compartment was loaded with 0.8 ml 10% FBS RPMI-1640 medium and incubated. After 24 h, cells passed through the microporous membrane