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Over-expression of SOCS-3 Gene Promotes IL-10 Production by JEG-3 Trophoblast Cells[☆]

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Abstract

Suppressor of cytokine signaling-3 (SOCS-3) plays an important role in negative regulation of inflammatory response. Evidence has shown that SOCS-3 and IL-10 expressions were significantly reduced in placental trophoblasts from preeclampsia. IL-10 is an anti-inflammatory cytokine. In this study, we sought to determine if enhance SOCS-3 expression could affect IL-10 production in placental trophoblasts. Placental JEG-3 cells were used. Over-expression of SOCS-3 was generated by transfection of JEG-3 cells with a green fluorescent protein (GFP) tagged SOCS-3 gene, SOCS-3/ZsGreen1, by siPORT lipid transfection. Cells transfected with ZsGreen1 vector only was used as control. Our results showed that IL-6 production was reduced in cells over-expressed with SOCS-3. Moreover, SOCS-3 transfected cells produced more IL-10 when stimulated with IL-6. The increased IL-10 production by JEG-3 cells was in a dose-dependent manner, $p < 0.05$. Our data suggested that enhanced SOCS-3 gene expression could promote IL-10 production by placental trophoblast cells, suggesting that SOCS-3 may play an important role in regulation of cytokine induced anti-inflammatory response in placental trophoblasts.

Keywords

SOCS-3; IL-6; IL-10; Trophoblasts

1. Introduction

Suppressors of cytokine signaling (SOCS) protein are a family of intracellular proteins that control cytokine signaling by suppressing cytokine signal transduction process. There are eight members of the SOCS family proteins, SOCS-1 to -7 and cytokine-inducible SH2-containing protein (CIS). Studies have shown that SOCS-1 and -3 specifically participate in regulation of Th1 and Th2 cytokine signaling [1]. For example, SOCS-1 is a key modulator of interferon- γ (IFN- γ) signaling. Mice lacking SOCS-1 exhibit deregulated responses to IFN- γ resulting in excessive T cell activation [2] and are hyper-responsive to viral infection [3]. SOCS-3 functions to control IL-6 induced Th2 associated response via its receptor

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gp130 [4]. In macrophages, SOCS-3 mediates interleukin-10 (IL-10) inhibition of tumor necrosis factor- α (TNF- α) and nitric oxide production [5].

An interesting study reported by Roberts et al. showed that mice with a deletion of SOCS-3 die at mid gestation (E11–13) due to placental insufficiency [6]. They observed that SOCS-3^{-/-} embryos were smaller than wild type but appeared normal. However, the placental spongiotrophoblast layer was significantly reduced and accompanied by increased numbers of giant trophoblast cells. The network of embryonic vessels and maternal sinuses was poorly developed. They concluded that the embryonic lethality is not due to anatomical defects of the embryo, but rather poor placental development that accounts for the developmental arrest and death [6], which suggests that SOCS-3 is an important molecule in the regulation of trophoblast function during placental development. Therefore, it is believed that SOCS-3 is critical for a successful pregnancy outcome.

IL-10 belongs to the Th2 cytokine family although it can be produced by both of Th1 and Th2 cells, as well as non-T cells [7]. An animal study has shown that fetal loss can be prevented by administration of IL-10 during pregnancy [8]. By acting on antigen presenting cells, IL-10 can inhibit cytokine production by Th1 cells [9]. In humans, there is defective or reduction of IL-10 production by placental trophoblasts from preeclampsia [10,11]. It is well accepted that increased inflammatory response occurs in the placenta of preeclampsia. Thus, lack of IL-10 expression or reduced IL-10 production in trophoblasts may contribute to increased inflammatory response in the placenta. Since SOCS-3 negatively regulates inflammatory cytokines and SOCS-3 was found to mediate IL-10 inhibition of TNF- α in macrophages [5], we attempted, in the present study, to investigate if IL-10 production was associated with SOCS-3 signaling in placental trophoblasts. We hypothesized that increased SOCS-3 signaling might promote IL-10 production by placental trophoblasts. Placental trophoblast cell line JEG-3 cells were used as a testing cell model and over-expression of SOCS-3 in trophoblast cells was generated by transfection of a green fluorescent protein (GFP) tagged SOCS-3 gene.

2. Materials and methods

2.1. Construction of SOCS-3/ZsGreen1 GFP vector

Vector pZsGreen1-N1 (Clontech, Palo Alto, CA), a vector fused with a green fluorescent protein (GFP) coding sequence, was used to construct SOCS-3 GFP vector, pSOCS-3/ZsGreen1. Briefly, open reading frame of human SOCS-3 was amplified from human cDNA by polymerase chain reaction (PCR) using oligonucleotide to create restriction sites for Nhe I and Kpn I at 5' and 3' end of SOCS-3 sequence. The primers, upstream: 5'-AGCGCTAGCACCATGGTCACCCACAGCAAGTTTCC-3' and downstream: 5'-GGTGGTACCCAAAGCGGGGCATCGTACTG-3', were designed based on NCBI accession number NM-003955 and synthesized by Integrated DNA Technologies (IDT) Coralville, IA. The PCR was performed using platinum pfx DNA polymerase (Invitrogen, Carlsbad, CA). PCR product and the pZsGreen1-N1 were digested with Nhe I and Kpn I (New England Lab, Ipswich, MA). After ligation, competent Ecoli-Top10 (Invitrogen) was transformed with plasmid and selected positive clones were amplified. SOCS-3 sequence was verified by Mclab (South San Francisco, CA).

2.2. Transfection of pSOCS-3/ZsGreen1 into JEG-3 cells

JEG-3 human choriocarcinoma cells (ATCC, Manassas, VA) were seeded in a 6-well plate in a density of 1.2×10^5 /well. Transfection was carried out when cells reached 40–50% confluent by siPORT lipid transfection reagent (Ambion, Austin, TX) and pSOCS-3/ZsGreen1 (1.5 μ g/per well). Cells were incubated with Opti MEM (Invitrogen) for 6 h and

then replaced with fresh Dulbecco's Modified Eagles Medium (DMEM, Sigma Chemical, St. Louis, MO) containing 10% FBS. Cells transfected with ZsGreen1-N1 vector only were used as a comparison. Successful transfection of SOCS-3 genes into trophoblasts was confirmed by positive green fluorescence reviewed under fluorescent microscope, mRNA expression by RT-PCR, and protein expression by SDS-PAGE.

For mRNA expression, an aliquot of total RNA (1 µg) per sample was used for reverse transcription with iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). First-strand cDNA (2 µl) was then used as a template for PCR by GoTaq PCR Core System I (Promega, Madison, MI). The upstream primer sequences used for detection of SOCS-3/ZsGreen1 and SOCS-3 are 5'-AGCGCTAGCACCATGGTCACCCACAGCAAGTTTCC-3' and downstream sequences are 5'-GTCAGCTTGTGCTGGATGAA-3' and 5'-GGTGGTACCCAAAGCGGGGCATCGTACTG-3', respectively. Primers used for detection of ZsGreen1 are 5'-CTGCATGTACCACGAGTCCA-3' and 5'-GTCAGCTTGTGCTGGATGAA-3'. β -actin was also amplified as an internal control. PCR products were separated by 1% agarose electrophoresis.

Protein expression was examined by Western blot with a rabbit polyclonal antibody against SOCS-3 (Santa Cruze, San Diego, CA) as the primary antibody and a horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz) as the secondary antibody. Membranes were also hybridized with β -actin antibody (Sigma) for a comparison.

2.3. Measurements of IL-6 and IL-10

IL-6 and IL-10 productions were measured in the trophoblast culture medium by enzyme-linked immunosorbent assay (ELISA). Both IL-6 and IL-10 ELISA kits were purchased from R&D system (Minneapolis, MN). The assay procedures were performed according to the manufacturer's instruction. The range of IL-6 standard curve was 0.6–600 pg/ml and the range of IL-10 standard curve was 2–2000 pg/ml. An aliquot of 100 µl sample was measured in duplicate for all samples. Within and between assay variations was less than 5% and 8%, respectively.

2.4. Statistical analysis

Data are expressed as mean $\pm$ SE. Paired *t*-test and one-way ANOVA was used for statistical analysis by computer software Statview (Cary, NC). Fisher's PLSD-test was used for post-hoc test. A *p* < 0.05 was considered statistically significant.

3. Results

3.1. Transfection of SOCS-3 gene into JEG-3 cells

Fig. 1A shows positive transfected cells exhibited green fluorescence under fluorescent microscope. The fusion protein is localized in the cytoplasm. Fig. 1B demonstrates GFP-tagged SOCS-3 gene is only present in pSOCS-3/ZsGreen1 transfected cells, whereas SOCS-3 expression can be detected in non-transfected cells, cells transfected with pZsGreen1-N1 and cells transfected with pSOCS-3/ZsGreen1. Positive ZsGreen1 expression is detected in cells transfected with pZsGreen1-N1 and pSOCS-3/ZsGreen1. Fig. 1C shows SOCS-3 protein expression. Endogenous SOCS-3 was detected approximately at 28 kDa in untransfected cells, cells transfected with pZsGreen1-N1, and cells transfected with pSOCS-3/ZsGreen1. In contrast, positive SOCS-3 fusion protein (at 65 kDa) was only detected in cells transfected with pSOCS-3/ZsGreen1. The band, at approximately 63–65 kDa, confirms the SOCS-3/ZsGreen1 protein (SOCS-3: 28 kDa; ZsGreen1: 25 kDa; linker: 12AA = 10 kDa).

3.2. Reduced IL-6 production in trophoblast cells over-expressed with SOCS-3

Since SOCS-3 plays its cytokine inhibitory role via IL-6/gp130/JAK pathway. We determined if over-expression of SOCS-3 in trophoblasts had any effects on IL-6 production. After 48 h of transfection, cells were incubated with fresh serum free DMEM for 2 and 6 h. Medium was collected and measured for IL-6 production. Cells transfected with pZsGreen1-N1 were used as control. As shown in Fig. 2A, cells transfected with SOCS-3 produced less IL-6 than the controls, $p < 0.05$. These data suggest that enhanced cellular SOCS-3 expression could reduce IL-6 production by trophoblast cells.

3.3. Increased IL-10 production by trophoblasts over-expressed with SOCS-3

Since SOCS-3 mediates IL-6/IL-6R signaling pathway [1,4], we further determined if SOCS-3 could regulate IL-10 generation in cells challenged with IL-6. Cells transfected with pZsGreen1-N1 were used as control. After 48 h of transfection, cells were briefly stimulated with IL-6 at concentrations of 1 and 10ng/ml for 10 min; medium was collected and measured for IL-10 production. We found that cells produced more IL-10 when they were challenged with a higher dose of IL-6 than the controls, $p < 0.05$ (Fig. 2B). Data are means from 5 independent experiments.

4. Discussion

SOCS-3 is a negative cytokine regulator via IL-6 and its receptor signaling transduction pathway [4]. Study has shown that IL-6 deficient mice exhibit severely impaired SOCS-3 expression in response to LPS stimulation [12]. Recently, we found that trophoblast SOCS-3 expression was reduced in placentas from women with preeclampsia [13], suggesting that insufficient expression of SOCS-3 or reduced SOCS-3 function may account for the reduced endogenous anti-inflammatory activity in placental trophoblasts in preeclampsia.

To determine if SOCS-3 plays a role in regulation of cytokine signaling in placental trophoblasts, JEG-3 cells were transfected with a GFP-tagged SOCS-3 gene. Using JEG-3 cells, not BeWo or JAR cells, is because JEG-3 cells have a higher degree of differentiation property [14,15]. Our results showed IL-6 production was reduced in trophoblasts over-expressed with SOCS-3, suggesting SOCS-3 could mediate cytokine IL-6 production.

Another finding of the present study is increased IL-10 production in SOCS-3 over-expressed trophoblasts when challenged with IL-6. IL-6 is considered as both a pro-inflammatory and an inflammatory cytokine, which is secreted by many types of cells including fibroblasts, endothelial cells, macrophages, lymphocytes, as well as placental trophoblasts. It exerts multifunction such as regulation of acute-phase proteins in the liver [16] and stimulation of multipotent colony formation in hematopoietic stem cells [17]. IL-10 is a Th2 cytokine. IL-10 is a key physiological negative regulator of macrophage activation and inflammatory response. An in vivo animal model has shown that IL-10 gene knockout mice develop chronic enterocolitis [18]. Administration of IL-10, however, ameliorates the disease in models of endotoxemia [19]. IL-10 was also found to protect against LPS-induced abortion and fetal growth restriction in mice [20]. In humans, reduced IL-10 expression and production was found in placentas from preeclampsia [10,11]. Induction of SOCS-3 is regulated via IL-6 receptor trans-signaling. The observation of increased IL-10 production in SOCS-3 over-expressed trophoblasts after IL-6 challenge is in line with the concept of pro-inflammatory role of IL-6 in trophoblasts and SOCS-3 signaling in regulation of Th2 cytokine production [21]. Our data also supports the idea that reduced SOCS-3 expression and activity could contribute to increased inflammatory response in placental trophoblasts. Whether reduced SOCS-3 expression [13] is responsible for down-regulation of IL-10 expression and production in preeclamptic placentas warrants further investigation.

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Fig. 1.

Transfection of SOCS-3 gene into JEG-3 trophoblasts. A: Images of JEG-3 cells transfected with pZsGreen1-N1 or pSOCS-3/ZsGreen1. a: cells transfected with pZsGreen1-N1 and b: cells transfected with pSOCS-3/ZsGreen1 (Bar = 25 micron). B: RT-PCR analysis of SOCS-3 mRNA expression. M: marker; lane 1: untransfected cells; lane 2: cells transfected with pZsGreen1-N1; and lanes 3, 4, 5 and 6: cells transfected with pSOCS-3/ZsGreen1 at 24 h, 36 h, 48 h and 60 h, respectively. mRNA expression of SOCS-3 can be detectable up to 60 h after transfection at our experimental condition. β -actin expression was used as an internal control. C: SOCS-3 protein expression by Western blot. SOCS-3/ZsGreen1 fusion protein was detected in cells transfected with pSOCS-3/ZsGreen1 using antibody against SOCS-3. Endogenous SOCS-3 expression was also detectable in JEG-3 cells, lane 1: untransfected cells; lane 2: cells transfected with pZsGreen1-N1; lane 3: cells transfected with pSOCS-3/ZsGreen1. β -actin expression indicates an equal loading of the samples. Molecular weight shows on the left. Arrow indicates non-specific bands.

Fig. 2.

Productions of IL-6 and IL-10 by JEG-3 cells transfected with pZsGreen1-N1 and pSOCS-3/ZsGreen1. A: IL-6 production. Cells transfected with pSOCS-3/ZsGreen1 produced less IL-6 than the cells transfected with pZsGreen1-N1. B: IL-10 production. Data are expressed as the ratio of IL-10 production by transfected to untransfected cells. Cells transfected with pSOCS-3/ZsGreen1 produced more IL-10, $*p < 0.05$. Data are expressed as mean $\pm$ SE from 5 independent experiments.