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Altered IL-6 receptor, IL-6R and gp130, production and expression and decreased SOCS-3 expression in placentas from women with preeclampsia

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Abstract

IL-6 and its receptor complex, IL-6 receptor (IL-6R) and gp130, are critical in induction of suppressor of cytokine signaling-3 (SOCS-3) protein, a negative cytokine regulator and anti-inflammatory mediator, in a biological system. Increased inflammatory response is believed to contribute to the placental dysfunction in preeclampsia (PE). However, it is not known if altered IL-6 receptor signaling and decreased SOCS-3 expression occur in placentas from PE. To study this, we examined IL-6, soluble IL-6R (sIL-6R) and soluble gp130 (sgp130) productions by villous tissues from normal and PE placentas. Hypoxia effects on IL-6, sIL-6R and sgp130 productions were determined. IL-6R, gp130 and SOCS-3 expressions were determined by immunohistochemical staining and by Western blot. Our results showed that under normoxic condition (21% O₂), villous tissues from PE placentas produced relative more sgp130, but significantly less IL-6 and sIL-6R ($p < 0.01$), than normal placental tissues. The ratio of sgp130/sIL-6R release was significantly higher by PE placentas than normal placentas, $p < 0.01$. Under hypoxic condition (2% O₂), IL-6 production was significantly reduced by both normal ($p < 0.01$) and PE ($p < 0.05$) placental tissues. Hypoxia promoted sgp130 release by normal, but not by PE, placental tissues. Reduced IL-6R and SOCS-3 immunostaining and expressions were found in PE placentas. We concluded that increased ratio of sgp130/sIL-6R production and/or reduced sIL-6R production combined with down-regulation of IL-6R and SOCS-3 expressions in trophoblasts may lead to less cytokine inhibitory activity in PE placentas, which may account for the increased placental inflammatory response in PE.

Keywords

IL-6R; gp130; SOCS-3; placenta; preeclampsia

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Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine, originally identified as a stimulating factor of B lymphocyte immunoglobulin production (1). Outside the hematopoietic system, IL-6 functions as a hepatocyte-stimulating factor and induces expression of various acute phase proteins. Recently, IL-6 was found to be instrumental in directing inflammatory response: switching from innate immunity to acquired immunity (2-4) via induction of suppressor of cytokine signaling-3 (SOCS-3) (5). IL-6 exerts biological function by binding to its receptors on target cells. It has two receptors: a cognate IL-6 receptor (IL-6R) and a trans-signal receptor gp130. Gp130 induced trans-signaling is critical for the induction of negative cytokine regulator SOCS-3 in a biological system (1,6). Like many membrane receptors, both IL-6R and gp130 have their soluble forms, sIL-6R and sgp130, that can be detected in the blood and in the extracellular fluids. Different effects of the two soluble receptors have been identified. Soluble IL-6R has agonistic effects on IL-6 (7,8), whereas sgp130 has been demonstrated to be an antagonist to IL-6/sIL-6R (9). Therefore, sgp130 generation or the ratio of sgp130 to sIL-6R could be significant in regulating IL-6 trans-signaling and its family protein molecules induced cell function.

The placenta is believed to play a central role in the pathophysiology of preeclampsia, a unique hypertensive and multi-system disorder during human pregnancy. Multiple lines of evidence support the notion that increased inflammatory response occurs in the placenta of women with preeclampsia. In vitro studies have also shown altered pro-inflammatory cytokine productions by placental tissues/trophoblast cells from women with preeclampsia compared to normal pregnant controls, including TNF- α , IL-6 and IL-8 (10,11). Cytokines produced by the placenta not only regulate trophoblast function *in situ* but may also contribute to their circulating levels during pregnancy. For example, IL-6 has been shown to induce leptin secretion and MMP-2 activation in primary first trimester cytotrophoblast cells in culture (12). It is believed that dysregulated cytokine production results in an excessive inflammatory response in preeclampsia and/or *vice versa* (13).

Several studies had shown placental tissue produces IL-6 (11,14,15), but no information is available about sIL-6R and sgp130 production by placentas from normal and preeclamptic pregnancies. To better understand mechanisms of IL-6 and its receptors, gp130 and IL-6R, in regulation of inflammatory response in the placenta, in the present study we determined IL-6, sgp130 and sIL-6R productions by villous explant tissue from normal and preeclamptic placentas. Placental tissue expressions of IL-6R, gp130 and SOCS-3 were also examined by immunohistochemistry and Western blot analysis.

Materials and Methods

Placental tissue collection

Placentas delivered by normal and preeclamptic pregnant women were obtained from the main hospital of Louisiana State University Health Sciences Center, Shreveport (LSUHSC-S). A total of 33 placentas (18 normal and 15 preeclamptic) were used in this study. The demographic data of study subjects are shown in Table 1. The diagnostic criteria for normal and preeclamptic pregnancies were based on ACOG guidelines (16). The study was approved by the Institutional Review Board (IRB) for Human Research at Louisiana State University Health and Sciences Center (LSUHSC) at Shreveport, LA. None of the study subjects had prolonged rupture of membranes or signs of infections. Freshly obtained placentas were processed immediately for explant culture, snap frozen, and fixed with 10% formalin for immunohistochemistry studies.

Placental explant culture

Placental explants were cultured with serum-free Dulbecco's Modified Eagle's Medium (DMEM, Sigma) as previously described (17). Briefly, an aliquot of 500mg of villous tissue was incubated with 5ml of serum free DMEM in duplicate. The incubation was carried out for 48 h at 37C in an incubator gassed with 95% air-5% CO₂ and lowered oxygen condition with 2% O₂, 5% CO₂ and 93% N₂ as previously described (18). For hypoxic culture, the culture plate was placed in a portable air chamber (Billups-Rothenberg, Del Mar, CA). The chamber, which has inflow and outflow valves, was flushed daily with 2% O₂, 5% CO₂ balanced with 93% N₂ for 2 minutes with flow rate at 10 liter/minute. Medium samples were collected at the end of incubation and stored at -80 C until assay. Tissue/Cell viability was examined by hematoxylin and eosin (H&E) staining of villous tissue sections in both normoxic and hypoxic cultured tissues fixed after 48 hours of incubation. Our results showed integrity of villous tissues after 48 hours of incubation, Figure 1 A and B.

Measurements of IL-6, sIL-6R and sgp130 production

Medium concentrations of IL-6, sIL-6R and sgp130 were measured by an enzyme-linked immunosorbent assay (ELISA). The duoSet ELISA kits for IL-6, sIL-6R and sgp130 were purchased from R&D Systems (Minneapolis, MN). The assay was carried out according to the manufacturer's instruction. The range of IL-6 standard curve is 0.6 – 600pg/ml; sIL-6R standard curve is 1-1,000pg/ml. The range of sgp130 standard curve is 10-10,000pg/ml. All samples were measured in duplicate at the same time. Within assay variations were <7% for all assays. Data are presented as pg/mg of wet tissue.

Immunohistochemistry

Paraffin embedded tissue sections were immunostained for IL-6R, gp130 and SOCS-3. Immunohistochemistry procedure was performed as previously described (19). Rabbit polyclonal antibodies against human IL-6R (C-20) (Santa Cruz Biotechnology, San Diego, CA), gp130 (H-255) (Santa Cruz), and SOCS-3 (ab16030) (Abcam Inc. Cambridge, MA) were used. Secondary antibody (goat anti-rabbit) and DAB chromogen ABC staining system was also purchased from Santa Cruz (sc-2018). Slides stained with a secondary antibody only or stained with isotype rabbit IgG (ab27478) (Abcam) were used as the negative control. The nuclei were counterstained by hematoxylin. Stained slides were examined by an Olympus microscope (Olympus IX 71). Images were captured by a digital camera with PictureFrame computer software (Uptronics Inc., Sunnyvale, CA) and recorded into a microscope-linked PC computer. An image of tissue sections stained with isotype IgG is shown in Figure 1C.

Protein expression

Total placental tissue protein was extracted by homogenizing of snap frozen tissues with cold protein lysis buffer containing 50mmol/l Tris-HCl (pH7.6), 1% Triton X-100, 0.5% NP-40, 1mmol/l phenylmethylsulfonyl fluoride (PMSF) and 0.5% mmol/l dithiothreitol (DTT). Protein expressions for IL-6R, gp130 and SOCS-3 were determined by Western blot analysis. An aliquot of 20µg total protein was run on SDS-PAGE under a reducing condition. The same antibodies were used as mentioned above for the immunostaining of villous tissue sections. β-actin expression was determined and used for internal control for each sample. The density was analyzed by NIH Image software 1.16.

Statistical analysis

Unpaired t-test and Mann-Whitney U test were used for data analysis of IL-6, sIL-6R and sgp130 production and expression between normal and preeclamptic groups by a computer

software program StatView (SAS, Cary, NC). A probability level of $p < 0.05$ was considered statistically significant. Data are presented as the means $\pm$ SE.

Results

IL-6, sgp130 and sIL-6R productions by placental villous tissue

We examined IL-6, sgp130 and sIL-6R productions by placental villous tissues cultured under normoxic and hypoxic conditions. After 48 hours of incubation, culture medium was collected and measured for IL-6, sgp130 and sIL-6R by ELISA. As shown in Table 2, under the normoxic culture condition, villous tissues from preeclamptic placentas ($n=5$) released significantly less IL-6 and sIL-6R than normal villous tissue ($n=7$), $p < 0.01$. Soluble gp130 release was slightly increased by preeclamptic tissues. As result, the ratio of sgp130 to sIL-6R release was significantly higher by preeclamptic than by normal villous tissues, 124.77 ± 12.24 vs. 49.08 ± 3.87 , $p < 0.001$, respectively (Table 2).

Compared to the normoxic culture condition, IL-6 production was significantly reduced in both normal and preeclamptic tissues when cultured under lowered oxygen condition, normal: 122.27 ± 17.83 pg/mg tissue and PE: 63.80 ± 16.25 pg/mg tissue, respectively (Table 2). Normal villous tissues, but not preeclamptic villous tissues, released more sgp130 when cultured under lowered oxygen condition, 152.77 ± 9.32 pg/mg tissue. Soluble IL-6R release was slightly increased by normal tissues although not statistically different, but reduced by preeclamptic tissues when cultured under lowered oxygen condition, 1.00 ± 0.17 pg/mg tissue (Table 2). As a result, under lowered oxygen condition the mean ratio of sgp130/sIL-6R release was increased approximately 35% by preeclamptic tissues (169.56 ± 30.52), whereas the ratio of sgp130/sIL-6R release by normal tissues (52.55 ± 4.17) was relatively stable.

Immunostaining of gp130 and IL-6R

IL-6R and gp130 immunostaining was examined in tissue sections from 19 placentas, 11 from normal and 8 from preeclamptic placental tissues. In the normal group, 7 were delivered < 37 weeks and 4 delivered > 37 weeks of gestation. In the preeclamptic group, 5 were delivered < 37 weeks and 3 delivered > 37 weeks of gestation. For those delivered < 37 weeks in the normal group, one was from placenta previa, two from twins, 3 due to preterm delivery, and one case had maternal congenital heart defect. None of them had signs of infection. The purpose of using preterm placentas in the normal group was to compare the staining results between normal and PE placentas matched with gestational age. As shown in Figure 2, both IL-6R and gp130 immuno-reactivity are mainly localized in the syncytio-trophoblasts layer of villous tissue. IL-6R, but not gp130, immunostaining was reduced in the syncytio-trophoblasts of placentas from women with preeclampsia (Figure 2 C and D) compared to those from normal pregnant controls, both < 37 weeks and > 37 weeks (Figure 2 A and B). The intensity of gp130 immunostaining in syncytio-trophoblasts was relatively similar between normal placentas (Figure 2 E and F) and preeclamptic (Figure 2 G and H) tissue sections.

Gp130 and IL-6R protein expressions

Placental tissue IL-6R and gp130 protein expressions were determined by Western blot, in which whole placental villous tissue homogenate was used, 5 from normal and 5 from preeclamptic placentas. β -actin expression was used as an internal control for each sample. We noticed that the relative density of IL-6R expression was significantly reduced in preeclamptic compared to normal placental tissue samples, 0.204 ± 0.026 vs. 0.706 ± 0.070 , $p < 0.01$, respectively. Gp130 expression was relatively consistent between preeclamptic and normal placental tissues, 1.006 ± 0.134 vs. 1.363 ± 0.048 , respective (Figure 3). These results

were consistent to the immunostaining data, in which, reduced IL-6R expression was down-regulation in preeclamptic placentas.

SOCS-3 expression

It is known that activation of IL-6R/gp130 pathway is critical to initiate SOCS-3 expression, which could, in turn, suppress cytokine induced down-stream inflammatory response. We then further examined SOCS-3 expression in placental tissues by immunostaining and by Western blot analysis. Immunostaining results are shown in Figure 4, upper panel. Similar to IL-6R and gp130, SOCS-3 immunostaining is mainly localized in the syncytiotrophoblast layer in the villous tissue. SOCS-3 immunostaining was much reduced in the villous tissue from preeclamptic placentas, both in preterm and term placentas (Figure 4 C and D), compared to those from normal placentas (Figure 4A: <37 weeks and Figure 4B: > 37 weeks). Reduced SOCS-3 protein expression in preeclamptic placental tissues was further confirmed by Western blot analysis (Figure 4, lower panel).

Discussion

In this study, we found that IL-6 production/release was significantly reduced by villous tissue from preeclamptic placentas when cultured under 21%O₂ condition, which is consistent with a previously published work (14). We also found decreased IL-6 production by both normal and preeclamptic placentas when tissues were cultured under hypoxic condition. Reduced IL-6 production could be significant and lead to a lack of cellular response to inflammatory stimulation in the placental trophoblasts in preeclampsia.

IL-6 is considered as both a pro- and an anti-inflammatory cytokine. It belongs to the Th2 cytokine family. Central to the IL-6 mediated responses are the corresponding receptors, not only on the target cells but also their soluble receptors in the extra-cellular fluid. IL-6 binds to both membrane-bound IL-6 receptor (IL-6R) and soluble IL-6R (sIL-6R) with similar affinities (20). Both the IL-6/IL-6R complex and the IL-6/sIL-6R complex are capable of eliciting IL-6 biological effects by binding to the trans-signal receptor gp130 in the cell membrane to initiate JAK phosphorylation and induce activation of SOCS-3 expression in target cells (21). However, when IL-6/sIL-6R binds to soluble gp130 (sgp130) in the extra-cellular fluid, such as in the circulation, and forms sgp130/IL-6/sIL-6R complex, IL-6/sIL-6R complex is no longer able to bind to membrane gp130. Thus, sgp130 could block IL-6 induced trans-signaling effects. Therefore, sgp130 is considered a native antagonist to IL-6 or IL-6/sIL-6R complex in a biological system (6,8,9,20).

One of the most significant findings of the present study is the increase in the ratio of sgp130/sIL-6R release by preeclamptic placentas. Increased sgp130/sIL-6R release by preeclamptic placentas suggests that antagonist effects of sgp130 to IL-6 or IL-6/sIL-6R complex may be important in the preeclamptic placentas. In addition, reduced villous production of sIL-6R in preeclampsia, regardless of the IL-6 levels, might further decrease the amount of IL-6/sIL-6R complex formation and increase the antagonist effect produced by sgp130. Through immunostaining of placental tissue sections, we found that IL-6 and gp130 receptors are mainly localized in the syncytiotrophoblast layer of villous tissue. We further noticed reduced IL-6R expression in trophoblast cells from preeclamptic placentas compared to those from normal placentas, as viewed by both immunostaining and Western blot data. These results suggest that both classic IL-6 signaling and IL-6 trans-signaling through cognate IL-6 receptors may be impaired in placental trophoblasts in preeclampsia.

The reason for selectively reduced sIL-6R release and IL-6R expression, compared to the sgp130 release and gp130 expression, in preeclamptic placentas is not known. Further study is needed to determine if reduced membrane-bound IL-6R is causal for the reduced sIL-6R

release from preeclamptic placentas. Regardless the reason, we believe that the ratio of sgp130/sIL-6R would present the balance between antagonistic and agonistic effects on IL-6 induced trans-signaling response in placental trophoblasts.

Another significant finding of the present study is the decreased SOCS-3 immuno-reactivity and protein expression in villous tissues from preeclamptic placentas. SOCS-3 is a key negative regulator of cytokine signaling (5,21). SOCS-3 also plays a fundamental role in the placental trophoblast development. Roberts et al reported that mice with a deletion of SOCS-3 die at mid gestation (E11-13) due to placental insufficiency (22). In their study, they found that the placental spongiotrophoblast layer was significantly reduced and the embryonic lethality is not due to anatomical defects of embryo, but rather poor placental development that account for the developmental arrest and death (22). Their study suggests that SOCS-3 is an important molecule in the regulation of trophoblast function during placental development. In general, cellular SOCS-3 expression can be induced by IL-6 trans-signaling pathway stimulation. SOCS-3 has a high affinity with phosphorylated gp130 on cell membrane. The induced SOCS-3 expression then block STAT3 phosphorylation (23), thus terminating or attenuating IL-6 and other cytokine signaling. Therefore, SOCS-3 deficiency is not only responsible for poor placental development, but may also contribute to inadequate anti-inflammatory response in placental trophoblasts, such as in the case of preeclampsia.

In contrast to IL-6 production, sgp130 release was increased in normal placentas when the tissues were cultured under lowered oxygen condition, which mimics the level of sgp130 release by preeclamptic tissues cultured under normoxic condition. Hypoxia had no effects on sIL-6R release by normal tissues, but could reduce sIL-6R release by preeclamptic tissues. The increased ratio of sgp130/sIL-6R release by preeclamptic, but not by normal, placental tissue under hypoxic condition further support the notion that hypoxia attributes to the pathophysiology of trophoblast function in preeclampsia.

Although the role of cellular regulation of chemokine/cytokine generation and anti-inflammatory response in preeclamptic placentas is not well understood, studies have shown that dysregulation of a number of placental cytokines is associated with defective trophoblast invasive potential in preeclampsia, such as TGF β -3 and TNF- α (24,25). At the present time, it is not known what modulates IL-6, sIL-6R and sgp130 releases in placental trophoblasts. However, findings of increased ratio of sgp130/sIL-6R production and reduced IL-6R and SOCS-3 protein expression in preeclamptic placentas could be significant regarding to the placental pathophysiology in preeclampsia. Whether impaired IL-6 trans-signal/SOCS-3 pathway regulation is an early event which leads to increased inflammatory response in placental trophoblasts in preeclampsia warrants further investigation.

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Figure 1.

Hematoxylin and eosin (H&E) staining, and isotype IgG staining of villous tissue sections. A and B: H&E staining. Villous tissue pieces were fixed with 10% formalin after 48 hours of culture and embedded with paraffin. Tissue sections (5 micron thickness) were H&E stained to evaluate the tissue viability. The H&E staining shows integrity of villous tissues after 48 hours of incubation, A: tissue cultured under 5%CO₂ and air; B: tissue cultured under 2%O₂/5%CO₂/93%N₂, respectively. C: isotype IgG staining as negative control. A and B: bar = 20 micron; C: bar = 50 micron, respectively.

Figure 2.

Immunostaining of IL-6 receptors IL-6R and gp130 in villous tissue sections from both normal and preeclamptic placentas. IL-6R: A to D; gp130: E to H. Normal placentas: A, B, E and F; Preeclamptic placentas: C, D, G and H. A, C, E and G are placentas delivered before 37 weeks of gestation; and B, D, F and H are placentas delivered after 37 weeks of gestation. IL-6R and gp130 are mainly localized in syncytio-trophoblast layer of the placenta. Immunostaining of IL-6R, but not gp130, was reduced in syncytio-trophoblast layer of preeclamptic placentas compared to normal placentas, in both < 37 weeks and > 37 weeks of samples. Bar = 20 micron.

Figure 3. Placental tissue IL-6R and gp130 expressions. IL-6R and gp130 expressions were determined in 5 normal and 5 preeclamptic placentas by Western blot analysis. β -actin expression was determined as internal control for each sample. The bar graph shows the relative density of IL-6R and gp130 protein expression after corrected with β -actin expression. IL-6R protein expression was significantly reduced in placentas from preeclampsia compared to normal controls, $p < 0.01$.

Figure 4. SOCS-3 immunostaining and protein expression in placental tissues from normal and preeclamptic placentas. SOCS-3 immunostaining is also mainly localized in the syncytiotrophoblast layer of the placenta. Similar to IL-6R, both SOCS-3 immunostaining (upper panel) and protein expression (lower panel) are reduced in preeclamptic placental tissue section compared to the normal controls. SOCS-3 immunostaining (normal: A and B; preeclampsia: C and D), Bar = 20 micron.

Table 1

Demographic characteristics for normal and preeclamptic pregnancies from which placentas were used in the study

Variables	Normal (n=18)	Preeclampsia (n=15)	p value
Maternal age (years)	25 ± 2	26 ± 2	nd
Racial Status			
White	4	1	---
Black	14	14	---
Gestational Age (weeks)	37 ± 2	34 ± 5	>0.05
Blood Pressure (mmHg)			
Systolic	124 ± 6	170 ± 4	<0.0001
Diastolic	74 ± 5	104 ± 4	<0.0001
Mode of Delivery			
Vaginal	6	2	---
Caesarean Section	12	13	---
Placental Weight (gram)	509.4 ± 84	493 ± 61	>0.05

Data presented as mean ± SD.

Table 2

Soluble IL-6R and sgp130 releases by placental villous tissues

	Normal (n=7)		PE (n=5)	
	Normoxia	Hypoxia	Normoxia	Hypoxia
IL-6	361.01 ± 15.73	122.27 ± 17.83 ^{##}	180.00 ± 40.43 ^{**}	63.80 ± 16.25 [#]
sgp130	131.52 ± 8.19	152.77 ± 9.32 [#]	152.94 ± 13.45	150.31 ± 8.54
sIL-6R	2.73 ± 0.19	2.98 ± 0.24	1.28 ± 0.19 ^{**}	1.00 ± 0.17
Ratio of sgp130 to sIL-6R	49.08 ± 3.87	52.55 ± 4.17	124.77 ± 12.24 ^{**}	169.56 ± 30.52

Data are expressed as mean ± SE, pg/mg wet tissue.

^{**}
p < 0.01: under normoxic condition, PE vs. normal.[#]
p < 0.05^{##}
p < 0.01: hypoxia vs. normoxia;