

Suppressors of cytokine signaling proteins in human preterm placental tissues

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Abstract

Decreased suppressors of cytokine signaling (SOCS) activity in human gestational tissues may play a part in the onset/progression of term labor. Since SOCS proteins negatively regulate cytokine-mediated inflammatory processes, we hypothesized that SOCS proteins are elevated in gestational tissues from spontaneous preterm deliveries with intrauterine infection. SOCS1, -2 and -3 mRNAs and proteins were detectable by RT-PCR and immunoblotting respectively, in preterm amnion, choriondecidua and placenta, irrespective of infection status. Immunoperoxidase staining localized SOCS1, -2 and -3 to all cell types of the gestational membranes, with infiltrating leukocytes reacting strongly in infected tissues. In villous placenta, SOCS was immunolocalized to the syncytiotrophoblast with marked staining of round mesenchymal cells, possibly Hofbauer cells. Nuclear SOCS staining was seen in amnion, chorion and placental syncytiotrophoblasts. SOCS proteins were, in general, significantly more abundant in placenta compared with amnion or choriondecidua. Placental SOCS1 and interleukin-1 β concentrations were positively correlated ($r^2=0.47$; $P<0.05$). However, no changes in SOCS levels in any tissues were observed with intrauterine infection. The relatively large amounts of SOCS proteins in the placenta may reflect a placenta-specific immuno-protective response to minimize the elaboration and effects of cytokines with potential to harm the placenta and fetus. Lack of labor-associated changes in SOCS levels suggests that the regulation of SOCS expression in preterm gestational tissues differs from those at term, perhaps reflecting roles in regulating placental somatotrophic responses.

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Introduction

The suppressors of cytokine signaling (SOCS) proteins were initially identified as cytokine-inducible inhibitors of intracellular signaling by a wide range of cytokine-related mediators including leukemia inhibitory factor (LIF), interleukin (IL)-6, IL-4, prolactin (PRL), growth hormone, interferon (IFN), and stem cell factor (Nicola & Greenhalgh 2000, Kile & Alexander 2001). The SOCS family is comprised of at least eight members, all containing a characteristic central SH2 domain and a carboxy terminal 'SOCS box'. SOCS proteins act as intracellular regulators of cytokine signaling by binding to members of the Janus kinase (JAK) family of protein tyrosine kinases, inhibiting kinase activity as well as subsequent phosphorylation and activation of downstream targets such as the receptor and signal transducers and activators of transcription (STATs). To date, only four members, CIS, SOCS1, SOCS2, and SOCS3, have been thoroughly studied (O'Shea *et al.* 2002, Kubo *et al.* 2003). SOCS1 and SOCS3 are

thought to inhibit cytokine signaling by binding to JAK kinases or to the JAK kinase/activated receptor complex and interfering with the catalytic activity of the kinase (Nicholson *et al.* 1999, Sasaki *et al.* 2000), whereas SOCS2 may act as a stimulator of signaling by some cytokines by suppressing the actions of SOCS1 (Pezet *et al.* 1999). Negative regulation of cytokine signaling by SOCS proteins may play a crucial role in both physiological and disease states by, for example, terminating responses to potentially damaging effects of cytokine action in inflammatory responses (Kubo *et al.* 2003).

A delicate and complex network of cytokines and growth factors within gestational tissues underlies the establishment and maintenance of a successful pregnancy. Inflammatory processes have long been implicated in the mechanisms of human parturition at term (Liggins 1981, Mitchell 1983). However, inflammatory activation prior to the onset of term parturition, as occurs during intrauterine infection, has been associated with preterm labor (PTL). While the causes of

spontaneous preterm birth are perceived to be multifactorial, intrauterine infection-driven PTL has frequently been associated with elevated levels of cytokines in amniotic fluid and fetal tissues. This is presumed to arise from a response of resident tissue cells or infiltrating immune cells to ascending pathogenic bacteria. Indeed, increased numbers of immune cells within the placenta and fetal membranes is an indicator of infection and these cells are known to produce large amounts of cytokines which may result in early onset labor (Mitchell *et al.* 1991, Gibbs *et al.* 1992).

We recently described an apparent withdrawal of SOCS proteins in gestational tissues with term labor, and hypothesized that a decrease in SOCS proteins in the term placenta could be part of the mechanism facilitating labor-associated inflammation, or part of a feed-forward mechanism for amplification of labor-associated processes through uninhibited cytokine effects (Blumenstein *et al.* 2002). The essential roles both of SOCS1 in the decidualization in rats (Barkai *et al.* 2000) and of SOCS3 in the regulation of cytokine signals involved in placental development (Roberts *et al.* 2001) are supportive of the notion that SOCS proteins could act as important regulators in human pregnancy and labor. However, the expression of SOCS proteins in human placental tissues throughout gestation has not been investigated, and the association between preterm labor or intrauterine infection and SOCS expression remains unknown.

The aim of the present study was to further investigate the role of SOCS proteins in the biomolecular processes leading to preterm birth, by exploring the association between intrauterine infection/chorioamnionitis and SOCS protein abundance and localization in gestational tissues delivered following spontaneous preterm labor.

Materials and methods

Tissues

Total RNA preparations and total cellular protein lysates were derived from a previously prepared tissue bank of gestational tissues (Keelan *et al.* 1999, Blumenstein *et al.* 2002) stored at -75°C . RNA and protein samples of amnion, choriondecidual, and villous placental origin used in this study were from preterm births with spontaneous PTL. The gestational age at delivery for the preterm group without infection averaged 32.2 ± 1.09 weeks (mean \pm S.D.) whereas the preterm group with infection delivered earlier at 28.1 ± 3.2 weeks. For the present study only singleton pregnancies were chosen. Placental tissues were obtained from the National Women's Hospital, Auckland, New Zealand with maternal consent under the approval of the Auckland Human Ethics Committees.

Assessment of infection and leukocyte infiltration

The PTL subgroups, with and without evidence of intrauterine infection, used in this study were derived from women presenting with spontaneous preterm birth, excluding complications by preeclampsia or intrauterine growth restriction. Evidence of intrauterine infection (PTL with infection) was defined as histological report (if available) indicating chorioamnionitis, definite or probable neonatal sepsis, or two or more of: maternal fever, maternal tachycardia, fetal tachycardia, uterine tenderness, neonatal sepsis and foul smelling liquor. Non-infected pregnancies (PTL no infection) were negative for all indices. In cases of absence of histological reports, leukocyte infiltration of the gestational membranes was additionally characterized by immunostaining with anti-CD45 leukocyte common antigen (Dako Corp., Carpinteria, CA, USA). The extent of CD45 positive cells in the gestational membranes was reported (Marvin *et al.* 1999) according to grades 0 to 4 for degree of infiltration of the gestational membranes, and + or – for each of the fetal membranes (Salafia *et al.* 1989).

Qualitative RT-PCR

First strand cDNA synthesis of amnion, choriondecidual and villous placental RNA was synthesized by reverse transcription of $1\text{ }\mu\text{g}$ total RNA each, utilizing 100 U Superscript II reverse transcriptase and oligo(d)T as primer (both from Life Technologies, Auckland, NZ) according to the manufacturer's protocol. A $2\text{ }\mu\text{l}$ sample of the RT reaction was then amplified in a total volume of $50\text{ }\mu\text{l}$ comprising $1\times$ PCR buffer, 1 U TAQ polymerase, 1 mM MgCl_2 , 0.4 mM dNTP mix (all supplied by Roche Molecular Biochemicals, Auckland, NZ) and $0.4\text{ }\mu\text{M}$ of each gene-specific primer pair for SOCS1 (forward: 5'-agaccctctctcacctcttg-3', reverse: 5'-ctgcacagc agaaaataaagc-3'), SOCS2 (forward: 5'-ctgccaccatttcgg acacc-3', reverse: 5'-cgctccctccccgccattcc-3') or SOCS3 (forward: 5'-gtcaccacagcaagttt-3', reverse: 5'-ctgagcgt gaagaagtgg-3'). PCR proceeded with the following profile: initial denaturation at 94°C for 5 min followed by 35–40 cycles at 94°C for 1 min, 58°C (SOCS1) or 65°C (SOCS2 and SOCS3) for 1 min, 72°C for 1 min with a final elongation at 72°C for 10 min. Amnion, choriondecidua, and villous placenta ($n=4$) from each condition (infection versus no infection) were amplified as above. PCR products were resolved on a 2% (w/v) agarose gel and visualized by ethidium bromide staining. cDNA synthesis was confirmed by RT-PCR (conditions as above with primer annealing at 65°C , amplification over 35 cycles) using specific primers to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5'-cat catctctgcccccttg-3', reverse: 5'-cctgtctcaccaccttcttg-3'). Identities of PCR products for SOCS1, SOCS2, and

SOCS3 were confirmed by sequencing the gel-purified PCR amplicons (The Centre for Genomics and Proteomics, School of Biological Sciences, University of Auckland, NZ; gel purification kit obtained from QIAGEN, Hilden, Germany) and subsequent BLAST analysis. For size determination of PCR products, a 123 bp ladder (Life Technologies) was used. As positive control for SOCS mRNA expression, the human mammary carcinoma cell line T47D was stimulated with human PRL for 2 h as described previously (Pezet *et al.* 1999). Human liver microsomal cell preparations served as positive control for SOCS2 mRNA expression. (Fig. 1A & B).

Western blot analysis

A 20 µg aliquot of total protein lysates (Keelan *et al.* 1999) from each tissue sample was separated on 12% NuPAGE Bis-Tris gels in 1 × MOPS running buffer (both obtained from Invitrogen, Auckland, NZ) and transferred to PVDF membrane (Amersham Pharmacia Biotech, Auckland, NZ). Immunodetection of SOCS1, SOCS2 and SOCS3 was achieved with appropriate antibodies to each protein Zymed; 1:100, (Innovative Science Ltd, Dunedin, NZ) goat anti-rabbit biotinylated secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA, USA; 1:3000) and biotinylated streptavidin-horseradish peroxidase complex (Amersham Pharmacia Biotech; 1:4500); visualization was by enhanced chemiluminescence (NEN, Boston, MA, USA) followed by exposure to X-ray film (Amersham Pharmacia Biotech). Specificity of detected protein bands was determined by pre-adsorption of antibodies with specific peptide immunogens to SOCS1, SOCS2 and SOCS3. Human peripheral blood lymphocytes (PBL) from a normal healthy donor were prepared using standard methods and were stimulated with 2 µg/ml phytohemagglutinin (PHA)-L (Roche) for 48 h in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 U/ml each of penicillin and streptomycin. Total cellular protein lysates were then prepared and used as a positive control for SOCS1 protein expression. As positive controls for SOCS2 and SOCS3 protein expression, human liver and murine lung tissue respectively, which express these two SOCS proteins constitutively, were used. For determination of protein sizes, a marker consisting of biotinylated low range proteins (BIORAD, Auckland, NZ) was run on the same gel as the samples, blotted and detected as for samples. Blots were scanned in a densitometer and band intensity was quantified using ImageQuant software (Molecular Dynamics; Amersham Biosciences, Auckland, NZ). After background subtraction, arbitrary units above zero were considered positive for SOCS protein expression.

Negative values were regarded as zero i.e. below the limit of detection.

Immunohistochemistry

Paraformaldehyde-fixed (4%), paraffin-embedded tissue blocks from placenta and gestational membranes (the same placentas as used to prepare the soluble tissue extracts) were sectioned at 7 µm and dewaxed/rehydrated prior to heat-induced antigen retrieval (microwave treatment for 5 min in 0.5 M Tris pH 10). Endogenous peroxidase activity was blocked with 3% H₂O₂ in 50% methanol for 20 min at room temperature. For some sections, amplification of SOCS signal was employed using the TSA biotin system kit (Perkin-Elmer Life Sciences, Boston, MA, USA), and those sections received an additional blocking step using the blocking reagent supplied in the kit. Sections were then incubated overnight at 4 °C in primary polyclonal antisera (1:100 dilution of SOCS antibody as detailed above) in the presence of 5% normal horse serum and 0.5% (v/v) Tween 20, followed by incubations at room temperature for 1–3 h with biotinylated secondary antibody (1:500 dilution; Jackson ImmunoResearch Laboratories), and for 1 h with extravidin peroxidase (Sigma Chemical Co., St Louis, MO, USA; 1:250). Immunoperoxidase staining was developed using diaminobenzidine (DAB). Sections were not counterstained. Digital photomicrographs were taken using a Nikon Eclipse E800 microscope fitted with a JBC TK-C1381 video camera and color-inverted to enhance visual clarity.

Cytokine assays

Interleukin-1β, IL-6 and IL-8 concentrations were measured by two-site ELISA according to published, validated procedures using commercially available matched-pair antibodies (R&D Systems, Minneapolis, MN, USA) as previously described (Keelan *et al.* 1999).

Statistical analysis

Densitometric values of SOCS protein expression from preterm labor samples were normalized to an appropriate positive control for SOCS (PBL for SOCS1, human liver for SOCS2, and PRL-stimulated T47D cells for SOCS3) to allow analytical comparison of SOCS abundance on multiple blots ($n=9$ individuals per group). For example, SOCS1 proteins of placenta and membranes were normalized to SOCS1 expression of PHA-stimulated PBLs analyzed on the same gel as the samples. Each lane of the PAGE gel received 20 µg total protein (samples and positive controls). Data are presented as mean (\pm standard error) band intensity relative to the appropriate SOCS control. Statistical

significance between means was determined by one-way ANOVA followed by post-hoc Dunnett's test. Significance was set at $P < 0.05$. Correlation between tissue SOCS protein expression and previously published cytokine concentrations (Keelan *et al.* 1999) was performed with GraphPad Prism version 4.01 (GraphPad Software Inc., San Diego, CA, USA) using Pearson correlation with a 95% confidence interval. Correlation with a P value < 0.05 was considered statistically significant.

Results

SOCS mRNA expression in preterm gestational tissues by RT-PCR

The mRNA transcripts for SOCS1 and SOCS2 were detectable by qualitative RT-PCR in preterm human amnion and choriodecidua from eight different individuals, four with evidence of intrauterine infection and four without infection (Fig. 1A). SOCS1 and SOCS2 were detectable at 202 bp and 176 bp respectively, regardless of the infection status. In the preterm membranes, amplification of SOCS3 (expected PCR product size 245 bp) by RT-PCR was problematic giving rise to smeary or very faint bands of the gel-separated PCR products (data not shown). We previously reported the presence of SOCS3 mRNA in amnion and choriodecidua at term, regardless of labor status (Blumenstein *et al.* 2002). However, SOCS3 expression in the preterm membranes by RT-PCR remained inconclusive despite the fact that several different primer pairs specific for SOCS3 were utilized and that it is detectable in extraplacental membranes at term. In the preterm placenta, all three SOCS mRNA transcripts were detectable, irrespective of infection status (Fig. 1B). Success of cDNA synthesis was verified by amplification of the housekeeping gene GAPDH and was detectable in all samples from amnion and choriodecidua (Fig. 1A) and except for one out of four placental samples without infection where GAPDH was weakly expressed (Fig. 1B).

Expression patterns of SOCS proteins in preterm gestational tissues with and without infection

In the amnion and choriodecidua Western blot analysis revealed the presence of SOCS1 and SOCS3 proteins of the expected molecular weight at 23 and 25 kDa respectively, which were expressed as single bands in all samples ($n=18$) regardless of infection status. Figure 2 shows a representative blot of SOCS1, -2 and -3 protein expression of three samples per group (infection versus no infection). A similar expression pattern in the membranes was found for SOCS2; however, in some blots a second immunoreactive SOCS2 protein was

detected which migrated slightly below the expected 22 kDa band. Both bands were abolished when antibody was pre-adsorbed with SOCS2-specific peptide confirming specificity. In the amnion and choriodecidua, relative expression of SOCS1, SOCS2, and SOCS3 proteins ranged from 2.6 to 15.3% of the appropriate positive control as determined by densitometric evaluation of the bands from placental, amnion and choriodecidua samples, $n=9$ per group (Fig. 3A-C). There were no significant differences in levels of SOCS proteins between membrane tissues, either with or without intrauterine infection.

In the villous placenta, SOCS1 and SOCS3 protein expression was detectable as a single band as described above, whereas SOCS2 protein appeared as a double band. In the tissues without infection, 3 out of 9 placental samples had detectable SOCS protein expression (Fig. 2), with levels of all three SOCS proteins averaging 24.4% of the appropriate control (Fig. 3A-C). In this group, levels of placental SOCS2 protein were greater than in amnion and choriodecidua (Fig. 3B). In the tissues with infection SOCS1, -2, and -3 proteins were detectable in four out of nine individual placentas, with relative expression levels ranging from 23 to 42% of the appropriate positive control for SOCS (Fig. 3A-C). In the infection group, SOCS2 protein was more abundant in the placenta compared with the membranes (Fig. 3B), while SOCS3 protein levels were greater in the placenta than in the amnion (Fig. 3C). However, there was no statistically significant difference in SOCS protein abundance between the two groups (with and without intrauterine infection) in any of the three tissues.

Correlation of SOCS protein levels with previously reported concentrations of cytokines from identical amnion, choriodecidua, and placental tissue samples (Keelan *et al.* 1999, 2003) revealed a significant correlation between SOCS1 protein expression and IL-1 β content in the placenta from preterm deliveries complicated with intrauterine infection ($r^2=0.47$; $P < 0.05$) (Fig. 4). There was no significant correlation between SOCS protein expression and IL-8 concentrations in any of the tissues investigated.

Immunolocalization of SOCS in preterm villous placenta

SOCS1, -2 and -3 immunostaining was detectable in all placental tissues and membranes from both PTL groups regardless of infection status or scale of infection ($n=6$ individual tissue sets per group). Figure 5 shows representative immunohistochemical slides of SOCS1 (Fig. 5A), SOCS2 (Fig. 5B) and SOCS3 (Fig. 5C) in the preterm placenta without infection in comparison with tissue sections incubated with the relevant pre-adsorbed SOCS antibody (Fig. 5D-F). In the placenta, SOCS1 showed weak syncytial staining with occasional nuclei

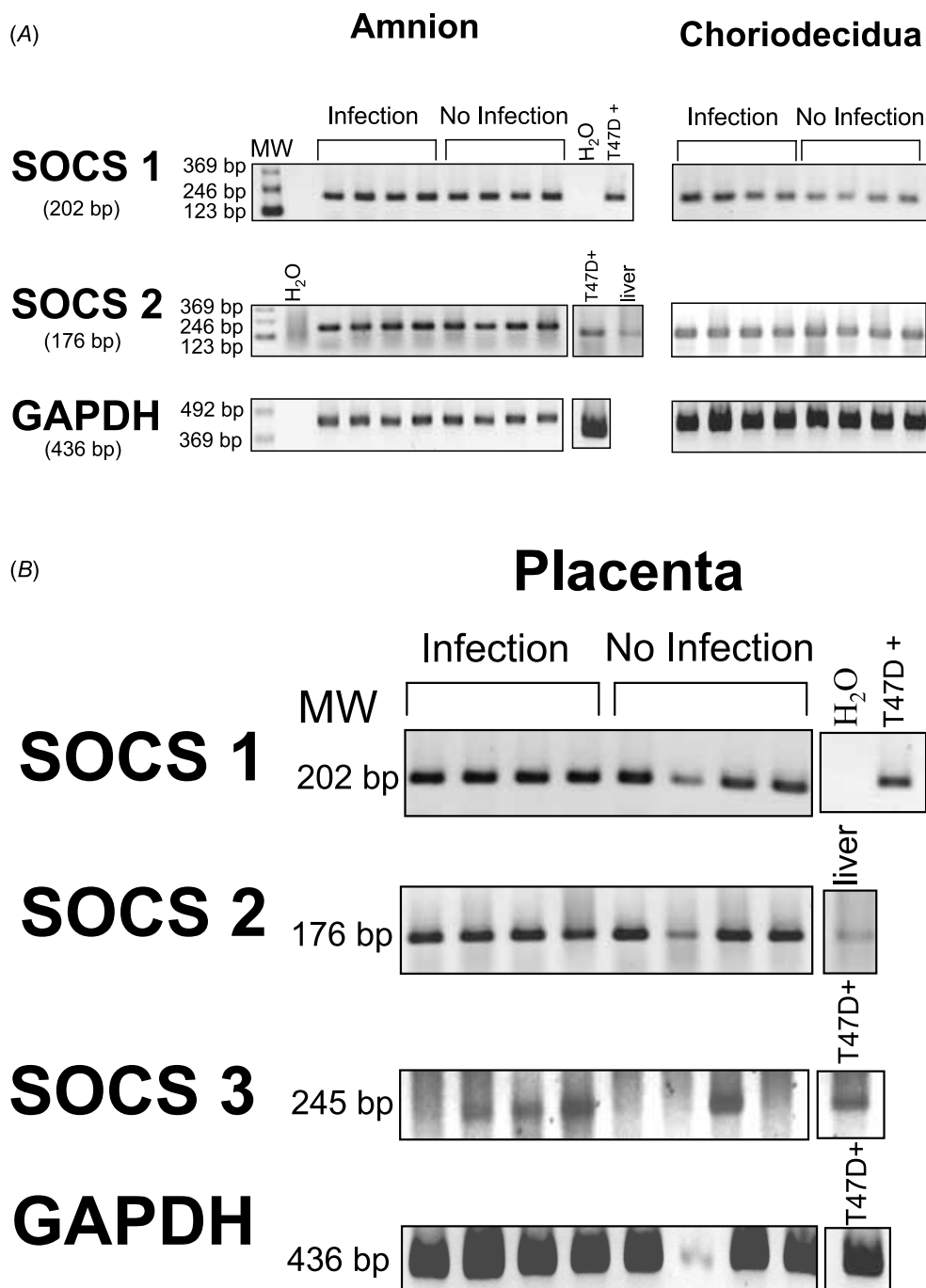


Figure 1 (A) RT-PCR analysis of SOCS expression in preterm human amnion and choriodecidua. Representative ethidium bromide stained gels showing a single PCR product for SOCS1 and SOCS2 in amnion and choriodecidua with and without infection. GAPDH was used as a control for cDNA synthesis; prolactin-stimulated T47D cells (T47D+) were used as a positive control for SOCS1, SOCS2, SOCS3 and human liver cells as positive control for SOCS2 mRNA. Sizes of expected PCR product for each mRNA are indicated to the left of the gel. (B) Reference RT-PCR analysis of SOCS expression in preterm placenta. A single PCR product for SOCS1, SOCS2 and SOCS3 was observed in placenta with and without infection. Controls as for (A). The expected sizes of PCR product are noted to the left of the gel.

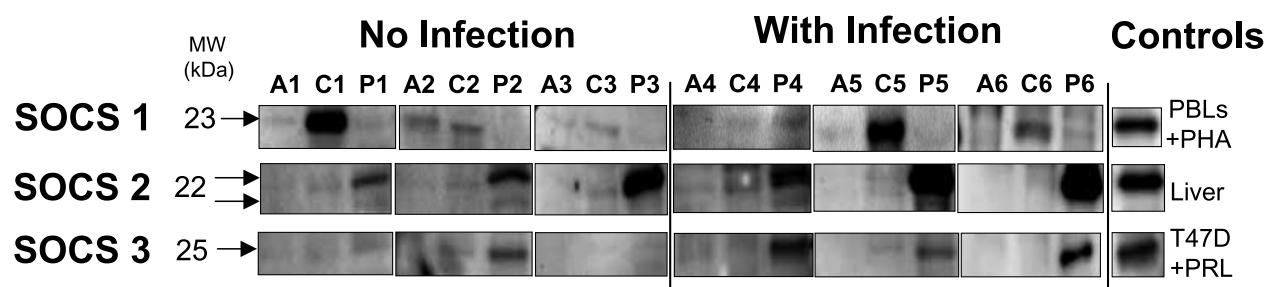


Figure 2 Protein expression of SOCS family members in human gestational tissues at preterm. Total cell lysates from amnion (A), choriodecidua (C) and villous placenta (P) from 9 different individuals per sample group (infection versus no infection) were loaded at 20 µg per lane and immunoblotted for SOCS1, SOCS2 and SOCS3 protein with respective antibodies as described in Materials and methods. Molecular masses of the proteins are indicated by arrows on the left, calculated from biotinylated protein markers run in parallel to the samples. SOCS1 and SOCS3 were expressed as single protein bands whereas SOCS2 appeared as two proteins.

being strongly stained; sporadic cytotrophoblasts were also strongly stained (Fig. 5A). Furthermore, SOCS1 staining of larger cells within the villi was detectable. These large cells exhibited Hofbauer-like morphological features, although attempts to identify the cell type by co-localization with anti-CD68 (specific for macrophages) was inconclusive. SOCS2 protein in the placenta (Fig. 5B) was strongly associated with the syncytia and appeared to be preferentially localized to the basement membrane. Strong SOCS2 staining was also observed in large mesenchymal cells, but none was observed in the cytotrophoblast. SOCS3 immunoreactive protein (Fig. 5C) was detected in syncytial and mesenchymal (possibly Hofbauer) cells but this was not basally located as seen in the SOCS2 slides. There was no detectable SOCS staining in any of the endothelial cells or fetal blood vessels. However, in placentas from preterm deliveries with infection, infiltrating neutrophils were evident which stained strongly for SOCS1, SOCS2 and SOCS3 (not shown). Overall, there were no obvious differences in immunoreactive SOCS protein localization between the infected and non-infected groups.

Immunolocalization of SOCS in preterm membranes

Figure 6 depicts results of the immunohistochemical analysis of SOCS proteins in preterm gestational membranes, here representing sections from the PTL group without infection. In the amnion (n=12), SOCS1, SOCS2 and SOCS3 staining was evident in the epithelial cells (Fig. 6A-F). SOCS staining was mainly cytoplasmic but in some cells scattered nuclear SOCS staining was observable. The mesenchyme of the amnion was also positive for all three SOCS proteins. In the chorion SOCS1, -2 and -3 staining was again mainly cytoplasmic but scattered strong nuclear staining was also seen. All three SOCS proteins were detected in the cytoplasm of all decidual cells. There was no change in SOCS immunostaining patterns with infection,

although preterm membranes with clinically manifest chorioamnionitis showed positive SOCS1, -2 and -3 staining of the infiltrating leukocytes (not shown). Tissue sections incubated with SOCS antibody pre-adsorbed to an appropriate peptide were negative for SOCS staining and are shown in Fig. 6 G-I.

Discussion

In this study we have described, for the first time, the presence of SOCS mRNA and proteins in preterm gestational tissues delivered after spontaneous labor and we have determined their abundance and localization with and without intrauterine infection. Regardless of infection status, mRNA for all three SOCS proteins were detectable in the placenta, although only SOCS1 and SOCS2 mRNAs were unequivocally detected in the membranes. Overall, SOCS protein levels were significantly higher in the preterm placenta compared with the membranes (amnion and choriodecidua), consistent with our previous findings in term tissues (Blumenstein *et al.* 2002), and supports the notion that the placenta may have more of a role in labor-associated immune responses than previously thought (Steinborn *et al.* 1995). These data are consistent with the work of Steinborn *et al.* (1995) who provided evidence of placental cytokine involvement in preterm labor, both with and without intrauterine infection. Although we have previously described the effects of labor on placental SOCS expression in term tissues, a deficiency in the present study is the lack of a non-labor control group of tissues delivered preterm. Unfortunately, due to the difficulty in obtaining tissues from normal pregnancies delivered preterm by Caesarean section without serious maternal disease or obstetric complications, factors that could independently alter SOCS expression, inclusion of an appropriate control group is not possible. This is an unavoidable limitation experienced in many studies of human pregnancy tissues.

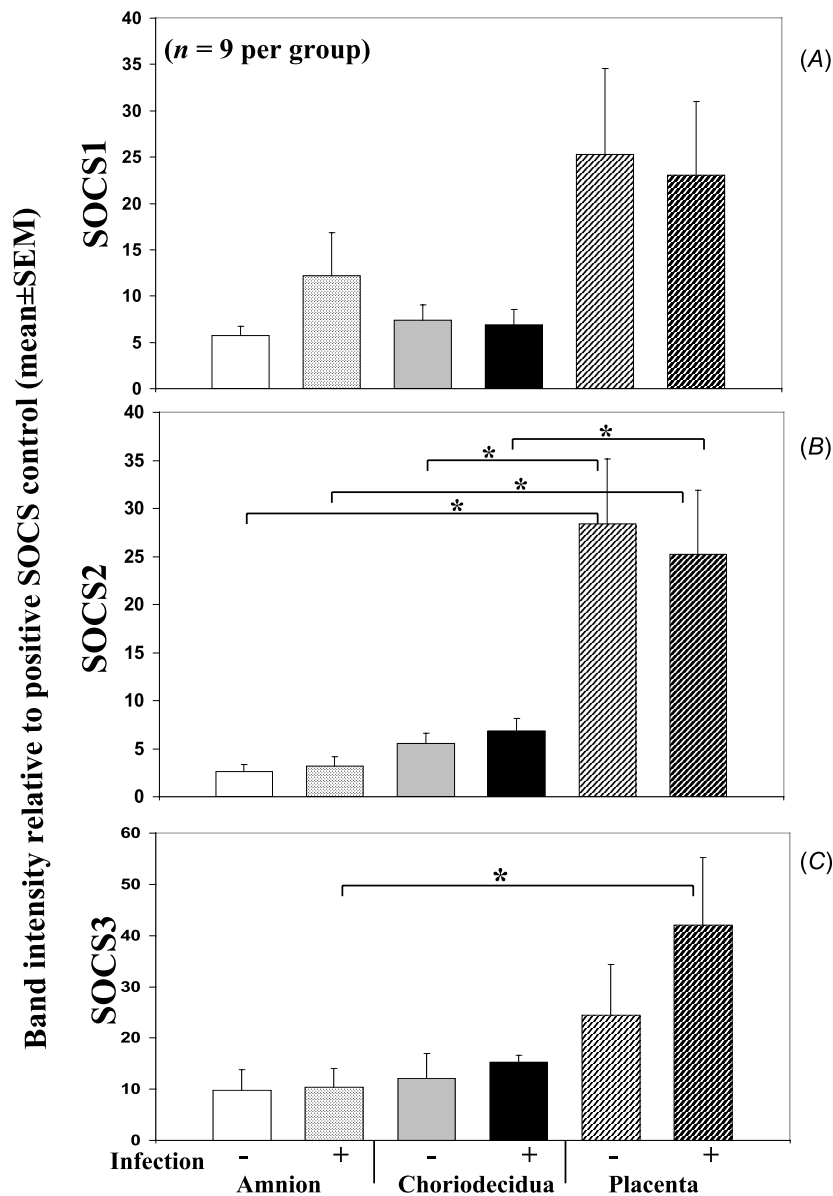


Figure 3 Expression patterns of SOCS proteins in preterm human gestational tissues with and without clinical manifestation of infection. The relative band intensity is normalized to a respective positive control for (A) SOCS1, (B) SOCS2 and (C) SOCS3. The graph shows normalized relative band intensities derived from Western blot analysis for SOCS1, -2 and -3 protein by human amnion, choriodecidua and placenta at preterm with (+) and without (-) infection ($n=9$ per group). Statistical significance between means was determined by ANOVA and post-hoc Dunnett's test with significance set at $P<0.05$.

We detected strong immunostaining in mesenchymal cells of placental villi irrespective of infection in both preterm labor groups. Unfortunately, we were unable to confirm whether these SOCS-positive cells were Hofbauer cells (placental macrophages), although this seems likely judging from their morphological appearance. While the mean gestational age at delivery of the

infected group was approximately 4 weeks earlier than the non-infected group, both groups of patients delivered well in advance of term (>5 weeks) and it is unlikely that this difference contributed towards the observed similarities in SOCS expression between the two groups. However, we cannot discount this possibility entirely, although there was no significant correlation between

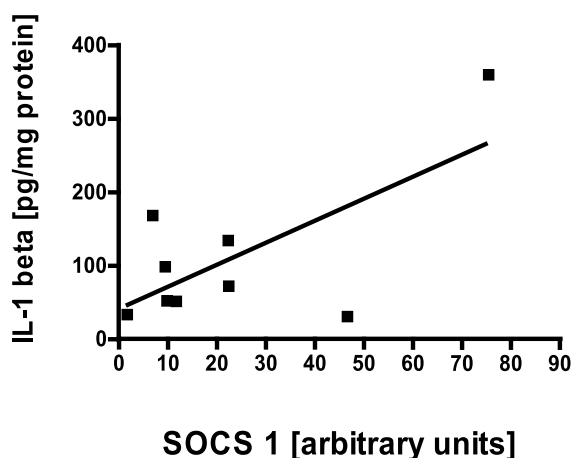


Figure 4 SOCS1 protein is correlated with IL-1 β in the preterm placenta complicated with infection. SOCS protein levels (relative expression) determined by Western analysis were significantly correlated with previously reported concentrations of IL-1 β from identical samples of placental tissue (Keelan *et al.* 1999) using Pearson correlation ($r^2=0.47$; Pearson $r=0.68$; $P<0.05$).

gestational age and any of the parameters measured. The relative abundance of SOCS expression within the placenta during pregnancy could reflect the importance of immune surveillance in the placenta, aimed at reducing the likelihood of maternal–fetal transmission of pathogens. Furthermore, the involvement of resident placental macrophages in the signaling pathways of the labor process cannot be excluded. Within this context, it is worth noting that SOCS3 can exert pro-inflammatory as well as anti-inflammatory effects (Kubo *et al.* 2003). In addition, SOCS2 can suppress SOCS1 expression, thereby minimizing its inhibitory effects on cytokine-induced inflammatory reactions, facilitating cytokine signaling and immune activation (Kubo *et al.* 2003).

An alternative interpretation of these data is that increased SOCS abundance may reflect attempts to suppress trophoblast cytokine production/effects to minimize placental (and fetal) damage. IFN- γ and tumor necrosis factor- α have both been shown to induce trophoblast apoptosis (Yui *et al.* 1994). SOCS1, in particular, limits IFN- γ signaling which may be beneficial in reducing placental cell death in response to immune activation.

All three SOCS proteins showed predominantly cytoplasmic localization by immunohistochemistry in villous placenta and gestational membranes, consistent with many reports in other tissues. However, sporadic nuclear staining was also observed, particularly in amnion epithelial cells, with more sporadic nuclear staining in the chorionic trophoblast and syncytium. This apparent presence of SOCS in the nucleus raises issues of translocation and functional significance with respect to the activation

of STATs. However, the causes and significance of this observation remain uncertain at the present time.

Previously, we reported that SOCS proteins in human gestational tissues are differentially regulated with the onset of labor at term and argued that reduced activity of negative regulators of cytokine activity (in this case SOCS) could be a trigger for labor and/or part of a positive feed-forward mechanism for amplification of labor (Blumenstein *et al.* 2002). To some extent the present findings in preterm tissues are inconsistent with these previously reported findings in term tissues. A lack of post-labor withdrawal of SOCS proteins from preterm membranes could reflect a lack of gestational maturity – either of the tissues themselves or of signals (paracrine or endocrine) derived from the fetoplacental unit that would normally trigger parturition at term but which are not functional in the pathological preterm delivery. Recent studies suggest that a change in estrogen and progesterone receptor ratios and signaling modifiers within intrauterine tissues may be responsible for triggering human labor, rather than a change in the concentrations of the circulating hormones themselves (Mesiano *et al.* 2002, Smith *et al.* 2002). SOCS gene expression can be modulated by steroid hormones such as estrogen (17 β -estradiol) as shown in a recent study by Leung *et al.* (2003). Crosstalk between steroid hormone and cytokine signaling pathways has also been reported (Stocklin *et al.* 1996). Hence, our observed lack of SOCS withdrawal in membranes delivered after preterm labor could reflect a lack of hormone receptor-mediated regulatory changes that would normally occur at term. Alternatively, in preterm tissues the effects of a labor-associated withdrawal of SOCS proteins might be countered by positive stimuli associated with infection/inflammation. This would result in increased or unchanged SOCS expression with considerable intersample variability, depending on the relative balance of the time from labor onset versus the duration, extent and time of onset of the infectious process.

Human parturition, both at term and preterm, has been likened to an inflammatory reaction/process (Bowen *et al.* 2002). SOCS proteins regulate signal transduction by several cytokines that have been reported to be present and affect human gestational tissues (Barkai *et al.* 2000, Nicola & Greenhalgh 2000), including the inflammatory cytokines IL-1 β , IL-6 and IL-8. Furthermore, elevated levels of these cytokines in fetal tissues and amniotic fluid have, in numerous studies, been associated with preterm birth, especially in pregnancies complicated by intrauterine infection (Keelan *et al.* 1997, 1999, Challis *et al.* 2000). The robust correlation between SOCS1 and IL-1 β (but not IL-8) protein levels in the villous placenta was an unanticipated finding of this study, particularly since it was not observed in the gestational membranes. This is despite the fact that, as detailed in previous studies, the cytokine

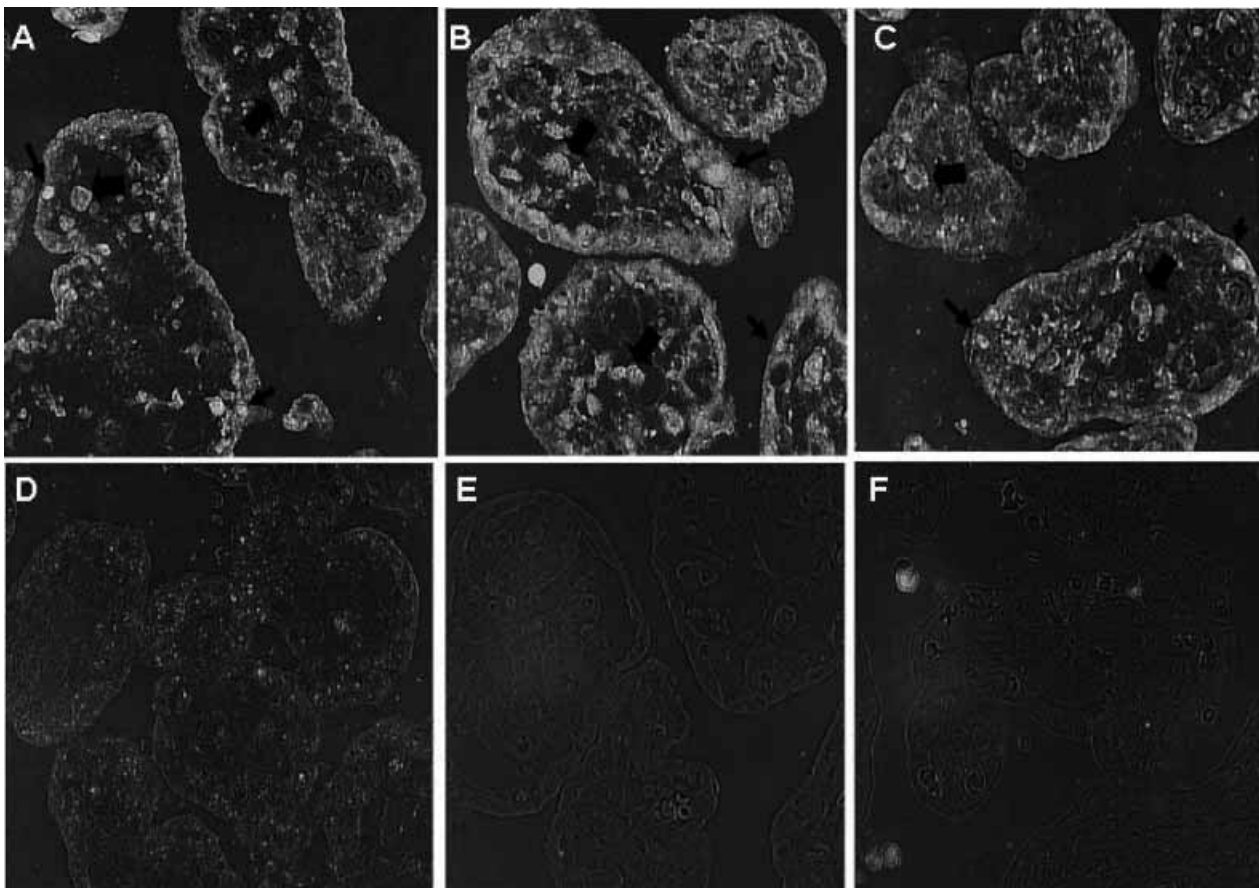


Figure 5 Immunolocalization of SOCS protein in preterm placenta. Representative examples of (A) SOCS1, (B) SOCS2 and (C) SOCS3 protein in placenta at preterm without infection. Bold block arrows point to mesenchymal staining within the villous core. Small arrows depict syncytial nuclear SOCS staining. Immunolocalization of SOCS proteins revealed no differences in staining patterns between placenta with and without infection. Peptide-neutralized controls were negative for SOCS staining (D-F). Slides photographed at $\times 400$ magnification.

response in the membranes was strongly associated with preterm labor and was correlated with the degree of chorioamnionitis, while in the placenta no such relationship was evident. The role of the villous placenta in response to infection and inflammatory cytokine output is controversial. Keelan *et al.* (1999, 2003) found no relationship between IL-1 β content and intrauterine infection in placental tissue delivered preterm. Steinborn *et al.* (1995), in contrast, reported elevated levels of IL-1 β in placental tissue cultures obtained from women without infection, but not those with confirmed chorioamnionitis, suggesting that preterm uterine activation signals are different in infected and uninfected women. The apparent discrepancy between the positive correlation between SOCS1 protein expression with IL-1 β in the face of unchanged placental IL-1 β levels with infection (Keelan *et al.* 1999, 2003) requires further elucidation. However, the large amounts of SOCS proteins in the placenta may suggest the presence of a pro-inflammatory cytokine

response. Alternatively, this phenomenon may reflect a key role for SOCS in the placenta in modulating the response to somatotrophic/metabolic, rather than inflammatory, mediators.

Leptin and growth hormone, for example, are both placental products that exert a range of actions on the placenta. Studies with SOCS3 gene deletions have revealed placental mal-development and perinatal death in the absence of SOCS expression (Roberts *et al.* 2001), demonstrating in principle the importance of SOCS in modulating responses to factors regulating growth, development and metabolism. It remains to be determined how the differential cellular localization of the various SOCS proteins in the placenta interacts with placental somatotrophic signals, although no doubt the system and its interaction with other cytokine receptor signals will be both subtle and complex.

SOCS2 expression can be induced by IL-1 β (Dogusan *et al.* 2000) while SOCS2 itself is capable of regulating

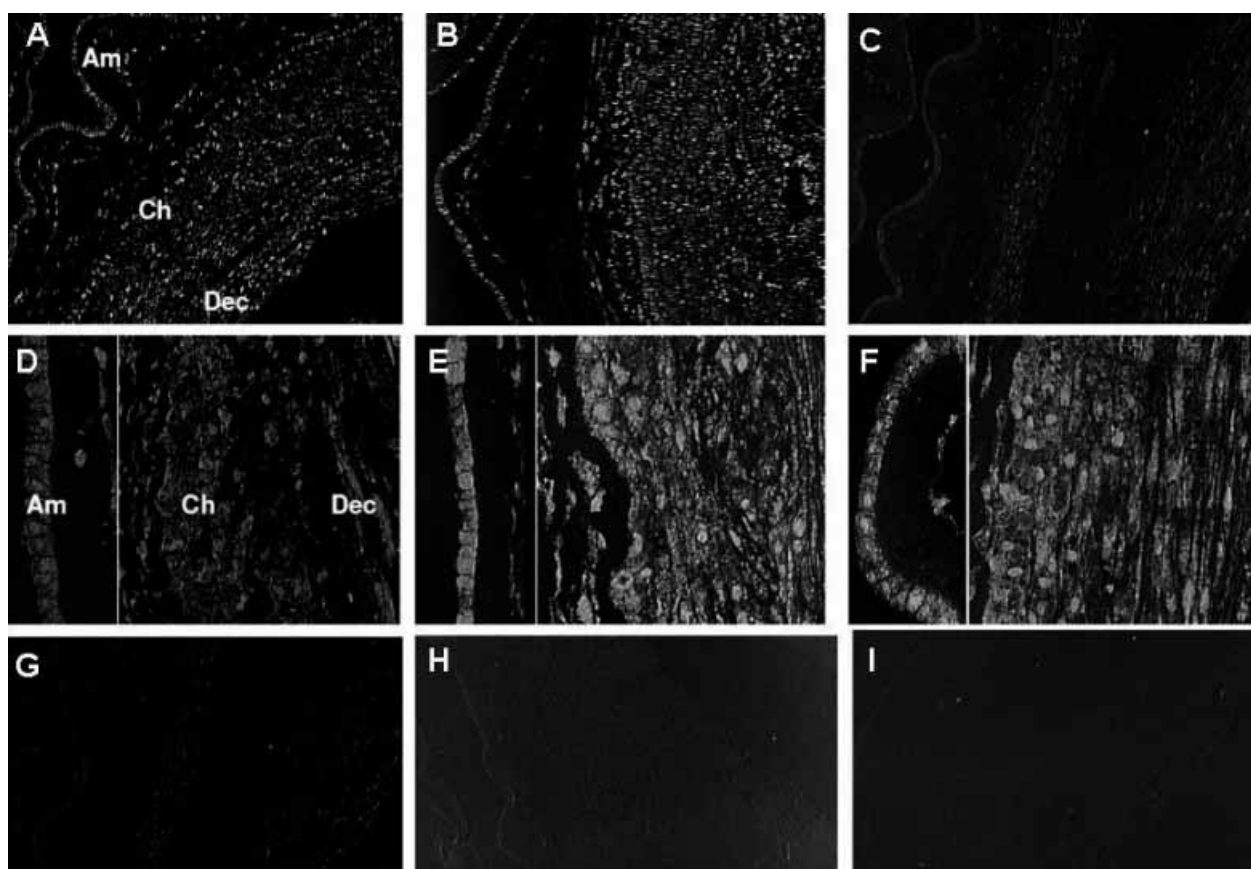


Figure 6 Immunolocalization of SOCS protein in preterm gestational membranes. Representative examples of (A and D) SOCS1, (B and E) SOCS2 and (C and F) SOCS3 protein in non-infected preterm amnion (Am), chorion (Ch) and decidua (Dec). SOCS-specific staining was abolished in the appropriate peptide antigen control (G-I). Preterm membranes showed cytoplasmic staining for all three SOCS, with nuclear staining evident particularly in the amnion epithelium and chorionic trophoblast. Immunolocalization of SOCS proteins revealed no differences in staining pattern between tissues with and without infection. Slides photographed at $\times 100$ (A-C and G-I) or $\times 400$ (D-F) magnification. A-C and D-F represent sections from different tissue blocks.

endogenous suppressors such as SOCS1 (Pezet *et al.* 1999). We speculate that inflammatory mediators arising from placental villitis might enter the circulation and activate placental macrophages, leading to IL-1 β release and subsequent SOCS activation as a feedback/response mechanism. This is supported by recent evidence of a pro-inflammatory cytokine response in the fetal placental vasculature whereby both the pro-inflammatory cytokines IL-6 and IL-8 and members of the SOCS family (SOCS2 and SOCS3, but not SOCS1) were detected in placental endothelium in the presence of placental vascular disease (Wang *et al.* 2003). The up-regulation of SOCS2 and SOCS3 indicates that these are the major negative regulators in umbilical placental microvessel endothelial cell activation pathways (Wang *et al.* 2003). SOCS2 and SOCS3 may, therefore, play a key role in the interaction of endothelial cells of the villous placenta with neighboring cells. In the present study, while no

endothelial SOCS staining was observed, strong immunostaining of villous mesenchymal cells (putative Hofbauer cells) may indeed indicate a pro-inflammatory cytokine response at preterm in the preterm placenta.

In conclusion, SOCS proteins are present in the preterm placenta and gestational membranes regardless of infection and are not abrogated with labor as previously shown at term. This finding suggests that the mechanisms that result in SOCS withdrawal with labor are absent or not sufficiently mature in spontaneous preterm deliveries. In addition to their roles in modulating endocrine/somatotropic signal within the placenta, SOCS proteins may serve as immunoprotective agents in the placenta to minimize the effects of cytotoxic cytokines with potential to cause harm to the feto-placental unit, or reflect enhanced immune surveillance aimed at preventing maternal-to-fetal transmission of pathogens.

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