Suppressors of cytokine signalling in ankylosing spondylitis and their associations with disease severity, acute-phase reactants and serum cytokines

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Abstract

Objective

To investigate the suppressors of cytokine signalling (SOCS1 and SOCS3) expression in peripheral blood cells in ankylosing spondylitis (AS), and their associations with clinical and laboratory manifestations.

Methods

The levels of SOCS1 and SOCS3 mRNA in peripheral blood mononuclear cells (PBMCs), T cells and monocytes were measured by RT-PCR in 53 AS patients and 31 healthy controls. Patient's serum IL-6, IL-10 and IL-17A levels were determined by ELISA. We evaluated patient's disease activity, functional ability and global assessment, and tested their ESR, CRP and IgA levels.

Results

Cellular SOCS1 expression did not show significant differences between AS patients and controls. However, T cells SOCS1 decreased significantly in the AS subgroup with lower ESR than controls (p=0.013). PBMCs (p=0.047) and T cells (p=0.035) SOCS1 decreased significantly in the AS subgroup with lower CRP than controls. Importantly, SOCS3 expression increased significantly in AS patients compared to the controls in PBMCs (p=0.025), T cells (p=0.003) and monocytes (p=0.009). Moreover, PBMCs SOCS3 correlated with ESR (r=0.297, p=0.031) and CRP (r=0.320, p=0.019). T cells SOCS3 correlated with BASFI (r=0.337, p=0.015), ESR (r=0.435, p=0.001) and CRP (r=0.300, p=0.029). Monocytes SOCS3 correlated with ESR (r=0.281, p=0.041) and IgA (r=0.426, p=0.006). Furthermore, T cells SOCS1 (r=-0.454, p=0.023) and T cells SOCS3 (r=-0.405, p=0.045) negatively correlated with serum IL-17A. Monocytes SOCS3 negatively correlated with serum IL-6 (r=-0.584, p=0.002).

Conclusion

The decreased SOCS1 and increased SOCS3 expression in AS PBMCs and T cells, and their correlation with patient's functional ability, acute-phase reactants and serum pro-inflammatory cytokines suggested that SOCS may participate in the pathogenesis of AS.

Key words

ankylosing spondylitis, SOCS, mononuclear cells, acute-phase reactants, cytokines.

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Introduction

Spondyloarthritis (SpA) is a chronic disease, characterised by inflammatory back pain, sacroiliitis, peripheral arthritis and enthesitis (1). It contains five major subtypes, including ankylosing spondylitis (AS), reactive arthritis, psoriatic arthritis, arthritis with inflammatory bowel disease and undifferentiated spondyloarthritis. The inflammatory process can also involve extra-articular organs, including eye, skin and gut. AS is the most common disease among the SpA family and the chronic inflammatory back pain with morning stiffness that improved after exercise is the major clinical symptom. AS occurs predominantly in young males and has a strong association with human leukocytes antigen (HLA)-B27. Long-term inflammation of the spines may result in the formation of syndesmophyte and the subsequent ankylosis of adjacent vertebral bodies, which leads to progressive loss of spinal mobility, risk of spinal fracture and reduced quality of life.

Suppressors of cytokine signalling (SOCS) proteins are intracellular inhibitors of cytokine signalling which activate the cells through the JAK-STAT pathway (Janus kinase-signal transducers and activators of transcription). The SOCS family consists of eight SOCS proteins so far, SOCS1-7 and CIS-1 (cytokine-inducible SH2-domain-1), and are the negative-feedback regulator of the JAK-STAT pathway (2-6). The SOCS family share a central SH2 (Src homology 2) domain, a highly conserved C-terminal region termed the SOCS box, and an amino-terminal region with a variable length and limited homology. SOCS protein inhibit the JAK-STAT pathway by several mechanisms, including suppression of the catalytic activity of JAK tyrosine kinases, competitive inhibition of STAT binding to the receptors and degradation of substrate through the ubiquitin pathway (7, 8).

Some studies investigated the association of SOCS gene in rheumatoid arthritis (RA) (9-12). However, the reports regarding the roles of SOCS in AS are very rare at present (13), and the peripheral blood cellular expression of SOCS1 and SOCS3 genes in the AS patients has not yet been reported. Therefore, a potential role of SOCS1 and SOCS3 in AS remain to be clarified. Several pro-inflammatory cytokines proved to be involved in the AS, and may be associated with the disease activity, such as IL-6 (14, 15) and IL-17A (16-19). Thus, the patient's disease severity, acute-phase reactants levels and serum levels of cytokines, containing IL-6, IL-10 and IL-17A were determined, and evaluated their associations with the peripheral blood cellular SOCS1 and SOCS3 expression in the AS patients.

Patients and methods

Patients

Blood samples were obtained from 53 Chinese AS patients who fulfilled the 1984 modified New York criteria (20) and visited the outpatient department of the Division of Allergy, Immunology and Rheumatology at Taipei Veterans General Hospital in Taiwan. As a control group, blood samples were obtained from 31 age and sex-matched Chinese healthy subjects. This research was approved by the ethics committee of Taipei Veterans General Hospital. Before study, informed consent was obtained from all the participants. Clinical and laboratory assessments were performed on the same day. We evaluated patient's disease severity, which includes disease activity, functional ability and patient's global assessments in the AS patients by using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (21), Bath Ankylosing Spondylitis Functional Index (BASFI) (22) and Bath Ankylosing Spondylitis Patient Global Score (BASG) (23), with visual analogue scales. The BASDAI, BASFI and BASG scores ranged from 0 to 10, and the higher scores indicated high disease activity, poor functional ability, and poor patient's global assessment, respectively. Acute-phase reactants, including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), as well as IgA levels were also measured in the AS patients. Table I shows the demographics and clinical characteristics of the 53 AS patients.

Cell preparations and culture conditions

To study the cellular mRNA expression

Table I. Demographic and disease characteristics of the 53 AS patients.

Characteristic	Total AS patients (n=53)
Age (years)	38.37 (13.26)
Male/Female	45/8
HLA-B27 (+)	90.6% (48/53)
ESR (mm/hour)	13.91 (12.36)
CRP (mg/dl)	1.16 (1.52)
IgA (mg/dl)	360.56 (150.92)
BASDAI	3.97 (2.27)
BASFI	3.00 (2.61)
BASG	4.42 (3.00)

Values are shown as mean (SD).

of SOCS1 and SOCS3 in the peripheral blood cells, including peripheral blood mononuclear cells (PBMCs), T cells and monocytes, quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed for the blood specimens from 53 AS patients and 31 healthy controls. PBMCs were obtained from anti-coagulated human blood by a density gradient centrifugation using Ficoll-Paque™ (GE Healthcare, Sweden). T cells and monocytes/macrophages were further purified from PBMCs using magnetic cell sorting technology according to manufacturer's instructions (BD Biosciences). CD3 (+) T cells were purified by negative selection using a cocktail of biotin-conjugated monoclonal antibodies (Abs), specific for non-T cells and BD IMag™ Streptavidin Particles Plus-DM (BD Biosciences, USA). PB monocytes were isolated by positive selection with Anti-Human CD14 Magnetic Particles-DM (BD Biosciences, USA).

Cellular RNA isolation and quantitative RT-PCR analysis

Total RNA extraction was performed according to the manufacturer's instructions using the NucleoSpin RNA II Kit. DNase digestion was performed on minispin columns according to the manufacturer's instructions using RNase free DNase. RNA was eluted in H_2O and stored at -70°C. Reverse transcriptase PCR (Clontech #639537) was performed to synthesise the single stranded complimentary DNA (cDNA) by random hexamer oligonucleotides in a final volume of 10 μL. Real-time PCR was performed using the Universal Master Mix. The Universal Master Mix (Roche) contains dNUTPs, $MgCl_2$, thermo stable DNA polymerase, and provides the necessary buffer requirements. Quantitative PCR analysis was performed by a microfluid technique using TaqMan real-time PCR using a Roche LightCycler 480 System. Universal ProbeLibrary kits were designed from Roche Website (http://www.universalprobelibrary.com). SOCS1 (Forward primer: 5'-cccctggttgttgtagcag-3', and Reverse primer: 5'-gtaggaggtgcgagttcagg-3'), SOCS3 (Forward primer: 5'-agacttcgattcgggacca-3', and Reverse primer: 5'- aacttgctgtgggtgacca-3') 100~200 nM, Probe 100 nM and Template cDNA 50ng were added in final volume 25ul. The temperature profile of the PCR amplification was 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 15s, and annealing and extension at 50°C for 1 minute. Expression of mRNA was corrected by house-keeping gene such as the TATA box binding protein (TBP). In order to validate our method, amplifications were performed on diluted samples, using primers for the reference TATA box binding protein (TBP) and the target genes (SOCS1, SOCS3).

Immunoassay of serum levels of IL-6, IL-10 and IL-17A

Samples of peripheral blood were allowed to clot and then were centrifuged at 1000 g for 10 minutes. The sera were frozen at -80°C immediately after sample collection. Serum samples from 25 of the 53 AS patients were obtained. Measurement of the serum levels of IL-6, IL-10 and IL-17A was performed with a commercial human ELISA kits (BioLegend, LEGEND MAX™, San Diego, CA), according to the manufacturer's instructions. The analytical standard range and sensitivity of the ELISA kits for serum cytokines are IL-6 (7.8–500 pg/mL, 1.6 pg/mL), IL-10 (3.9–250 pg/mL, 2 pg/mL) and IL-17A (3.9–250 pg/mL, 0.8 pg/mL).

Statistical analysis

Statistical analyses were carried out using the SPSS statistical package (SPSS for Windows, Chinese version 10.0.7C, SPSS Inc., 2000). The Mann-Whitney U-test was used to analyse group differences. Correlations between variables were determined by using the Spearman's Rank Correlation test. *P*-values <0.05 were regarded as significant.

Results

Peripheral blood cellular SOCS1 and SOCS3 mRNA expression in the AS patients and healthy controls

We compared the peripheral blood cellular mRNA expression levels of SOCS1 (Table II) and SOCS3 (Table III) between the 53 AS patients and the 31 healthy controls. There was a trend of lower cellular SOCS1 expression in the AS patients as compared to healthy controls in PBMCs (p=0.093), T cells (p=0.133) and monocytes (p=0.123), but the difference did not reach statistical difference. To test whether there were differences between cellular SOCS1 expression in the AS subgroups and the healthy controls, we divided the AS pa-

Table II. Comparison of peripheral blood cellular SOCS1 mRNA expression between the 53 AS patients and 31 healthy controls.

SOCS1 mRNA expression	AS patients (n=53)	Controls (n=31)	AS subgroups ESR <10 (n=26)	AS subgroups ESR ≥10 (n=27)	AS subgroups CRP <0.56 (n=26)	AS subgroups CRP ≥0.56 (n=27)
PBMCs	1.414 (0.336), 0.093	1.540 (0.288)	1.394 (0.338), 0.082	1.433 (0.339), 0.258	**1.386 (0.308)**, ***0.047****	1.441 (0.365), 0.374
T cells	1.340 (0.277), 0.133	1.446 (0.308)	**1.244 (0.239)**, ***0.013****	1.433 (0.283), 0.907	**1.273 (0.202)**, ***0.035****	1.405 (0.323), 0.634
Monocytes	0.374 (0.193), 0.123	0.397 (0.125)	0.382 (0.245), 0.086	0.367 (0.129), 0.354	0.399 (0.254), 0.163	0.351 (0.107), 0.215

Values are shown as Mean (SD.), *p*-value is determined by Mann-Whitney U-test as compared with healthy controls.
*Statistical significances are in bold characters. SOCS: Suppressors of cytokine signalling; PBMCs: peripheral blood mononuclear cells.

Table III. Comparison of peripheral blood cellular SOCS3 mRNA expression between the 53 AS patients and 31 healthy controls.

SOCS3 mRNA expression	AS patients (n=53)	Controls (n=31)	*p*-value
PBMCs	5.301 (1.615)	4.690 (1.546)	***0.025****
T cells	4.598 (1.812)	3.528 (0.843)	***0.003****
Monocytes	5.840 (2.245)	4.798 (1.514)	***0.009****

Values are shown as Mean (SD.). *p* is determined by Mann-Whitney U-test as compared with healthy controls. *Statistical significances are in bold characters. SOCS: Suppressors of cytokine signalling; PBMCs: peripheral blood mononuclear cells.

tients into two different subgroups with lower and higher values of ESR (<10 *vs*. ≥10 mm/hr, median), CRP (<0.56 *vs*. ≥0.56 mg/dl, median), BASDAI (<3.71 *vs*. ≥3.71, median), BASFI (<2.38 *vs*. ≥2.38, median) and BASG (<4.15 *vs*. ≥4.15, median), respectively. Interestingly, T cells SOCS1 expression showed significantly decreased in the AS subgroup with lower ESR levels (<10 mm/hr, p=0.013) than the healthy controls (Table II). In addition, there were significantly decreased cellular SOCS1 expression in the AS subgroup with lower CRP levels (<0.56 mg/dl) than the healthy controls in the PBMCs (p=0.047) and T cells (p=0.035) (Table II).

Importantly, the peripheral blood cellular SOCS3 mRNA expression showed significantly increased in the 53 AS patients than 31 healthy controls, in all PBMCs, T cells and monocytes (p=0.025, 0.003, and 0.009, respectively) (Table III).

Correlations between peripheral blood cellular SOCS1, or SOCS3 mRNA expression and clinical parameters in the 53 AS patients

Peripheral blood cellular expression of SOCS1 in PBMCs, T cells and monocytes did not show significant correlation with clinical parameters, such as ESR, CRP, BASDAI, BASFI, and BASG. However, T cells SOCS1 expression showed a trend of correlation with CRP levels (r=0.240, p=0.084) (Table IV).

Interestingly, PBMCs SOCS3 expression showed significant correlation with ESR (r=0.297, p=0.031), and CRP levels (r=0.320, p=0.019). Furthermore, T cells SOCS3 expression showed significant correlation with BASFI (r=0.337, p=0.015), ESR (r=0.435, p=0.001) and CRP levels (r=0.300, p=0.029). Monocytes SOCS3 expression showed significant correlation with ESR (r=0.281, p=0.041) and IgA levels (r=0.426, p=0.006) (Table IV).

In addition, the scores of BASDAI, BASFI and BASG showed significant correlation with each other parameters. T cells SOCS1 expression showed significant correlation with PBMCs (r=0.354, p=0.009) and T cells (r=0.484, p<0.001) SOCS3 expression. T cells SOCS3 expression showed significant correlation with monocytes SOCS1 expression (r=0.312, p=0.023).

Correlations between peripheral blood cellular SOCS1, or SOCS3 mRNA expression and serum cytokines in 25 of the 53 AS patients

Correlations between peripheral blood cellular SOCS1 or SOCS3 mRNA expression and serum cytokines, including IL-6, IL-10 and IL-17A in 25 of the 53 AS patients are shown in Table V. T cells SOCS1 expression showed a negative correlation with serum IL-17A levels with statistical significance (r=-0.454, p=0.023). PBMCs (r=-0.565, p=0.003) and T cells (r=-0.405, p=0.045) SOCS3 expression showed a negative correlation with serum IL-17A levels with statistical significance. Taken together, T cells SOCS1 and SOCS3 expression had a negative correlation with serum IL-17A levels. Monocytes SOCS3 expression showed a negative correlation with serum IL-6 levels with statistical significance (r=-0.584, p=0.002). However, cellular SOCS1 and SOCS3 expression did not show significant correlation with serum IL-10 levels.

Discussion

Our results did not show the difference of cellular SOCS1 expression between the AS patients and healthy controls, however there was a trend of lower cellular SOCS1 expression in the AS patients as compared to healthy controls in PBMCs (p=0.093). However, T cells SOCS1 expression showed significantly decreased in the AS subgroup with lower ESR levels (<10 mm/hr, p=0.013) as compared to healthy controls. Besides, PBMCs (p=0.047) and T cells (p=0.035) SOCS1 expression showed significantly decreased in the AS subgroup with lower CRP levels (<0.56 mg/dl) than the healthy controls. Taken together, peripheral blood cells demonstrated a reduced SOCS1 expression in the AS patients with lower systemic inflammation, but not showed in the higher systemic inflammation group. Recent one

Table IV. Correlations between peripheral blood cellular SOCS1 and SOCS3 mRNA expression and clinical parameters in the 53 AS patients.

Cellular mRNA expression	BASDAI	BASFI	BASG	ESR	CRP	IgA
PBMCs SOCS1	0.011 *(0.939)*	0.052 *(0.714)*	-0.086 *(0.547)*	0.033 *(0.817)*	0.109 *(0.435)*	-0.077 *(0.632)*
T cells SOCS1	0.084 *(0.555)*	0.134 *(0.344)*	0.055 *(0.701)*	0.202 *(0.147)*	0.240 *(0.084)*	-0.086 *(0.594)*
Monocytes SOCS1	-0.015 *(0.916)*	-0.131 *(0.355)*	-0.213 *(0.134)*	0.091 *(0.518)*	-0.034 *(0.812)*	-0.133 *(0.406)*
PBMCs SOCS3	-0.105 *(0.458)*	0.092 *(0.516)*	0.042 *(0.769)*	**0.297 *(0.031*)***	**0.320 *(0.019*)***	-0.009 *(0.957)*
T cells SCOS3	0.206 *(0.142)*	**0.337 *(0.015*)***	0.245 *(0.083)*	**0.435 *(0.001*)***	**0.300 *(0.029*)***	0.133 *(0.408)*
Monocytes SCOS3	0.219 *(0.118)*	0.200 *(0.154)*	0.171 *(0.231)*	**0.281 *(0.041*)***	0.154 *(0.269)*	**0.426 *(0.006*)***

Values are shown as r (*p*-value). r is determined by Spearman's Rank Correlation test. *Statistical significances are in bold characters. SOCS, Suppressors of cytokine signalling; PBMCs, peripheral blood mononuclear cells.

Table V. Correlations between peripheral blood cellular SOCS1 or SOCS3 mRNA expression and serum cytokines in 25 of the 53 AS patients.

Cellular mRNA expression	Serum IL-6	Serum IL-10	Serum IL-17A
PBMCs SOCS1	-0.266 *(0.198)*	-0.204 *(0.327)*	-0.182 *(0.385)*
T cells SOCS1	-0.178 *(0.394)*	-0.178 *(0.395)*	**-0.454 *(0.023*)***
Monocytes SOSC1	-0.228 *(0.274)*	0.286 *(0.165)*	-0.302 *(0.142)*
PBMCs SOCS3	-0.150 *(0.474)*	-0.142 *(0.50)*	**-0.565 *(0.003*)***
T cells SOCS3	-0.277 *(0.181)*	0.117 *(0.579)*	**-0.405 *(0.045*)***
Monocytes SOSC3	**-0.584 *(0.002*)***	0.146 *(0.458)*	0.073 *(0.728)*

Values are shown as r (*p*-value). r is determined by Spearman's Rank Correlation test.
*Statistical significances are in bold characters. SOCS: Suppressors of cytokine signalling; PBMCs: peripheral blood mononuclear cells.

study showed that SOCS-1 methylation could be found in serums samples from AS patients but not normal controls, and the methylation of SOCS-1 significantly associated with disease severity, sacroiliitis and CRP (13). In an animal study, increased arthritis severity has been shown in mice lacking SOCS1 and IFN-γ (24). Mice lack of SOCS1 died within the first few weeks of life from autoimmune disease, such as liver necrosis and mononuclear cell infiltration into various organs (25, 26). In OA patients, the SOCS2 and CIS-1 mRNA levels were reduced in OA condrocytes as compared to control samples, while SOCS1 and SOCS3 showed similar expression patterns in OA and control chondrocytes (27, 28). The dysregulation of SOCS1 expression may possible participate in the pathogenesis of AS, which results in chronic arthritis.

Cellular SOCS3 expression showed significantly increased in the AS patients than the healthy controls, in all PBMCs, T cells and monocytes (p=0.025, 0.003 and 0.009, respectively) in our study. Moreover, PBMCs SOCS3 expression showed mild correlation with ESR, and CRP levels. T cells SOCS3 expression showed mild to moderate correlation with BASFI, ESR and CRP levels. Monocytes SOCS3 expression showed mild to moderate correlation with ESR and IgA levels. These results indicated that SOCS3 could be up-regulated in response to the AS disease process, and the levels of SOCS3 expression may reflect the patient's systemic inflammation and functional ability. Our study also showed that T cells SOCS1 expression correlated significantly with T cells SOCS3 expression, and the T cells SOCS1 expression showed a trend of correlation with CRP levels. Isomäki P *et al*. showed that SOCS1 and SOCS3 levels were significantly increased in PBMCs from RA patients when compared with healthy volunteers (9). Van de Loo *et al*. demonstrated that the expression of SOCS3 mRNA was significantly enhanced in cartilage chondrocytes from OA and RA patients compared with cartilage from patients with femoral neck fracture (29). Previous studies have demonstrated the overexpression of SOCS in autoimmune and allergic disease, such as SOCS3 increased in the intestinal mucosa from patients with Crohn's disease and ulcerative colitis (30), levels of SOCS1, SOCS2 and SOCS3 elevated in the skin from patients with psoriasis or contact dermatitis (31), and SOCS3 were up-regulated in the synovium and synovial fluid mononuclear cells from acute gouty arthritis (32). SOCS3 increased in peripheral blood T cells from patients with asthma and atopic dermatitis (33). SOCS1 or SOCS3 mRNA levels were varied in different rheumatic disease, such as AS, RA, and OA etc. suggested the SOCSs expression may play different roles at different time, and different disease activity in those inflammatory joint diseases.

Our data demonstrated that T cells SOCS1 mRNA expression had a negative correlation with serum IL-17A levels (r=-0.454, p=0.023). PBMCs and T cells SOCS3 mRNA expression also showed a negative correlation with serum IL-17A levels (r=-0.565, p=0.003; r=-0.405, p=0.045, respectively). For serum IL-6 levels monocytes SOCS3 mRNA expression showed a negative correlation (r=-0.584, p=0.002). IL-17 is the cytokine produced from a distinct subset of CD4 T helper cell, the Th17 cells, and associated with inflammation, autoimmunity and against infection (34, 35). IL-6 is produced by T cells (36), monocytes (37) and fibroblasts (38), and is a pro-inflammatory cytokine (39-41), inducing various inflammatory cell proliferation and differentiation (42-44). Previous reports had revealed that serum cytokines, including IL-6 and IL-17 were significantly higher in the AS patients than the healthy controls (16-18), particularly in the AS patients with high disease activity (14, 19). Several cytokines, such as IL-6, IL-10 and IFN-γ signal through the JAK-STAT pathway, which is negatively regulated by the suppressors of cytokine signalling (SOCS) proteins (45, 46). Cellular SOCS1 and SOCS3 expression is tightly connected to upstream cytokine stimulus, and result in termination of cytokine-mediated signals. The results of the negative correlation between serum IL-17A or IL-6 levels with cellular SOCS mRNA expression in our study may possibly reflect inadequate suppression of pro-inflammatory cytokines in the AS patients with active disease. However, the detail regulation between the peripheral blood cellular SOCS expression and the serum cytokines, such as IL-6 and IL-17A still remained further investigation.

In conclusion, the decreased cellular SOCS1 mRNA expression in the AS subgroup implies that the dysregulation of SOCS1 may involve in the development of AS. The increased cellular SOCS3 mRNA expression in the AS patients and its correlation with acute-phase reactants suggest that SOCS3 is up-regulated to negative feedback control of the disease inflammation. The cellular SOCS1 and SOCS3 mRNA expression had negative correlation with serum pro-inflammatory cytokines may reflect an inadequate regulation of active inflammation in the AS patients. The expression of SOCS1 and SOCS3 changed in AS, and may take participation in the pathogenesis of AS.

Our study was a preliminary investigation of SOCSs in AS, and focused on the expressions of SOCSs in peripheral blood mononuclear cells. Further studies to investigate the tissue expressions of SOCSs in AS pathologic sites and more experiments comparing with other

inflammatory joint diseases are needed to clarify the roles of SOCSs in AS.

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