Suppressor of cytokine signaling 1 inhibits pulmonary inflammation and fibrosis

Taku Nakashima, MD,[a] Akihito Yokoyama, MD,[b] Yojiro Onari, MD,[a] Hiroyasu Shoda, MD,[b] Yoshinori Haruta, MD,[a] Noboru Hattori, MD,[a] Tetsuji Naka, MD,[c] and Nobuoki Kohno, MD[a] *Hiroshima, Kochi, and Osaka, Japan*

Background: Suppressor of cytokine signaling (SOCS) proteins are inhibitors of cytokine signaling. Our previous study suggested that SOCS1 regulates collagen synthesis by lung fibroblasts, suggesting a role of SOCS1 in the pathophysiology of pulmonary fibrosis.

Objectives: We sought to investigate the role of SOCS1 in pulmonary inflammation and fibrosis *in vivo*.

Methods: SOCS1-haplodeficient mice treated with bleomycin (BLM) were evaluated for pulmonary inflammation and fibrosis compared with wild-type mice. The human study group was composed of 18 patients with interstitial lung disease. Lung specimens obtained by means of open lung biopsy were investigated to determine whether the severity of fibrosis was associated with decreased SOCS1 expression. Finally, we further analyzed the effect of exogenous SOCS1 on BLM-induced lung injury based on adenoviral *SOCS1* gene transfer to the lung.

Results: SOCS1-haplodeficient mice treated with BLM showed markedly enhanced pulmonary inflammation and fibrosis compared with wild-type mice. Using human lung specimens, we found that *SOCS1* mRNA levels inversely correlated with duration of the disease. SOCS1 expression was significantly less in lung tissue from patients with idiopathic pulmonary fibrosis (IPF) compared with that in non-IPF patients. Moreover, SOCS1 expression was significantly less in severe fibrotic lesions (lower lobe) than in less fibrotic lesions (upper lobe). Adenoviral *SOCS1* gene transfer to murine lungs significantly decreased lymphocytic inflammation, pulmonary fibrosis, and mortality because of BLM-induced lung injury. Exogenous SOCS1 inhibited expression of various cytokines, including TNF-α, which might play a key role.

Conclusions: These results suggest that SOCS1 might act as a suppressor for pulmonary fibrosis. SOCS1 might be a target of IPF treatment. (J Allergy Clin Immunol 2008;121:1269-76.)

Key words: *Pulmonary fibrosis, Suppressor of cytokine signaling, tumor necrosis factor α, adenoviral vector, bleomycin*

Abbreviations used
BALF: Bronchoalveolar lavage fluid
BLM: Bleomycin
CHP: Chronic hypersensitivity pneumonitis
ILD: Interstitial lung disease
IPF: Idiopathic pulmonary fibrosis
NSIP: Nonspecific interstitial pneumonia
SOCS: Suppressor of cytokine signaling
WT: Wild-type

Pulmonary fibrosis is a disease of known and unknown cause characterized by lung destruction and dysfunction. The typical and most common phenotype is idiopathic pulmonary fibrosis (IPF). There has been no effective therapy for this lethal disease, and the prognosis of IPF is extremely poor.[1] A variety of cytokines and chemokines are involved in its pathophysiology. Of these, T_H2 cytokines are now considered to be dominant.[2]

The animal model most frequently used to study human pulmonary fibrosis is bleomycin (BLM)-induced lung fibrosis in rodents.[3,4] Like human IPF, murine BLM-induced lung injury is associated with the T_H2-dominant cytokine pattern.[2] In addition, we previously demonstrated that adoptive transfer of T_H1 clones leads to reversible alveolitis but not fibrosis.[5] T_H2 cells and T_H2 cytokines might be essential for induction of pulmonary fibrosis because inhibition of the T_H2 cytokines diminished fibrosis in this model.[6]

Suppressor of cytokine signaling (SOCS) family molecules inhibit cytokine signals by regulating the Janus kinase–signal transducer and activator of transcription pathway. SOCS, induced by various cytokines and hormones, regulates not only cytokine signals but also T_H1/T_H2 cell differentiation.[7] At present, there are 8 SOCS molecules; cytokine-inducible SH2 domain–containing protein and SOCS1 to SOCS7. The role of SOCS1 in T_H cell differentiation is not fully understood; however, a previous report suggested that SOCS1 is dominantly expressed in T_H1 cells.[8] In addition, SOCS1 is an inhibitor of profibrotic cytokines, such as IL-4 and TNF-α.[9] Transduction of TNF-α signal can be inhibited by SOCS1 but not by other SOCS proteins.[9] Therefore SOCS1 might suppress pulmonary fibrosis through inhibiting profibrotic cytokines.

We previously demonstrated that higher amounts of type I collagen and lower levels of *SOCS1* mRNA were produced by fibroblasts from lungs of patients with IPF than from healthy lungs. Furthermore, the deficiency of SOCS1 in murine fibroblasts resulted in increased collagen production, whereas overexpression of SOCS1 suppressed collagen production *in vitro*.[10] From these findings, we hypothesized that SOCS1 would be involved in the pathophysiology of lung fibrosis.

From [a]the Department of Molecular and Internal Medicine, Graduate School of Biomedical Sciences, Hiroshima University; [b]the Department of Hematology and Respiratory Medicine, Kochi University; and [c]the Laboratory for Immune Signal Project, National Institute of Biomedical Innovation, Osaka.

Supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication October 6, 2007; revised January 9, 2008; accepted for publication February 1, 2008.

Available online March 20, 2008.

Reprint requests: Akihito Yokoyama, MD, Department of Hematology and Respiratory Medicine, Kochi University, Kohasu Oko-cho, Nankoku-sity, Kochi 783-8505, Japan. E-mail: ayokoyama@kochi-u.ac.jp.

0091-6749/$34.00

doi:10.1016/j.jaci.2008.02.003

Because the role of SOCS1 in this critical disease has not been examined *in vivo*, we conducted the present study. We first examined the effects of SOCS1 on BLM-induced pulmonary fibrosis by using SOCS1-haplodeficient mice. Then we explored *SOCS1* mRNA expression in human lung tissues from patients with various types of pathologically proved lung fibrosis. Finally, we examined the effects of *SOCS1* gene transfer on BLM-induced pulmonary fibrosis to evaluate its possible use as therapy.

METHODS

Animals and BLM exposure

This study was approved and conducted in accordance with the guidelines of the animal ethics committee of Hiroshima University. Because SOCS1-deficient mice die within 3 weeks after birth, SOCS1 heterozygotic mice were used to evaluate BLM-induced lung injury.[11] For a separate analysis of the effect of exogenous SOCS1 by means of adenoviral gene transfer, female C57BL/6J mice aged 6 weeks were purchased from Japan Charles River Laboratories (Kanagawa, Japan). All animals were maintained in a specific pathogen-free environment.

For each experiment, age- and weight-matched groups of mice were used. At day 0, after achievement of pentobarbital-induced anesthesia, mice were instilled with BLM (Nippon Kayaku Co, Tokyo, Japan) intratracheally, as previously described.[12] The dose of BLM was 1.5 mg/kg dissolved in sterile PBS (pH 7.4) in all experiments, except the survival-monitoring experiment, in which BLM at a higher dose (3 mg/kg) was used, and mortality was assessed for 20 days. Control mice received PBS alone.

Human lung tissue

Our human study protocol was approved by the Institutional Review Board of Hiroshima University. Between January 2005 and March 2007, lung tissues from 18 Japanese patients (7 female and 11 male patients; age, 43-73 years) with interstitial lung diseases (ILDs) were obtained by means of open lung biopsy performed for diagnostic purposes at Hiroshima University Hospital. From 8 patients with lung cancer, histologically normal lung tissues were obtained from sites distant from the tumors and used as control specimens. Clinical, radiologic, and pathologic analyses revealed a diagnosis of IPF in 10 of the 18 patients, idiopathic nonspecific interstitial pneumonia (NSIP) in 5 patients (2 with cellular and 3 with fibrotic NSIP), and chronic hypersensitivity pneumonitis (CHP) in 3 patients. In 7 of the 10 patients with IPF, 2 of the 5 patients with NSIP, and 3 of the 3 patients with CHP, both upper and lower lung tissues were obtained. The diagnoses of IPF, idiopathic NSIP, and CHP were done by an expert lung pathologist according to guidelines.[1,13] After obtaining informed consent, these lung tissues were stored at −80°C until total RNA extraction (as described below).

Construction and preparation of adenoviral vector expressing SOCS1

Recombinant E1-deleted adenoviral vectors carrying murine *SOCS1* cDNA under the control of a cytomegalovirus promoter (Ad-SOCS1) were generated as previously described.[14] An adenoviral vector expressing the gene for murine β-galactosidase (Ad-LacZ) was used as a control. These viruses were grown in 293 cells and purified by using a commercially available kit (ViraKit AdenoMini Kit; Nacalai Tesque, Inc, Kyoto, Japan). After purification, these viral titers were determined by means of plaque titration in 293 cells and stored at −80°C. A dose of 0.1×10^9 plaque-forming units of vector was diluted in 50 μL of PBS and instilled intranasally 48 hours before the BLM exposure (at day −2), as previously described.[15]

Analysis of bronchoalveolar lavage fluid

Mice were killed by means of cervical dislocation after achievement of anesthesia at days 7 and 14. At the time of death, bronchoalveolar lavage fluid (BALF) was obtained by using a previously described method.[5] The concentrations of murine TNF-α and TGF-β1 in the BALF supernatant were quantified with ELISA kits (R&D Systems, Minneapolis, Minn).

Hydroxyproline assay

Hydroxyproline content of whole mouse lungs was assayed in each group 14 days after BLM administration to evaluate the total amount of lung collagen by using a previously described method.[12]

Purification of RNA and real-time quantitative RT-PCR

Murine left lungs were harvested and stored at −80°C at days 0 (before BLM treatment), 1, 3, and 7. Total RNA was isolated from these murine lungs and human samples by using TRIzol reagent (Invitrogen, Carlsbad, Calif) and purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). After this, total RNA was reverse transcribed into cDNA, and real-time quantitative RT-PCR was performed with sequence-specific TaqMan primers and probes, the ABI Prism 7500 Fast sequence detector, and SDS analysis software (Applied Biosystems, Foster City, Calif). Mouse samples were analyzed for SOCS1, SOCS3, IL-4, IL-5, IL-10, IL-13, IFN-γ, TGF-β1, TNF-α, monocyte chemoattractant protein 1, eotaxin, and 18S ribosomal protein, and human samples were analyzed for SOCS1 and 18S by using TaqMan Pre-Developed primers and probes (Applied Biosystems), as previously described.[16] The measured mRNA levels were expressed relative to the internal reference 18S mRNA level and further adjusted to the level in the control group at day 0 (which was taken to be one).

Histology

After BALF collection and left lung resection for RNA analysis, the right lung was removed and fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. The sections were stained with hematoxylin and eosin, or elastica-Masson trichrome. X-gal staining was done in the early (day 3) and late (day 14) phases of BLM treatment with the β-Galactosidase Staining Kit (Mirus Bio Corp, Madison, Wis) to locate and assess gene expression of the adenoviral vector. The paraffin sections were further processed for immunohistochemistry by using rabbit anti-SOCS1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), according to a previously described method.[17]

Statistical analysis

Data are shown as means ± SEMs. Differences between groups were analyzed by using the Mann-Whitney *U* test. Differences between paired parameters were tested with the Wilcoxon test. Correlation coefficients for the markers were calculated by using Spearman rank correlation coefficient analysis. The survival function of each group was evaluated with the Kaplan-Meier method, and differences between 2 groups were evaluated with the log-rank test. A *P* value of less than .05 was accepted as statistically significant. All analyses were performed with a statistical software package (SPSS for Windows, version 12.0; SPSS, Inc, Chicago, Ill).

RESULTS

Kinetics of SOCS1 expression in BLM-injured lungs

The time course of *SOCS1* mRNA expression before and after BLM administration is shown in Fig 1, *A*. *SOCS1* mRNA was rapidly induced after BLM treatment from day 1 and maintained to day 3 in wild-type (WT) mice. In $SOCS1^{+/-}$ mice, SOCS1 was also quickly induced after BLM instillation; however, the expression level of SOCS1 was significantly lower in $SOCS1^{+/-}$ mice than in WT mice throughout the time course. The SOCS1 expression was also evaluated by means of immunohistochemistry (Fig 1, *B*). Enhanced expression of SOCS1 was observed in epithelial cells and infiltrated mononuclear and polynuclear cells in mice