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SOCS1 hypermethylation mediated by DNMT1 is associated with lipopolysaccharide-induced inflammatory cytokines in macrophages

Chang Cheng[a,b], Cheng Huang[a,b], Tao-Tao Ma[a,b], Er-Bao Bian[a,b], Yong He[a,b], Lei Zhang[a,b], Jun Li[a,b,*]

[a] School of Pharmacy, Anhui Medical University, Hefei 230032, China
[b] Institute for Liver Diseases of Anhui Medical University, Hefei, China

HIGHLIGHTS

- SOCS1 hyper-methylation is detected in LPS-induced RAW264.7.
- SOCS1 methylation is restored after treatment with 5-azadC.
- DNMT1 is involved in SOCS1 hypermethylation during RAW264.7 activation.
- JAK2/STAT3 was correlated with SOCS1 due to its methylation.

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ABSTRACT

Macrophages activation which releases the pro-inflammatory cytokines is an essential event in the process of inflammation. SOCS1 has been shown to act as a negative regulator of cytokine signals and plays a key role in the suppression of tissue injury and inflammatory diseases. DNA methylation mediated by specific DNA methyltransferases1 (DNMT1) which contributes to the epigenetic silencing of multiple genes. SOCS1 promoter hypermethylation is by far the best categorized epigenetic change in tumors. Our study with a view to investigate whether the loss of SOCS1 due to SOCS1 promoter methylation was involved in the course of inflammatory cytokines released from lipopolysaccharide (LPS)-stimulated macrophages. Here, we found that treatment of LPS-induced RAW264.7 macrophage cells with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-azadC) reduced aberrant promoter hypermethylation of SOCS1 and prevented the loss of the expression of SOCS1 in macrophages which secret inflammatory cytokines. Knockdown of DNMT1 gene not only attenuated the SOCS1 gene promoter methylation but also up-regulated the expression of SOCS1 in activated RAW264.7 cells. Furthermore, silencing of DNMT1 prevented the activation of JAK2/STAT3 pathway in LPS-induced RAW264.7 cells. These studies demonstrated that DNMT1-mediated SOCS1 hypermethylation caused the loss of SOCS1 expression results in negative regulation of activation of the JAK2/STAT3 pathway, and enhanced the release of LPS-induced pro-inflammatory cytokines such as TNF-α and IL-6 in macrophages.

1. Introduction

Inflammation is a complex defense reaction, the aim of which is to neutralize an insult and restore normal tissue structure and function (Hirahara et al., 2013). An uncontrolled and prolonged inflammatory reaction may underlie the pathogenesis of chronic diseases (Scrivo et al., 2011). Macrophages are the most important cells in promoting the process of the inflammation and play a crucial role in secreting inflammatory cytokines after activation (Valledor et al., 2010; de Geus and Vervelde, 2013). Many factors such as bacterium, hepatitis B virus, alcohol activate macrophages to release inflammatory cytokines. Moreover, lipopolysaccharide (LPS), one of the components of the outer wall of Gram-negative

Abbreviations: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; SOCS, suppressor of cytokine signaling; JAK2/STAT3, kinase-signal transducers2 and activators of transcription3; DNMT1, DNA methyltransferase 1; MSP, methylation-specific PCR; RAW264.7, mouse macrophage cell line; 5-azadC, 5-aza-2′-deoxycytidine; RT, reverse transcription; siRNA, short interfering RNA; RNAi, RNA interference.

* Corresponding author at: Anhui Medical University, School of Pharmacy, Mei Shan Road, Hefei 230032, Anhui Province, China. Tel.: +86 551 5161001; fax: +86 551 5161001..
E-mail addresses: chengchang730@gmail.com (C. Cheng), lj@ahmu.edu.cn (J. Li).

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bacteria, is the most important mediator to activate resident macrophages, which release symbolic pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) that promote tissues damage and chronic diseases (Odegaard and Chawla, 2011; Gyorfy et al., 2013). Thus, we choose incubation of macrophages with LPS to imitate classical inflammation model in vitro. Furthermore, macrophages with the stimulation of LPS dramatically activate the Janus kinase-signal transducers2 and activators of transcription3 (JAK2-STAT3) pathway to produce inflammatory cytokines (Huang et al., 2010) while the loss of phosphorylation JAK2/STAT3 participates in preventing inflammatory cytokines secretion in macrophages (Minogue and Lynch, 2012). This suggests that LPS induces the production of pro-inflammatory mediator in macrophages via the JAK2/STAT3 pathway.

A family named suppressor of cytokine signaling (SOCS) has emerged as critical regulators of cytokine-mediated signaling in diverse tissues. These proteins are relatively small molecules containing a central src homology2 (SH2) domains and a C-terminal SOCS box (Palmer and Restifo, 2009). SOCS1, a member of SOCS family, located on chromosome16p12-p13.1, act in a negative feedback loop to attenuate signaling via the JAK2/STAT3 pathway that is important in the transmission of cytokine signals from the cell surface to the nucleus. SOCS1 interacts with JAK2 through its SH-2 domain to inhibit its phosphorylation, and prevents activation of STAT3 as well as down-regulates JAK2/STAT3 signaling pathway, thereby regulating inflammatory cytokine secretion in macrophages (Davey et al., 2006; Fujimoto and Naka, 2010; Tamiya et al., 2011). The loss of SOCS1 promotes JAK2 and STAT3 activation, leading to accumulation of pro-inflammatory cytokines (Hashimoto et al., 2009; Tajiri et al., 2012) while over-expression of SOCS1 inhibits JAK2/STAT3 signal pathway, which likely contributes to the loss of pro-inflammatory cytokines expression (Lesinski et al., 2010). However, the expression of SOCS1 could be caused by promoter methylation in a variety of diseases (Sutherland et al., 2004a,b; Hussain et al., 2011; Um et al., 2011; Souma et al., 2012; Capello et al., 2013).

DNA methylation involves the addition of a methyl group to the carbon 5 position of the cytosine ring in the CpG dinucleotide, leading to a conversion to methylcytosine (Jones, 2012). DNA methyltransferases (DNMTs) are key regulators of DNA methylation and have crucial roles in epigenetic modification, especially DNA methyltransferases1 (DNMT1) has enzymatic activity affecting maintenance of DNA methylation (Denis et al., 2011). Previous studies suggest that the DNA methylation mediated by specific DNMT1, results in the epigenetic silencing of multiple genes which are implicated in cancers (He et al., 2011; Wu et al., 2011; Krifa et al., 2013; Lubecka-Pietruszewska et al., 2013). The latest study found that the increase in DNMT1 is associated with the aberrant DNA methylation of a key gene involved in ulcerative colitis (Li et al., 2012). But there is no report concerning DNMT1 involved in hypermethylation of the SOCS1 gene in macrophages.

In the present study, we demonstrate that DNA hypermethylation at specific CpG sites of SOCS1 is related with DNMT1 in LPS-induced macrophages. To gain a further understanding of the epigenetic mechanisms about SOCS1 expression in LPS-induced macrophages, we use the methylation-specific PCR (MSP) to analyze the methylation status of SOCS1. We have shown that expression of the SOCS1 silenced by methylation could be detected in LPS-stimulated RAW264.7. Treatment with the DNA methylation inhibitor 5-aza-2-deoxycytidine (5-azadC) diminished LPS-exposed aberrant hypermethylation of the SOCS1 gene. DNMT1 knockdown with RNAi restored the expression of SOCS1 and prevented its hypermethylation. So our results may provide evidence for the underlying mechanisms of the epigenetic regulation of the SOCS1 gene in activated macrophages.

2. Materials and methods

2.1. Cell line and reagents

The RAW264.7 mouse macrophage cell line was obtained from the American Type Culture Collection (Rockville, MD). 5-azadC (5-aza-2′-deoxycytidine), dimethylsulfoxide (DMSO) and LPS (Escherichia coli 055:B5), were purchased from Sigma Inc. (St. Louis, MO, USA). Rabbit anti-SOCS1 polyclonal antibodies were purchased from Cell Signaling (Beverly, MA, USA). Mouse monoclonal antibodies against DNMT1 and β-actin were purchased from Santa Cruz (California, USA) and Biotechnology Boster (Wuhan, China). JAK2 antibodies, phospho-JAK2 antibodies, STAT3 antibodies, and phospho-STAT3 antibodies were obtained from anbobio (Shanghai, China). DNMT1, SOCS1, TNF-α, IL-6 and β-actin primers were produced from Shanghai Sangon Biological and Technological Company (Shanghai, China). DNA extraction kit was acquired from Axygen. Enzyme Linked Immunosorbent Assay (ELISA) kit was acquired from shanghai Hushang Biotechnology Co. Ltd (Shanghai, China). Secondary antibodies for goat anti-mouse immunoglobulin (Ig)G horse radish peroxidase (HRP), and goat anti-rabbit IgG HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

2.2. Cell treatment conditions

RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum, supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were grown in a humidified 5% CO_2 −37 °C atmosphere. RAW264.7 cells were pretreated in serum-free DMEM for 24 h. Then, they were treated with LPS 1 μg/ml.

2.3. Assays for cytokines

The amount of TNF-α and IL-6 produced by mouse macrophages was measured in RAW264.7 cell culture supernatant. RAW264.7 cells were stimulated with 1 μg/ml LPS for 24 h, the supernatants were harvested. Concentration of TNF-α and IL-6 in culture supernatant were measured using mouse TNF-α and IL-6 ELISA kits according to the manufacturer's instructions (BD bioscience). The ELISA data representing mean values (6SD) were obtained in duplicate from at least three independent experiments.

2.4. 5-aza-2′-deoxycytidine treatment

For treatment with 5-azadC, RAW264.7cells were seeded overnight in culture dishes and treated with 1 μg/ml LPS and 2 μmol 5-azadC (Sigma, St Louis, MO) for 24 h (Sigma-Aldrich, St. Louis, MO). RAW264.7cells were harvested for supernatant, genomic DNA, total cellular RNA, and whole cell protein extractions.

2.5. Methylation-specific polymerase chain reaction

High-molecular-weight genomic DNA was isolated by standard protocols from RAW264.7 cell lines using the QIAamp DNA mini kit (Axygen). The methylation-specific PCR (MSP) was performed to analyze the methylation status of SOCS1 CpG island. Briefly, treatment of DNA with bisulfite (which resulted in conversion of unmethylated cytosine to uracil, but unaffecting methylated cytosine) was performed with a commercially available kit (CpGenome DNA modification kit; Intergen, New York, NY). MSP primers were designed for the amplification of SOCS1 CpG-island: one specific to methylated DNA and the other specific to unmethylated DNA. The primer sequences used to amplify the methylated SOCS1 gene were 5′-TTCGCGTGTATTTTTAGGTCGGTC-3′ (forward) and 5′-CGACACAACTCCTAC AACGACCG-3′ (reverse) and primer sequences used for unmethylated SOCS-1 were 5′-TTATGAGTATTTGTGTGTATTTTT-3′ (forward) and 5′-CGACACAACTCCTACAACGACC G-3′ (reverse). The primers used in the present study detect specifically the promoter sequence of the SOCS1 gene rather than that of the SOCS1 pseudogene. MSP was performed in a thermal cycler with the following cycling conditions: 95 °C for 12 min, 35 to 45 cycles of 95 °C for 45 s, specific annealing temperature for 30 s, 72 °C for 30 s, and a final extension of 10 min at 72 °C. The polymerase chain reactions for SOCS1-M and SOCS1-U were carried out in a 50 μl volume containing 1× polymerase chain reaction buffer (15 mmol/l $MgCl_2$), 2.5 mmol/lmixture of dNTPs, 10 pM of each primer, 4 U HotStart Taq DNA polymerase (Qiagen, Frankfurt, Germany), and 25 ng to 50 ng of bisulfite-modified DNA. Methylation-specific PCR experiments were performed at least in duplicate.

2.6. Small interfering RNA analysis

Transfection of the RAW264.7 cells was carried out with lipofection technique, using Lipofectamine™ 2000. The experiment was conducted according to the manufacturer's protocol (Invitrogen). Briefly, Approximately 2×10^5 RAW264.7cells were seeded in 6-well plates and incubated 37 °C for 24 h before RAW264.7cells were cultured in serum-free DMEM for 12 h, then subjected to