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# p21$^{waf1/cip1}$ is down-regulated in conjunction with up-regulation of c-Fos in the lymphocytes of rheumatoid arthritis patients☆

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**Abstract**

Features characteristic to rheumatoid arthritis (RA) including synovial overgrowth and joint destruction are experimentally produced by augmenting c-*fos* gene expression. We show that cyclin dependent kinase inhibitor *p21$^{waf1/cip1}$*, that inhibits cell proliferation, is down-regulated in conjunction with up-regulation of c-*fos* in the lymphocytes of patients with RA. As to the mechanism of down-regulation of *p21$^{waf1/cip1}$* gene expression, transfection studies in U937 cells showed that c-*fos* down-regulated phosphorylation and dimerization of signal transducers and activators of transcription (STAT) 1, thereby inhibiting interferon γ-induced transactivation of *p21$^{waf1/cip1}$*. Phosphorylation of STAT1 was indeed decreased in the lymphocytes of patients with RA. Thus, under overexpression of c-*fos* gene, c-Fos inactivates STAT1 to down-regulate *p21$^{waf1/cip1}$* gene expression in the lymphocytes of patients with RA, and in this way may enhance proliferation of lymphocytes.


*Keywords:* c-Fos; Rheumatoid arthritis; p21$^{waf1/cip1}$; STAT1; IFNγ

Elevated expression of genes that are transcriptionally induced by phorbol ester is among the events required for tumor progression [1,2]. Previous studies have shown that one of such genes, immediate early c-*fos* gene, was significantly up-regulated in rheumatoid arthritis (RA) [3–5], and the symptoms characteristic to rheumatoid joint destruction including synovial overgrowth and periarticular osteoporosis are experimentally reproduced by augmenting c-*fos* gene expression [6–11]. Increased c-Fos/activator protein-1 (AP-1) is responsible not only for arthritic joint destruction mediated by synovial mesenchymal cells [6,11], but also significantly modulates the immune response and cell cycle progression of T cells [12,13], implicating that overexpression of c-Fos/AP-1 may significantly affect cell cycle progression of lymphocytes.

We have studied transcriptional regulation of *p21$^{waf1/cip1}$* gene expression with reference to the overexpression of c-*fos* gene. p21$^{waf1/cip1}$ is a cyclin dependent kinase (Cdk) inhibitor playing an essential role in cell cycle progression by inhibiting Cdk2, Cdk3, Cdk4, and Cdk6 to halt cellular G1/S transition [14]. p21$^{waf1/cip1}$ mediates growth arrest and apoptosis of cells in response to a variety of stimuli including DNA damage and cellular terminal differentiation [15]. Previous studies have shown that expression of *p21$^{waf1/cip1}$* gene is transcriptionally regulated through p53-independent and -dependent pathways [14–16]. Transactivation of *p21$^{waf1/cip1}$* gene is regulated independently of p53 by the signal transducers and activators of transcription (STATs) that are conditionally activated in response to interferon (IFN) γ stimulation from cell surface [17–20]. After receptor ligation, Janus kinase (Jak) 1/Jak2

☆ *Abbreviations:* AP-1, activator protein-1; Cdk, cyclin dependent kinase; IFNγ, interferon-γ; Jak, Janus kinase; RA, rheumatoid arthritis; OA, osteoarthritis; CBP, CREB binding protein; SIE, sis-inducible element.

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is phosphorylated and the Jak1/Jak2 subsequently phosphorylates STAT1 [21]. STAT1 then dimerizes to move into nuclei and binds onto the consensus STAT-responsive element in the promoter region of *p21$^{waf1/cip1}$* to enhance *p21$^{waf1/cip1}$* gene expression [20,21]. As to the other, p53-dependent pathway, the promoter of *p21$^{waf1/cip1}$* gene contains two conserved p53-responsive elements through which p53 transactivates the *p21$^{waf1/cip1}$* gene [22] by the help of p53 coactivator, p300/CBP, that had histone acetyltransferase activity [23].

In the present study, we found that p21$^{waf1/cip1}$ was markedly decreased in face of increased c-Fos in the lymphocytes of patients with RA. Further, IFNγ-induced signaling pathway, phosphorylation and dimerization of STAT1 were down-regulated by over-expressed c-*fos* gene. The result was discussed in relation to the contribution of p21$^{waf1/cip1}$ to lymphocyte proliferation.

## Materials and methods

*Cells.* Samples of synovium and peripheral blood lymphocytes were obtained from RA patients who met the American College of Rheumatology (formerly, the American Rheumatism Association) diagnostic criteria [24], osteoarthritis (OA) patients, and healthy individuals. Peripheral blood lymphocytes were isolated by using Lymphoprep (Nycomed, Olso, Norway). Synovial cells dispersed by treating with collagenase and trypsin were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FCS (Whittaker M. A. Bioproducts, Walkersville, MD), 50 units/ml penicillin and 50 μg/ml streptomycin [25]. After overnight culture on a 10 cm culture dish (Asahi Techno Grass, Tokyo, Japan), non-adherent lymphocyte population was separated and cultured for additional 48 h. U937 cells (RCB0435, Riken, Tsukuba, Japan) were maintained in RPMI 1640 medium (Nissui) supplemented with 10% FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin.

*Plasmids and transfection.* Human c-*fos* expression vector 1.4fpH8 and control pH8 contained the c-*fos* cDNA under the control of murine leukemia virus long terminal repeat [8]. U937 cells ($5 \times 10^6$ cells) in 250 μl RPMI 1640 medium supplemented with 20% FCS were reacted with 10 μg of 1.4fpH8. Electroporation was carried out in a 4 mm-gap cuvette at 200 V, 950 μF using Gene Pulser (Bio-Rad, Hercules, CA). The U937 transformants were cultured at 37 °C overnight before stimulation.

*Western blot.* Synovial or peripheral blood lymphocytes were suspended in 100 μl of lysis buffer 1 [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor complex (Complete Protease Inhibitor; Roche Diagnostics, Mannheim, Germany)]. After sonication, the protein fraction was obtained by centrifugation at 15,000*g* for 10 min. Ten micrograms of protein was separated by SDS–PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Membrane was incubated for 2 h with anti-p21$^{waf1/cip1}$ Ab (H-164, Santa Cruz Biotech, Santa Cruz, CA) or c-Fos (C-19, Santa Cruz). The U937 transformants ($2 \times 10^6$ cells) or peripheral blood lymphocytes ($3 \times 10^6$ cells) stimulated with IFNγ (100 ng/ml, PeproTech, Rocky Hill, NJ) were suspended in 100 μl of lysis buffer 2 (20 mM Hepes–KOH, pH 7.9, 0.2% Nonidet P-40, 10% glycerol, 400 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM sodium vanadate, and Complete Protease Inhibitor) [19]. Whole-cell extract was obtained by centrifugation at 15,000*g* for 10 min. Ten micrograms of protein was separated by SDS–PAGE and transferred to Immobilon-P membrane. Membrane was incubated for 2 h with anti-c-Fos Ab, anti-STAT1 Ab (E-23, Santa Cruz), and anti-phospho STAT1 Ab (Sigma–Aldrich, St. Louis, MO) that recognized 701 phosphotyrosine or anti-actin Ab (C-11, Santa Cruz), followed by reaction with HRP-conjugated anti-rabbit Ig (NA934, Amersham–Pharmacia Biotech., Buckinghamshire, England) or anti-goat IgG (Sigma) and ECL enhanced chemiluminescence reagent (Amersham).

*Quantitative RT-PCR.* Total RNA was isolated using RNeasy total RNA kit (Qiagen, Hiden, Germany) from U937 transformants stimulated with IFNγ for 24 h. The mRNA of these cells was reverse transcribed in a 80 μl reaction containing 2.5 μM of random hexamer, 5.5 mM $MgCl_2$, (1× RT buffer, 200 μM of each dNTP, and 0.4 units/μl RNase inhibitor. After adding 1.25 units/μl MultiScribe reverse transcriptase, this was incubated at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min (RT solution). The RT solution for *p21$^{waf1/cip1}$* and 18S *rRNA* was quantified by PCR using TaqMan PCR Core Reagent Kit (Perkin–Elmer, Norwalk, CT) in Applied Biosystem PRISM 7700 Sequence Detector (Perkin–Elmer) [26]. The primers and TaqMan probes were: 5′-ACTGTGATGCGCTAATGGCG-3′ (forward), 5′-AAGTCACCCTCCAGTGGTGTCT-3′ (reverse), and 5′-CCCGTGAGCGATGGAACTTCGACTTT-3′ (TaqMan probe) for *p21$^{waf1/cip1}$*; 5′-GGTTGATCCTGCCAGTAGCAT-3′ (forward), 5′-ATCCAAGTAGGAGAGGAGCGAG-3′ (reverse), and 5′-TAAGTACGCACGGCCGGTACAGTGAA-3′ (TaqMan probe) for 18S *rRNA*. A 25 μl reaction containing 5.5 mM $MgCl_2$, 200 μM of each dATP, dCTP, and dGTP, 400 μM of dUTP, 0.01 units/μl AmpErase UNG, 1× TaqMan Buffer A, 0.025 units/μl AmpliTaq Gold DNA polymerase, 300 nM each forward and reverse primers, 200 nM TaqMan probe, and 5 μl RT solution was carried out at 50 °C for 2 min and at 95 °C for 10 min, followed by amplification at 95 °C for 15 s and at 60 °C for 1 min for 40 cycles. For normalization, the mRNA of *p21$^{waf1/cip1}$* was divided by that of 18S *rRNA*.

*Electrophoretic mobility shift assay.* U937 transformants ($1 \times 10^7$ cells) were suspended in 200 μl of lysis buffer 2. Whole-cell extract (20 μg) was incubated with 1 pmol of digoxigenin (DIG)-labeled oligonucleotide probe for 30 min in a 20 μl reaction containing 20 mM Hepes–KOH, pH 7.9, 10% glycerol, 20 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μg BSA, and 0.5 μg of poly(dI–dC)·poly(dI–dC) (Amersham). Double-stranded probe for SIE (sis-inducible element) of STAT1 binding motif was 5′-GTGCATTTCCCGTAAATCTTGTCTACA-3′ and 3′-CACGTAAAGGGCATTTAGAACAGATGT-5′. Probes were end-labeled with DIG-11-ddUTP (Roche Diagnostics). Samples were run on a 4% polyacrylamide gel using 1× TGE buffer (50 mM Tris, 380 mM glycine, and 2 mM EDTA). Oligonucleotides were transferred to a positively charged-nylon membrane (Roche Diagnostics). DIG-labeled oligonucleotides were visualized by incubation with alkaline phosphatase-labeled $F(ab)_2$ anti-DIG Ab, and chemiluminescence reaction using 100 μg/ml CSPD substrate (Tropix).

## Results

### *p21$^{waf1/cip1}$ in the lymphocytes of patients with RA*

Western blotting showed that the amount of p21$^{waf1/cip1}$ was significantly decreased or almost absent in the lymphocyte population of rheumatoid synovium as compared with OA (Fig. 1A). In the peripheral blood lymphocytes of patients with RA as compared with healthy control, p21$^{waf1/cip1}$ was also significantly decreased or almost absent, while c-Fos was increased (Fig. 1B).

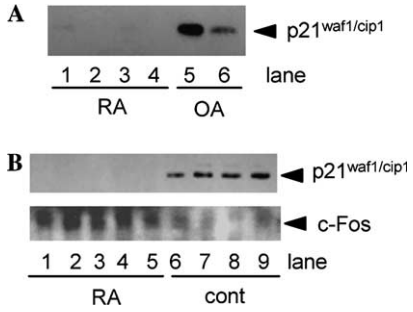


Fig. 1. Western blot for $p21^{waf1/cip1}$ and c-Fos. (A) $p21^{waf1/cip1}$ expressed in the synovial lymphocytes of patients with rheumatoid arthritis (RA) (lanes 1–4) and osteoarthritis (OA) (lanes 5 and 6). Each lane represented different individuals. (B) $p21^{waf1/cip1}$ and c-Fos in the peripheral blood lymphocytes of patients with RA (lanes 1–5) and healthy controls (cont, lanes 6–9).

### *c-Fos inhibits the transactivation of $p21^{waf1/cip1}$ through STAT1*

We have studied the pathway of transactivation of $p21^{waf1/cip1}$ through IFNγ and STAT1 in U937 cells. STAT1 was normally phosphorylated after IFNγ stimulation, and the peak phosphorylation level was achieved at 0.5 h after stimulation in U937 cells (Fig. 2A). Western blot experiment using cell lysates obtained 0.5 h after stimulation with IFNγ showed that phosphorylation of STAT1 as induced by IFNγ was significantly inhibited when c-Fos was simultaneously increased (Fig. 2B). Electrophoretic mobility shift assay (EMSA) using the $p21^{waf1/cip1}$ promoter oligonucleotides showed that STAT1 as detected by the probe for sis-inducible element (SIE) was increased after IFNγ stimulation (Fig. 2C, lanes 1 vs 2), whereas this increase was inhibited by co-expressed c-*fos* gene (Fig. 2C, lanes 3 vs 4). Quantitative RT-PCR showed that expression of $p21^{waf1/cip1}$ mRNA in U937 cells as stimulated by IFNγ was also significantly inhibited by co-expressed c-*fos* gene (Fig. 2D, lanes 3 vs 4).

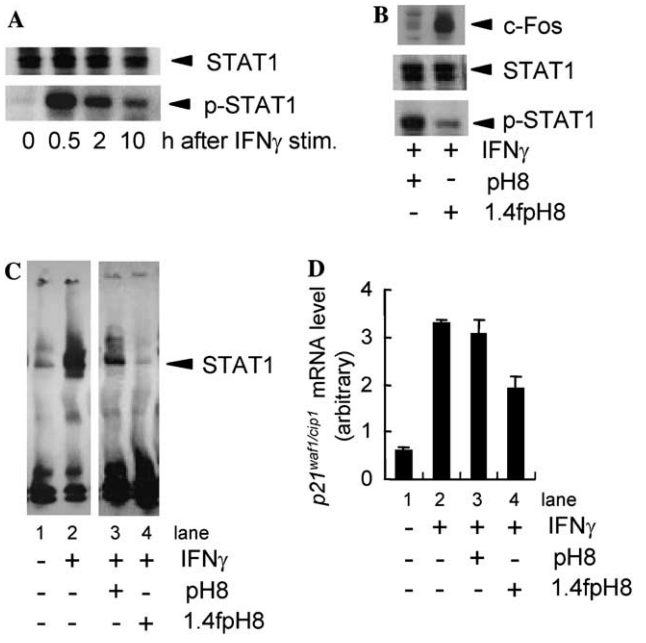


Fig. 2. IFNγ-induced transactivation of $p21^{waf1/cip1}$. (A) Western blot for STAT1 and phosphorylated STAT1 (p-STAT1) after stimulation with 100 ng/ml of IFNγ for indicated time periods. (B) Western blot for c-Fos, STAT1, and p-STAT1 in U937 transformants containing either control pH8 or c-*fos*-expressing 1.4fpH8 vectors 0.5 h after stimulation with IFNγ. (C) Electrophoretic mobility shift assay (EMSA) for STAT1. U937 transformants containing pH 8 or 1.4fpH8 vectors were stimulated with 100 ng/ml IFNγ (lanes 3 and 4). Cell extracts obtained 6 h after stimulation were assayed for EMSA using SIE probe. (D) Quantitative RT-PCR for $p21^{waf1/cip1}$ mRNA in U937 cells. U937 cells with or without containing pH 8 or 1.4fpH8 vectors were stimulated with 100 ng/ml IFNγ for 24 h (lanes 2–4). Each column represented the mean ± SD of three experiments.

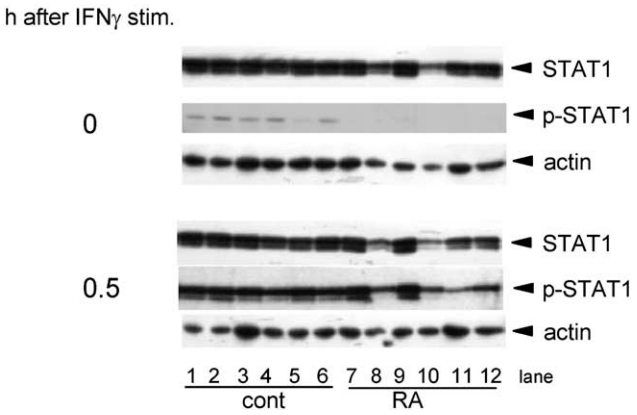


Fig. 3. Western blot for STAT1 and p-STAT1 in the peripheral blood lymphocytes of healthy controls (lanes 1–6) and patients with RA (lanes 7–12) before and 0.5 h after stimulation with IFNγ. Each lane represented different individuals.

### *Down-regulation of STAT1 in RA lymphocytes*

In the peripheral blood lymphocytes of patients with RA, while the amount of STAT1 was similar or somewhat decreased, phosphorylation of STAT1 was significantly decreased or almost absent in the patients with RA as compared with healthy controls (Fig. 3). The amount of STAT1 as well as the extent of phosphorylation of STAT1 tended to be lower in 4 out of 6 patients with RA as compared with healthy controls after stimulation with IFNγ.

## Discussion

There are two pathways in the transcriptional control of $p21^{waf1/cip1}$ gene expression [14–16]. With regard to the first p53-dependent pathway of $p21^{waf1/cip1}$ gene expression, the profile of transactivation of $p21^{waf1/cip1}$ was studied in COS7 cells using luciferase reporter (WWP-Luc) containing the 2.4 kb promoter region (−2320/+11)

of mouse *p21$^{waf1/cip1}$* gene. The transactivation of *p21$^{waf1/cip1}$* as induced by p53-expression vectors and/or p300-expression vectors was not significantly altered by the co-expressed c-*fos* gene, suggesting that c-Fos did not significantly affect the p53-dependent pathway of *p21$^{waf1/cip1}$* gene expression (data not shown).

With regard to the pathway of transcriptional regulation of *p21$^{waf1/cip1}$* gene expression, which normally operates after stimulation with mitogens or cytokines including IFNγ [19,20], we confirmed that *p21$^{waf1/cip1}$* gene expression was normally increased in response to IFNγ stimulation in U937 cells. There are three potential STAT-binding sites called SIE in the promoter region of *p21$^{waf1/cip1}$* gene [19]. By using this SIE sequence as a probe, we showed that not only the phosphorylation but also the binding of STAT1 as induced by IFNγ was inhibited by c-Fos. Phosphorylation of STAT1 before and after stimulation with IFNγ was decreased in the lymphocytes of the patients with RA as compared with healthy control. Considering the fact that p21$^{waf1/cip1}$ was decreased and c-Fos was increased in the lymphocytes of patients with RA, it was likely that transactivation of *p21$^{waf1/cip1}$* gene was decreased in line with the down-regulation of STAT1 in face of increased c-Fos in the lymphocytes with RA (Fig. 4). Although the molecular mechanism responsible for the down-regulation of STAT1 by c-Fos is unclear at present, Servidei et al. [27] have shown that while SHP-2 negatively regulates STAT binding activity, it simultaneously up-regulates the AP-1 binding activity by controlling the levels of c-Fos. Although SHP-2 is a tyrosine phosphatase that is implicated to play important roles in a variety of cellular signaling cascades [28], we confirmed in the present study that the level of SHP-2 protein did not differ significantly between the PBMC with RA and healthy controls (data not shown).

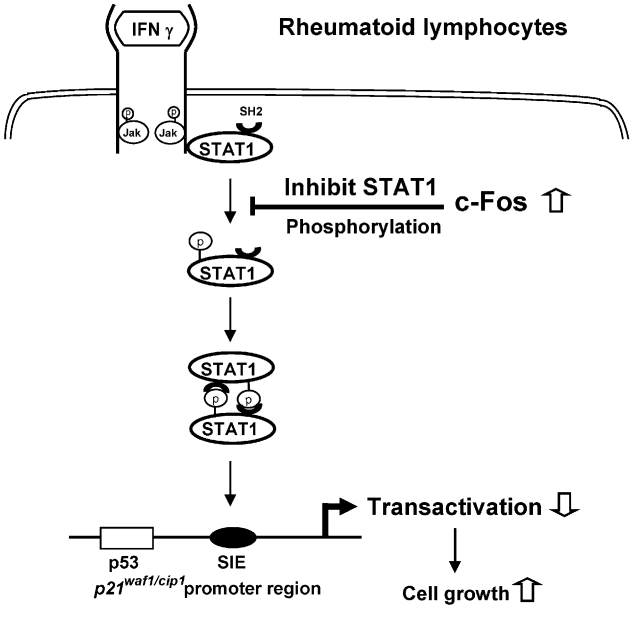


Fig. 4. Schematic representation of c-Fos and transcriptional control of *p21$^{waf1/cip1}$* gene expression in rheumatoid lymphocytes. Under overexpression of c-*fos* gene, c-Fos inactivates STAT1 to down-regulate *p21$^{waf1/cip1}$* gene expression in the lymphocytes of patients with RA, and in this way may enhance proliferation of lymphocytes.

As p21$^{waf1/cip1}$ plays as a Cdk inhibitor to halt cellular G1/S transition, loss of function of p21$^{waf1/cip1}$ should be intimately associated with abnormal checkpoint control and genomic instability in the cell [14,15]. We previously found that T lymphocytes overexpressing c-*fos* gradually went into aberrant mitosis because of the increase of wee1 kinase that halted mitotic cell division [13]. It thus appears that over-expressed c-*fos* in rheumatoid lymphocytes down-regulates *p21$^{waf1/cip1}$* gene expression to act as an accelerator to pass through cellular G1/S transition. On the other hand, over-expressed c-*fos* up-regulates *wee1* kinase to act as a brake to halt cellular G2/M transition. This 'tumor-like' cell growth in RA [29] may significantly influence the sequelae of rheumatoid inflammation.

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