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Gene Transfer of a Cell Cycle Modulator Exerts Anti-Inflammatory Effects in the Treatment of Arthritis

Yoshinori Nonomura, Hitoshi Kohsaka, Kenji Nagasaka, and Nobuyuki Miyasaka

Forced expression of a cyclin-dependent kinase inhibitor gene, p21\textsuperscript{Cip1} in the synovial tissues was effective in treating animal models of rheumatoid arthritis. Synovial hyperplasia in the treated joints was suppressed, reflecting the inhibitory effect of p21\textsuperscript{Cip1} on cell cycle progression. Additionally, lymphocyte infiltration, expression of inflammatory cytokines, and destruction of the bone and cartilage were inhibited. To determine why the cell cycle regulator gene exerted such anti-inflammatory effects, we investigated gene expression by rheumatoid synovial fibroblasts with or without the p21\textsuperscript{Cip1} gene transferred. We have found that p21\textsuperscript{Cip1} gene transfer down-regulates expression of various inflammatory mediators and tissue-degrading proteinases that are critically involved in the pathology of rheumatoid arthritis. These molecules included IL-6, -8, type I IL-1R (IL-1R1), monocyte chemoattractant protein-1, macrophage inflammatory protein-3α, cathepsins B and K, and matrix metalloproteinases-1 and -3. Down-regulation of IL-1R1 by p21\textsuperscript{Cip1} resulted in attenuated responsiveness to IL-1. Inhibition of the inflammatory gene expression by p21\textsuperscript{Cip1} was seen even when IL-1 is absent. This IL-1R1-independent suppression was accompanied by reduced activity of c-Jun N-terminal kinase, which was associated with p21\textsuperscript{Cip1}, and inactivation of NF-κB and AP-1. These multiple regulatory effects should work in concert with the primary effect of inhibiting cell cycle in ameliorating the arthritis, and suggest a heretofore unexplored relationship between cyclin-dependent kinase inhibitor gene and inflammatory molecules.


S
ynovial tissue from healthy individuals consists of a single layer of synovial cells without infiltration of inflammatory cells. In rheumatoid synovial tissue, lymphocytes and macrophages are recruited and activated, and these activated macrophages release high concentrations of inflammatory cytokines. In response to these cytokines, synovial fibroblasts proliferate vigorously and form villous hyperplastic synovial tissues. These fibroblasts secrete inflammatory mediators, which further attract inflammatory cells and stimulate growth of the synovial fibroblasts as well as that of vascular endothelial cells (1). These activated macrophages and fibroblasts produce tissue-degrading proteinases (2). Thus, the invasive hyperplastic synovial tissue, termed pannus, is directly responsible for structural and functional damage of the affected joints.

Therapeutic intervention against rheumatoid arthritis (RA)\textsuperscript{3} could be aimed at any one of these steps. Recently developed biological reagents that block activities of TNF-α have proved to be beneficial in clinical settings. However, they and other conventional drugs do not necessarily control synovial inflammation and hyperplasia in all patients. We hypothesized that the proliferation of the synovial fibroblasts is a common outcome of the multiple inflammatory processes in RA. If synovial fibroblasts become refractory to the proliferative stimuli, the tissue-degrading pannus should not develop. This idea led us to explore new therapeutic approaches that directly control synovial cell proliferation (3–5). The molecules we have focused on are cyclin-dependent kinase inhibitors (CDKIs). These intracellular proteins inhibit kinase activity of cyclin/cyclin-dependent kinase (CDK) complexes that are required for cell cycle progression (6).

Our previous studies have shown that CDKIs p16\textsuperscript{NK4a} and p21\textsuperscript{Cip1} are not expressed in vivo in the rheumatoid synovial tissues, but readily induced in vitro in cultured rheumatoid synovial fibroblasts (RSF). Induction of p16\textsuperscript{NK4a} is characteristic of RSF (3). In vitro inducibility of p16\textsuperscript{NK4a} and p21\textsuperscript{Cip1} suggested to us that their induction in vivo in the rheumatoid joints could be an ideal approach to suppression of the proliferative synovitis. This was substantiated by intraarticular transfer of the p16\textsuperscript{NK4a} or p21\textsuperscript{Cip1} gene to rodent models of RA (4, 5). These gene therapies suppressed synovial hyperplasia and also inhibited lymphocyte infiltration and destruction of the bone and cartilage of the treated joints. Expression of inflammatory cytokines such as IL-1, -6 and TNF-α was suppressed even in the small amount of hyperplastic synovial tissues that remained after the gene transfer (4). These data argued that induction of CDKI ameliorated the arthritis not only by inhibition of cell cycle but by other unknown functions that suppressed the inflammatory network in the arthritic joint.

Unlike the other CDKIs, p21\textsuperscript{Cip1} binds to various molecules related to gene expression and exerts differential effects on different cells (7). However, little is known about the effects of p21\textsuperscript{Cip1} on gene expression in the inflamed tissues. We show here that up-regulated expression of the p21\textsuperscript{Cip1} gene in RSF suppresses expression of various inflammatory molecules that play critical roles in the pathology of RA. Manipulation of these multiple...
molecular events should contribute to the therapeutic effects of p21\textsuperscript{Cip1} gene therapy.

Materials and Methods

Cell culture and recombinant adenoviruses

Synovial tissues were obtained from patients who had responded poorly to anti-rheumatic drugs and underwent joint replacement or synovectomy for active rheumatoid synovitis at Tokyo Medical and Dental University Hospital (Tokyo, Japan), Tokyo Metropolitan Bokuto, or Fuchu Hospital (Tokyo, Japan). The patients fulfilled the American College of Rheumatology criteria for classification of RA (5). All patients gave their consent for all procedures in the present studies, which were also approved by the ethics committee of Tokyo Medical and Dental University. From villous and congestive synovial tissues, RSF were isolated and cultured as described elsewhere (5). They were used at passages 3–11. RSF were infected with the AxCAp21 adenovirus, containing a human p21\textsuperscript{Cip1} gene (5, 9), or Ax1w1 adenovirus (Riken Gene Bank, Saitama, Japan), which lacks insert genes, at 50 multiplicity of infection. Some RSF were stimulated by 5 ng/ml TNF-\alpha (Genzyme, Cambridge, MA), 5 ng/ml IL-1\beta (PeproTech, Rocky Hill, NJ), and 25 \mu M indomethacin (Sigma-Aldrich, St. Louis, MO). In preliminary experiments, 5 ng/ml was determined to be the optimal concentration for each cytokine to stimulate RSF. RNeasy kit (Qiagen, Valencia, CA) with DNase I treatment was used to isolate total RNA. For ELISA, the virus-infected RSF were cultured for three days, transferred to microwells at 1.0 \times 10^5 cells/ml, and incubated for 12 h. After replacement of the culture medium, RSF were further cultured for 24 h with 10% serum alone, 5 ng/ml IL-1\beta together with 25 \mu M indomethacin, 5 ng/ml TNF-\alpha, a combination of IL-1\beta, indomethacin, and TNF-\alpha, or 5 \mu g/ml LPS of Escherichia coli O55:B5 (Sigma-Aldrich). One hundred ng/ml IL-1 receptor antagonist (IL-1ra) (R&D Systems, Mckinley, MN), which was sufficient for the inhibition of 10 pg/ml IL-1\beta, was added to some wells. The culture supernatants were collected after 24 h. For Western blotting, RSF were lysed for protein extraction at three days after the adenoviral infection (5). To assess transcription factor and c-Jun N-terminal kinase (JNK) activities in RSF that were incubated for 30 min in the medium containing 10% FBS with or without supplementation of 5 \mu g/ml LPS, nuclear extracts and cell lysates were prepared using Nuclear Extract Kit (Active Motif, Carlsbad, CA) or SAPK/JNK assay kit (Cell Signaling, Beverly, MA). The effects of the p21\textsuperscript{Cip1} gene were studied at three days after the adenoviral infection.

Northern blot analyses

Northern blotting was conducted as described elsewhere (10). Human monocyte chemotactarpt protein (MCP)-1 cDNA (No. 65933, American Type Culture Collection, Manassas, VA), human GAPDH cDNA (Life Technologies, Rockville, MD), and PCR products of type I IL-1R (IL-1R), cathepsins B and K, and matrix metalloproteinases (MMP)-1 and -3 were used as probes. Fragments of IL-1R, cathepsins B and K, and MMPs-1 and -3 cDNA were generated with RT-PCR using cDNA derived from RSF. PCR was conducted with Taq polymerase (Life Technologies) and sets of specific primers: human IL-1R-specific primers (11), human MMP-1-specific primers (12), human MMP-3-specific primers (12), human cathepsin B-specific primers (5'-TAG GAT GTG CCT GCC ATG AT-3' (sense) and 5'-CCA CGG CAG ATT AGG TCT TT-3' (antisense)) and human cathepsin K-specific primers (5'-AAC GAA GCA AGA CAA CAG ATT TCC-3' (sense), 5'-GAT TGG GCC TGC ATG AAC A-3' (antisense)). Annealing temperatures were 59°C for IL-1R, and cathepsins B and K, 55°C for MMP-1 and -3 cDNA. The products were purified and labeled with [\textsuperscript{32}P]dATP (Amersham Biosciences, Buckinghamshire, UK) and hybridized with the Northern blot membranes. Digital image files were generated with Phosphorimaging Screens and the BAS-2500 PhosphorImager, and analyzed with MacBas2 2.5.2 software (Fuji Film, Kanagawa, Japan).

Western blot analyses and immunoprecipitation

Rabbit anti-human IL-1R\alpha Abs, rabbit anti-human Toll-like receptor (TLR)-4 Abs, and rabbit anti-human p21\textsuperscript{Cip1} Abs (sc-688, sc-10741 and sc-387, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary Abs for Western blot analyses. HRP-conjugated anti-rabbit IgG polyclonal Abs (NA-934, Amersham Biosciences) were used as the secondary Abs. Bound Abs were visualized with ECL or ECL-plus (Amersham Biosciences). Signal intensities were quantified with NIH Image version 1.62 (National Institutes of Health, Bethesda, MD). JNKs 1–3 were immunoprecipitated using mouse anti-human JNK2 Ab (sc-7345, Santa Cruz Biotechnology) (13).

ELISA

ELISA kits for IL-1\beta, IL-6, IL-8, MCP-1, TNF-\alpha (BioSource International, Camarillo, CA), IL-1\alpha, macrophage inflammatory protein (MIP)-3\alpha (R&D Systems), MMP-1 (Amersham Biosciences) and MMP-3 (Fuji Chemical, Toyama, Japan) were used to quantify the protein levels in the culture supernatants.

Multiwell colorimetric transcription factor assays and JNK kinase assay

Using Trans AM AP-1/c-Jun, NF-kBp50, and p65 Transcription Factor Assay Kits (Active Motif), the nuclear extracts of RSF were examined for DNA binding activities of AP-1 and NF-kB (14). SAPK/JNK Assay Kit (Cell Signaling) was used to examine whole cell lysates for their JNK kinase activities to phosphorylate c-Jun substrates. The amount of the c-Jun substrate was standardized by immunoblotting with anti c-Jun Ab (sc-44, Santa Cruz Biotechnology).

Statistics

Signal intensity ratios of Northern and Western blot analyses, and protein concentrations were compared with a paired Student’s t test using StatView-5.0f software (SAS Institute, Cary, NC).

Results

p21\textsuperscript{Cip1} suppresses IL-1R1 and IL-6 expression by RSF

RSF samples derived from rheumatoid joints were cultured in vitro. Expression of p21\textsuperscript{Cip1} was not detected in any of the samples. They were infected with the AxCAp21 adenoviruses or the Ax1w1 adenoviruses. At three days postinfection, when the AxCAp21-infected RSF express p21\textsuperscript{Cip1} at the highest level, the cells were harvested for RNA and protein extraction.

In preliminary experiments using a few RSF samples and commercial DNA array systems, MCP-1, IL-1R1, and cathepsins B and K genes, which are related to RA pathology, showed a tendency to be down-regulated by the p21\textsuperscript{Cip1} gene transfer. Indeed, Northern blot analysis revealed that the IL-1R1 mRNA expression was significantly reduced in RSF overexpressing p21\textsuperscript{Cip1}, compared with those infected with the control adenoviruses (Fig. 1A).

Reflecting this, Western blot analyses of the total cell lysates showed that the IL-1R1 protein expression was reduced in the RSF expressing p21\textsuperscript{Cip1} (Fig. 1B).

The DNA array analyses suggested no differential expression of IL-6 in the unstimulated RSF. However, when RSF were stimulated by TNF-\alpha and IL-1\beta before the DNA array analysis, IL-6, as well as IL-8, MCP-1, MIP-3\alpha, MMP-1, MMP-3, and cathepsin K genes showed a tendency to be down regulated by p21\textsuperscript{Cip1}. This was consistent with the fact that TNF-\alpha and IL-1, both of which are critically involved in activating RSF in the rheumatoid joints, stimulate RSF to promote secretion of various cytokines including IL-6 (15). The unstimulated RSF did not release IL-1\alpha, IL-1\beta, or TNF-\alpha above the lowest limit of detection in the ELISA (3.9 pg/ml). These facts implied that the suppression of IL-6 in the stimulated RSF could be attributable to the down-regulation of IL-1R1. To address this issue, RSF were stimulated independently with IL-1\beta, TNF-\alpha, or a combination of the two. The culture supernatants were examined for the IL-6 concentration with ELISA. Each stimulation promoted IL-6 production. The effects of IL-1\beta were suppressed significantly by p21\textsuperscript{Cip1} while the effects of TNF-\alpha were not attenuated (Fig. 1C). Effects of the adenoviral infection alone on the IL-6 secretion were minimal (Fig. 1D). Thus, the down-regulation of IL-1R1 was biologically relevant to the suppression of IL-6.

To determine whether other pathways that regulate IL-6 production are affected, RSF were stimulated with LPS. Western blot analysis confirmed that Toll-like receptor (TLR)-4, which is a receptor for LPS, was not down-regulated by p21\textsuperscript{Cip1} (Fig. 1E). Nevertheless, the p21\textsuperscript{Cip1} expression suppressed the IL-6 production.
that was induced by LPS (Fig. 1C). Again, the culture supernatants of the LPS-stimulated RSF did not contain detectable amounts of IL-1 (<3.9 pg/ml) or TNF-α (<1.7 pg/ml). To eliminate the effect of a trace amount of IL-1 that might possibly have been secreted with the LPS stimulation, 100 ng/ml IL-1ra, a competitive inhibitor of IL-1α and IL-1β, was added to the culture. This treatment did not alter the results whereas the same concentration of IL-1ra suppressed the IL-6 production by RSF that were stimulated with 10 pg/ml IL-1β (data not shown).

**p21**<sup>Cip1</sup> suppresses inflammatory chemokine expression by RSF

The p21<sup>Cip1</sup>-induced reduction of the MCP-1 mRNA expression by unstimulated RSF was elucidated by Northern blot analyses (Fig. 2A). This was reflected in the reduced MCP-1 concentration in the culture supernatants of the p21<sup>Cip1</sup>-expressing RSF. As was the case in the IL-6 expression, addition of IL-1ra did not alter the results (Fig. 2B).

ELISA of MCP-1 in the culture supernatants of RSF stimulated with IL-1β and TNF-α validated the stimulatory effects of these cytokines, and also suppression by p21<sup>Cip1</sup> (Fig. 2C). The effect of IL-1β was significantly suppressed by the p21<sup>Cip1</sup> expression, while that of TNF-α was unchanged (Fig. 2C). These results confirmed the biological significance of the IL-1RI1 down-regulation. Furthermore, LPS-stimulated RSF to increase MCP-1 production. This was suppressed by p21<sup>Cip1</sup>. Addition of IL-1ra did not attenuate the LPS-induced production of MCP-1. Thus, the suppression in this setting was also independent of IL-1 (Fig. 2C).

In accordance with the results of the preliminary DNA array analysis, MIP-3α or IL-8 protein levels were suppressed by p21<sup>Cip1</sup> in the culture supernatants of RSF only when they were stimulated with IL-1β and TNF-α. The effect of IL-1β was significantly reduced by the p21<sup>Cip1</sup> expression, while that of TNF-α was unchanged. LPS also stimulated MIP-3α production. This IL-1-independent effect was partially inhibited by p21<sup>Cip1</sup>. Similarly, the production of IL-8 was increased both by IL-1β and TNF-α. The effect of IL-1β but not that of TNF-α was inhibited by p21<sup>Cip1</sup>. LPS exerted a stimulatory effect on IL-8 production comparable to that of TNF-α, which was inhibited significantly by p21<sup>Cip1</sup>.

**p21**<sup>Cip1</sup> suppresses expression of tissue-degrading proteases

Northern blot analyses confirmed that p21<sup>Cip1</sup> suppresses expression of cathepsins B and K in the unstimulated RSF, and that of MMP-1 and -3 in the stimulated RSF (Fig. 3, A–D).

These changes were reproduced when the concentrations of MMP-1 and -3 in the culture supernatants were determined (Fig. 3, E and F). Neither MMP was detected in the supernatants of the unstimulated RSF. MMP-1 production was increased by IL-1β and TNF-α. The combination of these two cytokines had a synergistic effect. The effects of IL-1 were significantly suppressed by p21<sup>Cip1</sup>. As was the case in the cytokine production, LPS increased production of MMP-1. This effect was partially suppressed by p21<sup>Cip1</sup>. The production of MMP-3 was increased by IL-1β and LPS. TNF-α alone had no apparent effect but showed a synergistic effect with IL-1β. Again, the effect of IL-1β was suppressed by p21<sup>Cip1</sup>, and the effect of LPS was abrogated completely by p21<sup>Cip1</sup>.

**FIGURE 1.** Suppression of inflammatory cytokine and cytokine receptor expression by p21<sup>Cip1</sup>. A, RNA from the p21<sup>Cip1</sup>-adenovirus-infected (p21) and control adenovirus-infected RSF (control) were examined for IL-1RI1 and GAPDH mRNA expression by Northern blot analysis. Representative blots of one of three samples are shown in the upper panel. Signal intensities of IL-1RI1 were normalized with those of GAPDH (relative mRNA levels), and are shown in the lower panel. The columns and bars represent the mean and SD of three samples. Mean reduction by p21<sup>Cip1</sup> was 48.2%. Statistical evaluation was conducted by paired Student’s t-test. *, p < 0.05. B, The cells from the same donor were examined for IL-1RI1 and CDK4 protein expression by Western blot analysis. Representative blots of one of three samples are shown in the upper panel. Signal intensities of IL-1RI1 protein were normalized with those of constitutively expressed CDK4 (relative protein levels), and are shown in the lower panel. Mean reduction of expression by p21<sup>Cip1</sup> was 67.6%. **, p < 0.01. C, RSF infected with the p21<sup>Cip1</sup> adenoviruses (open columns), and infected with the control adenoviruses (solid columns) were cultured without stimulation (–) or stimulated with IL-1β (IL-1), TNF-α (TNF), or combination of IL-1β and TNF-α (IL-1+TNF) for 24 h. RSF were stimulated with LPS for 24 h in a separate set of experiments, where some RSF were treated with IL-1ra after the infection with the control adenoviruses (hatched column) or with the p21<sup>Cip1</sup> adenoviruses (dotted column). IL-6 in the culture supernatants was measured by ELISA. Rep- resentative data of three independent experiments are shown. Columns and bars show the mean and SD of triplicate cultures. Mean reduction of expression by p21<sup>Cip1</sup> in IL-1, IL-1+TNF, LPS, and LPS+IL-1ra were 77.4, 64.3, 69.4, and 64.3%, respectively. *, p < 0.05 and ***, p < 0.005. D, RSF infected with the p21<sup>Cip1</sup> adenoviruses (open columns), and infected with the control adenoviruses (solid columns) and noninfected RSF (gray column) were cultured without stimulation (–) or stimulated with IL-1β (IL-1) or TNF-α (TNF) for 24 h. IL-6 in the culture supernatants was measured by ELISA. Representative data of two experiments are shown. Columns and bars show the mean and SD of triplicate cultures. No statistical differences were found between IL-6 production by control-virus infected RSF and that by non-infected RSF. E, Expression of TLR4 and CDK4 proteins was analyzed by Western blot analysis. Representative blots of one of three samples are shown. The relative protein levels of TLR4 to CDK4 are shown as columns and bars. TLR4 protein level was not suppressed by p21<sup>Cip1</sup>.
p21<sup>Cip1</sup> inhibits DNA binding activity of AP-1 and NF-κB

p21<sup>Cip1</sup> down-regulated expression of MCP-1 and cathepsins B and K in the unstimulated RSF. Although it did not suppress expression of TLR4, it suppressed the LPS-dependent up-regulation of many inflammatory mediators. This has led us to assume that p21<sup>Cip1</sup> should directly inhibit nonreceptor, intracellular molecules. Since promoter activity of the inflammatory mediator genes that were suppressed by p21<sup>Cip1</sup> is controlled mostly by NF-κB and AP-1 transcription factors, we investigated the DNA binding activities of these factors in the unstimulated and LPS-stimulated RSF. Multicell colorimetric assays to quantify DNA binding activity of the transcription factors showed that activity of AP-1 was down-regulated by p21<sup>Cip1</sup> in the unstimulated RSF. Stimulation
with LPS. Immunoblotting of the precipitants with anti-

**FIGURE 4.** Suppression of AP-1 and NF-κB transcription factors by p21Cip1. RSF infected with the p21Cip1 adenoviruses (p21) or control adenoviruses (control) were cultured with or without LPS stimulation. The DNA binding activities of AP-1 and NF-κB in the adenoviruses-infected RSF relative to those of the uninfected RSF without stimulation are shown (A). Columns and bars show the mean and SD of triplicate cultures. p21Cip1 expressed adenovirus expressing activity of AP-1, but did not suppress that of NF-κB. Mean reduction in expression of AP-1 was 47.3%. In the LPS-stimulated RSF, p21Cip1 suppressed DNA binding activities of AP-1, NF-κB, and p65. Mean reduction in expression of AP-1, NF-κB, and p53 by p21Cip1 was 47.4, 52.6, and 41.3%, respectively, α, p < 0.05. p21Cip1 in the p21Cip1-expressing RSF stimulated with LPS (p21) was coimmunoprecipitated with anti-JNK Ab (α-JNK), but not with control IgG (IgG). No precipitation was found when RSF were infected with the control viruses (control) (B). Phosphorylation of c-Jun (p-c-Jun) was suppressed in RSF infected with the p21Cip1 viruses (p21) but not in RSF infected with the control viruses (control). Reduction in expression of relative p-c-Jun levels to whole c-Jun (pan-c-Jun) was 57% (C). The results are representatives of two independent experiments.

Discussion

Since the primary function of CDKIs is the inhibition of kinase activity of CDKs, the anticipated effect of the p21Cip1 expression in RSF was suppression of cell cycle progression. Indeed, RSF infected with the AxCAp21 viruses did not respond in vitro to proliferative stimuli by proinflammatory cytokines or by growth factors (4). In vivo transfer of the p21Cip1 gene into the arthritic joints of RA model rats suppressed synovial hyperplasia and cell cycle progression of the synovial fibroblasts (5).

However, the present study has revealed that p21Cip1 exerts multiple auxiliary effects: down-regulation of cytokine, chemo-

**FIGURE 5.** Multiple effects of p21Cip1 on RSF. p21Cip1 inhibited kinase activity of cyclin/CDK complexes, and down-regulated IL-1R1 expression and DNA binding activities of NF-κB and AP-1. These effects were mediated at least by binding of p21Cip1 to cyclin/CDK complexes and JNK, and resulted in inhibition of proliferation and in suppression of IL-6, -8, MCP-1, MIP-3α, cathepsins B and K, and MMP-1 and -3 expression.
(24). Cathepsin B might contribute to rheumatoid joint damage by degrading collagen (25, 26, 27). Cathepsin K is not only expressed by osteoclasts, but also by synovial fibroblasts, contributing to bone destruction in the rheumatoid joints (28, 29). Down-regulation of these proteinases in the p21Cip1-expressing RSF was consistent with the remarkable inhibition of bone and cartilage degeneration observed in the p21Cip1 gene therapy.

Expression of IL-1R1 was suppressed by p21Cip1. IL-1 is one of the critical cytokines in the rheumatoid inflammation. It enhances migration of inflammatory cells into the synovial tissues and stimulates production of cytokines, chemokines and MMPs (15). Its blockade by an antagonist ameliorates RA (30–32). We saw that IL-1α promotion of IL-6, IL-8, MCP-1, MIP-3α, and MMP-1 and -3 release from RSF was significantly suppressed by p21Cip1. These results argue that down-regulation of IL-1R1 must be functionally relevant to the therapeutic effects.

MCP-1 and cathepsin B and K expression was suppressed even when RSF were not stimulated. Conventional ELISA detected no IL-1 in the culture supernatant of the unstimulated RSF. The blockade of IL-1 with IL-1ra did not affect the results. Thus, the suppression observed in the unstimulated RSF was not mediated by the down-regulation of IL-1R1. LPS also up-regulated MCP-1 expression, and induced expression of IL-6, IL-8, MIP-3α, and MMP-1 and -3. This was not accompanied by reduced expression of TLR4, which is a signaling receptor for LPS. The LPS-stimulated RSF under these conditions did not release a detectable level of IL-1 into the culture supernatants, and IL-1 blockade by IL-1ra ameliorates RA (30–32). Thus, interaction of p21Cip1 with mitogen-activated protein kinases including JNK could activate NF-κB.

p21Cip1 also suppressed expression of cathepsin B (45, 46). Thus, interaction of p21Cip1 with metion-activated protein kinase might account for the reduced activity of AP-1 and NF-κB.

Chang et al. (47) used the DNA array technique to study effects of p21Cip1 on gene expression in HT1080 human sarcoma cell line; they observed that genes related to senescence or age-related diseases were induced. We have shown here that p21Cip1 expression modulates the expression of genes related to inflammation. Although, Chang et al. found up-regulation of the cathepsin B gene in HT1080 cells, the same gene was down-regulated in RSF. It is probable that the effects of p21Cip1 depend on the cell types.

In conclusions, p21Cip1 gene transfer to the RSF regulated expression of various genes. Its effects include down-regulation of cytokine, chemokine, cytokine receptor and proteinase expression. Down-regulation of IL-1R1, as well as inactivation of intracellular signaling pathways appeared to account for these effects. These collateral effects observed in the p21Cip1 gene transfer suggest new links between CDKIs and immunological effector molecules.

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