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

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Forced expression of a cyclin-dependent kinase inhibitor gene, p21Cip1, 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 p21Cip1 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 p21Cip1 gene transferred. We have found that p21Cip1 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 chemotactrant protein-1, macrophage inflammatory protein-3α, cathepsins B and K, and matrix metalloproteinases-1 and -3. Down-regulation of IL-1R1 by p21Cip1 resulted in attenuated responsiveness to IL-1. Inhibition of the inflammatory gene expression by p21Cip1 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 p21Cip1, 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. The Journal of Immunology, 2003, 171: 4913–4919.

Synovial 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) 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 p16INK4a and p21Cip1 are not expressed in vivo in the rheumatoid synovial tissues, but readily induced in vitro in cultured rheumatoid synovial fibroblasts (RSF). Induction of p16INK4a is characteristic of RSF (3). In vitro inducibility of p16INK4a and p21Cip1 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 p16INK4a or p21Cip1 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, p21Cip1 binds to various molecules related to gene expression and exerts differential effects on different cells (7). However, little is known about the effects of p21Cip1 gene expression in the inflamed tissues. We show here that up-regulated expression of the p21Cip1 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^{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 (8). 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 (3). They were used at passages 3–11. RSF were infected with AxCAp21 adenovirus, containing a human p21^{Cip1} gene (5, 9), or AxI1w1 adenovirus (Riken Gene Bank, Saitama, Japan), which lacks insert genes, at 50 multiplicity of infection. Some RSF were stimulated by 5 ng/ml TNF-α (Genzyme, Cambridge, MA), 5 ng/ml IL-1β (PeproTech, Rocky Hill, NJ), and 25 μM 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. RNasea 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 × 10^6 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-1β together with 25 μM indomethacin, 5 ng/ml TNF-α, a combination of IL-1β, indomethacin, and TNF-α, or 5 μg/ml LPS of Escherichia coli O55:B5 (Sigma-Aldrich). One hundred ng/ml IL-1α antagonist (IL-1ra) (R&D Systems, Mckinley, MN), which was sufficient for the inhibition of 10 pg/ml IL-1β, 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 (3). 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 μg/ml LPS, nuclear extracts and cell lysates were prepared using Nuclear Extract Kit (Active Motif, Carlsbad, CA) or SAPJKN Assay kit (Cell Signaling, Beverly, MA). The effects of the p21^{Cip1} gene were studied at three days after the adenoviral infection.

Northern blot analyses

Northern blotting was conducted as described elsewhere (10). Human monocyte chemotactic 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-1R1), cathepsins B and K, and matrix metalloproteinases (MMP)-1 and -3 cDNAs (12), human MMP-3-specific primers (12), human MMP-3-specific primers (12), human cathepsin B-specific primers (5'-TAG GAT CTG GCT TCC AAC AT-3′) (sense) and 5'-CCA CGG CAG ATT AGA TCT TT-3′ (antisense) and human cathepsin K-specific primers (5'-AAC GAA GCC AGA CAA CAG ATT TCC-3′) (sense), 5'-GAT TGG GCT GGC TGT AGT CAC A-3′ (antisense). Annaling temperatures were 58°C for IL-1R1, and cathepsins B and K primers, and 62°C for MMP-1 and -3 cDNA. The products were purified and labeled with [α-32P]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 MacBas 2.5.2 software (Fuji Film, Kanagawa, Japan).

Western blot analyses and immunoprecipitation

Rabbit anti-human IL-1R1 Abs, rabbit anti-human Toll-like receptor (TLR)-4 Abs, and rabbit anti-human p21^{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 MacBas Image software. 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 MacBas Image software. Western blot analysis confirmed that Toll-like receptor (TLR)4, which is a receptor for LPS, was not down-regulated by p21^{Cip1} (Fig. 1E). Nevertheless, the p21^{Cip1} expression suppressed the IL-6 production.
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-1R1 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-1R1 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-1R1 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-1R1 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 adenoviruses (dotted column). IL-6 in the culture supernatants was measured by ELISA. Representative 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 noninfected 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>.

**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-1R1 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-1R1 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-1R1 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-1R1 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 adenoviruses (dotted column). IL-6 in the culture supernatants was measured by ELISA. Representative 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 noninfected 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> 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-1R1 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 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>.
p21<sup>Crip1</sup> inhibits DNA binding activity of AP-1 and NF-κB

p21<sup>Crip1</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>Crip1</sup> should directly inhibit nonreceptor, intracellular molecules. Since promoter activity of the inflammatory mediator genes that were suppressed by p21<sup>Crip1</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. Multwell colorimetric assays to quantify DNA binding activity of the transcription factors showed that activity of AP-1 was down-regulated by p21<sup>Crip1</sup> in the unstimulated RSF. Stimulation

**FIGURE 2.** Suppression of inflammatory chemokine expression by p21<sup>Crip1</sup>. A, The AxCAp21 adenovirus-infected (p21) and control adenovirus-infected (control) RSF from three RA patients were examined for MCP-1 mRNA expression by Northern blot analysis. RSF were not stimulated with cytokines. Representative results of one of three samples are shown in the upper panel. Signal intensities of MCP-1 messages (relative mRNA levels) are shown as columns and bars, representing the mean and SD. Mean reduction of expression by p21<sup>Crip1</sup> was 57.0%. *, p < 0.05. B, RSF with or without p21<sup>Crip1</sup> expression (p21 and control) were cultured without cytokine stimulation. The culture medium was supplemented with FBS alone (solid columns) or with FBS plus IL-1ra (hatched columns). MCP-1 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. Mean reduction of expression of MCP-1 by p21<sup>Crip1</sup> was 83.8% (no IL-1ra) and 87.1% (IL-1ra). **, p < 0.01 and ***, p < 0.005. C, E, RSF infected with the p21<sup>Crip1</sup> adenoviruses (open columns) or control adenoviruses (solid columns) were cultured without stimulation (−) or stimulated with IL-1β (IL-1), TNF-α (TNF), a combination of IL-1β and TNF-α (IL-1+TNF), or LPS for 24 h. Some RSF infected with the control adenoviruses (hatched column), and with the p21 adenoviruses (dotted column) were treated with IL-1ra before LPS stimulation. MCP-1 (C), MIP-3α (D), and IL-8 (E) in the culture supernatants were measured by ELISA. Representative data of two or three experiments are shown. Columns and bars show the mean and SD of triplicate cultures. n. d., not detectable. Mean reduction in expression of MCP-1 by p21<sup>Crip1</sup> in IL-1, IL-1+TNF, LPS, and LPS + IL-1ra was 58.3, 57.5%, 84.7, and 83.9%, respectively. Mean reduction in expression of IL-8 by p21<sup>Crip1</sup> in IL-1, IL-1+TNF, and LPS was 48.4, 38.2, and 54.0% and that of MIP-3α was 66.6, 68.7, and 38.7%. **, p < 0.05 and ***, p < 0.005.

**FIGURE 3.** Suppression of tissue-degrading proteinase expression by p21<sup>Crip1</sup>. A and B, RSF infected with the AxCAp21 adenoviruses (p21) or control adenoviruses (control) were cultured without cytokine stimulation, and examined for cathepsins B (A) and K (B) mRNA expression by Northern blot analysis. Representative blots are shown in the upper panels. Northern blots of cathepsin B showed dual bands representing two transcripts 4.0 and 2.2 kb long (48). The relative levels of mRNA to those of GAPDH mRNA are shown as columns and bars, representing the mean and SD of three samples derived from different patients. Mean reduction in expression of cathepsins B and K by p21<sup>Crip1</sup> was 47.8 and 75.0%, respectively, **, p < 0.01 and ***, p < 0.005. C and D, RSF stimulated with IL-1β and TNF-α were examined for MMP-1 (C) and -3 (D) mRNA expression by Northern blot analysis. Representative blots of one of three samples are shown in the upper panels. The relative levels of mRNA of MMP-1 and -3 are shown as columns and bars, representing the mean and SD of three samples. Mean reduction in expression of MMP-1 and -3 by p21<sup>Crip1</sup> was 78.6 and 82.6%, respectively, **, p < 0.01 and ***, p < 0.005. E and F, RSF infected with the p21<sup>Crip1</sup> adenoviruses (open columns), and with the control adenoviruses (solid columns) were cultured without stimulation (−) or stimulated with IL-1β (IL-1), TNF-α (TNF), a combination of IL-1β and TNF-α (IL-1+TNF), or LPS for 24 h. MMP-1 and -3 in the culture supernatants were measured by ELISA. Representative data of two experiments are shown. Columns and bars show the mean and SD of triplicate cultures. Unstimulated RSF produced no detectable MMP-1 or -3. Mean reduction in expression of MMP-1 by p21<sup>Crip1</sup> in IL-1, IL-1+TNF, LPS, and LPS + IL-1ra was 70.9, 65.3, and 29.5%, respectively, and that of MMP-3 was 92.6, 71.5, and 88.3%, respectively. **, p < 0.01 and ***, p < 0.005.
with LPS. Immunoblotting of the precipitants with anti-joints of RA model rats suppressed synovial hyperplasia and cell proliferation stimuli by proinflammatory cytokines or by growth infected with the AxCAp21 viruses did not respond in vitro to two independent experiments. The results are representatives of control viruses (control). Reduction in expression of relative p-c-Jun levels was 47.4, 52.6, and 41.3%, respectively. *, p < 0.05, p21Cip1 in the absence of LPS (p21) was down-regulated by p21Cip1 in the adenoviruses-infected cells, which results in reduction of the JNK enzymatic activity that indeed associated with JNK (Fig. 4B). Phosphorylation of c-Jun substrates showed that kinase activity of JNK was suppressed in the p21Cip1-expressing RSF stimulated with LPS (p21) was communoprecipitated 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-
MCP-1 and cathepsin B 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, MMP-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.

The IL-1R-independent suppression was accompanied by reduced activity of NF-κB and AP-1. In the rheumatoid synovial tissues these factors activate transcription of various inflammatory cytokines, chemokines, and proteins including those analyzed in the present studies (17, 24, 34–42). In agreement with our observation, constitutive expression of MCP-1 by mesangial cells required activation of AP-1 (43). Notably, the promoter of the promoter of the IL-6 gene has two AP-1-like binding sites (44). This suggests that the repressed activity of AP-1 might contribute to the down-regulation of IL-1R1.

Depending on the cell type, p21Cip1 binds to a variety of intracellular proteins other than CDKs. These include signal transduction molecules and transcription factors (7). We have shown that p21Cip1 expression regulates NF-κB and AP-1. This is important in the control of inflammatory responses.

In conclusion, 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 collaborative effects observed in the p21Cip1 gene transfer suggest new links between CDKIs and immunological effector molecules.

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