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*J Immunol* 2003; 171:4913-4919; doi: 10.4049/jimmunol.171.9.4913

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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, p21<sup>Cip1</sup>, 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<sup>Cip1</sup> 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<sup>Cip1</sup> gene transferred. We have found that p21<sup>Cip1</sup> 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, IL-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<sup>Cip1</sup> resulted in attenuated responsiveness to IL-1. Inhibition of the inflammatory gene expression by p21<sup>Cip1</sup> 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<sup>Cip1</sup> expression, 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 cell-cyclingdependent kinase inhibitor gene and inflammatory molecules.

molecular events should contribute to the therapeutic effects of p21Cip1 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 (To-kyo, 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 (9). Human cyclooxygenase-2 (COX-2) cDNA was generated with RT-PCR using cDNA derived from RSF. PCR was conducted with Oligo (antisense) primers (5′-CCG TAC ACC TTC CTA TCT TCG-3′ (sense) and 5′-CCA CCG CAG ATT AGA TCT TT-3′ (antisense)). Annealing temperatures were 58°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, rabbit anti-human p21Cip1 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-1β, IL-6, IL-8, MCP-1, TNF-α (BioSource International, Camarillo, CA), IL-1α, macrophage inflammatory protein (MIP)-3α (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.

Multimwell colorimetric transcription factor assays and JNK kinase assay

Using Trans AM AP-1/c-Jun, NF-κBp50, and p65 Transcription Factor Assay Kits (Active Motif), the nuclear extracts of RSF were examined for DNA binding activities of AP-1 and NF-κB (14). SAPJKNJNK Assay Kit (Cell Signaling) was used to examine whole cell lysates for their JNK kinase activities to phosphorylate c-Jun substrates. The amounts 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

p21Cip1 suppresses IL-1R1 and IL-6 expression by RSF

RSF samples derived from rheumatoid joints were cultured in vitro. Expression of p21Cip1 was not detected in any of the samples. They were infected with the AxCap21 adenoviruses or the Ax1w1 adenoviruses. At three days postinfecion, when the AxCap21-infected RSF express p21Cip1 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 p21Cip1 gene transfer. Indeed, Northern blot analysis revealed that the IL-1R1 mRNA expression was significantly reduced in RSF overexpressing p21Cip1, 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 p21Cip1 (Fig. 1B).

The DNA array analyses suggested no differential expression of IL-6 in the unstimulated RSF. However, when RSF were stimulated by TNF-α and IL-1β before the DNA array analysis, IL-6, as well as IL-8, MCP-1, MIP-3α, MMP-1, MMP-3, and cathepsin K genes showed a tendency to be down regulated by the p21Cip1 gene transfer. Indeed, Northern blot analysis revealed that the IL-1R1 mRNA expression was significantly reduced in RSF overexpressing p21Cip1, compared with those infected with the control adenoviruses (Fig. 1A).

Address this issue, RSF were stimulated independently with IL-1β, TNF-α, 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-1β were suppressed significantly by p21Cip1 while the effects of TNF-α were not attenuated (Fig. 1C). Effects of the adoviral 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 p21Cip1 (Fig. 1E). Nevertheless, the p21Cip1 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-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 proteinases**

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 MIP-1 and -3 in the culture supernatants were determined (Fig. 3, E and F). Neither MIP 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>.

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>.
**FIGURE 2.** Suppression of inflammatory chemokine expression by p21<sup>Cip1</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 normalized with those of GAPDH messages (relative mRNA levels) are shown as columns and bars, representing the mean and SD. Mean reduction of expression by p21<sup>Cip1</sup> was 57.0%. *, p < 0.05. B. RSF with or without p21<sup>Cip1</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 by p21<sup>Cip1</sup> was 83.8% (no IL-1ra) and 87.1% (IL-1ra). ***, p < 0.005 and ***, p < 0.005. C-F, RSF infected with the p21<sup>Cip1</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>Cip1</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>Cip1</sup> in IL-1, IL-1+TNF, and LPS was 48.4, 38.2, and 54.6% 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>Cip1</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 (4B). 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>Cip1</sup> was 47.8 and 75.0%, respectively, ***, p < 0.005 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>Cip1</sup> was 78.6 and 82.6%, respectively, ***, p < 0.005 and ***, p < 0.005. E and F, RSF infected with the p21<sup>Cip1</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>Cip1</sup> in IL-1, IL-1+TNF, LPS, and LPS + IL-1ra was 92.6, 71.5, and 88.3%, respectively, and that of MMP-3 was 92.6, 71.5, and 88.3%, respectively, ***, p < 0.005; ***, p < 0.001; and ***, p < 0.005.

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. Multwell 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-
cycle progression of the synovial
joints of RA model rats suppressed synovial hyperplasia and cell
expression of the AP-1 activity was 47.3%. In the LPS-stimulated RSF,
p21
expression of the AP-1 activity was 47.4, 52.6, and 41.3%, respectively. * p<0.05. p21
expression was found when RSF were infected with the control viruses
infected with the control viruses (p21) but not in RSF infected with the
fibroblasts, were targeted by the intraarticular adenoviral gene transfer (15, 17). Alternatively, the down-regulation
and DNA binding activities of AP-1, NF-
expression has a wide array of
with LPS up-regulated activities of NF-κB p50, p65, and AP-1, all of which were down-regulated by p21
It was shown that p21
associates with JNK in other type of
cells, which results in reduction of the JNK enzymatic activity that
activates AP-1 (16). Using anti-JNK Ab, we immunoprecipitated
JNK in cell lysates of p21
expressing RSF that were stimulated with LPS. Immunoblotting of the precipitants with anti-
p21
Abs revealed that p21
indeed associated with JNK (Fig. 4B). Phosphorylation of c-Jun substrates showed that kinase activity of JNK was suppressed in the p21
expressing RSF (Fig. 4C). Thus, the IL-1R1-independent suppression should be at least partly due to down-regulation of these pathways.

**Discussion**

Since the primary function of CDKIs is the inhibition of kinase activity of CDKs, the anticipated effect of the p21
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 p21
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 p21
exerts multiple auxiliary effects: down-regulation of cytokine, chemo-
kine, cytokine receptor, and proteinase activity critically involved in the pathology of RA. We found previously that expression of proinflammatory cytokines such as IL-1, IL-6, and TNF-α was unexpectedly inhibited in vivo in the synovial tissues treated with p21
gene transfer (4). The present report provides molecular evidence showing that p21
expression has a wide array of anti-inflammatory and bone-protective effects. Down-regulation of IL-1R1, and also IL-1R-independent inactivation of intracellular signaling pathways appeared to account for these effects (Fig. 5). Finally, these effects suggest that the p21
gene transfer might ameliorate types of inflammatory arthritides other than RA.

The down-regulation of IL-6 observed in vivo was actually seen in vitro in p21
expressing RSF while the expression of IL-1 or TNF-α were not significantly modulated. It is possible that the decreased expression of IL-1 and TNF-α in the synovial tissues was due to their down-regulation in the synovial macrophages. The macrophages are the primary source of these cytokines and, together with synovial fibroblasts, were targeted by the intraarticular adenoviral gene transfer (15, 17). Alternatively, the down-regulation of IL-1 and TNF-α might result from en bloc suppression of the inflammatory cytokine/chemokine network, multiple members of which were suppressed by p21
.

IL-6, in the rheumatoid synovial tissues, derives from the activated synovial macrophages and fibroblasts, and stimulates local osteoclasts to resorb the bone matrices in the affected joints. It also stimulates T and B lymphocytes. This has made this cytokine the target of a new biological reagent that is currently in clinical trials (18, 19). IL-8 produced by the activated synovial cells contributes to recruitment of neutrophils and T lymphocytes and to neovascularization in the rheumatoid tissues (20). The other chemokines, MCP-1 and MIP-3α, both evoke migration and activation of lymphocytes and macrophages in the rheumatoid synovial tissues (21, 22). Blockage of MCP-1 receptor was effective in treating an animal model of RA (23). Thus, the cytokines and chemokines down-regulated by p21
all play crucial roles in the rheumatoid inflammation.

Tissue degrading enzymes, such as MMPs and cathepsins, are abundantly expressed in rheumatoid synovial tissues. MMP-1 and -3 degrade collagen and proteoglycans that compose the matrices of bone and cartilage. In addition, it has been proposed that MMP-3 cleaves many proMMPs in the initiation of the proteinase cascade in rheumatoid joints (2). Treatment to inhibit MMP-1 production prevented bone destruction in adjuvant arthritis of rats

**FIGURE 4.** Suppression of AP-1 and NF-κB transcription factors by p21
RSF infected with the p21
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. p21
suppressed DNA binding activity of AP-1, but did not suppress that of NF-κBp50 or p65 significantly in the unstimulated RSF. Mean reduction in expression of AP-1 activity was 47.3%. In the LPS-stimulated RSF, p21
suppressed DNA binding activities of AP-1, NF-κBp50, and p65. Mean reduction in expression of AP-1, NF-κBp50, and NF-κB p65 by p21
was 47.4, 52.6, and 41.3%, respectively. * p<0.05. p21
in the p21
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 p21
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.

**FIGURE 5.** Multiple effects of p21
on RSF. p21
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 p21
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.
multiple effects of p21<sup>Cip1</sup> gene therapy

(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 p21<sup>Cip1</sup>-expressing RSF was consistent with the remarkable inhibition of bone and cartilage degeneration observed in the p21<sup>Cip1</sup> gene therapy.

Expression of IL-1R1 was suppressed by p21<sup>Cip1</sup>. 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-1R1, IL-1α and -1β release from RSF was significantly suppressed by p21<sup>Cip1</sup>. 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 down-regulation of IL-1R1 was repressed activity of AP-1 (43). Notably, the promoter of the IL-8 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, p21<sup>Cip1</sup> binds to a variety of intracellular proteinases other than CDKs. These include signal transduction molecules and transcription factors (7). We have shown that p21<sup>Cip1</sup> blocks the activation of AP-1 (43). Notably, the promoter of the IL-1R1 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.

In conclusions, p21<sup>Cip1</sup> 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 p21<sup>Cip1</sup> gene transfer suggest new links between CDKIs and immunological effector molecules.

Acknowledgments

We thank Drs. T. Muneta, Y. Kuga, and J. Hasegawa for providing synovial samples; Drs. N. Terada and M. Ikeda for providing adenoviruses; Drs. R. Koike, H. Hagiya, T. Nanki, and H. Nishioka for their technical support and advice; and also Dr. N. Nishimura at Genetic Laboratory (Sapporo, Japan) performing DNA array experiments. We are grateful to Dr. T. Page for reviewing the manuscript.

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