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IL-6 and Matrix Metalloproteinase-1 Are Regulated by the Cyclin-Dependent Kinase Inhibitor p21 in Synovial Fibroblasts

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During the pathogenesis of rheumatoid arthritis (RA), the synovial fibroblasts increase in number and produce proinflammatory cytokines and matrix metalloproteinases (MMPs) that function to promote inflammation and joint destruction. Recent investigations have suggested that cell cycle activity and inflammation may be linked. However, little is known about the mechanisms responsible for the coordinate regulation of proliferation and the expression of proinflammatory molecules in RA synovial fibroblasts. Here, we demonstrate a 50 ± 10% decrease in the expression of p21, a cell cycle inhibitor, in the synovial fibroblast population from RA compared with osteoarthritic (OA) synovial tissue. Moreover, p21 positivity in the synovial fibroblasts inversely correlates with medium synovial lining thickness (r = −0.76; p < 0.02). The expression of p21 is also reduced in isolated RA synovial fibroblasts compared with OA synovial fibroblasts. Adenovirus-mediated delivery of p21 (Ad-p21) arrests both RA and OA synovial fibroblasts in the G0/G1 phase of the cell cycle without inducing cytotoxicity. However, the spontaneous production of IL-6 and MMP-1 is suppressed only in the Ad-p21-infected RA synovial fibroblasts, indicating a novel role for p21 in RA. Analyses of p21-deficient mouse synovial fibroblasts reveal a 100-fold increase in IL-6 protein and enhance IL-6 and MMP-3 mRNA. Restoration of p21, but not overexpression of Rb, which also induces G0/G1 cell cycle arrest, decreases IL-6 synthesis in p21-null synovial fibroblasts. Furthermore, in RA synovial fibroblasts the ectopic expression of p21 reduces activation of the AP-1 transcription factor. Additionally, p21-null synovial fibroblasts display enhanced activation of AP-1 compared with wild-type synovial fibroblasts. These data suggest that alterations in p21 expression may activate AP-1 leading to enhanced proinflammatory cytokine and MMP production and development of autoimmune disease.

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heumatoid arthritis (RA) is an autoimmune disease characterized by infiltration of lymphocytes and macrophages, and hyperplasia of the synovial lining. In RA, synovial fibroblasts markedly increase in number, display a transformed phenotype, and invade and destroy adjacent cartilage (1). In contrast, osteoarthritis (OA) synovial fibroblasts do not hyperproliferate or invade articular cartilage in vivo (2). Furthermore, RA synovial fibroblasts spontaneously secrete numerous cytokines and matrix metalloproteinases (MMPs) including IL-6, IL-8, MMP-1, and MMP-3, which may be regulated by NF-κB and/or AP-1 (3–7). In culture, IFN-γ inhibits TNF-α-induced RA synovial fibroblast cell cycle activity and MMP-1 production (8), and IL-4 suppresses proliferation and MMP-1 and 3 mRNA expression (9), suggesting that RA synovial fibroblast proliferation and secretion of proinflammatory mediators may be linked.

The mammalian cell cycle is divided into four stages, a growth phase (G1/G0), a synthesis phase (S), a second growth phase (G2), and mitosis (M). Progression through the different phases of the cell cycle is dependent on the activities of cyclin-dependent kinases (cdks) and their binding partners, cyclins. In G1/G0, hypophosphorylated retinoblastoma (Rb) is bound to the E2F transcription factor, thereby sequestering E2F and repressing its transcriptional activity (10, 11). Once Rb is phosphorylated, E2F is released to activate transcription of genes required for DNA synthesis during S phase (12). Recently, a new class of cell cycle regulatory proteins has been isolated, the cdk inhibitors. These inhibitors bind to cdk or cdk-cyclin complexes and inhibit kinase activity. The cdk inhibitors are grouped into two categories based on function and homology, although overexpression of any of the cdk inhibitors will induce G1 cell cycle arrest (13). The INK4 (inhibitors of cdk4) family of cdk inhibitors, including p15, p16, p18, and p19, bind to and inhibit cdk4 and cdk6. In contrast, the Cip/Kip (cdk2-interacting protein) family of cdk inhibitors (p21, p27 and p57) exhibits a broad specificity for cdk4.

Abbreviations used in this paper: RA, rheumatoid arthritis; Ad-β-Gal, vector expressing β-galactosidase; Ad-Rb, adenovirus vector expressing Rb; cdk, cyclin-dependent kinase; JNK, c-Jun NH2-terminal kinase; MMP, matrix metalloproteinase; OA, osteoarthritis; Rb, retinoblastoma.
The role of the cyclin-dependent kinase inhibitors in RA remains to be fully evaluated. RA and OA synovial fibroblasts and dermal fibroblasts express higher levels of p21 (15) following serum starvation, which induces quiescence, compared with serum-stimulated cells. Moreover, overexpression of p21 or p16 by adenoviral-mediated delivery (Ad-p21) suppresses synovial fibroblast growth in vitro (15, 16). Furthermore, overexpression of p21 inhibits experimental arthritis development (16, 17). RT-PCR analysis of the Ad-p21-infected joints reveals a decrease in pro-inflammatory molecule expression, including IL-1β, TNF-α, and IL-6 mRNA (17). However, only a small percentage of cells in the joint are infected by replication-defective adenoviruses (18), suggesting that inhibiting synovial fibroblast proliferation may not account for the amelioration of experimental synovitis by Ad-p21.

Further, the observed reduction in the cytokine profile following Ad-p21 suggests that p21 may have additional functional activities that inhibit the progression of arthritis.

Here, we demonstrate a direct role for p21 in suppressing cytokine and MMP production. The expression of p21 is decreased in RA compared with OA synovial lining, and lower levels of p21 are observed in RA compared with OA synovial fibroblasts. Ectopic expression is accomplished by employing replication-defective adenovirus to express human p21. Ad-p21 inhibits S phase entry in RA and OA synovial fibroblasts, but only suppresses IL-6 and MMP-1 production in RA synovial fibroblasts. To confirm that p21 is essential for the regulation of IL-6, synovial fibroblasts isolated from p21-null mice demonstrate a 100-fold increase in IL-6 production compared with wild-type synovial fibroblasts. Restoration of p21, but not overexpression of Rb, decreases IL-6 production in p21-null synovial fibroblasts. Furthermore, AP-1, but not NF-κB, activation is diminished in Ad-p21-transduced RA synovial fibroblasts. Additionally AP-1 activation is markedly enhanced in p21-null synovial fibroblasts compared with wild-type cells, suggesting that p21 inhibits IL-6 and MMP-1 synthesis through inhibition of the AP-1 transcription factor. These data suggest a novel role for p21 in suppressing proinflammatory cytokine and MMP production, independent of cell cycle inhibition.

Materials and Methods

**Immunohistochemistry**

Synovial tissue for immunohistochemistry was obtained at the time of arthroplasty from patients with the diagnosis of RA and from with OA. All patients met the American College of Rheumatology classification criteria for RA and OA, respectively (19, 20). Sections (5 μm) from synovial tissue fixed in methyl Carnoy were deparaffinized and blocked in 10% goat serum. Sections were incubated with rabbit anti-p21 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG (Sigma-Aldrich, St. Louis, MO). A biotinylated donkey anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was followed by alkaline phosphatase (BioGenex Laboratories, San Ramon, CA) conjugated to streptavidin. For dual immunohistochemistry, sections were incubated with mouse anti-CD68 Ab (DakoCytomation, Glostrup, Denmark), followed by peroxidase (BioGenex) conjugated to streptavidin, was used to detect primary Ab complexes. Visualization was accomplished using the Vector blue alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA), and counterstained with phloxine. For dual immunohistochemistry, sections were incubated with mouse anti-CD68 Ab (DakoCytomation, Glostrup, Denmark), and rabbit anti-p21 Ab. The expression of p21 was detected as described above. To detect CD68 expression a biotinylated goat anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories) was followed by peroxidase (BioGenex) conjugated to streptavidin, was used to detect primary Ab complexes. Visualization was accomplished using the 3-amin-9-ethyl-carbazole substrate kit (BioGenex). There was no counterstain in the dual-stained tissue sections. Specimens were examined and photographed on a Nikon ES400 microscope (Garden City, NY) equipped for phase contrast visualization. A pathologist blinded to the study (G.K.H.) determined the number of p21-positive/CD68-negative cells in the synovial lining and the medium synovial lining thickness by analyzing multiple sections to avoid sampling error for each tissue as previously described (21–24). Fifty cells per area were analyzed for RA synovial tissue sections (n = 4), and 20 cells/area were examined for OA synovial tissue sections (n = 4).

**Cell culture**

OA and RA synovial tissue samples were obtained from patients undergoing total joint replacement who met the American College of Rheumatology criteria (19, 20). Normal human synovial fibroblasts were obtained from arthroscopic knee biopsies (25). Isolated human and mouse synovial tissues were digested with collagenase, dispase, and DNase I, and single-cell suspensions were obtained (5, 26, 27). Synoviocytes were used from passages 3–9 in these experiments. A homogenous population was determined by flow cytometry (<1% CD11b, <1% phagocytic, and <1% FcγRII receptor positive) (22, 25, 28, 29). Human RA, OA, and normal synovial fibroblasts were cultured in 10% FBS/DMEM. For infections, cells were plated in growth medium (10% FBS) and allowed to attach before being transferred to low serum medium. Cultures were serum-starved in 0.5% FBS/DMEM for 24–48 h before infection. Cells were then counted, and cultures were incubated with a vector expressing β-galactosidase (Ad-β-Gal) (30), Ad-p21 (30–32), or an adenovirus vector expressing Rb (Ad-Rb) (33) for 12 h in low serum medium. At the end of the infection period the virus was removed by washing with PBS and returned to low serum medium for an additional 12 h. The cultures were then stimulated for 48 h by the addition of medium containing 10% FBS/DMEM. Replication-defective adenovirus (Ad5) vectors were propagated in the 293 embryonic kidney cell line (American Type Culture Collection, Manassas, VA) and purified by ultracentrifugation through cesium chloride gradients.

Plaque assay or serial dilutions assays were employed to determine the titers of viral stocks (34).

**Western blot analysis**

Whole-cell extracts were prepared as previously described (30, 32) from uninfected and infected cultures. Extracts (25 μg) were analyzed by SDSPAGE on 12.5% polyacrylamide gels and transferred to Immobilon-P (Millipore Corp., Bedford, MA) by semidy blotting. Filters were blocked for 1 h at room temperature in PBS/0.2% Tween 20/0.2% nonfat dry milk. The filters were then incubated with mouse anti-tubulin (Calbiochem, La Jolla, CA) Ab or rabbit anti-p21 Ab (Santa Cruz Biotechnology) at a concentration of 0.25–0.4 μg/ml. All primary Abs were incubated overnight at 4°C in PBS/0.2% Tween 20/0.5% nonfat dry milk. Filters were washed in PBS/0.2% Tween 20/2% nonfat dry milk and incubated with donkey anti-rabbit or anti-mouse secondary Ab (1/2000 dilution) conjugated to HRP (Amersham Pharmacia Biotech, Piscataway, NJ). Visualization of the immunocomplex was conducted by enhanced chemiluminescence (ECL Plus, Amer sham Pharmacia Biotech).

**Flow cytometry**

For cycle analysis and determination of subdiploid DNA content, cultures were harvested by trypsinization, fixed in 70% ethanol overnight, and stained with propidium iodide (Roche, Indianapolis, IN) as previously described (32). A gate was established to count 10,000 events for each sample. Flow cytometry was conducted at the Robert H. Lurie, Comprehensive Cancer Center, Flow Cytometry Core Facility, of Northwestern University Medical School (Chicago, IL).

**EMSA**

32P-labeled oligonucleotides containing the AP-1 binding sequence from the MMP-1 promoter (35) was used as a probe. DNA binding reactions were performed by incubation for 20 min at room temperature in a final volume of 20 μl. The reaction mixture contained 100 mM/l of NaCl, 20 mM/l of HEPES, 1 mM/l of EDTA, 4% glycerol, 5% (w/v) Ficoll, 0.25 μg of BSA, 1 μg of poly(dI-dC), 1 ng of 32P-labeled oligonucleotide, and 5–10 μl of the nuclear extract. Protein-DNA complexes were separated from free probe by electrophoresis on 5% polyacrylamide gels in 0.5× Tris-borate-EDTA at 160 V for 2–3 h. Gels were dried onto 3M paper (Whatman, Maidstone, U.K.) and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). For supershift assays, 1–2 μl of a polyclonal Abs against c-Fos or c-Fos (Santa Cruz Biotechnology) was incubated with the nuclear extract on ice for 30 min before addition of the labeled oligonucleotide to the binding reaction.

**RT-PCR**

RNA was isolated by the RNAzol B method (Tel-Test, Friendswood, TX) as described by the manufacturer. One microgram of total RNA was incubated in reaction buffer containing oligo(dT) primer, avian myeloblastosis virus reverse transcriptase. RNase inhibitor (recombinant RNAsin ribonuclease inhibitor), and dNTP mixture for 1 h at 42°C. The reaction was

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stopped by incubation at 94°C for 5 min. Semiquantitative PCR was performed using primers for human IL-6 (forward, 5'-ATGACCTCTCTCCACAAACGC-3'; reverse, 5'-GAAGAGCCCTCAGGCTGGACTG-3') (6), MMP-1 (forward, 5'-ATTITCCTCCTTCTGAAAACTT-3'; reverse, 5'-ATGCACAGCTTTCTCCAATT-3'), and G3PDH (Clontech, Palo Alto, CA). Mouse primers were the following: IL-6 (forward, 5'-GACAAAGCCAGGTCCTCAGAG-3'; reverse, 5'-CTAGGGTTTCGGAGTAGATGCACAGCTTTCCTCCACTG-3') (36) and MMP-3 (forward, 5'-CTCACAACATAAGCTGAGCT-3'; reverse, 5'-TCCAGGTGCATAGGCATG-3') (37). Cycling conditions included one initial denaturation cycle for 5 min at 94°C; 28 cycles of amplification for 2 min at 72°C, 1 min at 94°C, and 1 min at 50°C; and a final extension phase consisting of one cycle of 10 min at 72°C.

**ELISA**

For human IL-6, pro-MMP-1, and mouse IL-6, sandwich ELISAs were performed according to the manufacturer’s instructions, employing commercially available kits (R&D Systems, Minneapolis, MN). The sensitivity for human IL-6 is 18.8 pg/ml, that for mouse IL-6 is 0.4 ng/ml, and that for mouse IL-6 is 31.3 pg/ml. IL-6 production ranged from 0.1–1,175 pg/ml in OA synovial fibroblasts, from 1,175–1,540 pg/ml in OA synovial fibroblasts, and from 590–1,321 pg/ml in normal synovial fibroblasts. The range of pro-MMP-1 production from 0.1–23.0 ng/ml in RA synovial fibroblasts, from 1.3–35 ng/ml in OA synovial fibroblasts, and from 0.4–13.0 ng/ml in normal synovial fibroblasts. The ODs were read by a Microplate VersaMax reader (Molecular Devices, Menlo Park, CA). All data were normalized by cell number.

**Statistics**

Results were expressed as the mean ± SE. Differences between groups were analyzed using unpaired two-tailed Student’s t test. Wilcoxon rank-sum tests were performed by a statistician (E.S.) on RA and non-RA synovial fibroblasts.

**Results**

Expression of p21 is reduced in RA

RA is an autoimmune disease characterized by increased proliferation of synovial fibroblasts leading to hyperplasia of the synovial lining. Cell cycle activity is suppressed by p21, and synovial fibroblasts exhibit increased proliferation in RA; therefore, we examined whether p21 expression is decreased in RA synovial tissue. Indeed, a decrease in the percentage of p21-positive cells (blue nuclei per total cell number) was detected in the RA (Fig. 1A) compared with OA synovial lining (Fig. 1, A and D). The synovial lining thickness was also greater in RA compared with OA synovial tissue. The decrease in p21 was specific to the RA synovial lining, since the endothelial and smooth muscle cells lining the blood vessel walls in both RA and OA synovial tissue were p21 positive (compare Fig. 1, B and E). These data suggest that the reduced percentage of p21-positive cells in RA synovial lining is unique and is not due to a general decrease in p21-positive cells in multiple other cell types. A 50 ± 10% reduction in the percentage of p21-positive (blue nuclei) per CD68-negative (clear cytoplasm) cells was observed in RA compared with OA synovial lining cells (compare Fig. 1, C and F). Furthermore, there was an inverse correlation between p21-positive synovial fibroblasts in the synovial lining and medium synovial lining thickness (r = −0.76; p < 0.02). Collectively, these data suggest that p21 expression is reduced in RA compared with OA synovial lining.

Similar to the RA synovial lining fibroblasts, cultured serum-starved (Q) RA-synovial fibroblasts exhibited decreased p21 expression compared with OA synovial fibroblasts (Fig. 2, compare lanes 1 and 3 to lane 5). The expression of p21 remained weaker in proliferating (S) RA compared with OA synovial fibroblasts (Fig. 2, compare lanes 2 and 4 to lane 6), similar to a previously published study (15). These data are consistent with those in Fig. 1.
FIGURE 3. Ad-p21 induces G0/G1 cycle arrest in RA synovial fibroblasts. Seventy-two-hour serum-starved (0.5% FBS) cultures were transduced with Ad-β-Gal or Ad-p21 (500 multiplicity of infection virus particles determined by serial dilution assay) for 12 h, after which the virus was removed, and cultures were returned to low serum (0.5% FBS) medium for an additional 12 h. Growth medium (10% FBS) was then added for 48 h to allow cell cycle progression. Cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry. Values represent the mean ± SE and were compared for statistical significance by Wilcoxon rank-sum test.* p < 0.05 vs Ad-p21-infected cultures. No cell death was apparent even at 96 h postinfection in all cultures (not shown).

p21 induces cell cycle arrest in RA synovial fibroblasts

To determine the significance of decreased p21 expression in RA synovial fibroblasts, we ectopically expressed p21 in RA and OA synovial fibroblasts (not shown) and analyzed for cell cycle activity. p21 was introduced into cells using replication-defective E1− E3− adenovirus vector (Ad-p21), which readily and uniformly infects synovial fibroblasts (22). Ad-β-Gal was used as a negative control. As shown in Fig. 3, 66% of the Ad-β-gal-infected RA synovial fibroblasts cells were in G0/G1, whereas 92% of the Ad-p21-infected RA synovial fibroblasts cells were in G0/G1. At the same time, 14% of the Ad-β-gal-infected cells were in S phase compared with only 2% of the Ad-p21-infected cells (Fig. 3). Similar data were observed employing OA synovial fibroblasts (not shown). These data indicate that RA and OA synovial fibroblasts infected with Ad-p21 arrest in the G0/G1 phase of the cell cycle.

Suppression of spontaneous IL-6 and MMP-1 production by p21 in RA synovial fibroblasts

RA synovial fibroblasts not only display an increased growth rate, but they also spontaneously secrete IL-6 and MMP-1. Therefore, we examined the effects of Ad-p21 infection on IL-6 and MMP-1 secretion by RA and non-RA synovial fibroblasts. Synovial fibroblasts were infected with Ad-β-Gal or Ad-p21, and supernatants were isolated 72 h following infection. Ad-p21 reduced IL-6 and MMP-1 secretion in RA synovial fibroblasts by 53 ± 11% (p < 0.0022) and 84 ± 8% (p < 0.0079), respectively (Fig. 4A). In contrast, Ad-p21 had no effect on IL-6 and MMP-1 production in non-RA synovial fibroblasts (OA and normal synovial fibroblasts, n = 11; Fig. 4B). Ad-IκBα infection, which suppresses NF-κB activation (6, 38), inhibited IL-6 and MMP-1 in all RA, OA, and normal synovial fibroblast cultures examined (not shown), similar to published studies (6, 39). The Ad-IκBα data indicate that unlike the disease type-specific inhibition of IL-6 and MMP-1 by p21, the ectopic expression of IκBα inhibits cytokine and MMP-1 production regardless of the origin of the cell type.

Down-regulation of IL-6 and MMP-1 mRNA by p21

To characterize the mechanism for the reduction of IL-6 and MMP-1, IL-6 and MMP-1 mRNA accumulation in Ad-β-Gal- and Ad-p21-infected RA synovial fibroblasts was examined by semiquantitative RT-PCR. Ad-p21 decreased IL-6 and MMP-1 accumulation compared with Ad-β-Gal-infected cells (Fig. 5). These data suggest that p21 suppresses IL-6 and MMP-1 at the transcriptional level.

The p21-deficient synovial fibroblasts exhibit enhanced IL-6 and MMP-3 protein synthesis

To further define the role of p21 in suppressing IL-6 and MMP-1 expression, we examined synovial fibroblasts isolated from wild-type and p21-deficient (p21−/−) mice. However, MMP-1 was not
and IL-6 was measured 48 h following infection. Values represent the mean ± SE and were compared for statistical significance by unpaired two-tailed Student’s t test. The p values are vs p21-deficient cells.

B, Enhanced expression of IL-6 and MMP-3 mRNA in p21−/− synovial fibroblasts. RNA was isolated from wild-type and p21-null synovial fibroblasts and was analyzed for IL-6, MMP-3, and G3PDH expression by semiquantitative RT-PCR. The data are representative of three experiments.

examined, because to date the murine orthologue of human MMP-1 has not been reported. Therefore, MMP-3 was examined in all experiments using mouse cells. Wild-type knee synovial fibroblasts spontaneously expressed low levels of IL-6 (61 ± 27 pg/ml). In stark contrast, p21-deficient synovial fibroblast expressed high levels of IL-6 (6178 ± 615 pg/ml; p < 0.00001) as measured by ELISA (Fig. 6A). In accordance with the ELISA data, IL-6 and MMP-3 mRNA accumulation was lower in the wild-type compared with the p21-deficient synovial fibroblasts (Fig. 6B). These data suggest that intact p21 is required to suppress IL-6 and MMP-3 expression.

Overexpression of p21, but not Rb, restores IL-6 and MMP-3 to basal levels in p21-null synovial fibroblasts

The p21-deficient synovial fibroblasts were transduced with Ad-β-Gal or Ad-p21 to document that reconstitution of p21 is sufficient to suppress IL-6 and MMP expression. Ad-Rb, which suppresses the cell cycle, was used to examine whether cell cycle arrest is required for IL-6 and MMP-3 reduction. Restoration of p21 by Ad-p21 suppressed IL-6 production in p21−/− synovial fibroblasts (Fig. 7A). In contrast, Ad-Rb infection of p21-deficient synovial fibroblasts had no effect on IL-6 secretion. Similar to the ELISA data, Ad-p21 reduced IL-6 and MMP-3 mRNA accumulation compared with wild-type levels (compare Fig. 7B with Fig. 6B). Again, IL-6 and MMP-3 mRNA levels remained unchanged following Ad-Rb infection (Fig. 7B). Collectively, these data demonstrate that p21 suppresses IL-6 and MMP synthesis in synovial fibroblasts, independently of cell cycle arrest.

Suppression of the activation of AP-1 by p21

AP-1 has been shown to regulate IL-6 and MMP-1 expression (40–43). To further characterize the mechanism of suppression mediated by Ad-p21, EMSAs were employed. Ad-p21 markedly decreased the binding of AP-1 to DNA compared with Ad-β-Gal-infected RA-synovial fibroblasts (Fig. 8A). In contrast, Ad-p21 did not inhibit the binding of CEBP/β or NF-κB to DNA in RA synovial fibroblasts (not shown). Since ectopic expression of p21 reduced AP-1 DNA-binding activity (Fig. 8B), we examined AP-1 DNA-binding activity in p21-deficient compared with wild-type synovial fibroblasts. AP-1 DNA-binding activity was increased in p21−/− compared with wild-type synovial fibroblasts. Both anti-c-Fos and c-Jun Abs decreased the amount of bound oligonucleotide (Fig. 8B) in p21-deficient synovial fibroblasts, suggesting that the AP-1 complex in the p21-null synovial fibroblasts consists of c-Jun/c-Fos heterodimers. These data suggest that p21 suppresses AP-1 DNA-binding activity and subsequent IL-6 and MMP-1 expression.

Discussion

Overexpression of p21 or p16 ameliorates experimental arthritis in multiple animals models and is associated with reduced IL-1β, TNF-α, and IL-6 mRNA (15–17). However, replication-defective adenoviruses delivered to the whole synovium nonspecifically infect lymphocytes, macrophages, fibroblasts, osteoclasts, and neutrophils. We demonstrated that <15% of the total cells in the synovium are infected by standard replication-defective adenoviruses (18). Collectively, these data suggest that inhibition of proliferation through p21 overexpression may not totally account for the observed reduced synovitis in experimental models of arthritis (15–17). Thus, in addition to preventing cell cycle progression, p21 may inhibit the expression of pro-inflammatory mediators. Here, we demonstrate a direct role for p21 in suppressing spontaneous IL-6 and MMP-1 production in RA synovial fibroblasts. In contrast, the overexpression of p21 has no effect on IL-6 or MMP-1 production in normal and OA synovial fibroblasts. Although it is unlikely, replication-defective adenoviruses may infect RA synovial fibroblasts more readily than OA synovial fibroblasts.
However, equal viral titers of Ad-p21 are employed in RA, normal, and OA synovial fibroblasts, which induces similar levels of protein expression (not shown) and equal inhibition of the cell cycle in the G0/G1 phase (Fig. 3). One potential explanation may be that the levels of p21 in normal and OA synovial fibroblasts may be at a maximum, and any increase in the expression of p21 has no effect on the additional functions of p21, including cytokine and MMP production. Alternatively, a factor that is present only in RA synovial fibroblasts may be required for p21-mediated suppression of IL-6 and MMP-1. Collectively, these data demonstrate a novel function for p21 in RA synovial fibroblasts and suggest that up-regulating p21 in the synovium, particularly in the synovial fibroblast population, may be therapeutically beneficial for RA.

The expression of p21 was down-regulated in RA synovial lining fibroblasts in vivo and in vitro compared with OA. One of the potential regulators of p21 is p53 (44), a transcription factor that inhibits cell cycle activity and/or induces apoptosis in response to stress or damage (45, 46). Although p53 expression is markedly increased in RA synovial tissue compared with normal or OA synovial tissue (47, 48), somatic mutations in p53 were also reported in the RA synovium (49–52). These data suggest that a subset of synovial fibroblasts may possess an inactive form of p53. Over-expression of plasmids encoding the p53 mutants found in RA synovium suppresses wild-type p53 function, as indicated by the induction of known p53-regulated genes, including IL-6 (53, 54) and MMP-1 (55, 56), which are normally inhibited by wild-type p53. Additionally, mice deficient in p53 display enhanced experimental arthritis, elevated levels of IL-6 in the synovium, and increased spontaneous MMP-13 expression compared with wild-type mice (57). Thus, p21-deficient synovial fibroblasts behave similarly to the RA synovial fibroblasts that lack p53 or express the mutant forms of p53. Furthermore, ectopic expression of p21 or overexpression of wild-type p53 reduces IL-6 and MMP-1 expression in RA synovial fibroblasts (53–56, 58). Collectively, these data suggest that p21 may be one of the nodal points through which p53 inhibits cytokines and MMPs (Fig. 9).

The mechanism of p21-mediated down-regulation of IL-6 and MMP-1 is unclear. c-Jun NH2-terminal kinase (JNK), which phosphorylates the c-Jun component of AP-1 and enhances its activity by 30-fold (59, 60), is increased in RA compared with OA synovial fibroblasts (28). JNK-1- or JNK-2-deficient synovial fibroblasts fail to express constitutive MMP-3 or -13 expression (5). Furthermore, p21 binds to and inhibits JNK activity (61). Inhibition of JNK and subsequently decreased AP-1 activity prevents cartilage and bone destruction in the rat model of adjuvant-induced arthritis (5). Thus, JNK activation of AP-1 may be essential for promoting proinflammatory molecule production. Furthermore, c-Fos and c-Jun expression correlates with synovial hyperplasia in RA synovial tissue (62), and increased AP-1 DNA-binding activity is observed in RA compared with OA synovial tissue (63, 64). Addition of AP-1 oligonucleotides ameliorates joint destruction in experimental arthritis models (42). Thus, AP-1 is integral for the pathogenesis of RA. We showed that p21 inhibits the JNK/AP-1 pathway, indicating that p21 may inhibit IL-6 and MMP-1 through the suppression of JNK/AP-1. Ectopic p21 expression reduces JNK activity (not shown) and AP-1 activation in RA synovial fibroblasts, which corresponds to decreased IL-6 and MMP-1 mRNA and protein. Furthermore, p21-deficient synovial fibroblasts display elevated AP-1 DNA binding compared with wild-type cells. Anti-c-Fos and anti-c-Jun Ab markedly reduced binding to the AP-1 oligonucleotide employing p21-null synovial fibroblast nuclear extracts. Collectively, these data suggest that p21 may suppress IL-6 and MMP-1 through inhibition of JNK/AP-1 pathway (Fig. 9).

Recent investigations have demonstrated that mice deficient for p21 exhibit lupus-like disease at 9 mo of age (65, 66). However, it

![FIGURE 8. Decreased activation of AP-1 by p21 in RA and mouse synovial fibroblasts.](Image)

![FIGURE 9. Schematic of transformation of a normal synovial fibroblast to the RA synovial fibroblast.](Image)
is unknown whether p21-null mice develop spontaneous arthritis or enhanced/accelerated experimental arthritis. Thus, these data suggest a vital role for p21 in suppressing proinflammatory cytokines and MMPs, which contribute to the development of autoimmune disease.

References


