Adenoviral Transfer of Cyclin-Dependent Kinase Inhibitor Genes Suppresses Collagen-Induced Arthritis in Mice

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Adenoviral Transfer of Cyclin-Dependent Kinase Inhibitor Genes Suppresses Collagen-Induced Arthritis in Mice

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In rheumatoid synovial tissues, synovial fibroblasts are activated by proinflammatory cytokines and proliferate to develop hyperplastic pannus tissues, which irreversibly damage the affected joints. We recently reported that the cyclin-dependent kinase inhibitors p16INK4a and p21Cip1 are not expressed in vivo in rheumatoid synovial fibroblasts, but are readily inducible in vitro. Our results demonstrate that the ectopic expression of cyclin-dependent kinase inhibitors not only prevents synovial overgrowth but also ameliorates the proinflammatory milieu in the affected joints. The induction of p21Cip1 in rheumatoid synovial tissues by pharmacological agents may also be an effective strategy to treat rheumatoid arthritis.


Rheumatoid arthritis (RA) is characterized by the synovial inflammation of multiple joints. The affected synovial tissues contain activated macrophages, fibroblasts, and T and B lymphocytes. In response to proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, which are produced in situ, the synovial fibroblasts proliferate and release tissue-degrading enzymes (1, 2). The resulting hyperplastic synovial membrane, termed pannus tissue, irreversibly destroys the cartilage and bone of the affected joints.

We previously studied the expression of cyclin-dependent kinase inhibitors (CDKIs) in rheumatoid synovial tissues (3). CDKIs are a group of nuclear proteins that inhibit cyclin-dependent kinases (CDKs). CDKs are essential for the progression of the cell cycle, and the up-regulation of CDKI genes generally results in cell cycle arrest. CDKIs discovered to date fall into two groups, the Cip/Kip family with three members (p21Cip1, p27Kip1, and p57Kip2) and the INK4 family with four members (p15INK4b, p16INK4a, p18INK4c, and p19INK4d) (4). The expression of individual CDKIs is independently regulated, and each appears to play a unique role in controlling the cell cycle. In fibroblasts derived from rheumatoid synovial tissues, neither p16INK4a nor p21Cip1 is expressed in vivo, but both are readily induced when the growth of the fibroblasts is inhibited in vitro. The induction of p21Cip1 is also observed in nonrheumatoid fibroblasts, whereas the induction of p16INK4a is characteristic of rheumatoid synovial fibroblasts. The specific induction of p16INK4a in the rheumatoid fibroblasts prompted us to transfer the p16INK4a gene into the joints of rats with adjuvant arthritis (AA). This treatment suppressed the synovial hyperplasia and associated pathology of the arthritis (3). This was the first evidence that arthritis could be treated by inhibiting the cell cycle of the synovial fibroblasts.

The treatment of arthritis by the local transfer of the p16INK4a gene compares well with treatment by conventional anti-rheumatic drugs and recently developed biological reagents. They all aim primarily at the suppression of inflammatory mediators involved in RA. Most of the biological reagents neutralize proinflammatory cytokines such as TNF-α, IL-1, or IL-6 (5–9). Our approach was to directly inhibit the proliferation of the synovial fibroblasts to prevent destructive synovial hyperplasia without specifically attempting to interfere with the proinflammatory cytokines. We believe that synovial tissues overexpressing cell-cycle-suppressing genes would not invade the bone or cartilage despite the presence of the proinflammatory cytokines.

In this study, we examined the therapeutic effects of the forced expression of p21Cip1, as well as that of p16INK4a, in the inflamed synovial tissues of mice with collagen-induced arthritis (CIA). As stated before, both genes are inducible in rheumatoid synovial fibroblasts in vitro (3). p16INK4a binds to cyclin D and prevents it from forming a catalytically active kinase complex with CDK4 or CDK6. Thus, it inhibits the cell cycle at the G1/S transition (10–12). p21Cip1 inhibits a wide variety of cyclin/CDK complexes (13, 14). It also binds to and inactivates proliferating cell nuclear Ag, which activates DNA polymerase δ (15–18). Thus, p21Cip1 inactivates the kinase activity of cyclin/CDK complexes at every stage of the cell cycle, and it also inhibits DNA replication.

Abbreviations used in this paper: RA, rheumatoid arthritis; CDKI, cyclin-dependent kinase inhibitor; CIA, collagen-induced arthritis; AA, adjuvant arthritis.
Like rat AA, the murine CIA model used in this study demonstrates remarkable similarities to human RA (19–21). It was successfully treated by gene transfer of p21Cip1 as well as that of p16INK4a. This murine model allowed us to further investigate the molecular mechanism of the therapeutic effects of CDKI. We found these effects to be not only anti-proliferative but also anti-inflammatory.

Materials and Methods

Cells

Cultured fibroblasts were prepared from synovial tissues of patients with RA undergoing total joint replacement surgery or synovec-tomy at Nippon Medical School Hospital, Tokyo Metropolitan Bokuto Hospital, or Fuchu Hospital. Consent forms were completed by patients before surgery. RA was diagnosed according to the criteria of the American College of Rheumatology (22). The fibroblasts were cultured as described elsewhere (3).

Recombinant adenoviruses

Replication-defective adenoviruses containing a human p16INK4a gene (AxCAp16) or a human p21Cip1 gene (AxCAp21) were prepared as described previously (23). A recombinant adenovirus encoding Escherichia coli lac Z gene (AxCALacZ) was generously provided by Dr. Saito (University of Tokyo, Tokyo, Japan) (24). High-titer recombinant adenoviruses were prepared by amplification in 293 cells and purified by cesium chloride density-gradient centrifugation (25).

Cell proliferation assay

In vitro gene transfer of the recombinant adenoviruses and measurement of [3H]thymidine incorporation by adenovirus-infected cells were performed as described elsewhere (23).

Induction of CIA

Male DBA/1J mice were purchased from Japan Charles River Breeding Laboratories (Tokyo, Japan) and housed in the Animal Research Center of Tokyo Medical and Dental University. Bovine type II collagen (Collagen Research Center, Tokyo, Japan) was dissolved at 4 mg/ml in 0.1 M acetic acid, and then emulsified with an equal volume of CFA (Iatron, Tokyo, Japan). For the primary immunization, 100 μl of the immunogen were injected intradermally into 8-wk-old mice at the tail base. After 3 wk, the mice received the same dose of the immunogen s.c. The arthritis developed only 10 days after the second immunization. The mRNA of the immunogen was isolated from the synovial tissues with Isogen (Nippongene, Tokyo, Japan). For the primary immunization, 100 μl of the immunogen were injected into 8-wk-old mice at the tail base. After 3 wk, the arthritis developed in 10% phosphate-buffered formalin (pH 7.4), decalcified in 10% EDTA, and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin. For immunohistochemical analyses, synovial tissues from the knee joints were embedded in Tissue-Tek ornhite carbamyl transferease compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and stored at −80°C. Serial cryostat sections (8 μm) were air-dried, fixed with cold 4% phosphate-buffered paraformaldehyde (pH 7.4), and washed with 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% saponin. Saponin permeabilizes the membranes of cells and intracellular organelles, thereby allowing the detection of intracellular cytokines. This technique gives positive nuclear staining in conventional immunohistochemical analyses, thus showing that cytokines can be stained mostly around the nucleus (27,28). The sections were then incubated with 10% normal goat serum for 1 h at room temperature and treated with rabbit anti-human IL-1β Ab (LP-712; Genzyme, Cambridge, MA), rabbit anti-mouse TNF-α Ab (IP-400; Genzyme), or normal rabbit serum overnight at 4°C. They were subsequently incubated with biotinylated goat anti-rabbit IgG (Southern Bio-technology Associates, Birmingham, AL), treated with 0.3% hydrogen peroxide in methanol, and incubated with HRP-labeled streptavidin (Vector Laboratories, Burlingame, CA). Bound Abs were visualized with 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride in PBS (pH 7.4) and 0.02% hydrogen peroxide, then they were stained with hematoxylin.

Statistics

Statistical analyses were performed with StatView 4.5J software (Abacus Concepts, Berkeley, CA). Statistical differences of the ankle width and the paw width of the CIA mice were assessed by Student’s t test, and the disease scores by the Mann-Whitney U test.

Results

Adenoviral gene transfer of either p16INK4a or p21Cip1 inhibits synovial cell proliferation

The antiproliferative effects of the forced expression of p21Cip1 as well as p16INK4a on synovial cells were examined using human synovial fibroblasts. Synovial fibroblasts prepared from rheumatoid synovial tissues were infected with a recombinant adenovirus (AxCAp16, AxCAp21, or AxCALacZ containing the human p16INK4a, human p21Cip1, and E. coli lac Z gene, respectively), and their growth was stimulated with 10% FCS or 10 ng/ml platelet-derived growth factor. When the synovial cells were infected with AxCAp16 or AxCAp21, their proliferation was suppressed in a dose-dependent manner (Fig. 1). No effect was observed with AxCALacZ. The virus-infected cells were all viable when examined by trypan blue staining. The cell displayed no signs of apoptosis when the nuclei were stained with Hoechst 33258 or when the cellular DNA was analyzed with agarose gel electrophoresis (data not shown). Similar results were obtained when murine NIH3T3 fibroblastoid cells were infected with the same set of viruses and stimulated in the same manner (data not shown).

Effects of p16INK4a and p21Cip1 gene therapy on the pathology of CIA

The same set of adenoviruses was used to introduce the p16INK4a, p21Cip1, or lac Z gene in vivo into the synovial tissues of CIA mice. The mice were immunized twice with type II collagen to induce arthritis. Gene transfer into the joints was performed either on the same day as the second immunization and 10 days later, or only 10 days after the second immunization. The mRNA of the...
Effects of p16INK4a and p21Cip1 gene transfer on the expression of proinflammatory cytokines in CIA-affected joints

In CIA, as in RA, TNF-α and IL-1, which are mainly secreted from the synovial cells, largely account for the pathology of the arthritis (29–32). Thus, their expression level in the synovial tissues reflects the inflammatory milieu in the affected joints. We studied the expression of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in arthritic joints treated with AxCAP16, AxCAP21, AxCALacZ, or saline. Synovial tissues from the hind paws were collected on the day of the histopathological examination. From the CDKI-treated joints, synovial tissues from the joints with the residual inflammation (disease score ~3) were collected. When the mRNA expression levels of the cytokines were examined by RT-PCR, all of the cytokine mRNAs were found abundant in the AxCALacZ- and saline-treated synovial tissues (Fig. 4). In contrast, they were undetectable or barely detectable in the synovial tissues treated with AxCAP16 or AxCAP21. The amount of mRNA used for each RT-PCR was standardized with respect to the amount of the extracted RNA. The mRNA of GAPDH was detected at comparable levels in all of the joints.

We next performed an immunohistochemical analysis to detect cells expressing IL-1β or TNF-α in the synovial tissues. In the joints treated with AxCALacZ or saline, the cells expressing these cytokines were found mainly in the synovial lining layer and in the intimal synovial tissues (Fig. 5, A–F). In accord with previous observations (27, 28), these cytokines were localized primarily in the nuclear regions, but cytoplasmic staining was also found. In contrast, in the synovial tissues from the AxCAP16- and AxCAP21-treated joints, the staining of IL-1β and TNF-α was very faint (Fig. 5, G–L). Specimens incubated with normal rabbit serum and subsequently treated in the same manner yielded no nonspecific staining (Fig. 5, C, F, I, and L). Thus, the expression of proinflammatory cytokines in the CDKI-treated joints was markedly down-regulated.

Discussion

We have shown that the forced expression of either p16INK4a or p21Cip1 in the synovial tissues significantly alleviated CIA in mice. In gene transfer experiments, p21Cip1 was as effective as p16INK4a, which had previously been used to treat AA in rats (3). Neither gene was expressed in vivo in the inflamed synovial tissues, but both were readily inducible in cultured rheumatoid synovial fibroblasts (3). Thus, the forced expression of the CDKI genes that are potentially inducible in rheumatoid synovial tissues has now been shown to be effective in treating two rodent models of proliferative synovitis.

Although p21Cip1, in contrast to p16INK4a, inhibits the kinase activity of all CDKs as well as DNA replication, the physiological expression of p21Cip1 is usually transient. Because adenoviral gene transfer would be expected to overcome this constraint, we assumed that the overexpression of p21Cip1 would suppress cell proliferation more profoundly than that of p16INK4a. However, we observed that both CDKI genes equally suppressed both the in vitro proliferation of synovial fibroblasts and the pathology of CIA. Presumably, other CDKI genes would also suppress the arthritis if they were transferred into the arthritic synovial tissues. However, in this study, an emphasis was placed on the effects of
p16\(^{INK4a}\) and p21\(^{Cip1}\) because their endogenous expression can be artificially induced in rheumatoid synovial fibroblasts. This suggests that synthetic compounds that selectively induce the expression of p16\(^{INK4a}\) or p21\(^{Cip1}\) could act as therapeutic agents against RA. However, the systemic expression of p16 \(^{INK4a}\) or p21 \(^{Cip1}\) may provoke serious adverse effects by inhibiting cell division essential for natural cell turnover. Because the induction of p16\(^{INK4a}\), unlike that of p21\(^{Cip1}\), under growth inhibitory conditions is characteristic of rheumatoid synovial fibroblasts, the specific induction of p16\(^{INK4a}\) in rheumatoid synovial fibroblasts by pharmacological agents might be preferable.

The regulation of p16\(^{INK4a}\) gene expression is still too poorly understood to guide us in developing p16\(^{INK4a}\)-inducing compounds. p16\(^{INK4a}\) is usually expressed in terminally differentiated or replicative senescent cells (33–38). Although sustained activation of the Ras oncogene leads to the expression of endogenous p16\(^{INK4a}\), the physiological role of this induction seems to be limited to the inhibition of tumor development (39). UV irradiation, bleomycin, or actinomycin D induces transient or delayed expression of p16\(^{INK4a}\) in certain cell types (40, 41). In contrast, the expression of p21\(^{Cip1}\) is immediately though transiently induced by a p53 transcription factor in response to DNA damage (42, 43). Many compounds that induce p21\(^{Cip1}\) in various human cell lines have already been identified (44–46). Therefore, artificial induction of endogenous p21\(^{Cip1}\) might prove easier, although the expression should be made tissue specific.

It should be noted that the dose and timing of gene transfer were not necessarily optimized for practical clinical use as in our previous studies (3). Gene transfer was used to show the effect of CDKI gene expression in the synovial cells. Repeated adenoviral gene transfer could induce tissue injury through an immune reaction against viral gene products. Although the observation period was designed to be short to minimize the effects of secondary immune reactions, the remaining inflammation in the CDKI-treated joints might be related to such effects.

The forced expression of the CDKI genes in murine CIA had similar anti-arthritis effects to the forced expression of the p16\(^{INK4a}\) gene in rat AA. Pannus formation was significantly suppressed, and no destruction of the cartilage or bone was seen. Moreover, the infiltration of mononuclear cells into the synovial tissues was also suppressed. The p16\(^{INK4a}\) gene therapy of rat AA

**FIGURE 2.** Effect of p16\(^{INK4a}\) and p21\(^{Cip1}\) gene therapy on the progression of CIA. Mice with CIA were treated with AxCAp16 (■), AxCAp21 (△), AxCALacZ (○), or saline (●) either twice (arrows, on days 21 and 31) (A–C) or once after the onset of arthritis (arrow, on day 31) (D–F). The ankle width (A and D), paw width (B and E), and disease score (C and F) of the animals were measured on the indicated day. The points and bars in the graph represent the mean ± SEM of seven mice. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (AxCAp16 vs saline). #, p < 0.05; ##, p < 0.01; ###, p < 0.001 (AxCAp21 vs saline).
had profound effects even though not all synovial fibroblasts expressed the exogenous gene. We speculated that the expression of p16INK4a made the synovial cells refractory to proliferative stimuli and that they might also exert anti-inflammatory effects. This hypothesis was addressed in this study by examining the expression of IL-1 and TNF-α and that they might also exert anti-inflammatory effects. This hypothesis was addressed in this study by examining the expression of proinflammatory cytokines between CDKI-treated and control joints suggested that the difference in expression did not merely reflect quantitative differences. Indeed, CD14 mRNA could be readily detected in RNA extracted from CDKI-treated joints (data not shown).

To test whether gene transfer of the CDKIs suppresses the production of proinflammatory cytokines directly, murine RAW264.7 macrophage-like cells were infected with the AxCAp16, AxCAp21, or control adenoviruses, and stimulated with LPS or IL-1β for TNF-α secretion. ELISA of the culture supernatant revealed no suppressive effects of the CDKI expression (data not shown). Thus, the suppression of the cytokine production did not appear to be an immediate effect; possibly, an autocrine or paracrine mechanism operated. Because the expression of endogenous p16INK4a and p21Cip1 is associated with cellular senescence, the cells expressing exogenous p16INK4a or p21Cip1 may have exerted some inhibitory effects characteristic of senescent cells. This hypothesis agrees with our previous observation that not all of the synovial cells expressed the transgene after the gene transfer (3).

The immediate effect alone would have allowed the uninfected synovial cells to produce the proinflammatory cytokines. Thus, we believe that the reduction of the cytokine production was not merely secondary to the suppressed synovial proliferation or due to an immediate effect of the CDKI gene expression. Underlying mechanisms are now under investigation.

By adenoviral transfer of a cell cycle regulator gene, we originally intended to suppress the cell cycle of synovial fibroblasts. However, our results demonstrate that the forced expression of CDKI genes in the synovial cells exerted not only an antiproliferative effect but also an anti-inflammatory effect; it suppressed the production of proinflammatory cytokines. In this regard, our short-term observations revealed that the local expression of CDKI genes in joints with established CIA suppressed further synovial thickening but did not diminish it. However, in the long run, the treatment would probably alleviate persistent arthritis such as RA, because it down-regulates the production of proinflammatory cytokines.

It was reported that adenoviral gene transfer into the joints exerted its effects on the contralateral joints (50, 51). Thus, it might be possible that the recombinant CDKI adenoviruses suppressed general immune reactions, which could contribute to suppression of CIA. However, no significant differences were found in the joint swelling of the forelegs, which were not treated with the gene transfer (data not shown). Moreover, in the previous report, we treated rats with AA successfully by injecting the AxCAp16 adenoviruses into the right knees and the control adenoviruses into
the left knees of the same animals (3). These results argue that the therapeutic effects observed in this study are also attributable primarily to the local effect of the gene transfer.

We have shown that the forced expression of CDKI exerted both antiproliferative and anti-inflammatory effects, which would serve the ultimate goal of RA treatment, i.e., long-term prevention of joint destruction. Thus, the local induction of p21Cip1, as well as the ultimate goal of RA treatment, i.e., long-term prevention of antiproliferative and anti-inflammatory effects, which would serve mainly to the local effect of the gene transfer.

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References


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