Suppression of arthritis by forced expression of cyclin-dependent kinase inhibitor p21Cip1 gene into the joints

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Abstract

Rheumatoid synovial fibroblasts (RSF) express cyclin-dependent kinase (CDK) inhibitors p16INK4a and p21Cip1 when they are growth-inhibited in vitro. The induction of p16INK4a is characteristic of RSF and intra-articular p16INK4a gene therapy has been shown to suppress adjuvant arthritis (AA) of rats. The other inducible CDK inhibitor, p21Cip1, has multiple functions depending on the cell type. They include inhibition of CDK as well as promotion of active CDK complex formation and induction of apoptosis. This study is to discern the biological effects of p21Cip1 gene transfer into RSF and its therapeutic effects on AA. A recombinant adenovirus containing a human p21Cip1 gene and control adenoviruses were prepared. RSF infected with these viruses were examined for their cell growth. Apoptotic cell death was evaluated by nuclear staining and DNA fragmentation analysis. In vivo gene therapy of rat AA was carried out by intra-articular injection of the viruses. Severity of the arthritis was clinically scored. The treated joints were examined histologically and proliferating cell nuclear antigens (PCNA) were detected immunohistochemically. The adenoviral p21Cip1 gene transfer inhibited growth of RSF without inducing apoptosis. p21Cip1 gene therapy suppressed AA clinically and histologically. The effects were comparable to p16INK4a gene therapy. PCNA expression was reduced in the p21Cip1-treated joints. The adenoviral gene transfer of p21Cip1 ameliorated rat AA. The effect was attributable to inhibition of proliferation. Because p21Cip1 is induced more easily by many chemicals than p16INK4a, it also appears to be a feasible target in developing anti-rheumatic drugs.

Introduction

Rheumatoid arthritis (RA) is characterized by chronic inflammation at multiple joints. T and B lymphocytes and macrophages infiltrate into the affected synovial tissues, and promote release of a high concentration of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and IL-1 (1). In response to these cytokines, synovial fibroblasts proliferate vigorously and form pannus tissues, which destroy the cartilage and bone of the joints. In clinics, various drugs are used to control RA. Representative are conventional disease-modifying anti-rheumatic drugs including hydroxychloroquine, d-penicillamine, gold, sulfasalazine as well as immunosuppressive methotrexate. Although they do not have distinct target molecules known to be responsible for their anti-rheumatic effects, they appear to down-modulate the inflammatory processes (2). In contrast, newly developed drugs, such as leflunomide, cyclosporin A and FK506, inhibit intracellular molecules essential for activation of the immunocompetent cells involved in the rheumatoid synovitis. TNF-α antagonists (remicade and infliximab) neutralize the key inflammatory mediator released by the synovial cells. Many patients respond well to these drugs, but some are resistant or become so after some period of disease amelioration. Moreover, few have been proven effective in halting the disease progression in the long term. We assume that suppression of one inflammatory mediator might activate other mediators because of redundancy of the inflammatory pathways. Thus, suppression of the synovial cell proliferation itself seems to be the most effective therapeutic way to intervene in the joint destruction.

In general, cell cycle progression of eukaryotic cells is
regulated largely by kinase activity of cyclin/cyclin-dependent kinase (CDK) complexes. There are several subtypes of cyclins and CDK, which undergo cyclic activation to promote cell cycle progression (3). The kinase activity is inhibited by a group of intranuclear proteins termed CDK inhibitors (CDKI). Up to now, two CDKI families have been identified (3). They are the INK4 family, which consists of p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d}, and the Cip/Kip family, which consists of p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}. Previously, we investigated expression of cell-cycle proteins in rheumatoid synovial fibroblasts (RSF) (4). We found that p16\textsuperscript{INK4a} and p21\textsuperscript{Cip1} expression was readily induced \textit{in vitro} in RSF derived from joints with active synovitis. This induction was observed when growth of RSF was artificially inhibited. Of note, the induction of p16\textsuperscript{INK4a} was characteristic of RSF, but not of other types of fibroblasts. Subsequently, using adjuvant arthritis (AA) of rats, an animal model of RA, we showed that adenoviral transfer of the p16\textsuperscript{INK4a} gene to the synovial tissues suppressed pathology of the arthritis (4). This was the first evidence showing that cell-cycle control of synovial cells is effective for arthritis treatment.

The p16\textsuperscript{INK4a} protein binds specifically to CDK4 and CDK6, and inhibits formation of the CDK/cyclin complexes (3). Physiologically, it is expressed by replicative senescent cells and by terminally differentiated cells (5). Forced expression of the p16\textsuperscript{INK4a} gene induces cell-cycle arrest at G\textsubscript{1} phase and phenotypic changes characteristic of senescent cells (6).

The p21\textsuperscript{Cip1} protein, being the other one of the two inducible CDKI in RSF, inhibits a wide variety of cyclin/CDK complexes (3). Forced expression of p21\textsuperscript{Cip1} also arrests the cell cycle of normal and tumor cells at the G\textsubscript{1} phase (7). In fibroblasts, the expression level of p21\textsuperscript{Cip1} goes up with an increase of cell division and decreases when the cells approach senescence (8). In contrast, the p16\textsuperscript{INK4a} protein level gradually rises and stays high in the senescent cells. Thus, p16\textsuperscript{INK4a} gene expression appears to have a more direct impact in senescence induction (5). The biological function of p21\textsuperscript{Cip1} is more complex. In certain settings, p21\textsuperscript{Cip1} promotes formation of active kinase complexes by cyclin and CDK, forwarding rather than halting the cell cycle (9). Whereas the N-terminal domain interacts with CDK/cyclin complexes, the C-terminal domain binds to and inhibits proliferation cell nuclear antigen (PCNA), a subunit of DNA polymerase \(\delta\), which is essential for DNA replication and repair (10). Also, the expression of p21\textsuperscript{Cip1} enhances NF-\kappaB-dependent gene expression by inhibiting cyclin E/CDK2, which binds to complexes of p300/ CBP co-activators and NF-\kappaB (11). In this regard, NF-\kappaB is a critical transcription factor involved in rheumatoid synovitis (12). Whereas proinflammatory cytokines such as IL-1 and TNF-\(\alpha\) activate NF-\kappaB in rheumatoid synovial tissues, the production of these cytokines depends on NF-\kappaB (13,14). Moreover, forced expression of p21\textsuperscript{Cip1} induced apoptotic cell death of some cell lines (15–18).

Thus, p21\textsuperscript{Cip1} ectopically expressed in the inflamed synovial tissues may exert differential effects than p16\textsuperscript{INK4a} on the pathology of the arthritis. In the present study, we first transferred the p21\textsuperscript{Cip1} gene adenovirally into RSF. Subsequently, the gene was transferred \textit{in vivo} to synovial tissues of rats with AA, which had been treated successfully with p16\textsuperscript{INK4a} gene therapy. The results revealed that p21\textsuperscript{Cip1} is also a potentially ideal target for development of gene therapy as well as anti-rheumatic drugs.

**Methods**

**Recombinant adenoviruses**

A recombinant replication-defective adenovirus, AxCAp21, containing a CAG promoter and a human p21\textsuperscript{Cip1} gene was prepared (19). An AxCALacZ adenovirus containing a bacterial lacZ gene was a gift from Dr Saito (20). An Ax1w1 adenovirus without insertion was purchased from the Riken Gene Bank (Saitama, Japan). Previous studies showed that the product of the p21\textsuperscript{Cip1} gene in the AxCAp21 virus can inhibit CDK activity and thus keep pRB protein underphosphorylated (21).

**Cell culture**

Synovial tissues were obtained from five patients, under their consent, who underwent synovectomy or total knee joint replacement surgery for active rheumatoid synovitis at Nippon Medical School Hospital, Tokyo Metropolitan Bokuto or Huchu Hospital. They fulfilled the American College of Rheumatology criteria for classification of RA (22). RSF were isolated and cultured as described elsewhere (4). The cells at passages 3–6 were used for experiments.

**Cell proliferation assay**

\textit{In vitro} adenoviral infection was performed as described previously (19). Cell growth was assessed by incorporation of \[^{3}H\]thymidine. RSF were plated in 96-well plates, incubated in 10% FBS for 24 h and then infected with various concentrations of the adenoviruses for 1 h. Then the cells were incubated for 48 h. For the last 24 h, the cells were pulsed with \[^{3}H\]thymidine (Amersham, Little Chalfont, UK) and then incorporated radioactivities counted.

**Apoptosis assays**

A half million RSF were infected with \(5 \times 10^{7}\) p.f.u. of the AxCAp21 or Ax1w1 adenoviruses at 100 m.o.i. To induce apoptosis, the same number of cells were treated with 50 \(\mu\)M...
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Fig. 2. Apoptosis assays of RSF with forced p21Cip1 expression. (A–D) Hoechst 33258 staining of the nuclei. RSF treated with N-acetyllysphingosine displayed nuclear condensation and fragmentation characteristic of the apoptotic cells (A). The AxCap21-treated RSF (B) and Ax1w1-treated RSF (C) showed no signs of apoptosis compared to the non-treated RSF (D). (Original magnification ×400.) See text for quantitation of the apoptotic cells. (E) Agarose gel electrophoresis of cellular DNA. Total cellular DNA of non-treated RSF (lane 2), RSF 2 days (Lane 3) and 4 days (lane 4) after the AxCap21 infection, and RSF 2 days (lane 5) and 4 days (Lane 6) after the Ax1w1 infection were analyzed. The DNA of UV-treated HL-60 cells was fractionated to show typical nucleosomal DNA ladder (lane 7). Lane 1: DNA mol. wt marker (φX174 HaeIII digests).

N-acetyllysphingosine (Wako Pure Chemical, Osaka, Japan) in RPMI 1640 medium supplemented with 40 ng/ml platelet-derived growth factor (PDGF; Genzyme, Cambridge, MA) (23). After 4 days, the treated cells were isolated with trypsin–EDTA solution (Immunobiology Laboratories, Gunma, Japan), fixed with 1% glutaraldehyde in PBS and stained with Hoechst 33258 (Molecular Probes, Eugene, OR). To quantify the apoptotic cells, 500 nuclei were visually examined. Total cellular DNA was extracted from the treated cells and their fragmentation was analyzed with 2% agarose gel DNA electrophoresis.

Gene therapy of AA

Six-week-old male Lewis rats were immunized with 1 mg of Mycobacterium butyricum emulsified in 100 µl of mineral oil. Intra-articular gene transfer to the knee joints was carried out by injecting 1×10⁷ p.f.u. of the adenoviruses in 50 µl of saline. During the course of the disease, severity of the arthritis was clinically scored (24). Width of the knee joints at the lower edge of the patella was measured by a micrometer. At the end of the clinical observation, the joints were fixed for histological and immunohistochemical analyses. Injections of saline alone served as a control. All procedures in these animal experiments were reviewed and approved the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (approval no. 990107), and carried out under the control of the guidelines for animal experiments.

Histological analysis

The knee joints were embedded in paraffin wax after 10% PBS–formalin fixation and decalcification. Their sections (5 µm) were stained with hematoxylin & eosin. Thickness of the synovial tissues and cartilage was measured at the position close to the synovial attachment to the tibial head. Mononuclear cells in 1.8×10⁻⁸ m² of the synovial tissues were enumerated at the same position. Severity of the pannus invasion was scored as was performed previously (4).

Immunohistochemistry

For immunohistochemical analyses, the fixed sections (5 µm) were deparaffinized and treated by two rounds of microwave heating for 5 min in 10 µM sodium citrate (pH 6). They were incubated with 0.3% H₂O₂, with 10% normal goat serum in PBS and with an anti-PCNA mAb (PC10; Santa Cruz Biotechnology, Santa Cruz, CA) or a control mouse IgG antibody for 1 h. This antibody reacts with a PCNA peptide chain distinct from the binding site for p21Cip1, ruling out the possibility that binding of p21Cip1 to PCNA interferes with the antibody reactivity (25,26). They were subsequently incubated with a biotinylated goat anti-mouse IgG antibody (AP181B; Chemicon, Temecula, CA) and with horseradish peroxidase-labeled streptavidin (Southern Biotechnology Associates, Birmingham, AL). Bound antibodies were visualized with 0.02% 3,3′-diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin. The percentages of the PCNA⁺ cells in total cells [PCNA labeling indices (LI)] were calculated by examining 200 synovial cells (27).

Statistics

Statistical analyses were carried out with StatView-5.0J software (SAS, Cary, NC). The [³H]thymidine uptake of the
cultured RSF, as well as knee width, thickness of the cartilage and synovial membranes and PCNA LI of the AxCap21-treated and AxCALacZ-treated joints were compared with a paired t-test. The scores of pannus invasion were compared with a Mann–Whitney U-test. The percentages of the apoptotic RSF infected with the adenoviruses were compared with a t-test.

Results

Growth inhibition of RSF by the p21 Cip1 gene transfer

Using recombinant adenoviruses, the effect of the ectopic expression of p21 Cip1 on proliferation of RSF was evaluated. RSF were cultured to allow logarithmic growth with media containing 10% FBS. They were infected with the AxCap21 adenoviruses containing a human p21Cip1 gene or with the Ax1w1 adenoviruses containing no inserted gene. Western blot analysis of the total cell lysates of the infected cells showed that the p21Cip1 protein was expressed specifically by the AxCap21-infected cells but not by the Ax1w1-infected cells (data not shown). Proliferation of the cells was evaluated 24 h after the infection. Compared to the proliferation of the Ax1w1-infected RSF, that of the AxCap21-infected RSF was significantly suppressed by the viruses. The suppressive effect depended on the titer of the viruses (Fig. 1).

Apoptotic cell death of RSF with the overexpressed p21 Cip1 gene

Growth inhibition observed in the AxCap21-infected RSF could be due to apoptosis induced by the ectopic p21 Cip1 expression. Thus, RSF infected with the AxCap21 adenoviruses and those infected with the Ax1w1 adenoviruses were examined for apoptotic cell death.

As was reported previously, RSF undergo apoptotic cell death when they are treated with N-acetylsphingosine (23). The apoptotic RSF displayed characteristic morphological changes such as nuclear condensation and fragmentation when they were stained with Hoechst 33258 (Fig. 2A). The RSF treated with a growth-inhibitory concentration (100 m.o.i.) of AxCap21 or those with the same dose of Ax1w1 were also stained 4 days after the infection (Fig. 2B and C). They were compared with RSF cultured without the viruses (Fig. 2D). The percentages of apoptotic cells that showed characteristic nuclear changes were evaluated in three different samples for each type of the infection. The frequencies of the apoptotic cells in the non-infected, AxCap21-infected and Ax1w1-infected RSF were not significantly different; the means ± SEM were 0.52 ± 0.45, 0.69 ± 0.60 and 0.60 ± 0.67% respectively.

Nuclear DNA of RSF infected with the AxCap21 or control viruses was fractionated with agarose gel electrophoresis. Whereas DNA of the apoptotic HL-60 cells had typical nucleosomal DNA ladders, the AxCap21-infected or Ax1w1-infected RSF had no fragmented DNA (Fig. 2E). These results argue that the p21 Cip1 overexpression did not induce apoptotic cell death of RSF.

Treatment of rat AA with adenoviral p21 Cip1 gene transfer

Six rats were immunized with M. butyricum to induce AA. They were treated with injection of AxCap21 into the right knees and that of AxCALacZ into the left knees of the same animals. The gene transfer was carried out once (7 days after the immunization) or 3 times (8, 15 and 22 days after the immunization). Swelling of the knee joints was monitored during the disease course. The arthritis developed in all rats ~10 days after the immunization. Without treatment, the knee joints width became maximal ~20 days after the immunization and decreased afterwards. The knee joints treated with the control adenoviruses followed this course (Fig. 3). In comparison, the gene p21 Cip1 transfer significantly suppressed swelling of the knees whether it was carried out once or 3 times (Fig. 3, *P < 0.05). The therapeutic effect lasted throughout the experiment to a statistically significant extent when the gene transfers were repeated 3 times with an interval of 1 week between transfers. Although the gene transfer carried out

![Fig. 3. Effect of p21 Cip1 gene transfer on the joint swelling of rats with AA. The knee joints of rats with AA were treated with intra-articular injection of the AxCap21 (filled diamonds) or AxCALacZ (open diamonds) adenoviruses. The points and bars represent the mean ± SEM of six rats. Arrows represent the timing of the gene transfer. The gene transfer was carried out 3 times (A) or once (B). Spontaneous decrease of the joint around day 22 seemed to be attributed to fibrosis of the joints. The asterisks represent statistically significant differences (*P < 0.05) between the two groups.](image-url)
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Fig. 4. Effect of p21<sup>Cip<sup>1</sup> gene therapy on histopathology of AA. The arthritic joints treated 3 times with gene transfer were histologically examined 4 weeks after the immunization. The synovial tissue sections around the patellar ligaments were stained with hematoxylin & eosin. (A) The normal joint, (B) the saline-treated joint, (C) the AxCALacZ-treated joint, (D) the AxCAp21-treated joint, (E) the AxCALacZ-treated normal joint and (F) the saline-treated normal joint. p, patella; s, synovial tissue; f, femur; t, subpatellar tendon.

Effects of p21<sup>Cip<sup>1</sup> gene transfer on the histopathology of rat AA

The joints of the rats treated 3 times with the gene transfer were histologically examined 1 week after the last treatment. The synovial tissues of the knee joints from normal rats had one or two layers of synovial lining cells that were supported by loose fatty connective tissues (Fig. 4A). The arthritic joints treated with AxCALacZ or with saline had marked synovial thickening, which was accompanied by mononuclear cell

once also suppressed the joint swelling effectively, the effect became less clear after 2 weeks from the gene transfer.

To evaluate the effects of adenoviruses <i>per se</i>, the AxCALacZ viruses was injected into the right knee joints of normal rats. The same volume of saline was injected into the other knees. Either experiment induced no obvious joint swelling, and no difference in the width of the right and left knees was observed.
cells in the S phase, were calculated by examining three independent microscopic fields. They were significantly smaller in the AxCAP21-treated joints than in the AxCALacZ-treated joints (Fig. 6E).

Discussion

Our studies have shown that forced expression of the p21Cip1 gene inhibits cell growth of RSF without inducing their apoptotic cell death. In vivo transfer of the p21Cip1 gene into the synovial tissues suppressed the pathology of AA. These in vivo and in vitro effects were comparable to those of the p16INK4a gene transfer. Reduced frequency of the PCNA-expressing cells in the p21Cip1-treated synovial tissues showed that the cell cycling of the synovial cells was indeed inhibited in vivo.

As was reviewed before, both p16INK4a and p21Cip1 are associated with replicative senescence. However, their expression is differentially regulated. p21Cip1 inhibits a wider variety of CDKs than p16INK4a. Moreover, their other biological effects than inhibition of kinase activity are distinct (9,10,28). Disruption of the p16INK4a gene, but not that of the p21Cip1 gene, resulted in frequent tumor development in a murine model (29). Nevertheless, the p16INK4a and p21Cip1 gene therapy ameliorated AA equally. They inhibited synovial thickening, mononuclear cell infiltration, pannus formation and cartilage degeneration. Thus, cell cycle control of the synovial cells by the senescence-related CDK1 is effective to suppress pathology of the arthritis.

Generally, p21Cip1 keeps cells from undergoing the apoptosis when the cells respond to various apoptosis-inducing stimuli (30–32). However, some immortalized cell lines died with apoptosis when they overexpressed the p21Cip1 gene (15–18). The molecular events underlying the p21Cip1-induced apoptosis remain to be elucidated. In the rheumatoid synovial tissues, apoptotic cell death is observed commonly. However, when massive apoptosis of the synovial cells was induced artificially in animal models of RA, it ameliorated the pathology of the arthritis (33–35). Thus, p21Cip1 induction might have exerted therapeutic effects, at least partially, through induction of apoptosis.

In our experiments, the gene transfer was performed once or 3 times with an interval of 1 week between transfers. Repeated gene transfers were required to maintain the statistically significant therapeutic effects. This agrees with the observation that ectopic p21Cip1 gene expression after the in vivo gene transfer was detectable only for 2–3 weeks (data not shown). Thus, anti-arthritic effects required sustained expression of p21Cip1. It was reported that induction of the p21Cip1 gene in human fibrosarcoma cells affected expression of many genes (36). Although the adenoviral p21Cip1 gene transfer must have altered the gene expression by the synovial cells, the changes did not provide prolonged anti-rheumatic effects.

By p21Cip1 transfer, mononuclear cell infiltration was also ameliorated. Lymphoid cells are generally resistant to the adenoviral infection. Indeed, in our experimental procedures, the recombinant adenoviruses delivered the exogene mainly to the synovial lining cells, and to some of the fibroblast-like and macrophage-like cells at the sublining and stromal area.
Fig. 6. PCNA expression in the synovial tissues of the treated joints. The synovial tissues from the AxCAp21- (A and C) and AxCALacZ- (B and D) treated joints were stained with an anti-PCNA mAb (A and B) or with normal mouse IgG (C and D) as a control. Filled arrowheads and open arrowheads represent PCNA⁺ and PCNA⁻ nuclei respectively. PCNA LI were calculated, and are shown as columns and bars, representing the mean ± SEM (E). The difference was statistically significant ($P < 0.01$).

but not to lymphoid cells (4). Thus the reduction of the mononuclear cells should not be due to direct inhibition of their proliferation, but probably to overall suppression of the synovial inflammation.

It should be noted that the gene transfer protocol in the present study was not necessarily optimized. Adenoviral gene transfer was applied because it introduces exogenes to the synovial cells with high efficacy (37). The aim of the present studies was to investigate the therapeutic effect of the p21⁰ gene transfer in the inflamed synovial tissues. For actual application to human RA, the gene delivery method should be improved. Injection of the adenoviruses into joints could induce inflammatory responses (37). Indeed, we observed residual synovitis in the AxCAp21-treated joints and minimal mononuclear cell infiltration in the joints of normal rats treated with the control adenoviruses. Thus, the residual synovitis in AxCAp21-treated joints might be partly attributable to the inflammatory effects of the adenoviruses. Although the therapeutic effects of CDKI gene transfer surpassed the inflammatory effects of the adenoviruses, it is desirable to use less inflammatory variants of the adenoviruses (38).
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Little is understood about regulation of the p16INK4a gene expression. Inactivation of retinoblastoma gene product (pRB) by viral gene products promotes transcription of p16INK4a (39). However, responsive promoter elements for this induction are still unclear (39). A few methods that induce p16INK4a gene expression in pre-senescent cells include γ-irradiation, bleomycin and actinomycin D treatment of certain cell lines (5,40). In contrast, multiple compounds have been identified to induce p21Cip1 in many cell lines (41–45). The p21Cip1 gene is also induced by serum, growth factors and IL-6 in some cell lines (46,47). Thus, development of pharmaceuticals to induce p21Cip1 in vitro in the synovial tissues might be more feasible. However, unlike p16INK4a, p21Cip1 is readily induced in the fibroblasts of non-synovial origin (4,5,8). Since generalized p21Cip1 induction will probably impair homeostasis of the body, the induction should be confined within the inflamed synovial tissues by some means.

The gene therapy reported here aims at transforming synovial cells per se and is thus distinct from the previous trials, which were mostly to produce anti-inflammatory molecules in the joints (48–54). It was shown that the intra-articular delivery of the anti-inflammatory cytokine and cytokine antagonist genes could exert their effects on non-treated joints (54–56). Such a remote effect was not observed in our experiments (data not shown). However, this was expected because the products of the p21Cip1 gene should not be released from the virus-infected cells.

In theory, gene delivery of other cell-cycle regulators could be likewise effective in treating the arthritis. Nevertheless, as for the development of new anti-rheumatic drugs, p21Cip1 and p16INK4a appear to be attractive target candidates because they are inducible in RSF.

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Abbreviations

AA  adjuvant arthritis
CDK  cyclin-dependent kinase
CDKI  cyclin-dependent kinase inhibitor
LI  labeling indices
PCNA  proliferating cell nuclear antigen
PDGF  platelet-derived growth factor
RA  rheumatoid arthritis
RSF  rheumatoid synovial fibroblast
TNF  tumor necrosis factor

References


