**Keywords** Rheumatoid arthritis · Surgery · Arthroplasty · MHAQ · Cohort study

## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder which leads to varying degrees of functional impairment and disability. In spite of the recent progress in pharmacological treatment, some patients develop progressive joint destruction and eventually require surgery [1–4]. Total joint arthroplasty (TJA) is a highly successful procedure to treat destroyed joints in RA patients, and the effectiveness of the procedure on overall function and quality of life is now well established. Recently, the importance of certain inflammatory cytokines in the pathology of RA has become clear, and biologic agents targeting tumor necrosis factor-$\alpha$ (TNF-$\alpha$) are commonly used in clinical practice. There is accumulating evidence that the progression of joint erosion can be ameliorated by early and aggressive treatment using disease-modifying antirheumatic drugs (DMARDs) and biological agents [5–8], and therefore clinical remission and prevention of joint destruction have become realistic goals of RA treatment. Such a drastic evolution of the applicability of drug therapy now compels us to reconsider the proper indication and timing of surgical intervention in RA patients. To do this, it is important to verify the prevalence of TJA in RA patients undergoing conventional drug treatment. Previous studies have revealed that there are variations in the rates of TJA by region, sex, race and socioeconomic status [9–11]. However, a nationwide survey of the prevalence of TJA in Japanese RA patients has not been performed, in part because of the absence of a national registry of procedures or a national health insurance program in Japan. Since 2002, we have developed a nationwide observational cohort database of rheumatic diseases, NinJa (National Database of Rheumatic Diseases by iR-net in Japan), which is located in 33 institutions located throughout the country. The aim of the current study was to analyze the prevalence of TJA in RA patients on conventional drug treatment strategies in Japan by cross-sectional analysis using the NinJa database.

## Methods

### Data source

The data source employed in this study was a nationwide observational cohort database of rheumatic diseases in Japan, NinJa, which was previously described in detail [12]. The NinJa project was reviewed and approved by the National Hospital Organization research ethics committees, and all the patients participating in the study provided written informed consent. NinJa has been performing data collection from patients since 2002 in 33 institutions located throughout the country. All the patients included in the present study fulfilled the 1,987 classification criteria of the American College of Rheumatology. The data consist of two components; one is the patient information collected over the course of the year [outcome, death, hospitalization, operation, number of TJAs in large joints (hip, knee, shoulder, and elbow), malignancy, and tuberculosis], and the other is the information collected on an arbitrary day in daily clinical practice [the count of tender joints (TJC) and swollen joints (SJC), a modified health assessment questionnaire (MHAQ), patient's global and pain visual analog scales (VASs), doctor's VAS, ESR, CRP, disease activity score (DAS)28-ESR, DAS28-CRP, use of corticosteroids, DMARDs, and nonsteroidal anti-inflammatory drugs].

### Patients

To analyze the prevalence of TJA in large joints in RA patients, we examined the data for 5,177 RA patients who were enrolled in the NinJa database during the fiscal year of 2006 (from April 2006 to March 2007). The numbers of patients with disease durations of 0–5 years, 6–10 years, 11–15 years, 16–20 years, 21–25 years, 26–30 years and more than 31 years who had undergone TJA were counted, and the prevalences of TJAs were calculated.

### Analyses and statistics

Descriptive statistics were employed to analyze clinical information, demographic factors and other test data. Continuous variables were expressed as means and SD. Differences between groups were examined using a one-way analysis of variance (ANOVA) for continuous variables, or a $\chi^2$ test for categorized data when appropriate.

## Results

### The prevalence of TJA in Japanese RA patients

The proportion of operated patients increased in accordance with the duration of the disease (Fig. 1). The prevalences of TJA in the groups with disease durations of 0–5 years, 6–10 years, 11–15 years, 16–20 years, 21–25 years, 26–30 years and more than 31 years were 2.9%, 9.3%, 19.9%, 24,1%, 24.7%, 30.7% and 30.5%, respectively (Fig. 1). The prevalence of TJA increased in accordance with the disease duration. In particular, the prevalence markedly increased after ten years, and 5.5% of

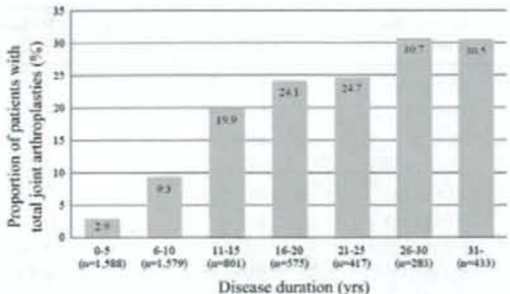


**Fig. 1** The relationship between the prevalence of joint replacement surgery and (the patient's) disease duration

the patients underwent TJA within ten years and 24.6% after more than ten years of disease duration, which was significantly different ($P < 0.001$).

Since the prevalence of TJA in patients markedly and significantly increased after ten years of disease, 2,695 patients with more than ten years of disease (patients >10 years) were subjected to further analysis (Fig. 1). There were 2,347 (87.1%) women and 348 men (12.9%). The mean age was 63.8 years old with a standard deviation (SD) of 10.8 (range 23–92), and the mean disease duration was 20.8 (SD 9.3) years (range 10–65). Of the 2,695 patients >10 years disease duration, 645 (24.6%) patients underwent 1,431 TJA in total. The number of surgeries performed per patient was one in 254 patients (39.3%), two in 240 patients (37.3%), three in 74 patients (11.5%), four in 67 patients (10.5%) and five in nine patients (1.4%). More than 60% of the patients who underwent TJA received two or more arthroplasty surgeries, and the mean number of surgeries per patient was 1.87. The patients with TJA exhibited a significantly longer disease duration, more TJC, and a higher proportion of oral corticosteroid usage than those without TJA. DAS28 was significantly higher in the patients with TJA than in those without (4.66 vs. 4.01, $P < 0.001$). In addition, ESR, CRP, MHAQ and pain VAS were significantly higher in the patients with TJA. In contrast, there was no significant difference in the age of disease onset, gender, SJC, DMARD frequency, or the daily dose of oral corticosteroid between the groups. These results indicate that the patients with a higher level of disease activity and poorer control of the disease require TJA.

## Discussion

In this cross-sectional study, we analyzed the prevalence of TJA in 5,177 Japanese RA patients who were enrolled in the NinJa database during 2006. The prevalence of TJA increased in accordance with the disease duration, and 5.5% of patients underwent TJA within ten years and 24.6% after more than ten years of disease duration. More than 60% of the operated patients with more than ten years of disease duration underwent more than one TJA, and the mean number of surgeries per patient was 1.87. These results are in line with the results of previous studies performed in European countries and in the USA [2–4, 13, 14]. Kapetanovic et al. reported that 24% of RA patients underwent primary TJA, and 46% of them underwent an additional arthroplasty during 16–20 years of follow-up [3]. Wolfe reported that 17.8% of RA patients underwent total joint arthroplasty over a mean disease duration of 15.9 years, and 25% of the patients seen within two years of disease onset were predicted to have a total joint arthroplasty within 23.1 years [4]. They also reported that the mean number of surgeries per patient was 1.85. Da Silva reported that 28.6% of RA patients underwent reconstructive surgery and Verstappen reported 27% in the Utrecht RA cohort [2, 14]. Based on a cross-sectional study, Hakala reported that 20% of RA patients required surgery within a decade of disease duration [13].

There are certain differences between this study and other reports. We were unable to find a gender difference in the prevalence of TJA, although some studies have reported that female RA patients have significantly more surgeries than male patients [2, 15]. Massardo et al. reported that the risk of joint surgery for RA-related joint disease was 1.4 times higher for women than men, mainly because of the greater frequency of small joint (hand and foot) surgery in women [15]. Da Silva also reported that female RA patients had more small joint surgeries than male patients [2]. James et al. commented that female gender was a risk factor for hand or foot joint surgery (the odds ratio being 3.2) [16]. We only analyzed the prevalence of large joint TJA and not that of small joint surgeries in the current study, which may explain the discrepancy between our results and the previous findings. James et al. also showed that a gender difference was not found in the prevalence of major surgeries (hip, knee, shoulder elbow replacement and spine surgery) (male 6.9% vs. female 7.3%) [16].

We were unable to find a correlation between the age of RA onset and the prevalence of surgery either. Consistent with our results, Kapetanovic also failed to show a gender or age difference between patients with and without large joint TJA [3]. This may be because surgeons avoid joint-sacrificing procedures such as TJA and instead select joint-preserving procedures, such as synovectomy, for younger patients. James et al. also reported that the ratio of intermediate to minor surgery (75.7%) in the patients with earlier onset (<45 years old) was larger than that (40.6%) in the patients with later onset (>60 years old) [16].

**Table 1** Demographic and disease characteristics for patients with more than 10 years of disease duration with or without total joint arthroplasty (TJA)

| | Patients with TJA ($n$ = 645) | Patients without TJA ($n$ = 2,050) | $P$ value |
|---|---|---|---|
| Age (years) | 65.1 (9.9) | 63.4 (11.0) | 0.001 |
| Gender | F = 586 | F = 1,761 | 0.346 |
| | M = 58 | M = 289 | |
| Age of disease onset (years) | 42.6 (12.1) | 43.2 (12.9) | 0.334 |
| Disease duration (years) | 22.5 (9.6) | 20.2 (9.1) | 0.001 |
| DAS28-ESR | 4.66 (1.19) | 4.01 (1.2) | 0.001 |
| Tender joint count | 6.3 (7.5) | 4.4 (6.3) | 0.001 |
| Swollen joint count | 3.3 (4.7) | 3.0 (4.0) | 0.075 |
| Pain VAS (cm) | 4.8 (2.5) | 3.7 (2.5) | 0.001 |
| MHAQ | 1.30 (0.80) | 0.72 (0.71) | 0.001 |
| ESR (mm/hr) | 53 (31) | 38 (26) | 0.001 |
| CRP (mg/L) | 16.4 (20.9) | 10.6 (15.0) | 0.001 |
| DMARDs usage (%) | 82.8 | 84.8 | 0.168 |
| Oral corticosteroid usage (%) | 74.2 | 65.4 | 0.001 |
| Daily amount of oral corticosteroid (mg/day) | 4.8 (3.4) | 4.7 (2.6) | 0.29 |

Data are presented as means (SD)

We found that the patients who had undergone TJA had higher disease activity. The mean DAS28 score of patients with TJA was 4.66, while the DAS28 of those without TJA was 4.01, which was significantly different ($P < 0.001$) (Table 1). The same was true of the other components such as pain VAS, MHAQ, ESR, CRP and the daily amount of oral corticosteroid (Table 1). These results suggest that patients who underwent TJA were under poorer disease control and suffered joint deterioration.

RA involves multiple joints, and therefore the indication of TJA should be determined not only by the function of the affected joints in isolation, but also the general status of the patient, including the functional conditions of other joints. We found a significant difference in MHAQ between patients >10 years with TJA and without TJA (1.3 vs. 0.72, $P < 0.001$). Consistent with our results, several previous reports have suggested that MHAQ is a risk factor for surgery in RA patients [3, 17–19]. As Anderson indicated in his review, "a patient who presents with a desire to improve his or her function is usually better motivated and will profit more by surgical intervention than an individual who is solely driven by an attempt to alleviate pain." [1].

Improved control of RA disease activity with new treatment strategies, such as early intervention using biologics combined with conventional DMARDs, is expected to reduce the extent of affected joints and the degree of joint destruction, and hence reduce the number of patients undergoing major joint surgery such as TJA. Several authors have in fact recently reported a decrease in orthopedic surgery with this type of aggressive treatment strategy [2, 20–23].

The limitation of the present study is that it is a retrospective cross-sectional analysis, and a prospective longitudinal study will need to be performed to identify the predictors of TJA in Japanese RA patients. In addition, most of the patients included in this study had been subjected to conventional treatment strategy and not to the early aggressive treatment recently recommended. However, the details of TJAs done under a conventional drug treatment strategy that are shown here provide basic data for discussing and ultimately constructing a new point of view concerning orthopedic intervention in the era of new drug treatment strategies in Japan.

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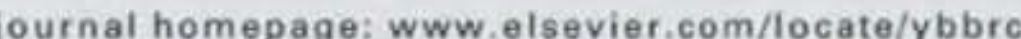

# Adiponectin stimulates IL-8 production by rheumatoid synovial fibroblasts

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ABSTRACT

The adipokines are linked not only to metabolic regulation, but also to immune responses. Adiponectin, but not leptin or resistin induced interleukin-8 production from rheumatoid synovial fibroblasts (RSF). The culture supernatant of RSF treated with adiponectin induced chemotaxis, although adiponectin itself had no such effect. Addition of antibody against adiponectin, and inhibition of adiponectin receptor gene decreased adiponectin-induced IL-8 production. Nuclear translocation of nuclear factor-kappa B was increased by adiponectin. The induction of interleukin-8 was inhibited by mitogen-activated protein kinase inhibitors. These findings suggest that adiponectin contributes to the pathogenesis of rheumatoid arthritis.

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Rheumatoid arthritis (RA) is a systemic, chronic, inflammatory disease that affects multiple joints. An important recent development in our understanding of RA is that patients with RA experience an acceleration of atherosclerosis that cannot be solely explained by the increased prevalence of traditional cardiovascular risk factors [1].

Adiponectin is an adipokine that is mainly secreted from white adipose tissue. It is believed to be beneficial because plasma levels of adiponectin are reduced in metabolic syndrome [2]. It has been reported that adiponectin exerts an anti-atherosclerotic effect [3,4]. Interestingly, recent studies have reported increased levels of adiponectin in RA patients [5,6], findings which appear paradoxical in light of the higher prevalence of atherosclerosis in RA. The existence of this paradox raises the possibility that adiponectin plays a novel role in the pathogenesis of RA. Moreover, the effect that adipokines have not only on the metabolic system, but also on inflammation, has recently become of clinical interest, and the role of adiponectin remains controversial [2,7,8].

RA is characterized by proliferative synovitis in multiple joints. The infiltration and activation of leukocytes results in progressive destruction of joint. Chemokines are pivotal in the recruitment of leukocytes and angiogenesis. In RA, IL-8 is an important chemokine, and high levels of IL-8 have been detected in RA patients [9,10]. In the present study, we examined the direct effect of adiponectin on IL-8 production by RSF.

## Materials and methods

*Reagents.* Reagents used in this study were purchased from the following sources; recombinant human full-length adiponectin from Biovendor (Brno, Czech Republic); recombinant human leptin from Sigma (Missouri, USA); recombinant human resistin from Axxora (CA, USA); human recombinant IL-8 from Chemicon (USA); Goat anti-human AdipoR1 and AdipoR2 antibodies from Santa Cruz Biotechnology (CA, USA); SB203580 from Sigma; SP600125 and PD98059 from Calbiochem (Darmstadt, Germany). Stock solutions were dissolved in phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO) when appropriate. The final concentration of DMSO in experiments was always <0.1% and control wells contained an equivalent concentration of the vehicle.

*Cell culture.* Rheumatoid synovial cells were prepared as described previously [11]. The synovial tissues were obtained from patients who fulfilled the 1987 revised criteria for RA [12] or patients who fulfilled the criteria for osteoarthritis [13]. Research protocol has approved by the ethics committee of Toho University (approval number: 16002, 19021). Synovial cells were cultured in RPMI 1640 with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) under the standard conditions.

*Evaluation of cytokine secretion.* RSF were cultured in 96-well plates for 24 h. The cells were then incubated with various concentrations of adipokines, human recombinant IL-1β (BD biosciences), or PBS in RPMI containing 1% FBS. Cytokine concentrations in the media were measured by enzyme-linked immunosorbent assay (ELISA) (IL-8, R&D systems, Minneapolis, USA; IL-1 β, IL-6, and TNF-α, BioSource International Inc., CA, USA).

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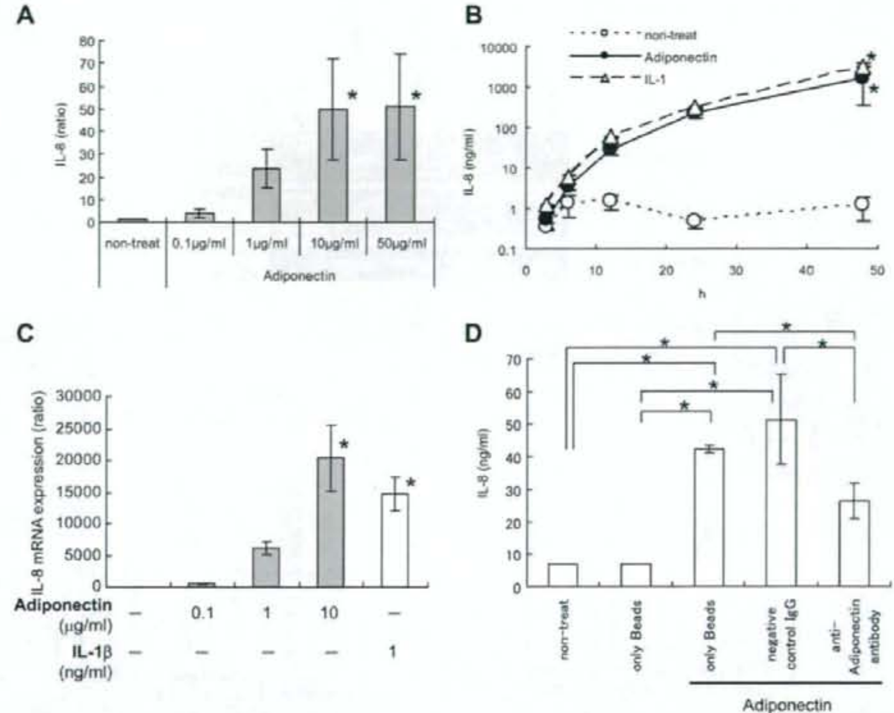


**Fig. 1.** IL-8 induction by adiponectin in RSF. (A) Cells were treated with each dose of adiponectin for 24 h and levels of IL-8 in culture supernatant were measured by ELISA. The IL-8 level of control (non-treat) was 0.11–10.03 ng/ml. $^*P < 0.05$ vs. non-treat. (B) Cells were treated with 10 μg/ml of adiponectin, 1 ng/ml of IL-1 (positive control), or PBS for 3, 6, 12, 24, or 48 h and levels of IL-8 were measured by ELISA. $^*P < 0.05$ vs. non-treat. (C) Expression of IL-8 mRNA as determined by real-time PCR. $^*P < 0.05$ vs. non-treat. (D) (From right) Adiponectin was incubated with anti-adiponectin antibody, negative control IgG, or PBS and with sepharose beads at 4 °C overnight. PBS incubated with/without sepharose beads were used as negative controls. Then supernatant was collected and added into cultured RSF. IL-8 concentration was measured by ELISA. $^*P < 0.05$.

*PCR.* Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen), and reverse-transcribed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). One microgram of each reverse-transcription product was used as a template. The primers of IL-8 (GenBank accession no. NM_000584.2) [14], AdipoR1 (GenBank accession no. NM_015999)/AdipoR2 (GenBank accession no. NM_024551) [15] and GAPDH (GenBank accession no. NM_002046) were purchased from Sigma. For real-time PCR, probes and primer pairs for IL-8 and β-actin were purchased from Applied Biosystems. The results were normalized for β-actin as an endogenous control.

*Western blot.* The cells were lysed in Triton lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and a protease inhibitor cocktail (Pierce Biotechnology). After electrophoresis of cell lysates, the SDS–PAGE separated proteins were electroblotted onto Immobilon-P poly (vinylidene difluoride) membranes with a semidry blotter (Atto, Tokyo, Japan). The membranes were probed with an appropriate primary antibody and a HRP-conjugated secondary antibody. Protein bands were detected with an enhanced chemiluminescence Western blot analysis system.

*Inhibition experiments of adiponectin with anti-adiponectin antibody.* Adiponectin was incubated with mouse anti-adiponectin monoclonal antibody (Chemicon), mouse monoclonal IgG1 negative control (Chemicon), or PBS and with Protein-G sepharose beads (GE Healthcare Bio-Sciences) at 4 °C overnight. Then supernatant was collected and added into RSF cultured in 96-well plates ($2 \times 10^4$ cells/well).

*RNA interference and transfection.* Small-interfering RNAs (*RNAi*) were designed and synthesized by Invitrogen. The sequences of the *RNAi* were as follows: AdipoR1: AAUAGACGGUGUGAAAGAGCCAGGA and UCCUGGCUCUUUCACACCGUCUAUU; AdipoR2: AGCAUGAUGGGCUUGUAAGAGAGGG and CCCUCUCUUACAAGCCCAUCAUGCU. The cultured RSF were transfected with 120 nM of *RNAi* by Lipofectamine RNAiMAX (Invitrogen). Cells transfected with non-functional *RNAi* (Invitrogen) were used as negative controls. Seventy-two hours after the transfection, cells were recultured for analysis by ELISA or PCR.

*Chemotaxis assay.* Polymorphonuclear cells (PMN) were prepared using Polymorphprep (Axis-Shield, Norway) and suspended in RPMI containing 1% FBS. For measurement of PMN migration, a 96-well chemotaxis chamber (Neuro Probe, USA) was used, as previously described [16]. The migrated cells were assessed by Cell Counting Kit (Dojindo Molecular Technologies, Kumamoto, Japan). The ratio of directed migration to random migration (*D*/*R* ratio) was calculated; *D*/*R* = total fluorescence of wells containing chemoattractant/total fluorescence of wells containing vehicle.

*Nuclear extracts and NF-κB assay.* Nuclear protein extraction from cultured RSF was performed with the nuclear extract kit (Active Motif, Tokyo, Japan). Nuclear extract was obtained from RSF treated with adiponectin (10 μg/ml), IL-1 β (1 ng/ml), or PBS for 3 h. Nuclear translocation of active NF-κB was assessed by ELISA (Active Motif, Tokyo, Japan).

*Statistical analysis.* All experiments were repeated as least three times using RSF obtained from three different patients. Differences

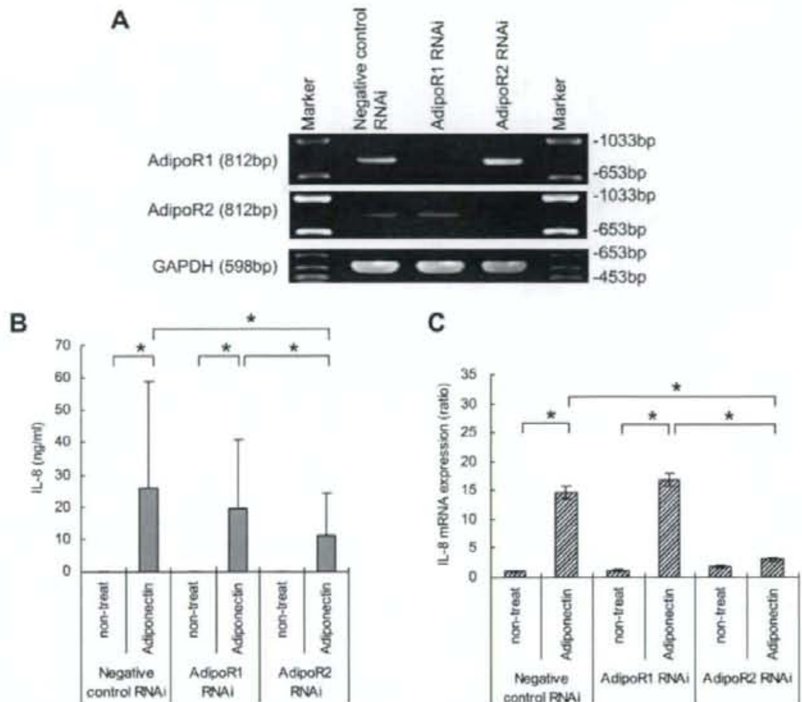


**Fig. 2.** Specific inhibition of adiponectin receptors by RNAi. (A) RSF were transfected with AdipoR1, AdipoR2, or Negative control RNAi as described (Methods), and mRNA levels of AdipoR1 or AdipoR2 was analyzed by RT-PCR. (B, C) After the transfection of RNAi, RSF were treated with 10 μg/ml of adiponectin or PBS for 24 h, and levels of IL-8 were measured by ELISA (B) and by real-time PCR (C). $^*P < 0.05$.

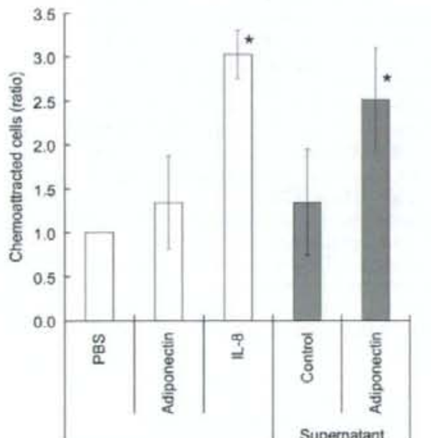


**Fig. 3.** PMN migration induced by supernatant of RSF treated with adiponectin. RSF were treated with 10 μg/ml of adiponectin or PBS; culture supernatants were collected after 24 h. Either 29 μl of vehicle, adiponectin (10 μg/ml), cell culture supernatants, or IL-8 dilutions (100 ng/ml) (positive control) was added to the bottom chamber, and 25 μl of PMN suspension was placed onto a filter. After incubation for 1 h, the $D/R$ ratio of cells that migrated into the bottom chamber was assessed as described (Methods). $^*P < 0.05$ vs. vehicle.

between groups were assessed by Tukey's test. The statistical analysis was performed using the SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

## Results

### *Expression of AdipoR1 and AdipoR2 on RSF*

To date, two specific adiponectin receptors—AdipoR1 and AdipoR2—have been cloned [17]. We performed RT-PCR and western blot to investigate whether the adiponectin receptors were present on RSF. Both mRNA and protein expression of these adiponectin receptors were detected in all RSF used in this study (data not shown).

### *Adiponectin-induced cytokine production by RSF*

After stimulation of RSF with adiponectin, leptin, or resistin, IL-8 levels in the culture supernatants were determined. A dose-dependent increase in IL-8 was detected in the culture supernatants of RSF treated with adiponectin, which suggests that adiponectin stimulated IL-8 production by RSF (Fig. 1A). A physiological concentration (10 μg/ml) of serum adiponectin induced the highest IL-8 production. However, physiological concentrations of leptin and resistin did not stimulate IL-8 production (data not shown). The IL-8 induction by adiponectin increased in a time-dependent manner (Fig. 1B). To confirm the IL-8 expression, real-time PCR were performed. Expression of IL-8 mRNA was dose-dependently increased by adiponectin (Fig. 1C). In addition to IL-

8 production. IL-6 production by adiponectin from RSF was observed (data not shown), as previously reported by others [18,19]. In contrast, neither of the cytokines IL-1β and TNF-α could be detected by ELISA.

IL-8 induction by adiponectin was inhibited by presence of anti-adiponectin antibody (Fig. 1D). To confirm IL-8 production by adiponectin, specific inhibition of adiponectin receptors expression was accomplished with RNAi (Fig. 2A). Treatment with AdipoR2 RNAi blocked the IL-8 production (Fig. 2B) and mRNA expression (Fig. 2C) by RSF, meanwhile AdipoR1 RNAi treatment had few effects.

We also examined the effect of adiponectin on IL-8 production in synovial fibroblasts obtained from patients with osteoarthritis. Adiponectin even concentration at 20 μg/ml weakly induced IL-8 production in synovial fibroblasts of osteoarthritis patients, which were less than one tenth when compared to the maximal responses of those in RSF (data not shown).

*Effect of adiponectin on chemotaxis of human polymorphonuclear cells*

We evaluated the biological effect of supernatant of RSF that were treated with adiponectin on chemotaxis of polymorphonuclear cells obtained from healthy human subjects. Adiponectin alone had no effect on chemotaxis; however, the culture supernatant of RSF treated with adiponectin induced significant chemotaxis of human polymorphonuclear cells *in vitro* (Fig. 3).

*Intracellular signaling*

As shown in Fig. 4A and B, nuclear translocation of p50 and p65 NF-κB was increased by adiponectin. A signal decrease was noted only in the presence of wild-type competitor oligonucleotides, which confirms that the assay specifically measures binding of p50 and p65 to their target sites. Other NF-κB family members, namely p52, RelB, and c-Rel, were not activated by adiponectin (data not shown).

The effects of SB203580, SP600125, and PD98059, which are MAPK inhibitors for p38, JNK, and ERK, respectively, were evaluated to investigate signal pathways (Fig. 5A–C). Production of IL-8 by adiponectin was significantly inhibited by the addition of SB203580 or SP600125. Although the effect of PD98059 was not significant, there was a tendency toward inhibition. These results suggest that adiponectin induces IL-8 from RSF via NF-κB and MAPK pathways.

## Discussion

In the present study, we demonstrated that adiponectin, but not leptin or resistin stimulated IL-8 production by RSF that express specific receptors for adiponectin. Addition of antibody against adiponectin, and inhibition of adiponectin receptor gene decreased adiponectin-induced IL-8 production. Supernatant obtained from RSF treated with adiponectin significantly induced chemotaxis of PMN; adiponectin alone had no such effect. These results support the hypothesis that adiponectin has a local proinflammatory role in RA. The core IL-8 promoter contains an NF-κB site and previous studies have highlighted the contribution of the MAPK pathways to IL-8 gene expression [20]. We noted that adiponectin induced nuclear translocation of activated p50 and p65 NF-κB and that MAPK inhibitors reduced adiponectin-induced IL-8 production by RSF.

Previous reports have shown that adiponectin exerts an anti-atherosclerotic effect on endothelial cells by inhibiting the expression of TNF-induced IL-8 [3] and adhesion molecules [4]. Adiponectin has also been found to inhibit endothelial NF-κB signaling [21], which may contribute to inhibition of monocyte adhesion to endothelial cells. These findings do not accord with our results, possibly because of differences in the cells and culture conditions used in the experiments. In contrast, Ehling et al. [18] showed that adiponectin induced IL-6 and matrix metalloproteinase-1 production by RSF, suggesting that adiponectin has the potential to promote arthritis, a findings confirmed by our data. Tang et al. [19] also showed that adiponectin induced IL-6 production by RSF. Although their reports indicated a significance of AdipoR1 in adiponectin-induced IL-6 production, our results suggested that AdipoR2 might be a major receptor involved in adiponectin-induced IL-8 production. Indeed, levels of adiponectin in synovial fluid and sera have been shown to be significantly

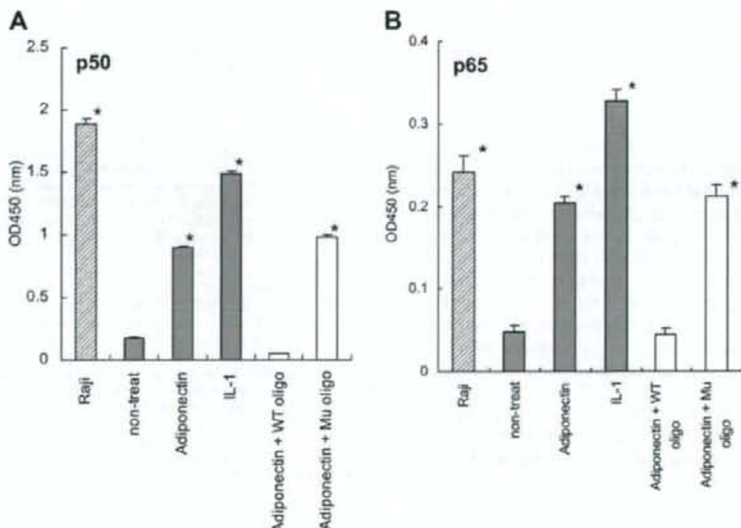


**Fig. 4.** Nuclear translocation of the activated NF-κB. Nuclear translocation of the activated (A) p50 and (B) p65 NF-κB were assessed by ELISA. RSF were treated with adiponectin, IL-1, or PBS for 3 h, and nuclear extracts were obtained. Raji nuclear extracts were used as positive controls. *$P < 0.05$ vs. non-treat. WT oligo, wild-type oligonucleotides; Mu oligo, mutated oligonucleotides.

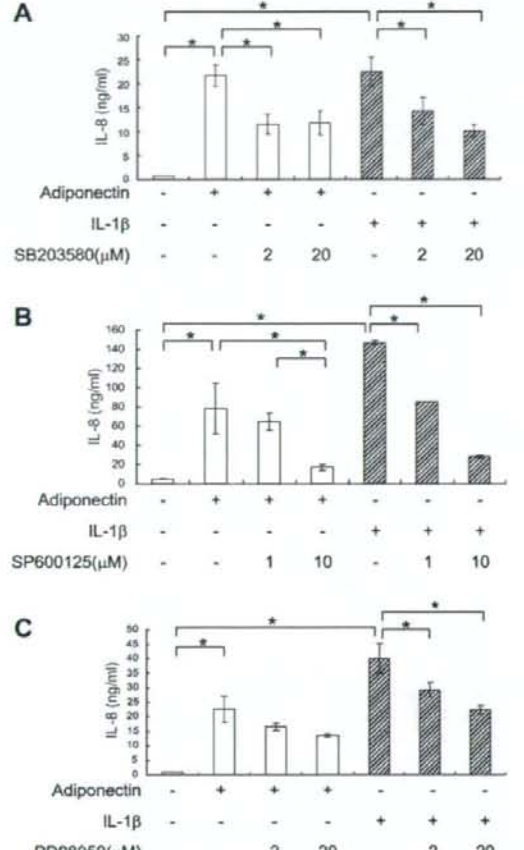


**Fig. 5.** Effect of MAPK inhibitors on adiponectin-stimulated IL-8 production. Cells were treated with (A) SB203580, (B) SP600125, or (C) PD98059 for 1 h prior to the treatment with 10 μg/ml of adiponectin, 1 ng/ml of IL-1, or PBS. IL-8 levels after 24 h were measured by ELISA. $^*P < 0.05$.

elevated in patients with RA [5,6,22], which indicates that adiponectin might have a role in the pathogenesis of RA.

Anti-TNF-α treatment has been reported to be associated with increase in adiponectin levels [23–25], however, this might merely reflect the fact that TNF-α negatively regulates adiponectin transcription [26]. In the present study, we examined the direct effect of adiponectin on RSF and found that adiponectin stimulated IL-8 production by RSF. This result supports the hypothesis that adiponectin alone has a proinflammatory effect on RSF. Some recent studies also have found that adiponectin promotes inflammation by inducing production of several proinflammatory cytokines [27,28].

In summary, we have shown that adiponectin significantly increases IL-8 production by RSF and supernatant of RSF treated with adiponectin induced PMN chemotaxis, suggesting that adiponectin might play a proinflammatory role in RA.

## Acknowledgments

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REVIEW ARTICLE

# Pro-apoptotic effect of nonsteroidal anti-inflammatory drugs on synovial fibroblasts

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**Abstract** Rheumatoid arthritis (RA) is a systemic inflammatory disease that mainly affects the articular synovial tissues. Although the etiology of RA has not yet been elucidated, physical and biochemical inhibition of synovial hyperplasia, which is the origin of articular destruction, may be an effective treatment for RA. Nonsteroidal anti-inflammatory drugs (NSAIDs) have long been used for the treatment of RA. The mechanism of action of NSAIDs generally involves the inhibition of cyclooxygenase (COX) at sites of inflammation. Thus, NSAIDs were not generally considered to have a so-called anti-rheumatic effect, including inhibition of progressive joint destruction and induction of remission. However, certain conventional NSAIDs and celecoxib, a selective COX-2 inhibitor, have been reported to inhibit synovial hyperplasia by inducing the apoptosis of human synovial fibroblasts. Therefore, it has been suggested that such NSAIDs may not only have an anti-inflammatory effect but also an anti-rheumatic effect. In this review, we summarize findings about the pro-apoptotic effect, in other words, anti-proliferative effect of NSAIDs on synovial fibroblasts from patients with RA.

**Keywords** Apoptosis · NSAIDs · Celecoxib · Synovial fibroblasts

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## Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease that mainly affects the articular synovial tissues. It is thought that an autoimmune response to the synovium is induced in RA patients when genetic factors are combined with various environmental ones, resulting in the occurrence of chronic inflammation. However, the etiology of RA has not yet been elucidated.

Guidelines for the management of RA by American College of Rheumatology have been published [1] and were also prepared in 2004 by a research group of the Japanese Ministry of Health, Labour and Welfare [2]. These guidelines cover a wide variety of drugs, including the new biological preparations, as well as glucocorticoids, disease-modifying anti-rheumatic drugs (DMARDs), and nonsteroidal anti-inflammatory drugs (NSAIDs) that have long been used for the treatment of rheumatic diseases [3]. Although the role of NSAIDs in the treatment of RA has been decreasing, these are still convenient drugs to employ for their anti-inflammatory and analgesic effects.

The mechanism of action of NSAIDs generally involves the inhibition of cyclooxygenase (COX) at sites of inflammation. As a result, these drugs exhibit a therapeutic effect by inhibiting the production of inflammatory mediators known as prostaglandins (PGs), including $PGE_2$ and $PGI_2$. In the treatment of RA, NSAIDs are used as symptomatic therapy with analgesic and anti-inflammatory effects mediated via the inhibition of COX. However, other mechanisms of action of NSAIDs except for COX inhibition have also been discussed [3]. For instance, certain NSAIDs have been reported to inhibit inflammation by suppression of nuclear factor (NF)-$\kappa$B due to I$\kappa$B-kinase $\beta$ inhibition in mononuclear cells [4]. In this review, we summarize data about the

pro-apoptotic effect of NSAIDs on synovial fibroblasts of patients with RA.

## Synovial proliferation and apoptosis

Healthy synovial tissue is essential for normal joint function. In RA, hyperplasia of the synovium and formation of granulation tissue (pannus) occur together with the infiltration of inflammatory cells, such as T cells and macrophages (Fig. 1). Activated pannus may eventually cause the destruction of bone and cartilage via the release of various mediators, including inflammatory cytokines such as interleukin (IL)-1$\beta$ and tumor necrosis factor (TNF)-$\alpha$ [5]. Synovial hyperplasia may be due to an increase in the proliferation of cells composing the pannus. This has been suggested by detection of the increased expression of various markers of proliferation and growth factors, including platelet-derived growth factor, basic fibroblast growth factor, and transforming growth factor-$\beta$ [6]. Another reason for the formation of pannus might be a decrease of apoptosis [7]. Physical and biochemical inhibition of synovial hyperplasia, which is the initial factor leading to articular destruction, may be effective treatments for RA [8].

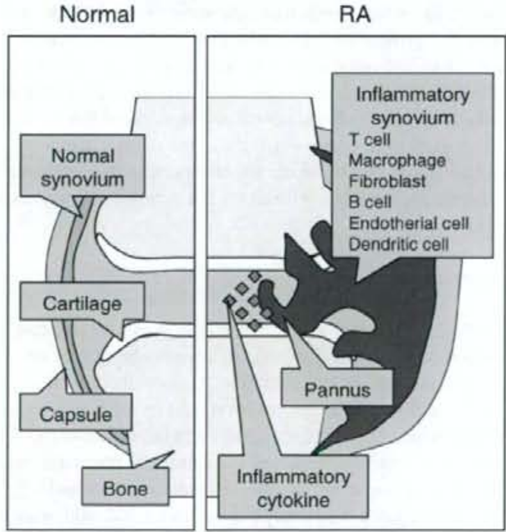


**Fig. 1** Synovial proliferation in rheumatoid arthritis (RA). In articular joint of RA, hyperplasia of the synovium and formation of granulation tissue (pannus), which were composed of inflammatory cells, such as T cells, macrophages, and B cells, secrete inflammatory mediators to articular cavity

Several molecules, which induce apoptosis in vitro have been reported as preventing agents in in vivo experimental models of RA. Peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) is an intranuclear transcription factor that promotes differentiation of adipocytes [9]. It also inhibits production of proinflammatory cytokines by macrophage [10]. On the other hand, Kawahito et al. [11] reported that articular destruction is significantly inhibited by administration of 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$), an endogenous ligand of PPAR$\gamma$, by induction of apoptosis on synovial fibroblasts in experimental arthritis of rats. Administration of an anti-Fas antibody that also induces apoptosis inhibits arthritis in mice [12, 13]. Stimulation of RA synovial fibroblasts by macrophage inhibitory factor (MIF) reduces apoptosis, while MIF knock-out mice have less severe arthritis due to increased apoptosis in the synovium [14]. Although these findings suggest the possibility of achieving an anti-rheumatic effect by inhibiting hyperplasia of the synovial tissues, there have been no clinical studies of pro-apoptotic agents such as PPAR$\gamma$ ligands and anti-Fas antibodies targeting this endpoint.

## Pro-apoptotic action of NSAIDs on synovial fibroblasts

### Conventional NSAIDs

First, we found that some of the conventional NSAIDs (inhibitors both of COX-1 and COX-2), indomethacin, diclofenac, oxaprozin, and zaltoprofen, all inhibited the proliferation of RA synovial fibroblasts by the induction of apoptosis, which was confirmed by detection of DNA fragmentation [15] (Fig. 2). On the other hand, ketoprofen, acetaminophen, and NS-398, a selective COX-2 inhibitor, did not induce apoptosis of RA synovial fibroblasts. Since PPAR$\gamma$ ligands such as 15d-$PGJ_2$ and troglitazone have a pro-apoptotic effect on synovial fibroblasts [11], we hypothesized that the mechanism of these pro-apoptotic NSAIDs was mediated by PPAR$\gamma$ activation.

Some conventional NSAIDs, such as ibuprofen, indomethacin, flufenamic acid, and fenoprofen, are known to cause the transcriptional activation of PPAR$\gamma$ in C3H10T1/2 mouse fibroblast cells [16]. In addition, activation of PPAR$\gamma$ was obtained by ibuprofen, indomethacin, and fenoprofen in human monocytes [10]. Kawahito et al. [11] reported that 15d-$PGJ_2$, an endogenous ligand of PPAR$\gamma$, ameliorate experimental arthritis of rats. Therefore, we investigated the effect of NSAIDs on PPAR$\gamma$ activity in RA synovial fibroblasts, one of the target cells in RA (Fig. 3). PPAR$\gamma$ activation was measured by luciferase reporter gene assay. Indomethacin, diclofenac, oxaprozin, and zaltoprofen induced PPAR$\gamma$ activation, while ketoprofen, acetaminophen, and NS-398,

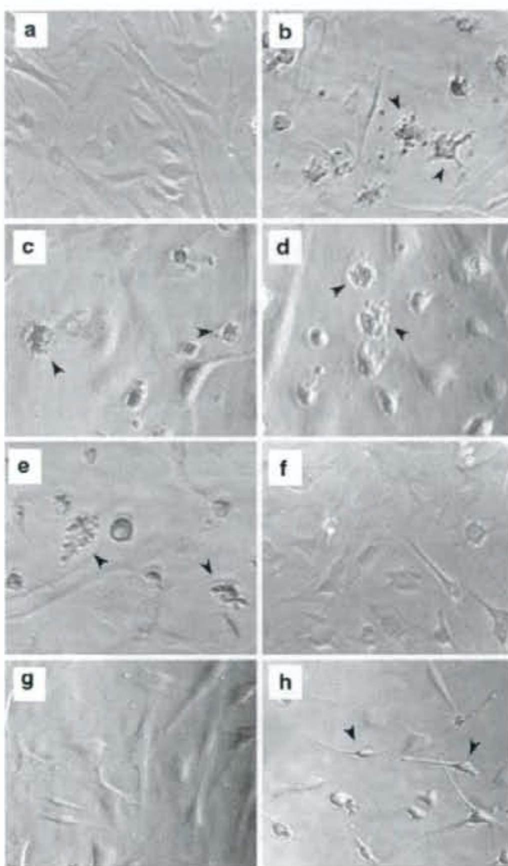


Fig. 2 Morphology of NSAID-treated rheumatoid synovial fibroblasts. Rheumatoid synovial cells were untreated (**a**) or treated with 300 μM indometacin (**b**), 100 μM diclofenac (**c**), 300 μM oxaprozin (**d**), 300 μM zaltoprofen (**e**), 300 μM ketoprofen (**f**), 300 μM acetaminophen (**g**), or 300 μM NS-398 (**h**) for 24 h. Cell morphology was observed with a light microscope. *Arrows* indicate representative morphological changes of synovial fibroblasts (×60). Reprinted from Yamazaki et al. [15], with kind permission from American Society for Pharmacology and Experimental Therapeutics

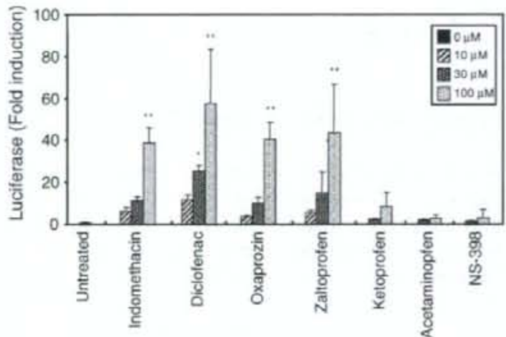


Fig. 3 Effect of NSAIDs on activation of peroxisome proliferator-activated receptor γ (PPARγ) in rheumatoid synovial cells. Rheumatoid synovial cells were cotransfected with a PPAR response element-driven luciferase reporter plasmid, PPARγ expression plasmid, and internal control plasmid. The transfected cells were treated with NSAIDs for 18 h. The fold-induction of luciferase activity is relative to untreated control cells. Data are the mean ± SD for triplicate cultures. Results are representative of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.001$ versus untreated control cells. Reprinted from Yamazaki et al. [15], with kind permission from American Society for Pharmacology and Experimental Therapeutics

which do not induce apoptosis of RA synovial fibroblasts, did not promote PPARγ activation. Furthermore, the ability of NSAIDs and PPARγ ligands to stimulate the activation of PPARγ correlated with their ability to decrease cell viability and ability to induce DNA fragmentation in synovial fibroblasts.

Then we studied sodium salicylate and aspirin, which are the historical NSAIDs, to assess their apoptosis-inducing effect on RA synovial fibroblasts [17]. At relatively higher concentrations comparable to those that cause COX inhibition, sodium salicylate and aspirin induced apoptosis of these cells. It was also suggested that these drugs induced apoptosis via a mechanism independent of COX inhibition. Sodium salicylate and aspirin are known as potent inhibitors of the transcription factor NF-κB [18], and it has been shown that inhibition of the NF-κB pathway by pyrrolidinedithiocarbamate or N-acetylcysteine is linked to the induction of apoptosis in a variety of cells [19–21]. However, our additional study revealed that these inhibitors of NF-κB did not cause apoptosis of RA synovial fibroblasts [17]. Moreover, salicylates did not promote the activation of PPARγ in our experiments, so the mechanism of their pro-apoptotic effects on RA synovial fibroblasts is still unknown.

## Celecoxib

COX-2 is an isozyme of COX that is markedly induced by inflammatory stimuli and is considered to be closely related to the process of inflammation. Celecoxib, which selectively inhibits COX-2, was developed by investigating the 3D structure of COX-2. In addition to inhibition of COX-2, celecoxib has been reported to inhibit the proliferation of various cancer cells, mainly by inducing apoptosis [22]. We investigated the effect of selective COX-2 inhibitors on apoptosis in RA synovial fibroblasts [23] (Fig. 4). Among six selective COX-2 inhibitors (celecoxib, etodolac, meloxicam, nimesulide, NS-398, and rofecoxib), only celecoxib induced the apoptosis of RA synovial fibroblasts, whereas the other COX-2 inhibitors did not. This indicated

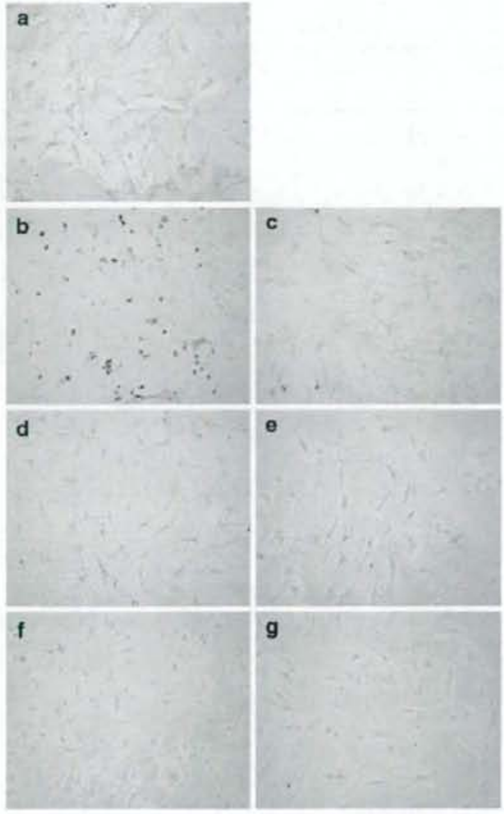


Fig. 4 Detection of apoptosis in RA synovial fibroblasts, by the TUNEL assay. Cells were incubated for 24 h. **a** Without any COX-2 inhibitors or with **b** celecoxib (40 μM), **c** etodolac (100 μM), **d** meloxicam (100 μM), **e** nimesulide (100 μM), **f** NS-398 (100 μM), or **g** rofecoxib (100 μM). Apoptotic cells exhibiting TUNEL staining are brown; normal cells counterstained with methyl green are blue (×200). Reprinted from Kusunoki et al. [23], with kind permission from John Wiley & Sons, Inc.

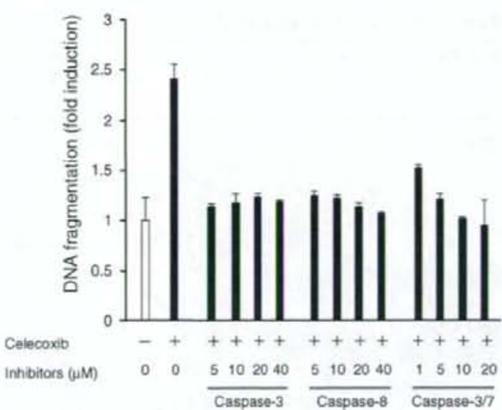


Fig. 5 Effect of caspase inhibitors on celecoxib-induced DNA fragmentation in RA synovial fibroblasts. Cells were incubated with celecoxib (40 μM) and with caspase inhibitors [Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (a caspase-8 inhibitor), and Z-VAD-FMK (a caspase-3/7 inhibitor)] for 24 h at the indicated concentrations, after which DNA fragments in the cytoplasm were measured by enzyme immunoassay. The fold-induction of DNA fragmentation is shown relative to the control value (untreated cells). Representative results from two independent experiments are shown; values are the mean and SD from triplicate cultures. Reprinted from Kusunoki et al. [23], with kind permission from John Wiley & Sons, Inc.

that the pro-apoptotic effect of celecoxib on RA synovial fibroblasts was independent of COX-2 inhibition. This pro-apoptotic effect was suppressed by caspase inhibitors (Fig. 5). In addition, celecoxib did not cause transcriptional activation of PPARγ in RA synovial fibroblasts.

Epidemiological studies have shown that chronic intake of aspirin is associated with a reduction in the incidence of colorectal cancer [24]. NSAIDs have also been shown to exert a pro-apoptotic effect on various cell lines, particularly colon cancer cells [25]. We previously investigated the pro-apoptotic effect of six selective COX-2 inhibitors indicated above on human colorectal cancer cells, and found that only celecoxib induced apoptosis again, which was induced via a mechanism that was unrelated to COX inhibition [26]. We found that celecoxib reduced the phosphorylated Akt, an anti-apoptotic molecule, in colon cancer cell lines [26]. Several NSAIDs such as indomethacin [27], dicrofenac [28], salicylic acid [29], etodolac [30], nimesulide [31], and NS-398 [32] inhibited Akt activation in vitro experiments using cancer cell lines. Celecoxib alters intracellular calcium by inhibiting $Ca^{2+}$ ATPases in the endoplasmic reticulum [33], and blocks TNF-induced activation of NF-κB [34]. However, these intracellular changes induced by NSAIDs were not consistent among several different cell types. The mechanisms of pro-apoptotic effects of NSAIDs on cancer cells as well as synovial fibroblasts are still remained to be studied.

## TT101, a new derivative of celecoxib

Although celecoxib suppressed the proliferation of RA synovial fibroblasts and induced apoptosis at the optimal concentrations were higher (10–40 μM) compared with those for COX-2 inhibition (0.01–10 μM) [23]. The mean maximum plasma concentration of celecoxib in healthy volunteers was reported to be 1.4, 2.5, and 7.7 μM after single doses of 100, 400, and 800 mg, respectively [35], showing that insufficient concentrations for pro-apoptotic effect on RA synovial tissue. Therefore, we tried to develop potent inducer of apoptosis by modification of the

**Table 1** Properties of bioactivities of several NSAIDs

| NSAIDs | Structure | Pro-apoptotic effect on RSF | Activation of PPARγ | Inhibitory effect of Akt activation | COX-1 $IC_{50}$ (μM) | COX-2 $IC_{50}$ (μM) | COX-1/COX-2 ratio |
|---|---|---|---|---|---|---|---|
| Indomethacin | | ++ [15] | + [15] | + [27] | 0.013 [15] | 0.044 [15] | 0.30 |
| Diclofenac | | ++ [15] | + [15] | + [28] | 0.076 [44] | 0.026 [44] | 2.92 |
| Oxaprozin | | ++ [15] | + [15] | NT | 2.2 [15] | 36 [15] | 0.061 |
| Zaltoprofen | | ++ [15] | + [15] | NT | 1.3 [15] | 0.34 [15] | 3.82 |
| Ketoprofen | | − [15] | − [15] | − [38] | 0.11 [15] | 0.88 [15] | 0.13 |
| Acetaminofen | | − [15] | − [15] | − [39] | 42 [15] | 11 [15] | 3.81 |
| Aspirin | | ++ [17] | − [17] | − [38] | 1.7 [45] | 7.5 [45] | 0.23 |
| Salicilic acid | | ++ [17] | − [17] | + [29] | 4,956 [45] | 34,440 [45] | 0.14 |

**Table 1** continued

| NSAIDs | Structure | Pro-apoptotic effect on RSF | Activation of PPARγ | Inhibitory effect of Akt activation | COX-1 $IC_{50}$ (μM) | COX-2 $IC_{50}$ (μM) | COX-1/COX-2 ratio |
|---|---|---|---|---|---|---|---|
| Celecoxib | | ++ [23, 36] | − [23] | + [26] | 82 [44] | 0.0032 [36] | 25,625 |
| TT101 | | +++ [36] | NT | − [36] | NT | 0.31 [36] | NT |
| TT201 | | + [36] | NT | − [36] | NT | 0.13 [36] | NT |
| SC236 | | ++ [36] | + [40] | + [41] | NT | 0.0071 [36] | NT |
| Rofecoxib | | − [23] | NT | NT | >100 [44] | 0.048 [36] | >2,083 |
| Etodolac | | − [23] | NT | + [30] | >100 [44] | 53 [44] | >1.89 |
| Meloxicam | | − [23] | − [42] | NT | 37 [44] | 6.1 [44] | 6.07 |

**Table 1** continued

| NSAIDs | Structure | Pro-apoptotic effect on RSF | Activation of PPARγ | Inhibitory effect of Akt activation | COX-1 $IC_{50}$ (μM) | COX-2 $IC_{50}$ (μM) | COX-1/ COX-2 ratio |
|---|---|---|---|---|---|---|---|
| Nimesulide | | − [23] | − [43] | + [31] | 10 [45] | 1.9 [45] | 5.26 |
| NS-398 | | − [15, 23, 36] | − [15, 23] | + [32] | 125 [44] | 0.012 [36] | 10,416 |

*NT* not tested

**Fig. 6** Chemical structure of celecoxib and its derivatives

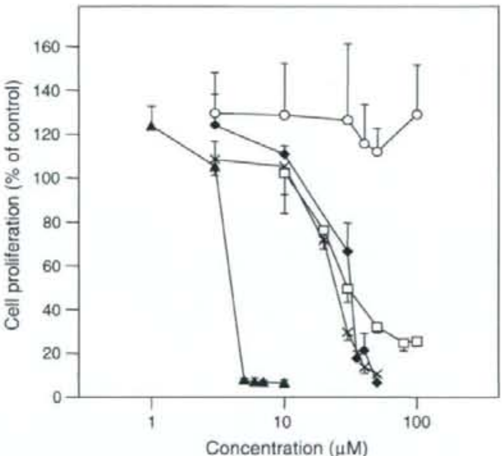


**Fig. 7** Effect of the each drug on the proliferation of synovial fibroblasts obtained from patients with RA. Cells were incubated with celecoxib (*closed diamonds*), TT101 (*closed triangles*), TT201 (*open squares*), SC-236 (*crosses*), or rofecoxib (*open circles*) for 24 h. Then proliferative activity was estimated from the nuclear incorporation of BrdU and was expressed as a percentage of the control value (untreated cells). Data are the mean ± SD for triplicate cultures, and representative results from three independent experiments are shown. Reprinted from Kusunoki et al. [36], with kind permission from American Society for Pharmacology and Experimental Therapeutics

structure of celecoxib. We synthesized two celecoxib derivatives (TT101 and TT201) and analyzed their pro-apoptotic effect on RA synovial fibroblasts [36].

We summarized properties of several NSAIDs from the view point of bioactivities and structures (Table 1). The sulfonamide group of celecoxib was changed to an N-(2-aminoethyl)-sulfonamide group when developing TT101, whereas the tolyl group in the terminal aromatic ring of celecoxib was changed to an aminophenyl group to create TT201 (Fig. 6). Interestingly, TT101 was more potent with respect to suppression of hyperplasia (Fig. 7) and induction of apoptosis (Fig. 8) in RA synovial fibroblasts when compared to celecoxib. NSAIDs without sulfonamide group,

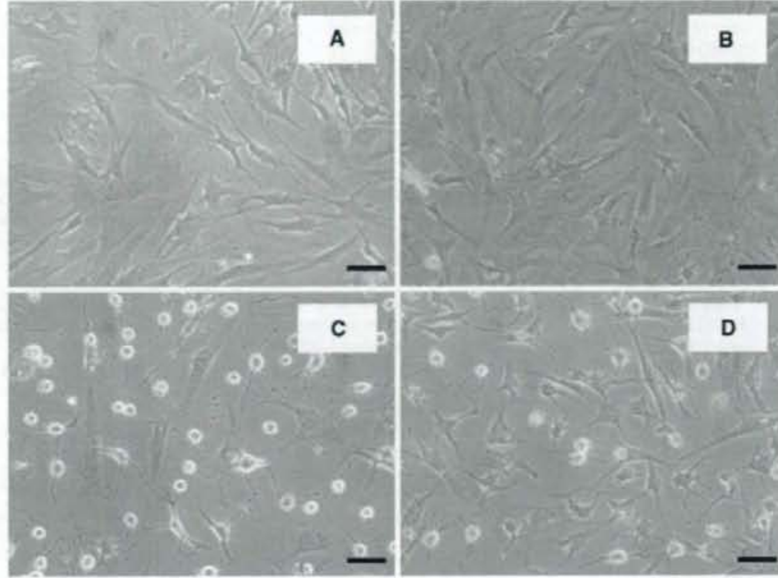


**Fig. 8** Morphological changes of the synovial fibroblasts from RA patients (**a** and **c**) or osteoarthritis patients (**b** and **d**) as observed by light microscopy. Cells were incubated for 24 h without (**a** and **b**) or with (**c** and **d**) TT101 at a concentration of 7 μM. *Bar* 60 μm. Reprinted from Kusunoki et al. [36], with kind permission from American Society for Pharmacology and Experimental Therapeutics

such as indomethacin, diclofenac, oxaprozin, zaltoprofen, also induced apoptosis in RA synovial fibroblasts. However, they activated PPAR$\gamma$ in the cells, while celecoxib did not. A pro-apoptotic effect of TT201 was weaker than that of celecoxib. Therefore, conformations of TT101 and celecoxib except sulfonamide group are possibly important to maintain pro-apoptotic effect. We also measured the COX-2 inhibitory effect of these compounds in RA synovial fibroblasts and found that the order of potency for the COX-2 inhibition by these drugs was celecoxib > TT201 > TT101 [36]. The potent pro-apoptotic effect of TT101 was also observed in colon cancer cell lines [37]. Although the mechanism of action of TT101 remains unclear, it may have potential as a novel anti-proliferation drug for rheumatoid synovial fibroblasts and colon cancer cells.

## Conclusion

Pro-apoptotic effects of NSAIDs including conventional NSAIDs, celecoxib and a derivative of celecoxib were reviewed. Although additional studies are needed, these results suggest that the induction of apoptosis caused by some NSAIDs may help to prevent the degradation of articular cartilage in RA after the inhibition of synovial hyperplasia and pannus formation.

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**Competing interests** S.K. has served as consultants to and/or received honoraria from Pfizer Japan (Tokyo), the manufacture relatives of celecoxib, and Astellas Pharma (Tokyo), the selling company of celecoxib. N.K. and S.K. hold a patent for TT101 and TT201. R.Y. has no competing interests.

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