Research of apoptosis inducing effect on synovial fibroblasts from rheumatoid arthritis by celecoxib, its derivative, and triptolide

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List of publications

As for each chapter of this dissertation, the content reported to the following science journals is mainly composed.

Chapter 1

Kusunoki N., Yamazaki R., Kawai S., Induction of apoptosis in rheumatoid synovial fibroblasts by celecoxib, but not by other selective cyclooxygenase 2 inhibitors., Arthritis Rheum., 46, 3159-3167, 2002.

Chapter 2

Kusunoki N., Ito T., Sakurai N., Suguro T., Handa H., Kawai S., A novel celecoxib derivative potently induces apoptosis of human synovial fibroblasts., J Pharmacol Exp Ther., 314, 796-803, 2005.

Chapter 3

Kusunoki N., Yamazaki R., Kitasato H., Beppu M., Aoki H., Kawai S., Triptolide, an active compound identified in a traditional Chinese herb, induces apoptosis of rheumatoid synovial fibroblasts., BMC Pharmacol., 4, 2, 2004.

List of abbreviations

In this dissertation, following abbreviations were used.

$15dPGJ_2$	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
BCA	bicinchoninic acid
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
COX	cyclooxygenase
CRP	C-reactive protein
DMARDs	disease modifying anti-rheumatic drugs
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ESR	erythrocyte sedimentation rate
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
GTW	multi-glycoside chloroform/methanol extract of TWHF
HPLC	high performance liquid chromatography
IC_{50}	50 % inhibitory concentration
IL	interleukin
NF-κB	nuclear factor-κB
NSAIDs	nonsteroidal anti-inflammatory drugs
OA	osteoarthritis
PBS	phosphate-buffered saline (-)

PG	prostaglandins
PPARγ	peroxisome proliferator-activated receptor γ
PPRE	peroxisome proliferator response element
RA	rheumatoid arthritis
SEM	standard error mean
S.D.	standard deviation
TBS-T	Tris buffered saline containing 0.1 %(v/v) Tween 20
TNFα	tumor necrosis factor α
TRAIL	TNF-related apoptosis inducing ligand
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
TWHF	Tripterygium wilfordii Hook F
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt
Z-DEVD-FMK	benzyloxycarbonyl-Aspartic acid(OMe)-Glutamic acid(OMe)-Valine - Aspartic acid(OMe)-fluoromethyl ketone
Z-IETD-FMK	benzyloxycarbonyl-Isoleucine-Glutamic acid(OMe)-Threonine-Aspartic acid(OMe)-fluoromethyl ketone
Z-LEHD-FMK	benzyloxycarbonyl-Leucine-Glutamic acid(OMe)-Histidine-Aspartic acid(OMe)-fluoromethyl ketone
Z-VAD-FMK	benzyloxycarbonyl-Valine-Alanine-Aspartic acid(OMe)-fluoromethyl ketone

General Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease that mainly affects articular synovial tissue. It is thought that autoimmunity is induced in RA patients when hereditary factors are combined with various environmental ones, resulting in the onset of chronic inflammation. However, the etiology has not yet been elucidated completely, and prevention is still impossible. RA occurs all around the world and in every race. This disease can develop at any age. Since there is no reliable diagnostic test for RA, the incidence and prevalence of this disease have been investigated by using certain diagnostic criteria that were developed for the purpose of disease classification. The most commonly used criteria are the American Rheumatism Association Criteria for Classification of Rheumatoid Arthritis (revised in 1987)[1]. The prevalence of RA is estimated to be 0.3-1.5 %, although it varies depending on the strictness of the criteria used[2]. If this range is applied to the Japanese population, the number of RA patients in Japan can be estimated as 380,000 to 1,900,000. The results of a recent survey conducted by a research group of the Japanese Ministry of Health and Welfare suggested that RA patients account for 0.54 % of females and 0.11 % of males, and that the total number of RA patients is around 700,000.

Guidelines for treatment of RA were prepared and published in 2004 by a research group of the Japanese Ministry of Health, Labour and Welfare. These new guidelines cover a great variety of drugs, including the newly developed biological preparations, as well as steroids, disease-modifying anti-rheumatic drugs (DMARDs), and nonsteroidal anti-inflammatory drugs (NSAIDs) that have long been used for the treatment of rheumatic disease[3]. Although the importance of NSAIDs in the treatment of RA has been decreasing, they are still convenient drugs to employ for an anti-inflammatory and

analgesic effect.

The progression of articular lesions in RA can be classified into three stages: onset of autoimmunity, synovial inflammation, and articular destruction. With the onset of autoimmunity, there is accumulation of immunocompetent cells, such as lymphocytes (mainly $CD4^+$ T cells) and macrophages in the synovium, which is followed by the development of synovial inflammation. Synovial inflammation is classified into acute inflammation and chronic hyperplasia of the synovial membrane associated with neovascularization. Synovial tissue is essential for normal joint function. In RA, there is hyperplasia of the synovium and formation of granulation tissue (pannus) together with infiltration of inflammatory cells, such as T cells and macrophages. Formation of pannus is presumably ascribable to an imbalance between the factors promoting hyperplasia, including various cytokines, and antihyperplastic factors that include the induction of apoptosis[4]. Activated pannus may eventually cause the destruction of bone and cartilage via the release of various mediators, including inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)α[5]. Effective drugs for each of these stages of joint involvement have been developed. Physical and biochemical inhibition of synovial hyperplasia as the starting point of articular destruction may be an effective radical treatment for RA[6].

It was reported that articular destruction was significantly inhibited when 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ$_2$), an endogenous ligand of peroxisome proliferator-activated receptor (PPAR)γ, which is an intranuclear transcription factor that induces apoptosis of RA-derived synovial fibroblasts, was administered to rats with arthritis[7], while arthritis in mice was inhibited by administration of an anti-Fas antibody that induces apoptosis[8]. Although these findings suggest the possibility of

achieving an antirheumatic effect by inhibiting hyperplasia of the synovial membrane, there have been no clinical studies targeting this endpoint.

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 prostaglandin (PG)s, including PGE_2 and PGI_2 (representative inflammatory mediators). In the treatment of RA, NSAIDs are used as adjuvant therapy with an analgesic and anti-inflammatory effect via inhibition of COX. These agents were not considered to have a so-called anti-rheumatic effect, including inhibition of progressive joint destruction and induction of remission, unlike some anti-rheumatic drugs. However, certain NSAIDs have been reported to inhibit synovial hyperplasia by inducing the apoptosis of human synovial fibroblasts. We previously found that some conventional NSAIDs (diclofenac, indometacin, oxaprozin, and zaltoprofen) induced the apoptosis of synovial fibroblasts from RA patients via activation of PPARγ and thus inhibited hyperplasia of these cells[9]. We also demonstrated that aspirin and sodium salicylate could induce apoptosis of RA synovial fibroblasts, although only at higher concentrations[10]. As mentioned above, several NSAIDs have not only exhibited an analgesic/anti-inflammatory effect, but have also show an inhibitory effect on synovial fibroblast hyperplasia in several fundamental studies. However, these drugs only exhibited an apoptosis-inducing effect when administered at a relatively high concentration. Therefore, if agents with a stronger activity were developed, it will be possible to inhibit synovial hyperplasia.

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 three-dimensional

structure of COX and it has been marketed in more than 70 countries. In addition to inhibition of COX-2, celecoxib has been reported to inhibit the proliferation of cells from various cancers mainly by inducing apoptosis[11-16]. We previously investigated the apoptotic effect of six selective COX-2 inhibitors on human colorectal cancer cells, and found that only celecoxib induced apoptosis, which was induced via a direct effect and was unrelated to COX inhibition[17].

Based on these findings, drugs inhibiting the hyperplasia of synovial fibroblasts from RA patients were investigated in the present study. In chapter 1, six selective COX-2 inhibitors (including celecoxib) were compared with respect to the induction of apoptosis and inhibition of PGE_2 production. In chapter 2, a novel agent (TT101) that was developed based on investigation of celecoxib, was assessed for the same parameters. In chapter 3, the apoptotic activity of triptolide, an active ingredient of Tripterygium wilfordii Hook F (TWHF: used clinically in China for its antirheumatic activity) was investigated using synovial fibroblasts from RA patients.

Chapter 1 Effects of selective COX-2 inhibitors

1. Introduction

Arachidonic acid is released from the cell membrane phospholipids by phospholipase A_2. COX is a rate-limiting enzyme that metabolizes arachidonic acid to various physiologically active substances such as PGs. Several studies have shown that COX has 2 isoenzymes, which differ with respect to their basal expression, tissue localization, and induction during inflammation[18,19]. The existence of 2 COX isoenzymes with differing characteristics may help to explain differences in the pharmacological profiles of various NSAIDs and may also have important clinical consequences. COX-1 is constitutively expressed by various cells, whereas COX-2 is an inducible enzyme and is found predominantly at sites of inflammation. It has been proposed that the inhibition of COX-1 in gastric mucosal cells and the resultant decrease of PG synthesis leads to weakening of local mucosal protective mechanisms and accounts for the significant gastrointestinal toxicity of NSAIDs[20]. COX-2 shows an increase in activity with the onset of inflammation that leads to increased production of PGs, so it should theoretically be a better therapeutic target than COX-1[21].

The recently developed selective COX-2 inhibitors (coxibs) are now being used as anti-inflammatory agents to treat patients with RA and/or osteoarthritis (OA), and are associated with a lower incidence of gastrointestinal complications compared with conventional NSAIDs. Two large-scale clinical trials of celecoxib[22] and rofecoxib[23] have provided evidence that these coxibs can reduce the incidence of severe upper gastrointestinal toxicity in patients with RA and/or OA.

It is generally accepted that NSAIDs regulate inflammation via the inhibition of COX-2, but some novel mechanisms of action have recently been discussed. Jiang[24] et

al. found that activation of PPARγ, a nuclear transcription factor, inhibited the production of cytokines in human monocytes. They reported that several PPARγ ligands, including NSAIDs such as indometacin, could activate this receptor. We recently found that several NSAIDs, such as indometacin, diclofenac, and oxaprozin, induced apoptosis of RA synovial fibroblasts in association with PPARγ activation[9]. In contrast, results reported by Yin[25] et al. suggest that aspirin and sodium salicylate may suppress inflammation not only by inhibition of COX, but also by inhibition of inhibitor of nuclear factor (NF)-κB kinase -β in Jurkat cells. These findings suggest the existence of other mechanisms besides COX inhibition for the antirheumatic activity of NSAIDs.

RA is characterized by extensive inflammation and proliferation of the synovium in various joints. Nishioka[26] et al. have suggested that stimulation of proliferation by TNFα and induction of apoptosis by Fas ligand play an important role in regulating the growth of rheumatoid synovial tissue. Although NSAIDs (even selective COX-2 inhibitors) are generally considered to have little disease-modifying effect in RA, some NSAIDs have been reported to suppress the proliferation of cells by induction of apoptosis. Selective COX-2 inhibitors are relatively safe drugs, even at higher doses. Accordingly, I investigated whether selective COX-2 inhibitors could cause apoptosis of RA synovial fibroblasts.

2. Results

2-1 Effects of selective COX-2 inhibitors on prostaglandin E_2 production.

I investigated the effect of six selective COX-2 inhibitors on PGE_2 production by synovial fibroblasts from patients with RA. As shown in Figure 1, selective COX-2 inhibitors (0.01-10 μM) caused suppression of PGE_2 production stimulated by the addition of 3 μM arachidonic acid, and the suppression was concentration dependent.

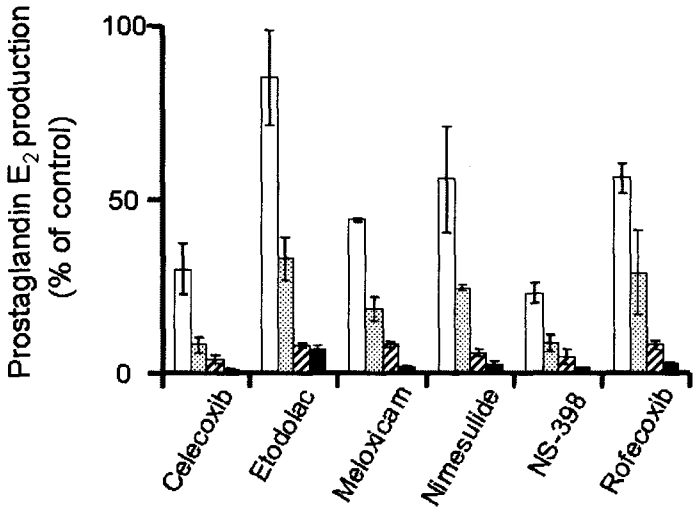

Figure 1. Effects of selective cyclooxygenase 2 (COX-2) inhibitors on prostaglandin E_2 (PGE_2) production by rheumatoid arthritis (RA) synovial fibroblasts. Cells were incubated for 1 hour at 37°C with various concentrations of each selective COX-2 inhibitor (open bars = 0.01 μM; dotted bars = 0.1 μM; hatched bars = 1 μM; solid bars = 10 μM). Arachidonic acid (3 μM) was then added and incubation was continued for another 30 minutes, after which the PGE_2 level in the culture medium was measured by enzyme immunoassay. Representative results from 3 independent experiments are shown; values are the mean ± S.D. from triplicate cultures. The PGE_2 level in the control cultures was 12.3 ± 1.3 ng/mL.

2-2 Effects of selective COX-2 inhibitors on cell proliferation

To determine whether selective COX-2 inhibitors had an antiproliferative effect on RA synovial fibroblasts, I examined the effect of these drugs on cell proliferation (DNA synthesis) by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) (Figure 2). Celecoxib suppressed the proliferation of RA synovial fibroblasts in a concentration

dependent manner and strongly inhibited cell proliferation, while etodolac, meloxicam, nimesulide, and NS-398 had some inhibitory effect at higher concentrations. Rofecoxib at concentrations up to 100 μM had no effect on cell proliferation.

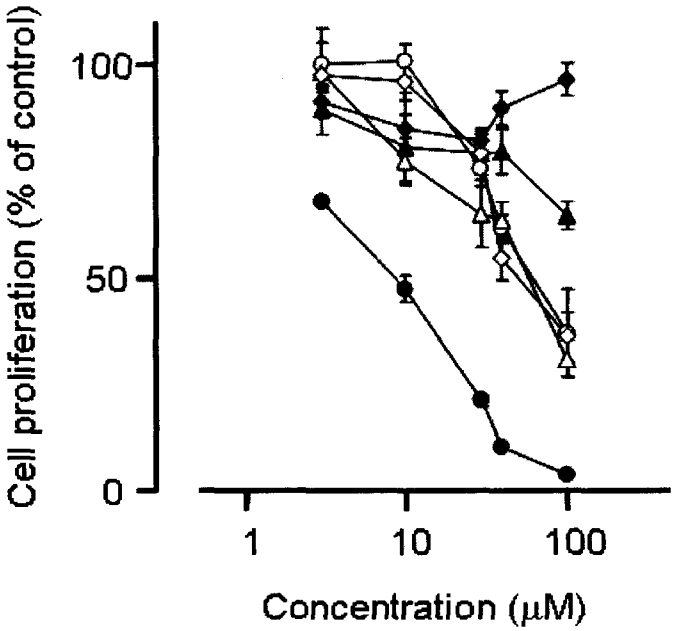

Figure 2. Effects of selective COX-2 inhibitors on proliferation of RA synovial fibroblasts. Cells were incubated for 24 hours with each inhibitor (at 3, 10, 30, 40, and 100 μM). Cell proliferation was then estimated from the incorporation of 5-bromo-2'-deoxyuridine (BrdU). Representative results from 4 independent experiments are shown; values are the mean ± S.D. from triplicate cultures. ● = celecoxib; ○ = etodolac; ▲ = meloxicam; △ = nimesulide; ◆ = rofecoxib; ◇ = NS-398.

2-3 Effects of selective COX-2 inhibitors on cell viability

Since some selective COX-2 inhibitors suppressed the proliferation of RA synovial fibroblasts, I next evaluated the effect of these drugs on cell viability, using a sulfonated tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-1) assay. Celecoxib reduced cell viability in a concentration-dependent manner (Figure 3). In contrast, etodolac, meloxicam, nimesulide, NS-398, and rofecoxib at concentrations up to 100 μM had little effect on cell viability.

Since the incubation time can also influence cell viability, I examined the effect of celecoxib and rofecoxib (at 30 μM) on the viability of RA synovial fibroblasts after incubation for 96 hours. Cell viability was reduced to 21 % after incubation with celecoxib, versus 98 % after incubation with rofecoxib.

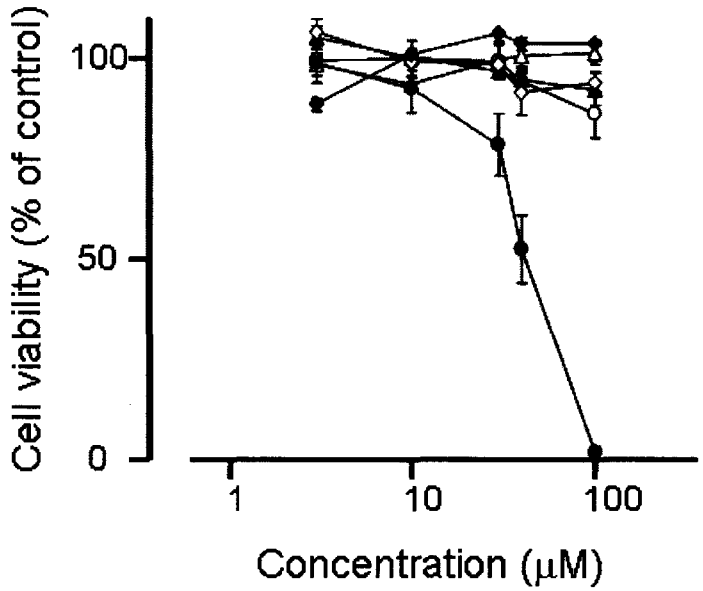

Figure 3. Effects of selective COX-2 inhibitors on viability of RA synovial fibroblasts. Cells were incubated for 24 hours with each inhibitor (at 3, 10, 30, 40, and 100 μM). Viability was then measured by the WST-1 assay. Representative results from 4 independent experiments are shown; values are the mean ± S.D. from triplicate cultures. ● = celecoxib; ○ = etodolac; ▲ = meloxicam; △ = nimesulide; ◆ = rofecoxib; ◇ = NS-398.

2-4 Effects of selective COX-2 inhibitors on DNA fragmentation

To determine whether the decrease in RA synovial fibroblast viability was caused by apoptosis, I examined the effect of selective COX-2 inhibitors on DNA fragmentation, a hallmark of apoptosis, using a quantitative enzyme-linked immunosorbent assay (ELISA) that specifically detected cytoplasmic histone–associated DNA fragments, mononucleosomes, and oligonucleosomes. As shown in Figure 4, celecoxib induced DNA fragmentation, with the peak effect occurring with a concentration of 40 μM. With

celecoxib at 100 μM, DNA fragments were released into the culture medium (data not shown), indicating that the cells had undergone secondary (type 2) necrosis, which usually occurs after apoptosis during *in vitro* experiments. In contrast, the other selective COX-2 inhibitors did not induce DNA fragmentation at any concentration.

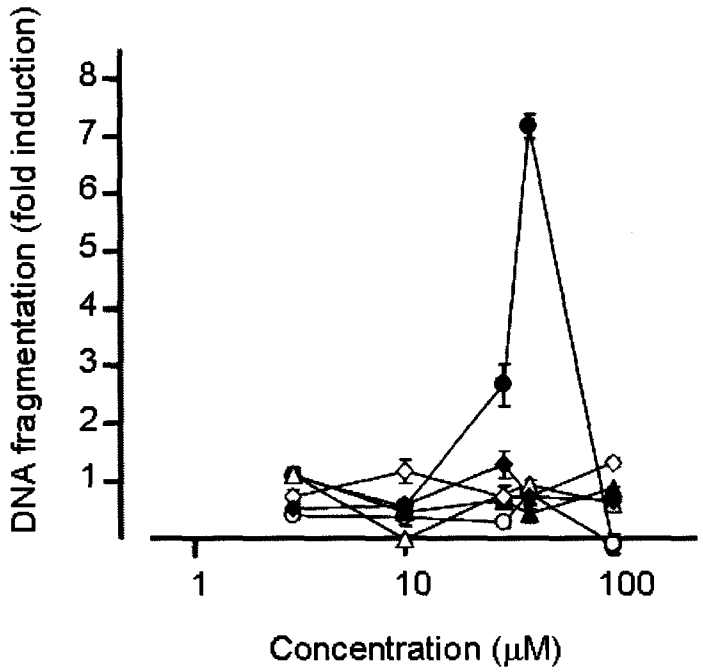

Figure 4. Effects of selective COX-2 inhibitors on DNA fragmentation in RA synovial fibroblasts. Cells were incubated for 24 hours with each inhibitor (at 3, 10, 30, 40, and 100 μM). DNA fragments in the cytoplasm were then measured by enzyme immunoassay. The fold induction of DNA fragmentation is shown relative to the control value (untreated cells). Representative results from 4 independent experiments are shown; values are the mean ± S.D. from triplicate cultures. ● = celecoxib; ○ = etodolac; ▲ = meloxicam; △ = nimesulide; ◆ = rofecoxib; ◇ = NS-398.

2-5 Effects of caspase inhibitors on celecoxib-induced apoptosis

To investigate whether the caspase cascade participated in the induction of apoptosis by celecoxib, I examined DNA fragmentation after RA synovial fibroblasts were cultured in the presence of celecoxib with or without caspase inhibitors. The proapoptotic effect of celecoxib (40 μM) was abolished by addition of caspase-3,

caspase-8, or caspase-3/7 inhibitors (Figure 5).

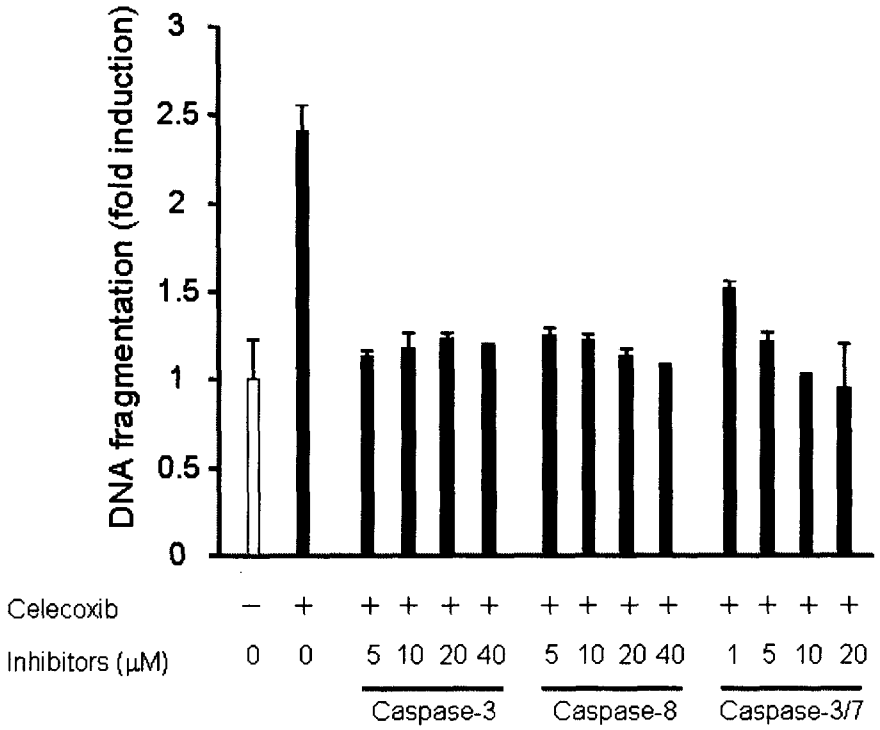

Figure 5. Effects of caspase inhibitors on celecoxib-induced DNA fragmentation in RA synovial fibroblasts. Cells were incubated with celecoxib (40 μM) and each caspase inhibitor (Z-DEVD-FMK [a caspase-3 inhibitor], Z-IETD-FMK [a caspase-8 inhibitor], and Z-VAD-FMK [a caspase-3/7 inhibitor]) for 24 hours 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 2 independent experiments are shown; values are the mean and S.D. from triplicate cultures.

2-6 Effect of interleukin-1β on celecoxib-induced apoptosis

The effect of IL-1β on celecoxib-induced DNA fragmentation was also determined. As shown in Figure 6, addition of IL-1β (1 ng/mL) to RA synovial fibroblasts cultures caused slight enhancement of celecoxib-induced apoptosis.

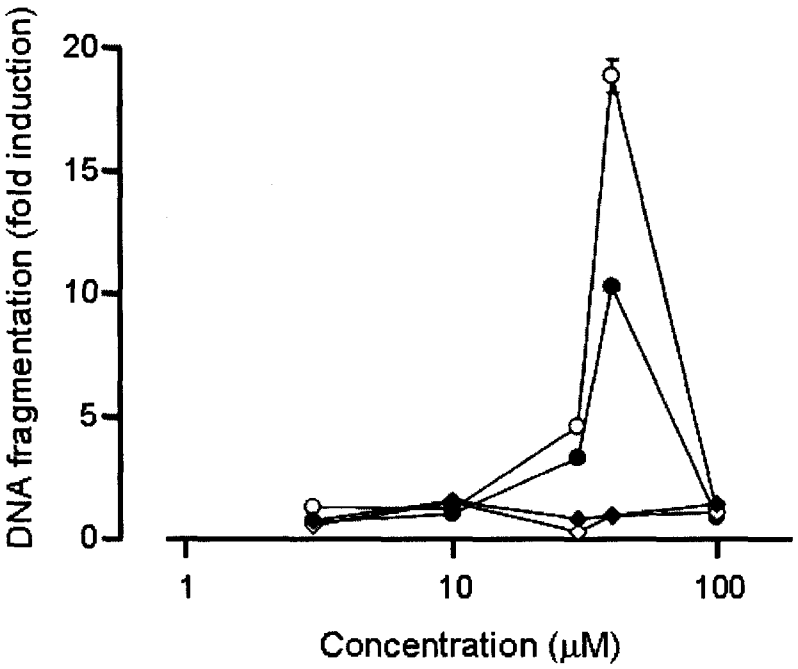

Figure 6. Effect of interleukin (IL) -1β on celecoxib-induced apoptosis in RA synovial fibroblasts. Cells were incubated for 24 hours with or without IL-1β(1 ng/mL), after which celecoxib (40 μM) or rofecoxib (100 μM) was added and DNA fragments in the cytoplasm were measured by enzyme immunoassay. The fold induction of DNA fragmentation is shown relative to the control value (untreated cells). Representative results from 2 independent experiments are shown; values are the mean ± S.D. from triplicate cultures. ● = celecoxib alone; ○ = celecoxib and IL-1β; ◆ = rofecoxib alone; ◇ = rofecoxib and IL-1β.

2-7 Detection of apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay

To confirm the proapoptotic effect of celecoxib, cell morphology was examined and the TUNEL assay was applied. Morphologic changes of RA synovial fibroblasts are shown in Figure 7. Cells treated with celecoxib became rounder, exhibited shrinkage, and became separated from adjacent cells. As shown in Figure 8, TUNEL positive cells, accounting for 38 % of the total cells present, were observed after incubation for 24 hours with 40 μM celecoxib. In contrast, TUNEL staining was negative when RA synovial fibroblasts were incubated with the other selective COX-2 inhibitors (100 μM)

and was also negative in untreated cells. These results suggested that celecoxib caused a decrease of RA synovial fibroblast proliferation by inducing apoptosis.

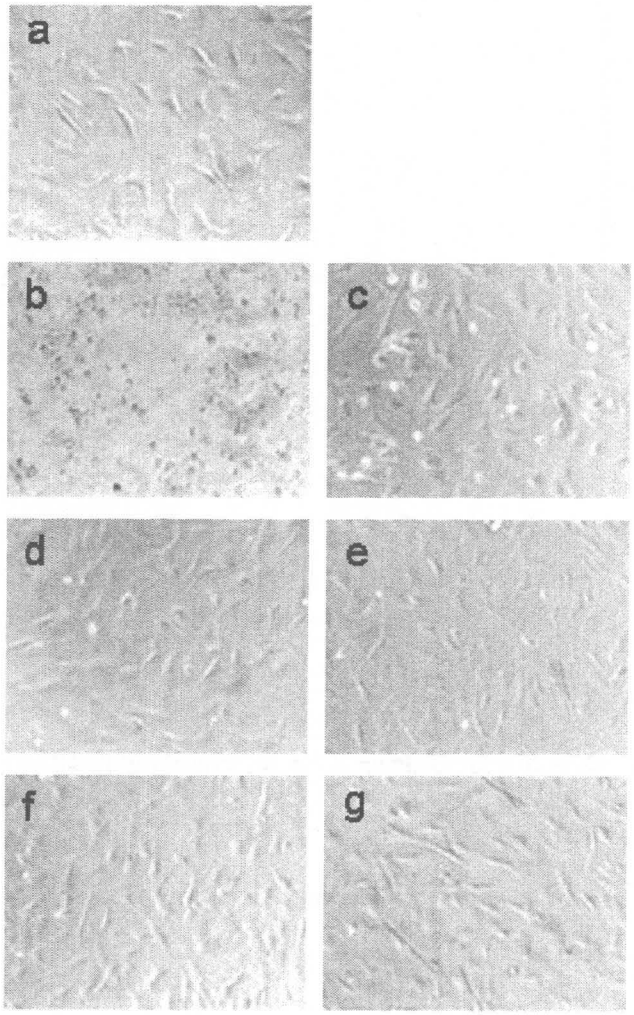

Figure 7. Morphologic changes in RA synovial fibroblasts. Cells were incubated for 24 hours **a**, without any COX-2 inhibitors or with **b**, celecoxib (40 μM), **c**, etodolac (100 μM), **d**, meloxicam (100 μM), **e**, nimesulide (100 μM), **f**, NS-398 (100 μM), or **g**, rofecoxib (100 μM). Magnification × 200.

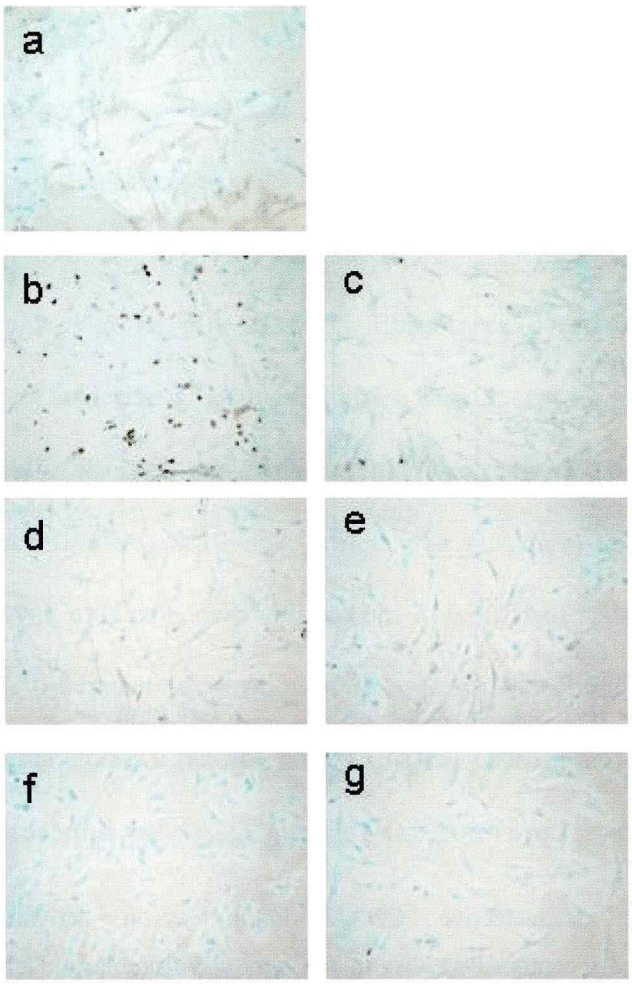

Figure 8. Detection of apoptosis in RA synovial fibroblasts, by the TUNEL assay. Cells were incubated for 24 hours **a**, without any COX-2 inhibitors or with **b**, celecoxib (40 μM), **c**, etodolac (100 μM), **d**, meloxicam (100 μM), **e**, nimesulide (100 μM), **f**, NS-398 (100 μM), or **g**, rofecoxib (100 μM). Apoptotic cells exhibiting TUNEL staining are brown; normal cells counterstained with methyl green are blue. Magnification × 200.

2-8 Effects of selective COX-2 inhibitors on peroxisome proliferator-activated receptor (PPAR)γ activation

To explore whether PPARγ activation was involved in the apoptotic effect of celecoxib, I performed a luciferase reporter gene assay in which RA synovial fibroblasts were co-transfected with a PPRE-driven luciferase reporter plasmid and a PPARγ expression plasmid. As shown in Figure 9, PPARγ ligands (troglitazone and

indometacin) induced significant PPRE-driven luciferase activity in this system. In contrast, celecoxib and NS-398 had no effect on PPARγ activity.

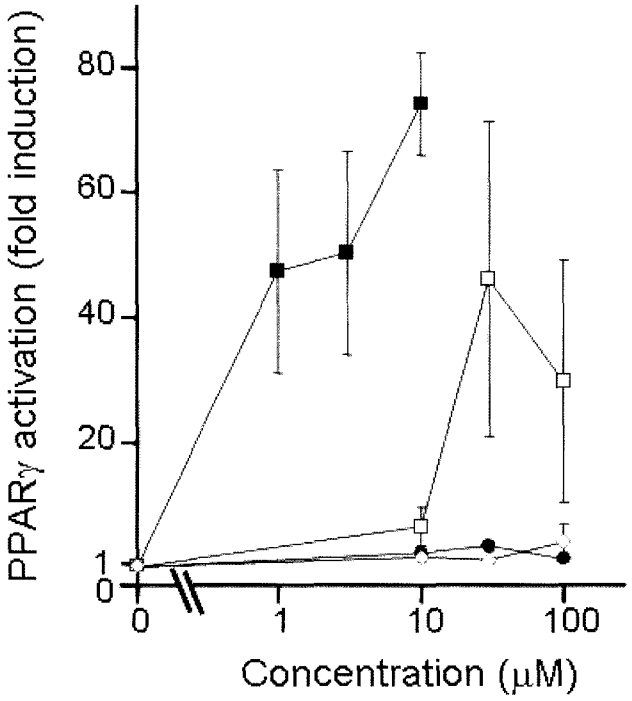

Figure 9. Effect of selective COX-2 inhibitors on activation of peroxisome proliferators-activated receptor (PPAR) γ in RA synovial fibroblasts. Cells were cotransfected with a peroxisome proliferator response element-driven luciferase reporter plasmid, a PPARγ expression plasmid, and an internal control plasmid. Transfected cells were then treated with known PPARγ ligands or selective COX-2 inhibitors, and luciferase activity was determined as described in Materials and Methods. The fold activation of PPARγ is shown relative to the control value (untreated cells). Representative results from 3 independent experiments are shown; values are the mean ± SEM from triplicate cultures. ● = celecoxib; ◇ = NS-398; ■ = troglitazone; □ = indometacin.

3. Discussion

In this chapter, I reported that the induction of apoptosis in RA synovial fibroblasts by celecoxib. It has previously been suggested that both COX-1 and COX-2 are expressed by human RA synovial fibroblasts, and de novo synthesis of COX-2 protein has been observed in RA synovial fibroblasts even without addition of stimulants[27, 28]. All 6 selective COX-2 inhibitors that I studied (celecoxib, etodolac, meloxicam, nimesulide, NS-398, and rofecoxib) caused a > 90 % decrease in PGE_2 production at a concentration of 10 μM, which was sufficient to almost completely inhibit COX-2. However, only celecoxib induced the apoptosis of RA synovial fibroblasts. In addition, long time (96 hours) incubation of RA synovial fibroblasts with rofecoxib did not affect cell viability. These results indicate that the induction of apoptosis by celecoxib was not associated with inhibition of COX-2 or suppression of PG production in RA synovial fibroblasts.

This study also showed that induction of DNA fragmentation in RA synovial fibroblasts by celecoxib was abolished after the addition of inhibitors of caspase-3, caspase-8, or caspase-3/7. Accordingly, the caspase cascade may play an important role in the induction of apoptosis of RA synovial fibroblasts by celecoxib

Stimulation by IL-1β had a weak enhancing effect on celecoxib-induced apoptosis of RA synovial fibroblasts. Tsuboi[29] et al. reported that IL-1β inhibited Fas antigen expression at the posttranscriptional level in RA synovial fibroblasts and protected these cells from Fas antigen–mediated apoptosis. However, I found that celecoxib-induced apoptosis was slightly enhanced by the addition of IL-1β. These different responses to IL-1β might be explained by differences in the inducer of apoptosis, i.e., anti-Fas antibody versus celecoxib.

PPARγ is expressed by human RA synovial fibroblasts, and specific ligands for this receptor (troglitazone and 15dPGJ$_2$) have been shown to inhibit the growth of these cells by inducing apoptosis[7,9]. Activation of PPARγ in monocytes is enhanced by some NSAIDs, such as indometacin, fenoprofen, and ibuprofen, while this receptor is activated in pre-adipocytes by indometacin, fenoprofen, ibuprofen, and flufenamic acid [24,30]. We recently found that induction of apoptosis in RA synovial fibroblasts by some NSAIDs, such as indometacin, diclofenac, and oxaprozin, was associated with PPARγ activation[9]. Therefore, I examined whether selective COX-2 inhibitors could activate this receptor in RA synovial fibroblasts. Celecoxib and NS-398 did not activate PPARγ, whereas indometacin and troglitazone did, as demonstrated in the luciferase reporter gene assay.

A recent clinical study showed that celecoxib could reduce the number of polyps in patients with familial adenomatous polyposis (FAP)[31]. It was reported that disruption of the COX-2 gene in the Apc$^{\Delta 716}$ knockout mouse, an animal model of human familial polyposis, reduced the number and size of intestinal polyps[32]. Treating Apc$^{\Delta 716}$ knockout mice with selective COX-2 inhibitors also reduced the number of polyps [33]. Recently, Sonoshita[34] et al. reported that homozygous deletion of the gene encoding EP2, a cell surface receptor for PGE$_2$, caused a reduction in the number and size of intestinal polyps in Apc$^{\Delta 716}$ knockout mice. Although the inhibition of polyp formation in these mice was incomplete even after COX-2 gene knockout, these findings suggest that PGE$_2$, a terminal product of arachidonic acid metabolism via COX-2, may play a key role in the progression of colon tumor proliferation.

In fact, 6 selective COX-2 inhibitors, celecoxib, etodolac, meloxicam, nimesulide, NS-398, and rofecoxib, have demonstrated an in vivo antitumor effect in animal models

of colon tumor[33,35-39]. The association of apoptosis with the antitumor effect was not reported, and this effect of selective COX-2 inhibitors might be explained, at least in part, by indirect mechanisms such as an anti-angiogenic action due to COX-2 inhibition[40,41]. However, it has been reported that celecoxib has a proapoptotic effect on cancer cells by COX-2-independent mechanisms, such as cell cycle arrest, blockade of Akt activation, and inhibition of basic fibroblast growth factor[42,43]. As discussed above, celecoxib inhibits the proliferation of RA synovial fibroblasts and induces apoptosis of these cells through COX-2-independent and PPARγ-independent mechanisms.

Although the significance of such a COX-2 independent mechanism for the induction of apoptosis in RA synovial fibroblasts remains to be studied, this unique action of celecoxib implies a disease-modifying effect in RA. In the present study, I found that celecoxib suppressed the proliferation of RA synovial fibroblasts and induced apoptosis at relatively high concentrations (10-30 μM) when compared with the optimal concentration for COX-2 inhibition (Figure 1). 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[44]. Therefore, this possible disease-modifying effect of celecoxib could hardly be expected to occur with standard oral administration. However, it might become possible to achieve such an effect if the drug is administered directly into the synovial space or via a new delivery system. Otherwise, a novel compound that induces apoptosis at clinically achievable concentrations by mimicking the action of celecoxib may possibly be of therapeutic value in RA.

4. Conclusion

Celecoxib suppressed the proliferation of RA synovial fibroblasts by COX-2–independent and PPARγ-independent induction of apoptosis.

Chapter 2 Effect of TT101, derivative of celecoxib

1. Introduction

Celecoxib is one of the NSAIDs that selectively inhibit COX-2. It is widely used for treatment of RA with the expectation that an anti-inflammatory effect will result from inhibiting the production of prostanoids, like PGE_2, through the suppression of COX-2 activity. In chapter 1, I had demonstrated that celecoxib induces the apoptosis of synovial fibroblasts obtained from patients with RA.

Apoptosis is considered to be one of the mechanisms regulating autoimmune diseases such as RA[45]. In the pathogenesis of RA, it is thought that the normal balance between proliferation and apoptosis of synovial fibroblasts is lost, leading to hyperplasia of these fibroblasts[4]. Activated synovial fibroblasts cause growth of synovium in the articular cavity along with angiogenesis, invade the adjacent bone, promote production of inflammatory cytokines by inflammatory cells, and cause cartilage and bone destruction[6]. Therefore, it has been shown that stimulation of the apoptosis of synovial fibroblasts might be useful for the treatment of RA[26, 46].

Recently, celecoxib was reported to cause a significant reduction in the number of colorectal polyps in patients with FAP[31], and it has attracted attention as an antiproliferative agent in animal and cell culture studies. We have previously shown that celecoxib induces the apoptosis of colorectal carcinoma cells[17]. In addition, several studies conducted by other investigators have demonstrated that celecoxib suppresses the proliferation of various cells by inducing apoptosis[11-16], suggesting that the proapoptotic action of celecoxib may be useful for the chemoprevention of tumorigenesis[13,15]. These effects may represent an action that is unique to the drug celecoxib rather than being a class effect of COX-2 inhibitors

In chapter 1, the concentration of celecoxib required to induce apoptosis of synovial fibroblasts obtained from patients with RA was slightly higher than the blood level of the drug achieved in healthy individuals[44]. Therefore, for adequately proapoptotic activity to achieve an antirheumatic effect in clinical use, celecoxib may need to be used administered at higher dose levels than those used clinically. Accordingly, I have attempted to develop a drug with more potent apoptosis inducing activity for this purpose. In this process, we synthesized two derivatives, TT101 and TT201, and analyzed these proapoptotic reactions on synovial fibroblasts.

2. Results

2-1 Effect of TT101 on cell proliferation

To determine whether TT101 and TT201 had an inhibitory effect on the proliferation of RA or OA synovial fibroblasts, I first examined the influence of these drugs on cell proliferation (DNA synthesis) by measuring the nuclear incorporation of BrdU (Figure 10). TT101 inhibited the proliferation of both RA synovial fibroblasts (Figure 10A) and OA synovial fibroblasts (Figure 10B) in a concentration-dependent manner. TT201, celecoxib, and SC-236 also inhibited cell proliferation, but their effects were weaker than that of TT101. Rofecoxib had no effect on cell proliferation up to a concentration of 100 μM.

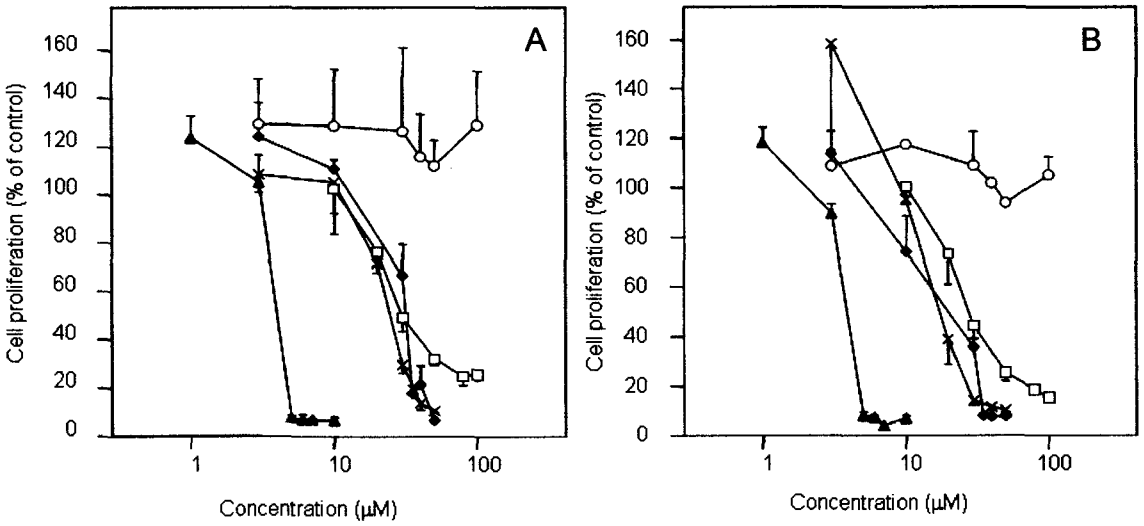

Figure 10. Effect of the each drug on the proliferation of synovial fibroblasts obtained from patients with rheumatoid arthritis (RA, panel A) and osteoarthritis (OA, panel B) incubated with celecoxib (◆), TT101 (▲), TT201 (□), SC-236 (×), or rofecoxib (○) for 24 hours. Then proliferative activity was estimated from the nuclear incorporation of BrdU, and was expressed as a percentage of the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown

2-2 Effect of TT101 on cell viability

To determine whether TT101 and TT201 had an influence on the viability of RA synovial fibroblasts and OA synovial fibroblasts, I evaluated cell viability using the WST-1 assay (Figure 11). TT101 caused a marked decrease of the cell viability of both RA synovial fibroblasts (Figure 11A) and OA synovial fibroblasts (Figure 11B) in a concentration-dependent manner. The order of potency of the effect of each drug on cell viability was as follows: TT101 > celecoxib = SC-236 > TT201 (for both types of cells). Rofecoxib had no effect at all on cell viability.

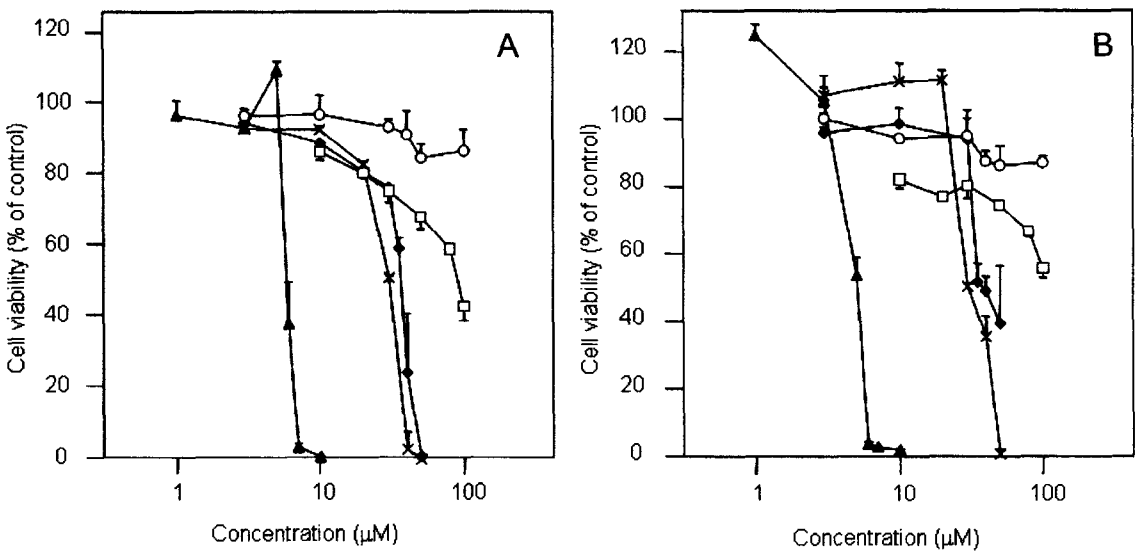

Figure 11. Effect of each test drug on the viability of synovial fibroblasts obtained from patients with RA (panel A) and OA (panel B). Cells were incubated with celecoxib (◆), TT101 (▲), TT201 (□), SC-236 (×), or rofecoxib (○) for 24 hours. Then cell viability was determined by the WST-1 assay and expressed as a percentage of the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown.

2-3 Effect of TT101 on DNA fragmentation

TT101 induced DNA fragmentation, the hallmark of apoptosis, in both RA synovial fibroblasts (Figure 12A) and OA synovial fibroblasts (Figure 12B), and its effect

was more potent than that of celecoxib or SC-236. TT201 also induced DNA fragmentation but was weaker than celecoxib. In contrast, rofecoxib did not induce any DNA fragmentation.

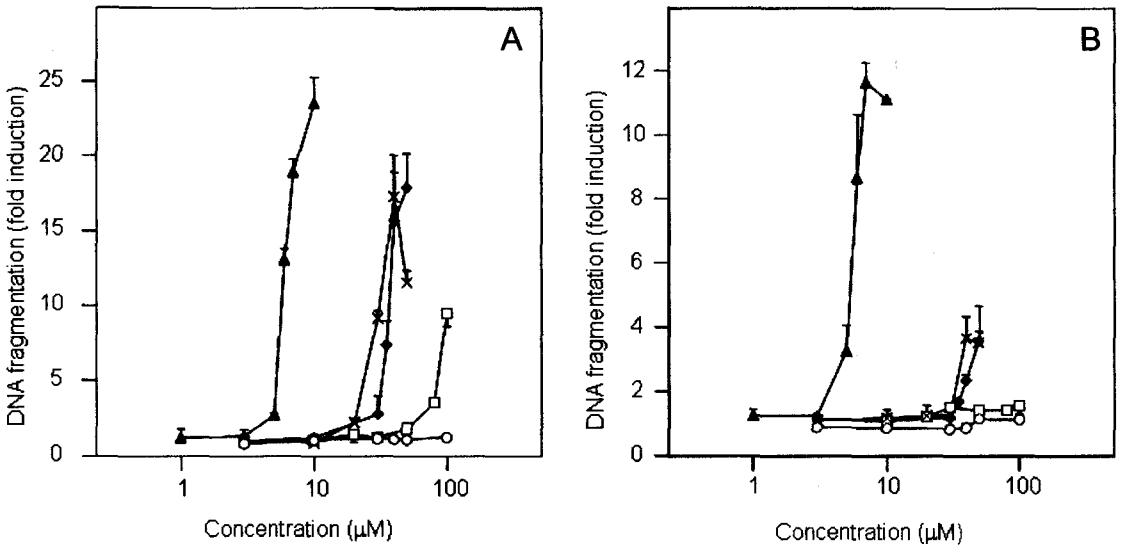

Figure 12. DNA fragmentation in synovial fibroblasts from RA patients (panel A) and OA patients (panel B). Cells were incubated with celecoxib (◆), TT101 (▲), TT201 (□), SC-236 (×), or rofecoxib (○) for 24 hours, after which cytoplasmic DNA fragmentation was measured by enzyme immunoassay and expressed relative to the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown.

The cell morphology was examined under a light microscope after exposure to TT101 (Figure 13). When the cells were incubated with 7 μM TT101 for 24 hours (Figure 13, C and D), distinctive morphological changes were observed, such as rounding and shrinkage, and the cells became detached from their neighbors.

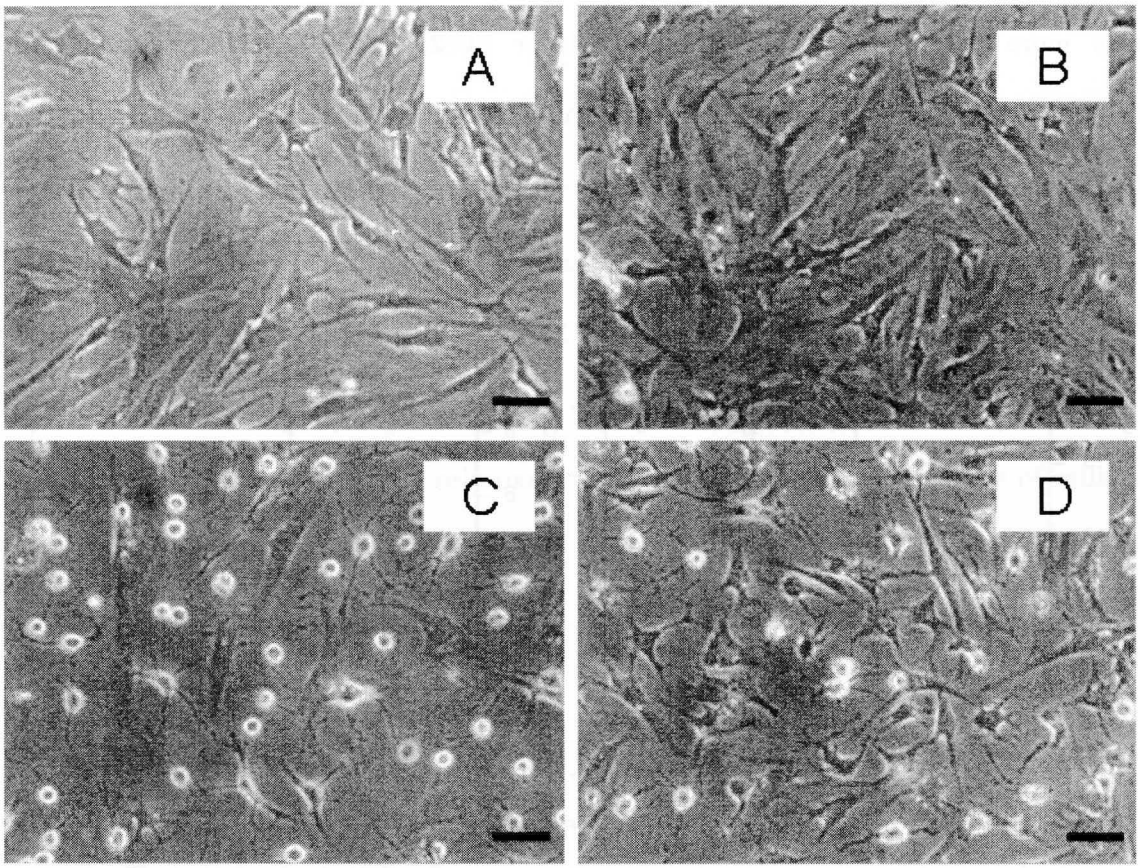

Figure 13. Morphological changes of the synovial fibroblasts from RA patients (panels A and C) or OA patients (panels B and D) as observed by light microscopy. Cells were incubated for 24 hours without (A, B) or with (C, D) TT101 at a concentration of 7 μM. Bar = 60 μm.

2-4 Effect of TT101 on caspase-3 activity

Caspases are responsible for many of the biochemical and morphological changes that occur during apoptosis, so I investigated whether TT101 induced the activation of caspase-3 (a terminal enzyme in the apoptotic pathway). As shown in Figure 14, incubation of RA synovial fibroblasts with 7 μM TT101 for 24 hours induced the activation of caspase-3, whereas this activation was completely blocked by incubation with Z-IETD-FMK or Z-LEHD-FMK (inhibitors of caspase-8 and -9, respectively).

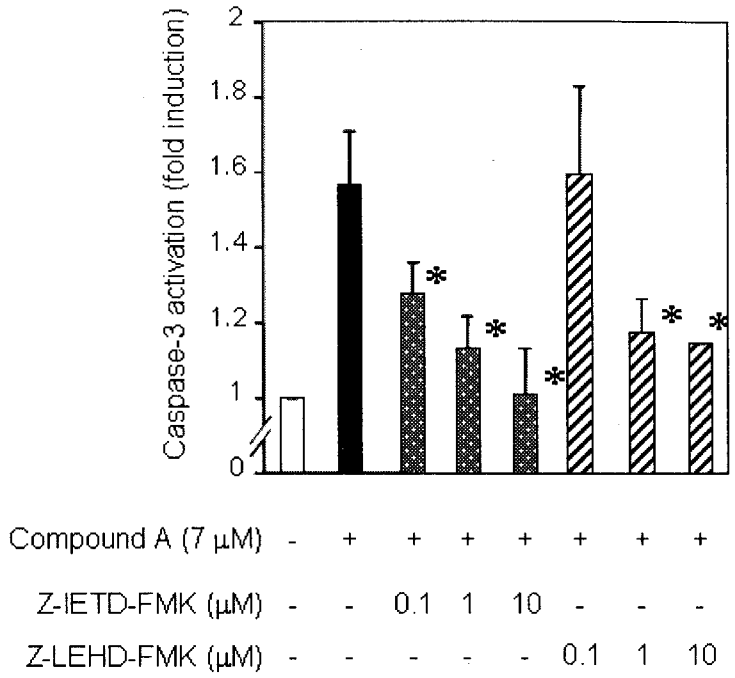

Figure 14. Effect of TT101 on caspase activity in RA synovial fibroblasts. Cells were cultured for 24 hours in the absence of any of agents, or with 7 μM TT101 alone, 7 μM TT101 plus Z-IETD-FMK (a caspase-8 inhibitor), or 7 μM TT101 plus Z-LEHD-FMK (a caspase-9 inhibitor), after which caspase activity was detected by using the CaspACE™ assay system (the absorbance was measured at 405 nm). Data are the mean ± S.D. from 3 independent experiments. *, $p < 0.01$ vs. cells treated with TT101 alone. Significance was evaluated by Tukey's multiple comparison test.

2-5 Effects of caspase inhibitors on TT101-induced apoptosis

Next, I examined the effects of these caspase inhibitors on the TT101-induced apoptosis (Figure 15). Induction of DNA fragmentation in RA synovial fibroblasts by TT101 was suppressed by Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK in a concentration-dependent manner.

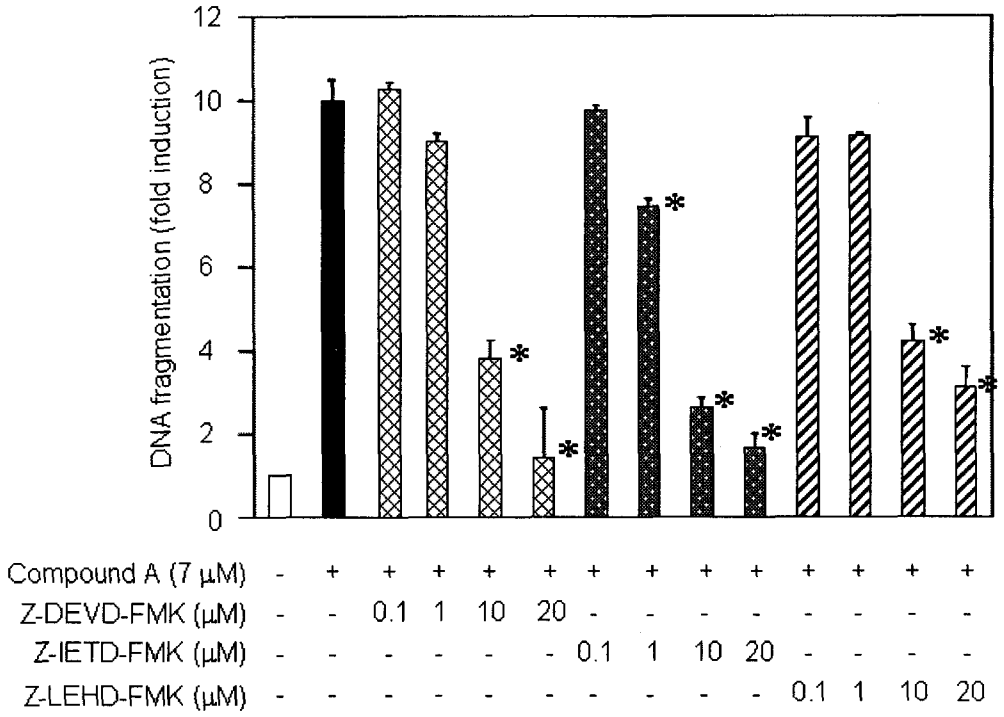

Figure 15. Effects of TT101 plus a caspase inhibitor on DNA fragmentation in RA synovial fibroblasts. Cells were incubated with TT101 with/without a caspase inhibitor for 24 hours, after which the cytoplasmic DNA fragmentation was measured by enzyme immunoassay and expressed relative to the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown. *, $p < 0.01$ vs. cells treated with TT101 alone. Significance was evaluated by Tukey's multiple comparison test.

2-6 Effect of TT101 on expression of apoptotic related protein

I examined the effects of the test drugs on the expression of Bcl-2, which is linked to inhibition of apoptosis[47]. Western blotting demonstrated expression of Bcl-2 by RA synovial fibroblasts, and little change in the level of expression was seen after treatment with any of the COX-2 inhibitors (Figure 16, top). I also studied the effects of the each drug on cleavage of BID (a substrate of caspase-8) in RA synovial fibroblasts, since truncated BID (tBID; 15 kDa) activates the mitochondrial proapoptotic pathway. Full-length BID was expressed in RA synovial fibroblasts, but none of the test drugs

caused cleavage of BID under the conditions tested (Figure 16, bottom).

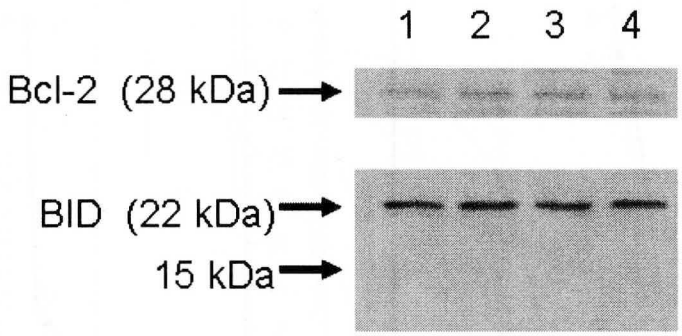

Figure 16. Effects of TT101 and celecoxib on expression of Bcl-2 and cleavage of BID. RA synovial fibroblasts were cultured for 6 hours without any agents (lane 1) or were incubated with 7 μM TT101 (lane 2), 100 μM TT201 (lane 3), or 40 μM celecoxib (lane 4). Protein extracts were prepared from the cells and subjected to Western blotting using an antibody that detected Bcl-2 (upper) or BID (lower), as described in Methods.

2-7 Effect of rofecoxib on TT101-induced apoptosis

To investigate whether the interaction between TT101 and the COX-2 was related to the induction of apoptosis, I studied the effect of pretreatment with another COX-2 inhibitor (rofecoxib) on TT101-induced apoptosis. One hour before the addition of TT101, rofecoxib was added to cultures of human synovial fibroblasts at a concentration that was sufficient to inhibit COX-2, and then DNA fragmentation was assessed as described above. As shown in Figure 17, TT101 induced the same extent of apoptosis after pretreatment with rofecoxib as that seen in the absence of COX-2 inhibition by rofecoxib; this was true for both in RA synovial fibroblasts (Figure 17A) and OA synovial fibroblasts (Figure 17B).

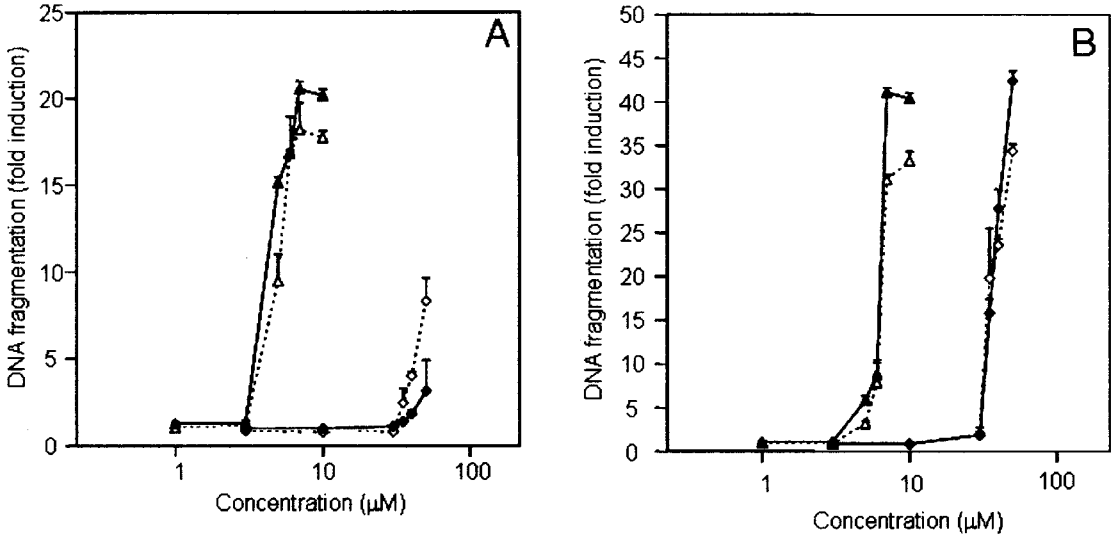

Figure 17. 7. Effect of rofecoxib on TT101-induced DNA fragmentation. RA synovial fibroblasts (panel A) and OA synovial fibroblasts (panel B) were incubated with (continuous line)/without (broken line) 1 μM rofecoxib for 1 hour, and then were incubated in the presence/absence of TT101 (triangles) or celecoxib (diamonds) for 24 hours. Cytoplasmic DNA fragmentation was measured by enzyme immunoassay and expressed relative to the control value (untreated cells). Data are means ± S.D. from triplicate cultures, and representative results from 3 independent experiments are shown.

2-8 Effect of TT101 on prostaglandin E_2 production

I investigated the effect of each test drug on the production of PGE_2 by RA synovial fibroblasts. As shown in Figure 18, all of the drugs suppressed the production of PGE_2 (stimulated by the addition 3 μM of arachidonic acid) in a concentration-dependent manner, but TT101 had the weakest suppressive effect on PGE_2 production.

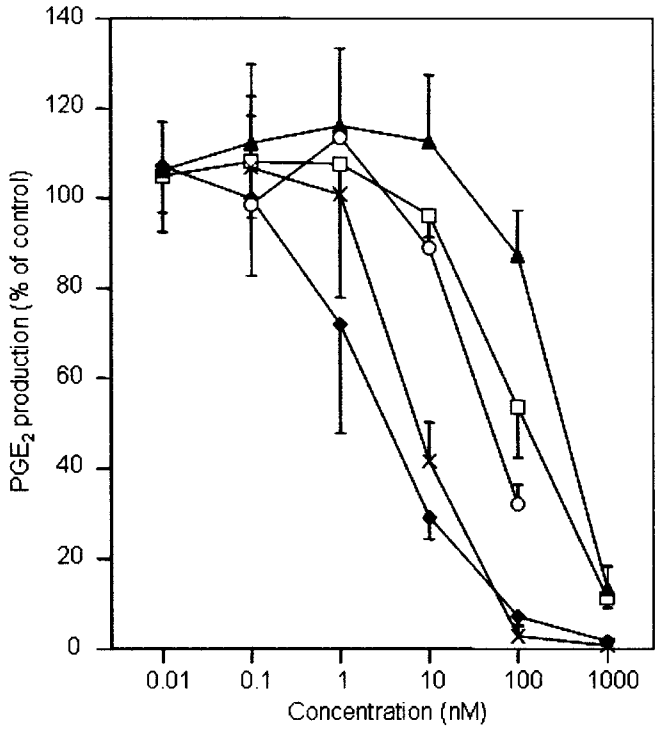

Figure 18. Effect of the test drugs on the production of PGE_2 by RA synovial fibroblasts. Cells were stimulated with IL-1β (1 μg/mL) for 24 hours, washed twice with PBS, and incubated for 1 hour at 37°C with various concentrations of celecoxib (◆), TT101 (▲), TT201 (□), SC-236 (×), or rofecoxib (○). Then arachidonic acid (3 μM) was added and incubation was continued for another 30 minutes, after which the PGE_2 level in the culture medium was measured by enzyme immunoassay. Representative results from 3 independent experiments are shown, and values are the mean ± S.D. from triplicate cultures. The PGE_2 level in control cultures was 38.0 ± 5.9 ng/mL.

2-9 Effect of TT101 on cell viability of U937

I examined the proapoptotic effect of TT101 on U937, a human monocyte cell line. As shown in Figure 19, TT101 as well as celecoxib induced cell death in U937 at almost the same potency as those in synovial fibroblasts.

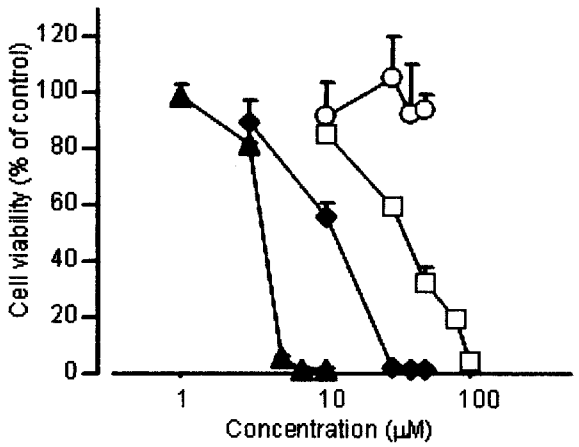

Figure 19. Effect of each test drug on the viability of U937 cells. Cells were incubated with celecoxib (◆), TT101 (▲), TT201 (□), or rofecoxib (○) for 24 hours. Then cell viability was determined by the WST-1 assay and expressed as a percentage of the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown.

3. Discussion

In this chapter, TT101, a novel celecoxib derivative, was shown to be a powerful inducer of apoptosis in both RA synovial fibroblasts and OA synovial fibroblasts. The potency of the inhibitory effect of TT101 on cell proliferation, evaluated in terms of the 50 % effective concentration, was about 5-fold stronger than that of celecoxib for RA synovial fibroblasts and 15-fold stronger for OA synovial fibroblasts. On the other hand, the potency of TT101 for inhibiting IC_{50} (50 % Inhibitory concentration) for COX-2 was about 70-fold weaker than that of celecoxib. Thus, the proapoptotic activity of TT101 was far stronger than that of celecoxib, whereas its COX-2 inhibitory activity was much weaker. The proapoptotic activity of TT101 was not altered by the binding of rofecoxib with COX-2 after pretreatment of cultured cells with adequate concentrations of rofecoxib (Figure 17). This allows us to rule out the possibility that binding of TT101 to COX-2 alters the structure of the enzyme and thus triggers apoptosis, suggesting that the mechanism of apoptosis induction by TT101 (as well as celecoxib) involves neither COX-2 inhibition nor structural changes to the COX-2 molecule.

I examined the proapoptotic effect of TT101 on U937, a human monocyte cell line. TT101 as well as celecoxib induced cell death in U937 at almost the same potency as those in synovial fibroblasts (Figure 19). A strong proapoptotic activity of TT101 on human synovial fibroblasts might be one of the therapeutic approaches for rheumatoid arthritis[26]; however, a major improvement of TT101 itself and/or a proper targeting technique for the drug will be necessary for the clinical application.

The study of adenocarcinoma cells by Yamazaki et al.[17] demonstrated that phosphorylation of Akt was only induced by celecoxib among the selective COX-2 inhibitors tested. Accordingly, the results of the present study using synovial fibroblast

and OA synovial fibroblast differ from those of previous studies conducted using tumor cell lines. Zhu et al.[48] synthesized various derivatives of celecoxib and examined each derivative for induction of the apoptosis of PC-3 human prostate cancer cells. They demonstrated that stronger inhibition of 3-phosphoinositide-dependent kinase-1, upstream kinase of Akt activation, was associated with stronger induction of apoptosis by celecoxib analogs. In the present study, however, phosphorylated Akt was detected in RA synovial fibroblasts, but it was unaffected by treatment with TT101 or celecoxib (data not shown). The different types of cells used in the two studies may explain the differences in the proapoptotic effects of celecoxib.

Apoptosis can be induced by internal (mitochondria-dependent) and external (death receptor-dependent) pathways[46]. In the mitochondria-dependent pathway, cytochrome c and apoptotic protease activating factor-1 are released from the mitochondria and then bind to pro-caspase-9 to produce active caspase-9. In the death receptor-dependent pathway, extracellular death ligands bind to receptors and cause activation of caspase-8. In this study showed that TT101 activated caspase-3, which is at the end of the caspase cascade. Also, the induction of DNA fragmentation induced by TT101 was suppressed by all of the caspase inhibitors tested (inhibitors of caspases-3, -8, and -9). These findings suggest that the proapoptotic activity of TT101 may involve two signal transduction pathways, i.e., both the internal (mitochondria-dependent) pathway and the external (death receptor-dependent) pathway.

The Bcl-2 family is thought to be involved in the regulation of these pathways [47]. Bcl-2 prevents various apoptotic mitochondrial changes, including cytochrome c release and loss of the membrane potential[49]. Similar to the observations obtained in our previous study using HT-29 cells[17], in which celecoxib did not affect Bcl-2

expression, TT101 did not alter the expression of this anti-apoptotic protein. BID is a specific substrate of caspase-8 involved in the external death receptor-dependent pathway[50,51]. Death receptor-dependent signals cause cleavage of cytosolic BID to release tBID, which tBID translocates to mitochondria and thus transduces apoptotic signals from the cytoplasmic membrane. Although TT101-induced DNA fragmentation was suppressed by the addition of a caspase-8 inhibitor, I found that BID was not degraded by TT101. It seems likely that the mechanisms of mitochondria-mediated apoptosis are not only regulated by Bcl-2 and BID but also by various other factors, including Bad, Bim, and Bcl-X_L[47]. I cannot rule out the involvement of such mechanisms in the induction of apoptosis by TT101. Further studies including in vitro experiments to investigate the mechanisms of TT101-induced apoptosis and in vivo experiments to examine the antirheumatic activity using animal arthritis models remain to be conducted.

We modified the sulfonamide group of celecoxib to an N-(2-aminoethyl)-sulfonamide group when developing TT101, whereas the tolyl group in the terminal aromatic ring of celecoxib was modified to an aminophenyl group to create TT201. In the case of SC-236, this region was changed to a chlorophenyl group. The structures of these celecoxib derivatives and rofecoxib are shown in Figure 20. Whereas TT101 had a strong proapoptotic effect, the proapoptotic activity of TT201 for RA and OA synovial fibroblasts was much weaker than that of celecoxib. This result suggests that the methyl group of celecoxib may be essential for a proapoptotic effect on synovial fibroblasts, when the inhibition of PGE_2 production via COX-2 inhibition was evaluated, TT201 was less potent than celecoxib, but IC_{50} of TT201 was comparable with that of rofecoxib. As suggested by Zhu et al. [48], the basic frame and electron

density of TT101 and TT201 may influence their apoptosis-inducing activity and COX-2 inhibitory activity.

4. Conclusion

TT101 potently suppressed the proliferation of RA and OA synovial fibroblasts by induction of apoptosis.

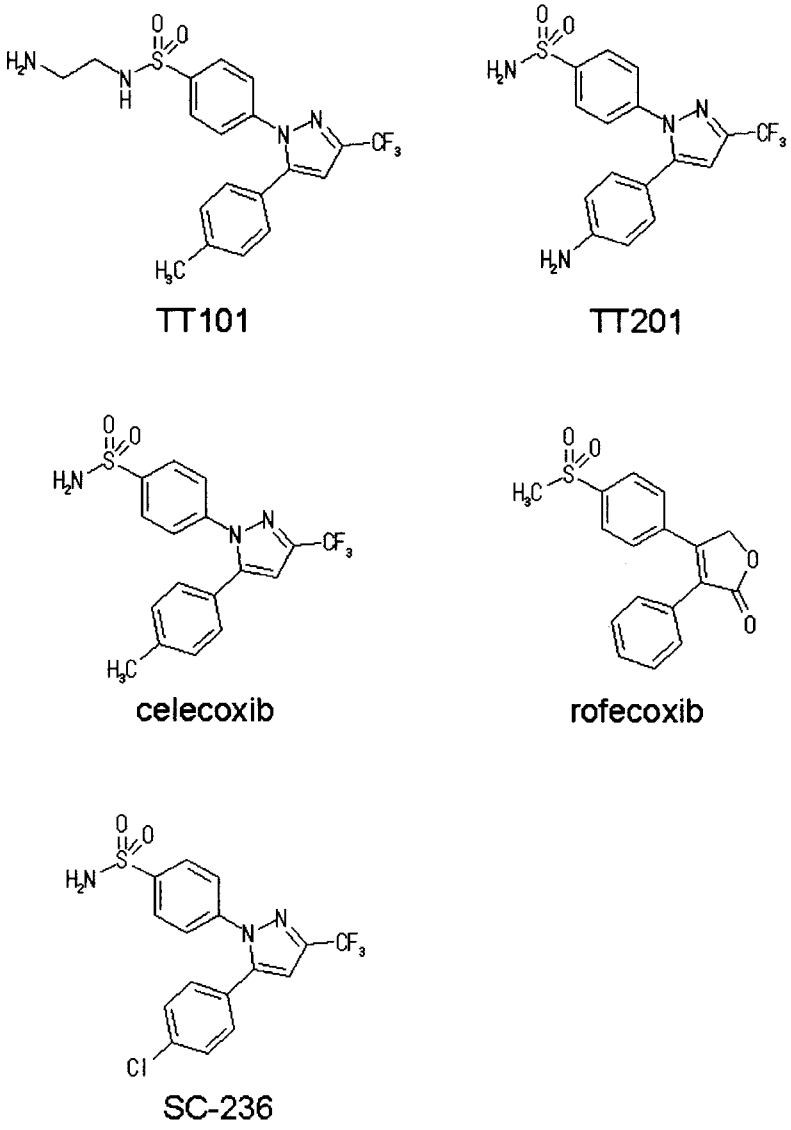

Figure 20. Chemical structures of the drugs used.

Chapter 3 Effect of Triptolide

1. Introduction

Extracts of Tripterygium wilfordii Hook F (TWHF) have been reported to show efficacy in patients with a variety of inflammatory and autoimmune diseases, including RA[52-54]. Previous studies have shown that several TWHF extracts can exert immunosuppressive and anti-inflammatory effects in vivo. A chloroform extract of TWHF suppresses type II collagen-induced arthritis in mice[55], while carrageenan-induced inflammation in rats is suppressed by the ethyl acetate extract of TWHF [56].

The mechanism of action of these TWHF extracts has been investigated in vitro. It has been shown that a multi-glycoside chloroform/methanol extract of TWHF (GTW) significantly inhibits proliferation and IL-2 productions by activated T cells[57]. GTW also inhibits the production of IL-1, IL-6, IL-8, TNF-α, and PGE_2 by human peripheral blood monocytes, as well as IgG, IL-2, and IL-4 production by human peripheral blood lymphocytes[58]. Likewise, a chloroform/methanol extract known as T2, significantly inhibits the release of PGE_2 and IL-2 from human peripheral blood mononuclear cells[59]. Maekawa et al.[60] have reported that the anti-rheumatic effect of GTW or TWHF might be partly mediated through inhibition of PGE_2 production by human synovial fibroblasts due to downregulation of IL-1β-induced COX-2 mRNA expressions, possibly via the inhibition of NF-κB activity. TWHF also inhibits T cell proliferation via the induction of apoptosis [57].

Triptolide is an active compound that was identified in extracts of TWHF[61,62], and its actions have been reported to be as follows: inhibition of IL-2 production by mouse T cell hybridomas[63], human peripheral blood lymphocytes, and Jurkat cells

through nuclear inhibition of transcriptional activation of NF-κB[64]; inhibition of vascular endothelial growth factor expression[65]; suppression of NF-κB in T lymphocytes[66]; inhibition of IL-8 expression in human bronchial epithelial cells[64]; reduction of PGE_2 production in human monocytes and RA synovial fibroblasts[67]; and inhibition of pro-matrix metalloproteinase-1 and -3 mRNA expression[68]. Taken together, these results suggest that triptolide may be an active compound from TWHF extracts with immunosuppressive and anti-inflammatory effects. However, it has not been clarified whether triptolide exerts a disease modifying effect on the pathophysiology of RA. Since induction of apoptosis in synovial fibroblasts may be a possible therapeutic strategy for RA, I examined whether triptolide could induce the apoptosis of RA synovial fibroblasts.

2. Results

2-1 Effect of triptolide on cell proliferation

First, I examined effect of triptolide on cell proliferation (DNA synthesis) by measuring the incorporation of BrdU (Figure 21) using an ELISA. Triptolide caused marked suppression of cell proliferation in a concentration-dependent manner and it was almost completely inhibited at 100 nM. In contrast, the other drugs that were tested, such as bucillamine, D-penicillamine, and methotrexate, did not suppress the proliferation of RA synovial fibroblasts. The mean (± S.D.) value of IC_{50} of 7 independent experiments was 20.4 ± 2.4 nM.

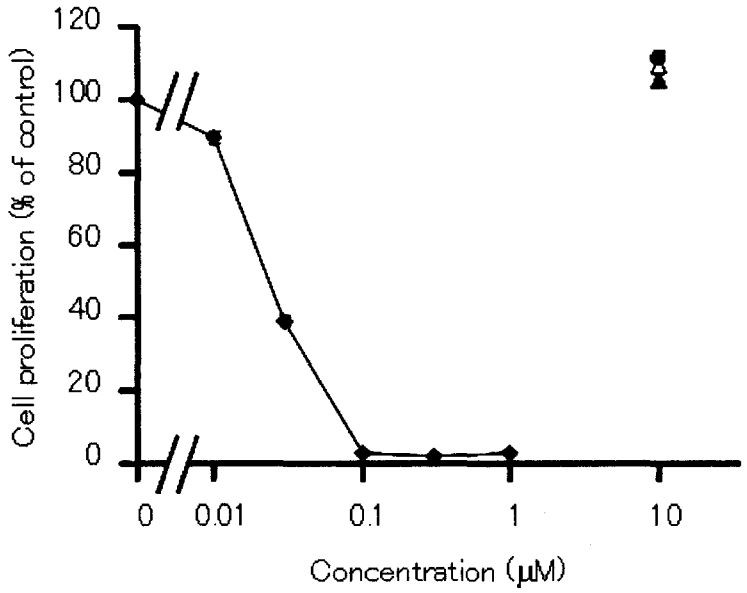

Figure 21. Effects of triptolide and anti-rheumatic drugs on the proliferation of RA synovial fibroblasts. Cells were incubated with triptolide (◆), bucillamine (▲), methotrexate (●), or D-penicillamine (△) for 24 hours. Then cell proliferation was estimated from the incorporation of BrdU and calculated as a percentage of the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures and representative results from 7 independent experiments are shown.

2-2 Effect of triptolide on cell viability

To determine whether triptolide had an influence on the viability of RA synovial

fibroblasts, I evaluated the effects of various anti-rheumatic drugs on cell viability using WST-1 assay. Triptolide caused a marked decrease of cell viability in a concentration dependent manner (Figure 22), while bucillamine, D-penicillamine, methotrexate, sulphasalazine, and sodium aurothiomalate did not decrease viability at a concentration of 10 μM. The mean (± S.D.) value of IC_{50} of 6 independent experiments was 74.3 ± 9.5 nM.

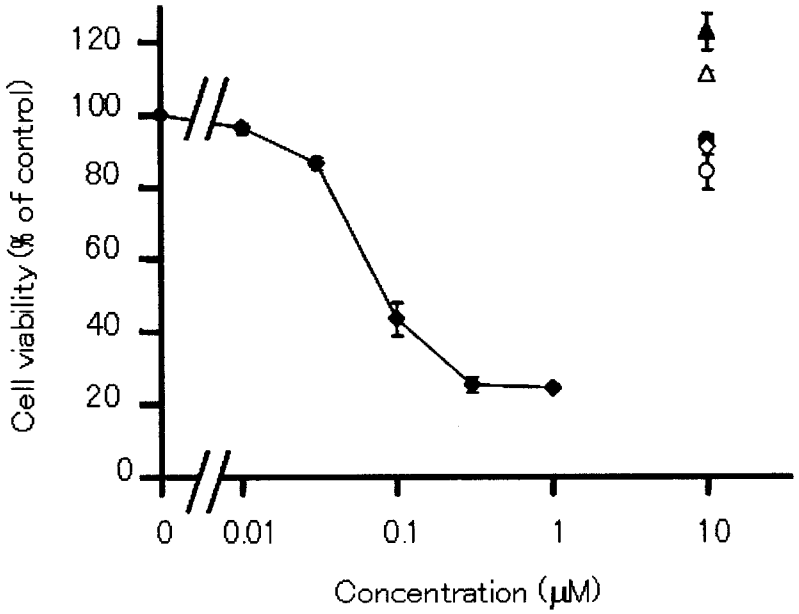

Figure 22. Effects of triptolide and anti-rheumatic drugs on the viability of RA synovial fibroblasts. Cells were incubated with triptolide (◆), bucillamine (▲), methotrexate (●), D-penicillamine (△), sodium aurothiomalate (◇), or sulphasalazine (○) for 24 hours. Then viability was measured by the WST-1 assay and calculated as a percentage of the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures and representative results from 6 independent experiments are shown.

2-3 Effect of triptolide on DNA fragmentation

To determine whether the decrease of viability and suppression of proliferation when RA synovial fibroblasts were treated with triptolide was related to apoptosis, I examined the effect of triptolide on DNA fragmentation (a hallmark of apoptosis). An ELISA that specifically detected cytoplasmic histone-associated DNA fragments,

mononucleosomes and oligonucleosomes, was used to quantitatively analyze DNA fragments. As shown in Figure 23, triptolide induced DNA fragmentation in cultured RA synovial fibroblasts, while bucillamine, D-penicillamine, and methotrexate did not affect cellular DNA. The mean (± S.D.) value of triptolide concentration at a half of maximum fold induction of 5 independent experiments was 35.9 ± 16.3 nM.

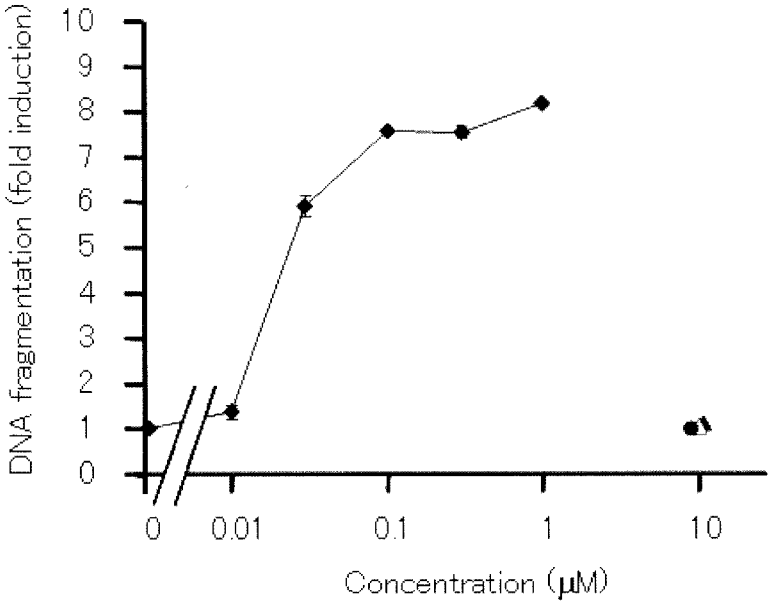

Figure 23. Effects of triptolide and anti-rheumatic drugs on DNA fragmentation of RA synovial fibroblasts. Cells were incubated with triptolide (◆), bucillamine (▲), methotrexate (●), or D-penicillamine (△) for 24 hours, after which fragmented DNA in the cytoplasm was measured by enzyme immunoassay and the fold induction of DNA fragmentation was calculated relative to the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures and representative results from 5 independent experiments are shown.

2-4 Detection of apoptotic cells by TUNEL assay

Cell morphology was observed with a light microscope after treatment of RA synovial fibroblasts with triptolide (Figure 24). When synovial fibroblasts were incubated with triptolide (100 nM) for 24 hours, distinctive morphological changes occurred, such as cellular rounding, shrinkage, and membrane blebbing, and the cells became separated from neighboring cells (panel b). The TUNEL assay was used to

confirm the apparent proapoptotic effect of triptolide. As shown in panel d, TUNEL-positive cells were observed after incubation with 100 nM triptolide for 24 hours, accounting for 66 % of the cultured RA synovial fibroblasts. These results suggested that triptolide caused a decrease of RA synovial fibroblasts proliferation by the induction of apoptosis.

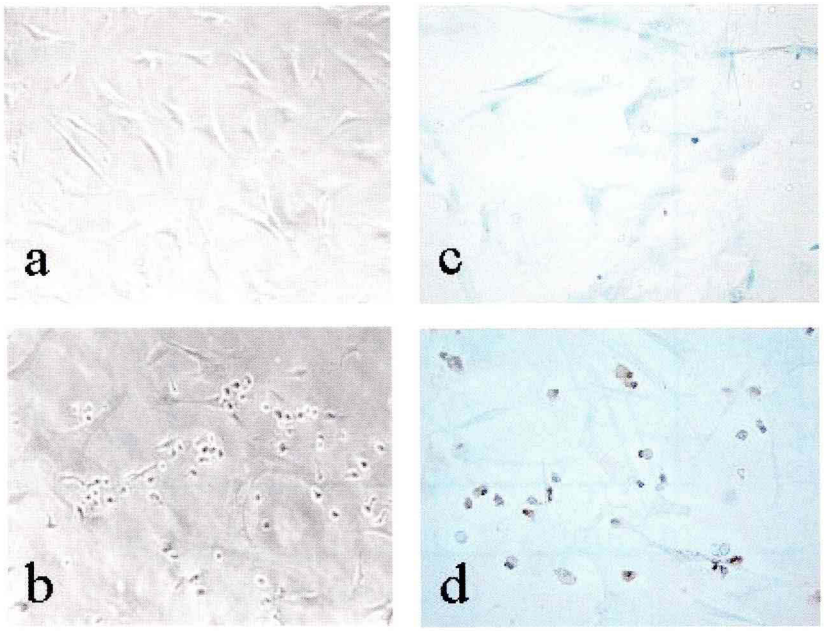

Figure 24. Morphological changes of RA synovial fibroblasts (a, b) and detection of apoptosis by the TUNEL assay (c, d). Cells were incubated for 24 hours without (a, c) or with (b, d) triptolide at 100 nM. Brown cells are apoptotic cells that show TUNEL staining, while blue cells are normal cells stained with methylgreen. Original magnification × 200.

2-5 Effect of triptolide on caspase-3 activity

Caspases are responsible for many of the biochemical and morphological changes that occur during apoptosis, so I investigated whether triptolide induced the activation of caspase-3 in RA synovial fibroblasts. As shown in Figure 25, incubation of RA synovial fibroblasts with 100 nM triptolide for 24 hours induced the activation of caspase-3, while activation was completely blocked by incubation with the pan-caspase inhibitor Z-VAD-FMK.

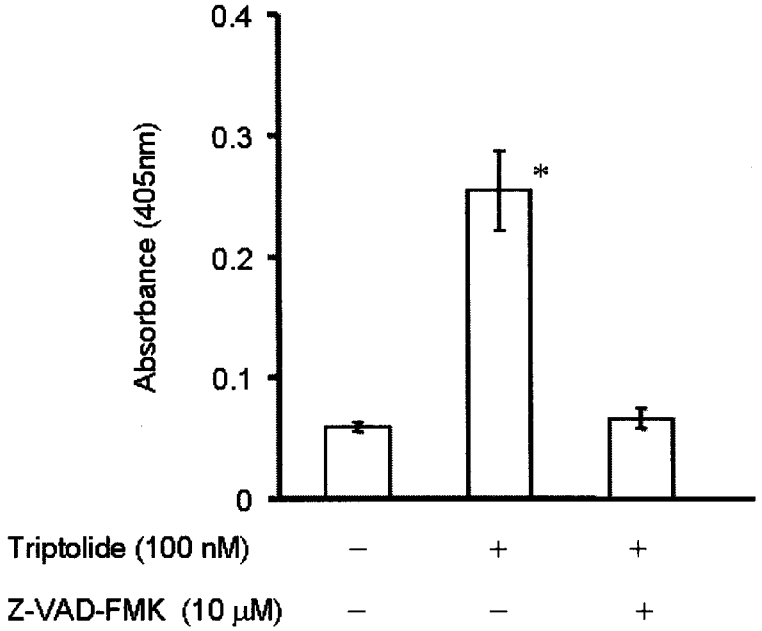

Figure 25. Effect of triptolide on caspase activity in RA synovial fibroblast. Cells were cultured without agents for 24 hours, or were incubated with 100 nM triptolide or 100 nM triptolide plus 10 μM Z-VAD-FMK, and then caspase activity was measured by the CaspACE™ assay system as the absorbance at 405 nm. Data are the mean ± S.D. for 3 independent experiments. *, $p < 0.01$ versus untreated control cells and versus cells treated with triptolide plus Z-VAD-FMK. Significance was evaluated by Student's t-test with a Bonferroni's correction.

2-6 Effects of caspase inhibitors on triptolide-induced apoptosis

I then examined effects of caspase inhibitors on triptolide-induced apoptosis in RA synovial fibroblasts. Triptolide-induced DNA fragmentation in RA synovial fibroblasts was suppressed by Z-DEVD-FMK, ZIETD-FMK, and Z-LEHD-FMK in concentration dependent manner (Figure 26).

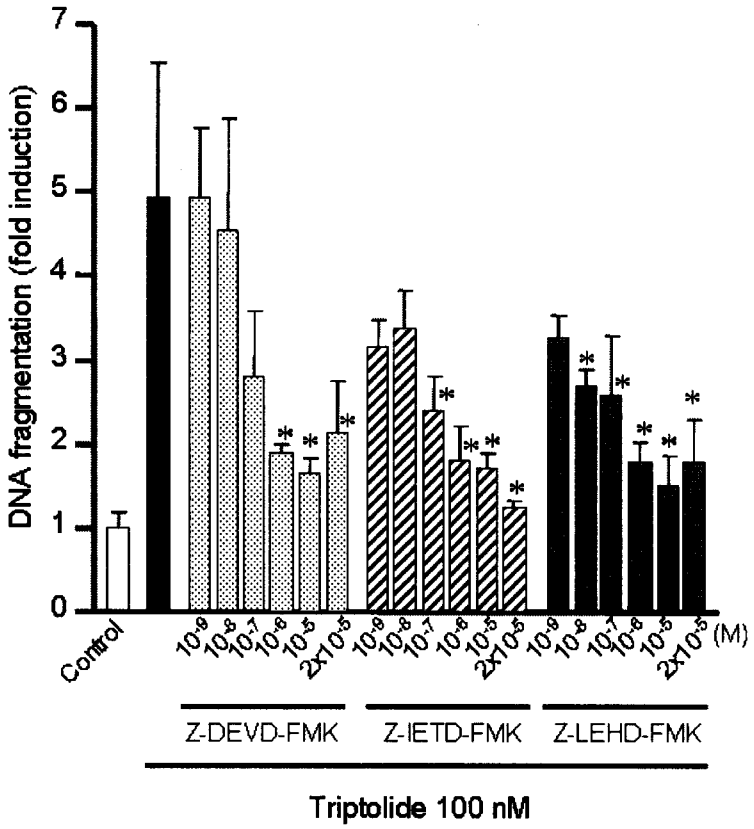

Figure 26. Effects of triptolide and caspase inhibitors on DNA fragmentation in RA synovial fibroblasts. Cells were incubated with triptolide with/ without caspase inhibitors for 24 hours, after which fragmented DNA in the cytoplasm was measured by enzyme immunoassay and the fold induction of DNA fragmentation was calculated relative to the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures and representative results from 3 independent experiments are shown. *, $p < 0.05$ versus cells treated with triptolide alone. Significance was evaluated by Student's t-test with a Bonferroni's correction in each caspase inhibitor study.

2-7 Effect of triptolide on PPARγ activation

To explore whether PPARγ activation was involved in the apoptotic effect of triptolide, I performed a luciferase reporter gene assay by co-transfection of RA synovial fibroblasts with a PPRE-driven luciferase reporter plasmid and a PPARγ expression plasmid (Figure 27). Incubation with $15dPGJ_2$, a PPARγ ligand, significantly induced PPRE-driven luciferase activity in this system. In contrast, triptolide had little influence on PPARγ activation despite causing the apoptosis of RA synovial fibroblasts.

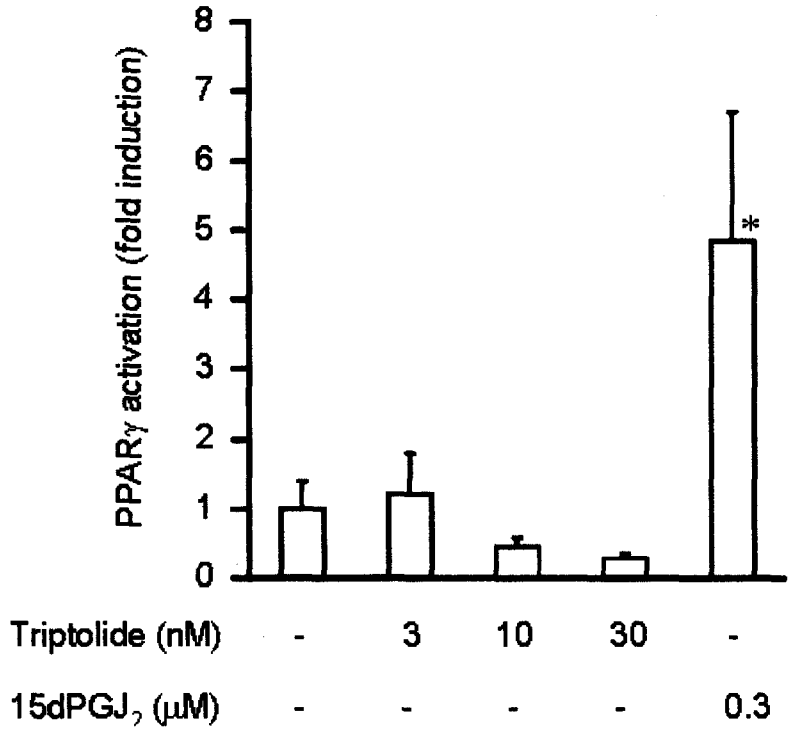

Figure 27. Effect of triptolide on activation of PPARγ in RA synovial fibroblasts. Cells were co-transfected with a PPRE-driven luciferase reporter plasmid, a PPARγ expression plasmid, and an internal control plasmid. Then the transfected cells were treated with known PPARγ ligand or triptolide and luciferase activity was determined as described in Materials and Methods. Data are the mean ± S.D. for triplicate cultures and representative results from 3 independent experiments are shown. *, $p < 0.05$ versus other groups. Significance was evaluated by Student's t-test with a Bonferroni's correction.

2-8 Effect of an extract of Tripterygium wilfordii Hook F. on DNA fragmentation

Since triptolide was regarded as a major active compound of GTW, an extract of TWHF, I examined whether GTW induced apoptosis in RA synovial fibroblasts. As show in Figure 28, GTW induced DNA fragmentation in RA synovial fibroblasts at 10 to 30 μg/mL. With GTW at 50 μg/mL, the cells had undergone secondary (type 2) necrosis, which usually occurs after apoptosis during *in vitro* experiments.

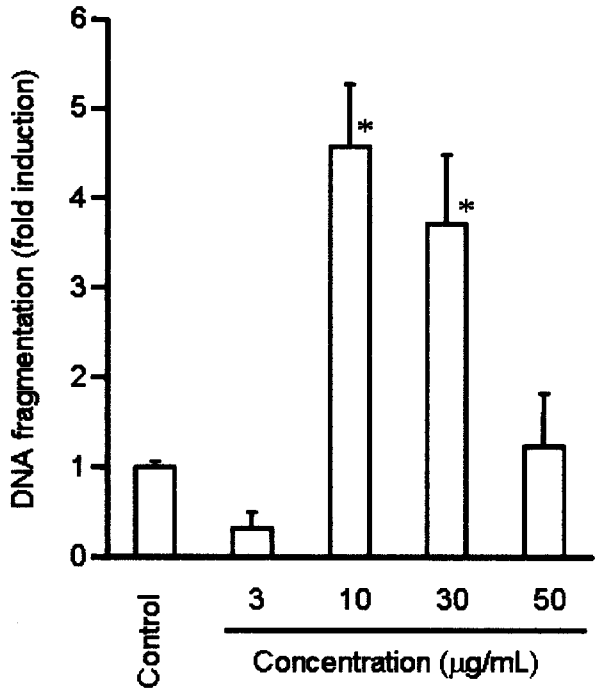

Figure 28. Effect of GTW on DNA fragmentation in RA synovial fibroblasts. Cells were incubated with GTW for 24 hours, after which fragmented DNA in the cytoplasm was measured by enzyme immunoassay and the fold induction of DNA fragmentation was calculated relative to the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures and representative results from 3 independent experiments are shown. *, $p < 0.01$ versus untreated control cells. Significance was evaluated by Student's t-test with a Bonferroni's correction.

3. Discussion

This study showed that triptolide, which has been identified as a major active compound in TWHF extract, induced the apoptosis of RA synovial fibroblasts. Triptolide as well as TWHF extracts have been shown to inhibit cell growth by induction of apoptosis in several kinds of lymphocytes and cancer cell lines, as reviewed by Chen et al.[69]. However, this is the first evidence that triptolide induces apoptosis of RA synovial fibroblasts.

Extracts of TWHF (T2 and GTW) and triptolide have very similar actions, such as suppressing the production of NF-κB by T lymphocytes[66], IL-8 by human bronchial epithelial cells[64], and PGE_2 by human monocytes and RA synovial fibroblasts[60,67]. Triptolide has been reported to display both immunosuppressive and anti-inflammatory effects[64,67,70,71]. These findings suggest that it is a major active compound of TWHF extracts.

I already showed in chapter 1 and chapter 2 that RA is characterized by extensive inflammation and proliferation of the synovium in various joints and synovial hyperplasia leads to joint destruction. Therefore, the identification of agents that induce apoptosis of RA synovial fibroblasts may be a key step toward the successful treatment of RA. Our finding that triptolide induces the apoptosis of RA synovial fibroblasts may help to explain the anti-rheumatic effect of TWHF extract in clinical studies[50]. Although I found that triptolide could induce the apoptosis of RA synovial fibroblasts, standard DMARDs like methotrexate and sulphasalazine did not induce apoptosis. Low-dose methotrexate and sulphasalazine have been reported to inhibit radiographic progression of joint changes in patients with RA[72,73]. I found that triptolide and GTW could induce the apoptosis of RA synovial fibroblasts, suggesting that triptolide might

have a stronger effect than conventional DMARDs. Tao et al.[54] studied the efficacy and safety of TWHF extract in patients with RA. They found that TWHF extract improved the symptoms of RA according to American College of Rheumatology criteria. In addition, it decreased C-reactive protein (CRP), rheumatoid factor, and the erythrocyte sedimentation rate (ESR), changes that were not seen during treatment with NSAIDs.

In the present study, activation of caspase-3 was increased when cells were treated with triptolide (Figure 25). This triptolide-induced activation of caspase-3 was suppressed by Z-VAD-FMK, a pan-caspase inhibitor. Therefore, induction of apoptosis by triptolide was dependent on the caspase-3 pathway. Our results showed those inhibitors of not only caspase-3, but also caspases-8 and -9 suppressed triptolide induced-DNA fragmentation in RA synovial fibroblasts (Figure 26). From these results, it suggested that the activation of caspase-3 followed activities of caspase-8 or -9 would take a part on triptolide inducing apoptosis. There are a lot of uncertain points of the details though this suggestion is similar to the one still shown in the chapter 2. Yang et al.[63] reported that over expression of Bcl-2, which inhibit mitochondrial intrinsic pathway, suppressed triptolide induced degradation of poly ADP-ribose polymerase, a caspase substrate, and apoptosis of T lymphocytes. In contrast, our data suggest that triptolide-induced apoptosis in RA synovial fibroblasts was performed by both intrinsic and extrinsic pathways.

On the other hand, it was reported that $15dPGJ_2$ and troglitazone, natural and synthetic ligand of PPARγ, respectively, could induce RA synoviocyte apoptosis in vitro[7]. We previously found that some NSAIDs induce the apoptosis of RA synovial fibroblasts in a COX-2-independent, but PPARγ-dependent, manner[9]. We also reported that celecoxib, a selective COX-2 inhibitor, induces apoptosis and suppresses the

proliferation of RA synovial fibroblasts without activation of PPARγ (chapter 1). In this chapter, however, triptolide did not induce PPARγ activation. Intracellular mechanism of proapoptotic action of triptolide except for PPARγ activation in RA synovial fibroblasts is to be studied.

There have been several reports suggesting the mechanism of triptolide-induced apoptosis in cancer cell lines. Jiang et al.[74] found that suppression of p53 expression by an antisense oligonucleotide could prevent triptolide induced apoptosis, while over-expression of dominant negative p53 abolished the inhibitory effect on NF-κB activation in human gastric cancer cells. In contrast, it was reported that triptolide has a broad spectrum of proapoptotic activity against several kinds of cancer cells that express wild-type or mutant forms of p53[75]. Lee et al.[76] suggested that NF-κB is an important factor for proapoptotic action of triptolide on cancer cell lines. Recently, Frese et al.[77] reported that TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis in lung cancer cells was sensitized by triptolide via activation of extracellular signal-regulated kinase (ERK)2, a member of the mitogen-activated protein kinase family. It might have to be examined whether such the already-known mechanism of triptolide inducing apoptosis takes part on RA synovial fibroblast.

In this study, GTW induced apoptosis in RA synovial fibroblasts in concentration dependent manner. It was reported that triptolide and tripdiolide were purified from aqueous extract of TWHF[70]. Although I did not examine experiments with tripdiolide, one of the major active compounds of GTW might be triptolide. Furthermore, studies should be conducted to clarify the mechanism of the proapoptotic action of triptolide in RA synovial fibroblasts.

4. Conclusion

I demonstrated that triptolide, an active compound, identified in a traditional Chinese herb, induced apoptosis of RA synovial fibroblasts.

Summary

Among the various mechanisms underlying the therapeutic effect of drugs treating RA, induction of apoptosis in synovial cells was investigated in the present study and the following findings were obtained.

In chapter 1, six selective COX-2 inhibitors were investigated with respect to the induction of apoptosis. As a result, it was confirmed that only celecoxib induced the apoptosis of RA synovial cells. The other selective COX-2 inhibitors that were tested did not induce apoptosis, and induction of apoptosis in RA synovial cells was not observed even after incubation for a long period with rofecoxib at a concentration high enough to inhibit COX-2. Therefore, it was shown that the apoptotic effect of celecoxib on RA synovial cells was not related to inhibition of COX-2 and was unique to this drug. However, celecoxib only induced apoptosis of RA synovial cells at a higher concentration than the blood level achieved after administration to healthy volunteer at the usual clinical dose[44], so it was considered necessary to take additional measures in order to obtain an antirheumatic effect via the induction of apoptosis.

Therefore, to find drugs with a stronger apoptotic effect than celecoxib, TT101 and TT201 (celecoxib derivatives) were investigated with respect to induction of apoptosis in RA synovial cells during chapter 2. As a result, TT101 was found to have a stronger apoptotic effect on RA synovial cells than celecoxib, while the effect of TT201 was weaker than that of celecoxib. It was also shown that the inhibitory effect of TT101 on PGE_2 production was weaker than that of celecoxib and that the apoptotic effect of TT101 was unrelated to inhibition of COX-2. To clarify the cellular selectivity of the apoptotic effect of TT101, its activity was also investigated using OA synovial cells and U937 cells. As a result, TT101 was shown to markedly induce apoptosis in OA synovial

cells and U937 cells as well as RA synovial cells, so induction of apoptosis by TT101 showed no cellular selectivity among the cell types investigated in the present study.

In chapter 3, triptolide (an active ingredient of TWHF, which is a herbal medicine used clinically in China to treat autoimmune diseases such as RA and control post-transplantation rejection) was investigated to assess its induction of apoptosis in RA synovial cells. As a result, it was shown that triptolide induced apoptosis in RA synovial cells. As reported previously, the main pharmacological effect of TWHF was on cells with a role in the process of inflammation, including T cells. Since the present study showed that triptolide (an active ingredient of TWHF) induces apoptosis of RA synovial cells, it can be suggested that TWHF has a new mechanism of action, which is inhibition of the abnormal proliferation of synovial tissue that plays an important role in the pathogenesis of RA.

These results indicate that some of NSAIDs and ingredients of Chinese herbal medicines may exhibit an antihyperplastic effect by promoting the apoptosis of RA synovial cells. Clinical application of such an antihyperplastic effect on synovial cells for the treatment of RA will require further research. However, unlike the well-known anti-inflammatory effect of these agents, the apoptotic effect revealed by the present study could control the mechanism involved in the pathogenesis of articular disease in RA, which expands the possibility for clinical application of these drugs. Furthermore, detailed investigation of the structures of celecoxib, TT101, and triptolide may lead to a new class of RA therapeutics that act by inducing the apoptosis of RA synovial cells.

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Microbiology, Kitasato University School of Medical Science) for suggestions about the methodology of the experiments, as well as Ms. Sonoko Sakurai for secretarial assistance.

Materials and Methods

1. Chapter 1

1-1 Materials

Celecoxib

4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (synthesized using previously reported methods and obtained from Pharmacia K. K., Tokyo, Japan), molecular weight; 381.38.

The purity of these celecoxib preparations was analyzed by highperformance liquid chromatography (HPLC) (1100 series; Agilent, Palo Alto, CA) using a Supelcosil LC-DP column (250 × 4.6 mm internal diameter; Sigma, St. Louis, MO) packed with 5 μm–diameter particles as the stationary phase and a mobile phase of 20 mM potassium phosphate (pH 3.0) / methanol (MeOH) / acetonitrile (MeCN) (6/3/1) at a flow rate of 1.15 mL/minute and a temperature of 60 °C with ultraviolet (UV) detection at 215 nm. The retention times of the synthesized and the Pharmacia-obtained celecoxib preparations were identical (25 minutes), and their purities were 99.95 % and 99.94 %, respectively.

Etodolac

1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-acetic acid (Wyeth Lederle Japan, Tokyo, Japan), molecular weight; 287.35.

Meloxicam

4-Hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide (Nippon Boehringer Ingelheim, Osaka, Japan), molecular weight; 351.41.

Nimesulide

N-(4-Nitro-2-phenoxy-phenyl)methanesulfonamide, (Cayman Chemical, Ann Arbor, MI), molecular weight; 308.31.

NS-398

N-[2-(Cyclohexyloxyl)-4-nitrophenyl]-methanesulfonamide, (Cayman Chemical), molecular weight; 314.4.

Rofecoxib

4-[4-(Methylsulfonyl)-phenyl]-3-phenyl-2(5*H*)-furanone (synthesized using previously reported methods[78]), molecular weight; 314.36.

Indometacin

1-(4-Chlorobenzoyl)-5-methoxy-2-methylindol-3-acetic acid (Sigma Chemical Co., St. Louis, MO), molecular weight; 357.8.

Troglitazone

5-[[4-[(3,4-Dihydro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-yl)methoxy]-phenyl]methyl]-2,4-thiazolidinedione (Sankyo Co., Ltd., Tokyo, Japan), molecular weight; 441.55.

NSAIDs and troglitazone were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. The final concentration of DMSO was 0.1 % (v/v) in the cell culture medium.

RPMI 1640 medium, penicillin/streptomycin solution, fetal bovine serum (FBS), and 0.25 % trypsin/EDTA were purchased from Gibco BRL (Gaithersburg, MD). Phosphate-buffered saline (PBS) (-) were purchased from Takara Shuzo Co., Ltd, (Shiga, Japan). Z-DEVD-FMK (a caspase-3 inhibitor) and Z-IETD-FMK (a caspase-8 inhibitor) were purchased from R&D Systems (Minneapolis, MN). Z-VAD-FMK (a caspase-3/7 inhibitor) was purchased from Promega (Madison, WI). IL-1β was purchased from

Genzyme (Cambridge, MA). All other chemicals were purchased from Wako (Osaka, Japan).

1-2 Cell culture

RA synovial tissue specimens were obtained during total knee replacement surgery in patients with RA that fulfilled the revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association)[1,79,80]. The protocol for this study was approved by the St. Marianna University Ethics Committee, and all patients gave written consent to the use of their tissue for this research. RA synovial fibroblasts were prepared from synovial tissue as described previously[27]. Synovial tissue was digested for 2 hours with 0.2 % (w/v) bacterial collagenase (Immuno-Biological Laboratories, Gunma, Japan) and then was suspended in RPMI 1640 with 10 % (v/v) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37 °C in 5 % CO_2 for several days, after which nonadherent cells were removed. Fibroblast-like adherent cells from the first or second passage were used as RA synovial fibroblasts.

1-3 Assay of prostaglandin E_2 production

RA synovial fibroblasts were plated in 24-well plastic plates (1×10^5 cells/well) and cultured for 24 hours. After washing with PBS (9.57 mM, pH 7.35-7.65), the cells were incubated for 1 hour at 37°C with various concentrations of celecoxib, etodolac, meloxicam, nimesulide, NS-398, or rofecoxib in RPMI 1640 containing 1 %(v/v) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 5 % CO_2. Then, 3 μM arachidonic acid (Cayman Chemical) was added to each well. After incubation for 30 minutes, the culture medium was harvested using a syringe and filtered with a 0.22-μm filter (Millipore, Bedford, MA). The PGE_2 concentration in the

medium was measured using an ELISA kit according to the instructions of the manufacturer (Cayman Chemical). Each measurement was done in triplicate.

1-4 Cell proliferation

The proliferation of RA synovial fibroblast was estimated from the incorporation of BrdU. Cells (1×10^4 cells/well) were incubated in 96-well plastic plates with the test drugs in RPMI 1640 containing 1 % (v/v) FBS for 24 hours at 37 °C in an atmosphere of 5 % CO_2. After incubation, BrdU (10 μM) was added to the culture medium, and then the cells were incubated for another 18 hours. Subsequently, the cells were fixed and BrdU incorporation was determined with a Cell Proliferation ELISA Kit according to the instructions of the manufacturer (Roche Diagnostics, Mannheim, Germany). Each measurement was done in triplicate, and the results are presented as percentages relative to the value determined with untreated control cultures.

1-5 Cell viability

RA synovial fibroblasts (2×10^4 cells/well) were incubated at 37 °C in 96-well plastic plates with test drugs in RPMI 1640 containing 1 % (v/v) FBS in an atmosphere of 5 % CO_2. After 24-96 hours, cell viability was assessed by measuring mitochondrial NADH-dependent dehydrogenase activity with a Cell Counting Kit (Dojindo Molecular Technologies, Kumamoto, Japan) using WST-1. Each measurement was done in triplicate, and the results are presented as percentages relative to the value determined with untreated control cultures.

1-6 DNA fragmentation

RA synovial fibroblasts were plated in 96-well plastic plates (2×10^4 cells/well) and cultured for 24 hours. The cells were then incubated for a further 24 hours at 37 °C with test drugs in RPMI 1640 containing 1 % FBS (v/v). After incubation, DNA

fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics). Each measurement was done in triplicate, and the results are presented as the fold induction compared with untreated control cultures.

1-7 TUNEL assay

RA synovial fibroblasts (2.5×10^4 cells/well) were incubated in 8-well chamber slides (Iwaki, Chiba, Japan) for 24 hours at 37 °C with the test drugs in RPMI 1640 containing 1 % (v/v) FBS. Morphologic changes of the cells were observed under a microscope (BX51; Olympus, Nagano, Japan), after which the cells were fixed with 4 % (v/v) neutral buffered formalin for 10 minutes at room temperature. Subsequently, apoptotic synovial fibroblasts were identified with the TUNEL assay, using an Apoptosis in situ Detection Kit according to the instructions of the manufacturer (Wako). Sections were counterstained with methyl green (Wako) before observation.

1-8 Effects of caspase inhibitors on celecoxib-induced apoptosis

RA synovial fibroblasts were plated in 96-well plastic plates (2×10^4 cells/well) and cultured for 24 hours. The cells were then incubated for a further 24 hours at 37 °C with celecoxib with/without Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (a caspase-8 inhibitor), or Z-VAD-FMK (a caspase-3/7inhibitor) in RPMI 1640 containing 1 % (v/v) FBS. After incubation, DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics). Each measurement was done in triplicate, and the results are presented as the fold induction compared with untreated control cultures.

1-9 Effect of Interleukin-1β on celecoxib-induced apoptosis

RA synovial fibroblasts were plated in 96-well plastic plates (2×10^4 cells/well) and cultured for 24 hours. The cells were then incubated for a further 24 hours at 37 °C with/without IL-1β (1 ng/mL) in RPMI 1640 containing 1 % (v/v) FBS. After incubation,

celecoxib or rofecoxib were added to the culture medium, and then the cells were incubated for another 24 hours. Subsequently, the cells DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics). Each measurement was done in triplicate, and the results are presented as the fold induction compared with untreated control cultures.

1-10 Assay of transcriptional activation of PPARγ

A luciferase reporter plasmid, containing 4 copies of the PPRE of the acyl-coenzyme A oxidase gene promoter at the Nhe I restriction site in the firefly luciferase expression vector PGV-P2 (Toyo, Tokyo, Japan), was used to measure PPARγ activation[81]. RA synovial fibroblasts (6×10^4 cells/well) were seeded into 24-well culture plates in RPMI 1640 containing 10 % (v/v) FBS. After culture for 24 hours at 37 °C in an atmosphere of 5 % CO_2, the cells were co-transfected with the reporter plasmid (0.1 μg/ well), a PPARγ expression plasmid containing mouse PPARγ2 complementary DNA at the *Hind* III and *Xba* I restriction sites in the expression vector pRc/CMV (0.1 μg/well; Invitrogen, Groningen, The Netherlands), and an internal control plasmid (pRL-SV40) (0.01 μg/well; Promega), using Effectene Transfection Reagent (12 μg/mL) according to the instructions of the manufacturer (Qiagen, Hilden, Germany). Incubation was continued for a further 24 hours at 37 °C in an atmosphere of 5 % CO_2, and then the transfection mixture was replaced by RPMI 1640 containing 1 % (v/v) FBS with or without one of the test drugs. After an additional incubation for 18 hours at 37 °C in an atmosphere of 5 % CO_2, luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 luminometer, according to the instructions of the manufacturer (Turner Designs, Sunnyvale, CA). Each measurement was done in triplicate, and firefly luciferase activity was normalized to Renilla luciferase activity.

2. Chapter 2

2-1 Materials

TT101

N-(2-Aminoethyl)-4-[5-(4-tolyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (kindly provided by Professor H. Handa (Tokyo Institute of Technology, Yokohama, Japan)), molecular weight; 370.48

The purity of TT101 was > 99 %, as assessed by HPLC under the following conditions: MeOH solution (1 mg/mL) was injected into the L-column octadecylsilane (4.6 × 150 mm), and the mobile phase was MeCN / 20 mM phosphate buffer (pH 6.5) (35:65) at a flow rate of 1 mL/min. TT101 was detected with a UV detector at a wavelength of 254 nm and a retention time of 21.2 min. ^{1}H NMR (DMSO-d_6), δ 2.31 (s, 3H), 2.50 (br, 5H), 2.76 (t, J = 7.0 Hz, 2H), 7.20 (br, 5H), 7.55 (dd, J = 2.0, 6.0 Hz, 2H), and 7.84 (dd, J = 2.0, 6.0 Hz, 2H).

TT201

4-[5-(4-Aminophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (kindly provided by Professor H. Handa (Tokyo Institute of Technology, Yokohama, Japan)), molecular weight; 370.48, m.p., 202–203 °C.

The purity of TT201 was > 99 %, as assessed by HPLC under the following conditions: MeOH solution (1 mg/mL) was injected into the L-column octadecylsilane (4.6 × 150 mm), and the mobile phase was MeCN / 20 mM phosphate buffer (pH 6.5) (1:1) at a flow rate of 1 mL/min. TT201, as previously reported by Penning et al.[82], was detected with a UV detector at a wavelength of 254 nm and a retention time of 8.6 min. Rofecoxib was synthesized as

reported elsewhere[78].

Celecoxib

4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (Pfizer Japan Inc., Tokyo, Japan), molecular weight; 381.38.

SC-236

4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (Pfizer Japan Inc., Tokyo, Japan), molecular weight; 347.83.

Rofecoxib

4-[4-(Methylsulfonyl)-phenyl]-3-phenyl-2(5*H*)-furanone (synthesized using previously reported methods[80]), molecular weight; 314.36.

NSAIDs were dissolved in DMSO to make stock solutions. The final concentration of DMSO was 0.1 % (v/v) in the cell culture medium. Z-DEVD-FMK, Z-IETD-FMK, IL-1β, FBS, RPMI 1640 medium, penicillin/streptomycin solution, 0.25 % trypsin/EDTA, and PBS were same in chapter 1. Z-LEHD-FMK (a caspase-9 inhibitor) was purchased from R&D Systems.

2-2 Cell culture of synovial fibroblast

Synovial fibroblast cells were prepared by the method of chapter 1.

2-3 Cell culture of U937

U937 cells were kindly provided by Professor H. Kitasato (Kitasato University School of Medical Science, Kanagawa, Japan). Cells were washed with RPMI 1640 containing 10 %(v/v) FBS, 100 units/mL penicillin, and 100 μg/mL. Then, the cells were incubated at 37 °C in 5 % CO_2 for several days.

2-4 Cell proliferation

The proliferation of RA or OA synovial fibroblast was estimated from the

incorporation of BrdU. Cells (1×10^4 cells/well) were incubated in 96-well plastic plates with the test drugs in RPMI 1640 containing 1 % (v/v) FBS for 24 hours at 37 °C in an atmosphere of 5 % CO_2. After incubation, BrdU (10 μM) was added to the culture medium, and then the cells were incubated for another 18 hours. Subsequently, the cells were fixed and BrdU incorporation was determined with a Cell Proliferation ELISA Kit according to the instructions of the manufacturer (Roche Diagnostics). Each measurement was done in triplicate, and the results are presented as percentages relative to the value determined with untreated control cultures.

2-5 Cell viability

RA or OA synovial fibroblasts (2×10^4 cells/well) were incubated at 37 °C in 96-well plastic plates with test drugs in RPMI 1640 containing 1 % (v/v) FBS in an atmosphere of 5 % CO_2. After 24 hours, cell viability was assessed by measuring mitochondrial NADH-dependent dehydrogenase activity with a Cell Counting Kit (Dojindo Molecular Technologies) using WST-1. Each measurement was done in triplicate, and the results are presented as percentages relative to the value determined with untreated control cultures.

2-6 DNA fragmentation

RA or OA synovial fibroblasts were plated in 96-well plastic plates (2×10^4 cells/well) and cultured for 24 hours. The cells were then incubated for a further 24 hours at 37 °C with test drugs in RPMI 1640 containing 1 % (v/v) FBS. After incubation, DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics). Each measurement was done in triplicate, and the results are presented as the fold induction compared with untreated control cultures.

2-7 Activity of caspase-3

RA synovial fibroblasts were incubated in tissue culture flasks in the presence or absence of TT101 and/or Z-IETD-FMK and Z-LEHD-FMK under the conditions described above. After 24 hours, cellular caspase-3 activity was measured by using the CaspACE assay system (Promega Corporation) in accordance with the manufacturer's instructions. Then the cells were washed in ice-cold PBS and resuspended in cell lysis buffer. After lysis of the cells by freezing and thawing, the lysates were centrifuged, and the supernatants were used as cell extracts. The protein content of each extract was determined by the bicinchoninic acid (BCA) protein assay method (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as the standard, and the protein content of was adjusted to 1 mg/mL.

2-8 Effects of caspase inhibitors on TT101-induced apoptosis

RA synovial fibroblasts were plated in 96-well plastic plates (2×10^4 cells/well) and cultured for 24 hours. The cells were then incubated for a further 24 hours at 37 °C with celecoxib with/without Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (a caspase-8 inhibitor), or Z-LEHD-FMK (caspase-9 inhibitor) in RPMI 1640 containing 1 % (v/v) FBS. After incubation, DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics). Each measurement was done in duplicate, and the results are presented as the fold induction compared with untreated control cultures.

2-9 Western blotting analysis

RA synovial fibroblasts were lysed in Chaps cell extract buffer (50 mM Pipes/HCl, pH 6.5, 0.1 % (w/v) Chaps, 5 mM dithiothreitol, 2 mM EDTA, 10 μg/mL pepstatin, 20 μg/mL leupeptin, and 10 μg/mL aprotinin) and then centrifuged at 14,000 rpm for 30 min to remove debris. Subsequently, the protein content of the supernatant was determined by the BCA protein assay (Pierce Biotechnology) with bovine serum

albumin as the standard, and the protein content of the extracts was adjusted to 1 mg/mL. Then the extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 15 % (w/v) acrylamide slab gels under reducing conditions. The proteins thus separated were electroblotted onto Immobilon-P poly (vinylidene difluoride) membranes (Millipore Corporation) with a semidry blotter (Atto Technology, Inc., Tokyo, Japan). After the membranes had been blocked in 10 mM TBS containing 0.1 %(v/v) Tween 20 (TBS-T) and 5 %(w/v) skim milk for 1 hour at room temperature, rabbit anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-BID polyclonal antibody (Cell Signaling Technology, Inc., Beverly, MA) was applied for 18 hours at 4 °C. Then the membranes were washed with Tris buffered saline-tween (TBS-T), and incubation with the secondary antibody (horseradish peroxidase conjugated goat anti-rabbit antibody at a dilution of 1:10,000 in TBS-T) was performed for 1 hour. After further washing with TBS-T, the protein bands were detected by using an enhanced chemiluminescence Western blot analysis system.

2-10 Effect of rofecoxib on TT101-induced apoptosis

RA synovial fibroblasts were plated in 96-well plastic plates (2×10^4 cells/well) and cultured for 24 hours. The cells were then incubated with/without rofecoxib for 1 hour, and TT101 was added to the medium for a further 24 hours at 37 °C. After incubation, DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics). Each measurement was done in triplicate, and the results are presented as the fold induction compared with untreated control cultures.

2-11 Assay of prostaglandin E_2 production

RA synovial fibroblasts were incubated in 24-well plates (5×10^5 cells/well) with IL-1β (1 ng/mL) for 18 hours washed with PBS and then exposed to the test drugs for 1

hour under the conditions described above. Then 3 μM arachidonic acid (Cayman Chemical) was added to the medium. After incubation for 30 minutes, the culture medium was harvested using a syringe and filtered through a 0.22-μm filter (Millipore Corporation). The PGE_2 concentration in the medium was measured using an ELISA kit (Cayman Chemical) in accordance with the manufacturer's instructions. Each measurement was performed in triplicate.

3. Chapter 3

3-1 Materials

Triptolide

(BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA), molecular weight; 360.4

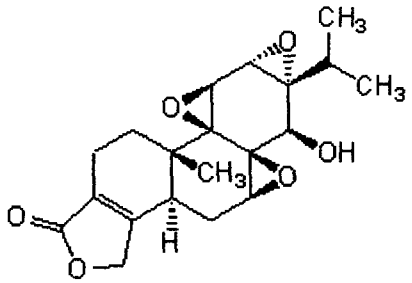

Figure 29. Chemical structure of triptolide

Bucillamine

N-(2-Mercapto-2-methyl-1-oxopropyl)-L-cysteine (Sigma Chemical Co.), molecular weight; 223.32

Methotrexate

N-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic acid (Wako Pure Chemical Industries, Osaka, Japan), molecular weight; 454.44

D-Penicillamine

3-Mercapto-D-valine (Sigma Chemical Co.), molecular weight; 149.21

Sodium aurothiomalate

Mixture of monogold monosodium monohydrogen(RS)-1-sulfidobutane-1,2-dioate and monogold disodium(RS)-1-sulfidobutane-1,2-dioate (Shionogi & Co. Ltd., Osaka, Japan), molecular weight; 390.08, 368.09, respectively

Sulphasalazine

2-Hydroxy-5-[4-(pyridin-2-ylsulfamoyl)phenylazo]benzoic acid (Sigma

Chemical Co.), molecular weight; 398.39

15dPGJ$_2$

11-Oxo-prosta-5Z,9,12E,14Z-tetraen-1-oic acid (Cayman Chemical), molecular weight; 316.4.

GTW was kindly given from Taizhou Pharmaceutical Co. (Jiang Su, China).

FBS, RPMI 1640 medium, penicillin/streptomycin solution, 0.25 % trypsin/EDTA, and PBS were the same as in chapter 1. Z-VAD-FMK was the same as in chapter 1. Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK were the same as in chapter 2. Test drugs except GTW were dissolved in DMSO as 1000 × stock solutions and then diluted with RPMI-1640 medium containing 1 % (v/v) FBS for cell culture experiments. GTW powder was dissolved in ethanol, filtrated, and then stored at -20 °C. Test drug solutions were prepared freshly on the day of use. The final concentration of DMSO or ethanol (just for GTW study) for all treatments (including control cultures) was 0.1 % (v/v).

3-2 Cell culture

Synovial fibroblast cells were prepared by the method of chapter 1.

3-3 Cell proliferation

The proliferation of RA synovial fibroblast was examined by the method which was same as in chapter 1.

3-4 Cell viability

RA synovial fibroblasts (2×10^4 cells/well) were incubated at 37 °C in 96-well plastic plates with test drugs in RPMI 1640 containing 1 % (v/v) FBS in an atmosphere of 5 % CO_2. After 24 hours, cell viability was assessed by measuring mitochondrial NADH-dependent dehydrogenase activity with a Cell Counting Kit (Dojindo Molecular Technologies) using WST-1. Each measurement was done in triplicate, and the results

are presented as percentages relative to the value determined with untreated control cultures

3-5 DNA fragmentation

The DNA fragmentation of RA synovial fibroblast was examined by the method which was same as in chapter 1.

3-6 TUNEL assay

TUNEL assay of RA synovial fibroblast was examined by the method which was same as in chapter 1.

3-7 Activity of caspase-3

RA synovial fibroblasts were incubated in tissue culture flasks in the presence or absence of triptolide and/or Z-VAD-FMK under the conditions described above. After 24 hours, cellular caspase-3 activity was measured by using the CaspACE assay system (Promega Corporation) in accordance with the manufacturer's instructions. Then the cells were washed in ice-cold PBS and resuspended in cell lysis buffer. After lysis of the cells by freezing and thawing, the lysates were centrifuged, and the supernatants were used as cell extracts. The protein content of each extract was determined by the BCA protein assay method (Pierce Biotechnology) with BSA as the standard, and the protein content of was adjusted to 1 mg/mL.

3-8 Effects of caspase inhibitors on triptolide-induced apoptosis

RA synovial fibroblasts were plated in 96-well plastic plates (2×10^4 cells/well) and cultured for 24 hours. The cells were then incubated for a further 24 hours at 37 °C with triptolide with/without Z-DEVD-FMK, Z-IETD-FMK, or Z-LEHD-FMK in RPMI 1640 containing 1 % (v/v) FBS. After incubation, DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics). Each measurement was done

in duplicate, and the results are presented as the fold induction compared with untreated control cultures.

3-9 Assay of transcriptional activity of PPARγ

RA synovial fibroblasts (6×10^4 cells/well) were seeded into 24-well culture plates in RPMI 1640 containing 10 % (v/v) FBS. After culture for 24 hours at 37 °C in an atmosphere of 5 % CO_2, the cells were co-transfected with the reporter plasmid, a PPARγ expression plasmid, and an internal control plasmid by the same method as in chapter 1. Incubation was continued for a further 24 hours at 37 °C in an atmosphere of 5 % CO_2, and then the transfection mixture was replaced by RPMI 1640 containing 1 % (v/v) FBS with or without one of the test drugs. After an additional incubation for 18 hours at 37°C in an atmosphere of 5 % CO_2, luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 luminometer, according to the instructions of the manufacturer (Turner Designs). Each measurement was done in triplicate, and firefly luciferase activity was normalized to Renilla luciferase activity.

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