Nonsteroidal Anti-Inflammatory Drugs Induce Apoptosis in Association with Activation of Peroxisome Proliferator-Activated Receptor γ in Rheumatoid Synovial Cells

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been reported to induce apoptosis in a variety of cell lines. In this study, we examined the effect of NSAIDs on the growth and apoptosis of synovial cells from patients with rheumatoid arthritis and analyzed the activation of peroxisome proliferator-activated receptor γ (PPARγ) as a possible mechanism of action of NSAIDs. Cell proliferation and viability were assessed from 5-bromo-2'-deoxyuridine incorporation and by 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)]-5-tetrazoliol-1,3-benzenesulfonate (WST-1) assay, respectively. The apoptosis of synovial cells was identified by DNA fragmentation assay and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. Indometacin, diclofenac, oxaprozin, and zaltoprofen reduced cell proliferation and induced apoptosis. Rheumatoid synovial cells expressed PPARγ mRNA, and the PPARγ ligands 15-deoxy-Δ12,14-prostaglandin J2 and troglitazone reduced the proliferation and induced apoptosis in synovial cells. Luciferase reporter assay demonstrated that not only PPARγ ligands but also NSAIDs, which could induce apoptosis, increased the activation of PPARγ in synovial cells. Furthermore, the ability of NSAIDs and PPARγ ligands to stimulate the activation of PPARγ correlated with their ability to decrease cell viability (r = 0.92, p < 0.01) and ability to induce DNA fragmentation (r = 0.97, p < 0.001) in synovial cells. These results suggest that PPARγ is an attractive target for induction of apoptosis in rheumatoid synovial cells and that the activation of the PPARγ pathway is associated with the apoptotic action of NSAIDs.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used in the treatment of rheumatoid arthritis because of their analgesic and anti-inflammatory activities. A major mechanism of the action of NSAIDs is generally thought to be inhibition of cyclooxygenase (COX) (Vane, 1971). COX is a key enzyme in catalyzing the conversion of arachidonic acid, which is released from the cell membrane, to prostaglandin (PG) G2 and H2. There are two isoforms of COX, COX-1 and COX-2 (Kujubu et al., 1991; Xie et al., 1991). COX-1 is constitutively expressed in a number of cell types and tissues and plays an important role in maintaining homeostasis. In contrast, COX-2 is induced in inflammatory cells by a variety of stimuli, including cytokines. These observations suggest that COX-2 plays a key role in controlling inflammation. In addition, studies have found that COX-2, but not COX-1, is markedly elevated in most colorectal adenocarcinoma tumors (Eberhart et al., 1994), indicating that COX-2 expression may play a central role in colorectal carcinogenesis.

Recent evidence suggests that NSAIDs have chemopreventive activity for colon cancer (Thun et al., 1991; Giardiello et al., 1993). NSAIDs also have been shown to exert apoptotic effects in a variety of cell lines, particularly colon cancer cells (Hanif et al., 1996; Shiff et al., 1996; Elder et al., 1997; Li et al., 2001), suggesting a possible mechanism for their chemopreventive activity. Although COX is the molecular target of most NSAIDs, not only a COX-dependent (Souza et al., 2000; Li et al., 2001) but also a COX-independent (Hanif et al., 2001) mechanism is involved.
to a protocol that was approved by the ethics committee of St. Marianna University, and all patients gave written consent to the use of their tissues for this research. Synovial tissues were digested for 2 h with 0.2% (w/v) bacterial collagenase and for 2 h with 0.125% (w/v) trypsin, and then were suspended in RPMI 1640 with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The cells were incubated at 37°C in 5% CO₂ for several days, and nonadherent cells were removed. The fibroblast-like adherent cells were used as rheumatoid synovial cells within two passages. Among the adherent cells, T cells (CD3+ and macrophage/microphages (CD14+) were not detected by two-color immunofluorescence and flow cytometry.

The human monocytic leukemia cell line U937 was obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂. U937 cells were used after treatment with 0.1 μM TPA for 24 h at 37°C in 5% CO₂.

**Drug Preparation.** Test drugs were dissolved in dimethyl sulfoxide as × 1000 stock solutions and then diluted with RPMI 1640 containing 1% FBS for cell culture experiments. The test drug solutions were prepared freshly on the day of testing. The final concentration of dimethyl sulfoxide for all treatments, including control culture conditions, was maintained at 0.1%.

**Cell Proliferation Assay.** The proliferation of rheumatoid synovial cells was evaluated from the cellular incorporation of 5-bromo-2′-deoxyuridine (BrdU). Rheumatoid synovial cells (1 × 10⁴ cells/well) on 96-well culture plates were treated with the test drugs in RPMI 1640 containing 1% (v/v) FBS at 37°C in 5% CO₂. After 24 h, BrdU (10 μM) was added to the culture medium and then incubated for another 16 to 18 h. The synovial cells were fixed and BrdU incorporation was determined with a Cell Proliferation Enzyme-Linked Immunosorbent Assay (ELISA) kit (Roche Applied Science, Mannheim, Germany) using peroxidase-conjugated anti-BrdU Fab fragments according to the manufacturer's instructions. The results are presented as a percentage of the value for control culture conditions.

**Cell Viability Assay.** Rheumatoid synovial cells (2 × 10⁴ cells/well) on 96-well culture plates were treated with the test drugs in RPMI 1640 containing 1% (v/v) FBS at 37°C in 5% CO₂. After 24 h, cell viability was measured as mitochondrial NADH-dependent dehydrogenase activity with a Cell Counting Kit (Roche Applied Science) using anti-histone mouse monoclonal antibody (clone MCA-33) as secondary antibody according to the manufacturer's instructions. The results are presented as a percentage of the control value. Cell morphology was also observed with a light microscope at 60× magnification.

**DNA Fragmentation Assay.** Rheumatoid synovial cells (2 × 10⁴ cells/well) on 96-well culture plates were treated with the test drugs in RPMI 1640 containing 1% (v/v) FBS at 37°C in 5% CO₂. After 24 h, the level of fragmented DNA in synovial cells, which is characteristic of apoptosis, was measured by DNA Cell Death Detection ELISA PLUS (Roche Applied Science) using anti-histone mouse monoclonal antibody (clone H11-4) as primary antibody and anti-DNA mouse monoclonal antibody (clone MCA-33) as secondary antibody according to the manufacturer's instructions. The results are presented as fold-induction compared with the control culture.

**Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay.** Rheumatoid synovial cells (6 × 10⁴ cells/well) on eight-well chamber slides (Iwaki, Chiba, Japan) were treated with the test drugs in RPMI 1640 containing 1% (v/v) FBS at 37°C in 5% CO₂. After 24 h, synovial cells were fixed with a 4% (w/v) formalin neutral buffer solution for 10 min at room temperature and then apoptotic synovial cells were identified by TUNEL assay using an Apoptosis In Situ Detection kit (Wako, Osaka, Japan) according to the manufacturer's instructions. The synovial cells were also counterstained using methyl green solution (Wako).
Reverse Transcription-Polymerase Chain Reaction (RT-PCR). PPARγ mRNA expression in rheumatoid synovial cells was determined by RT-PCR. Total RNA was extracted using Isogen (Nippon Gene Co., Tokyo, Japan) from the synovial cells. The cDNA synthesis and PCR amplification reactions were done using Ready-To-Go RT-PCR Beads (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. The primer sequences for PPARγ were 5'-TCTCTTCTTAATGGAAGACC-3' (sense) and 5'-GCATATTGAGATCCTGCCAC-3' (antisense), yielding a 474-bp PCR product. As a control, glyceraldehyde-3-phosphate dehydrogenase mRNA expression was also determined using the following primers: 5'-CCACCCATGCCAATTTCCATGGA-3' (sense) and 5'-TCTAGACGGGAGGTCAGGTCCACC-3' (antisense), yielding a 593-bp PCR product. The PCR protocol was 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, for 35 cycles. The PCR products were analyzed by electrophoresis using 2% agarose gels and were visualized by ethidium bromide staining and UV illumination.

Luciferase Reporter Assay. A luciferase reporter plasmid, which contains four copies of the peroxisome proliferator response element (PPRE) of the acyl-CoA oxidase gene promoter (Marcus et al., 1993) at the NheI restriction site in the firefly luciferase expression vector PGV-P2 (Toyo Ink, Tokyo, Japan) was used to measure the activation of PPARγ. Rheumatoid synovial cells (6 x 10⁴ cells/well) were seeded in 24-well culture plates in RPMI 1640 containing 10% (v/v) FBS. After culture for 24 h at 37°C in 5% CO₂, the synovial cells were cotransfected with the reporter plasmid (0.1 µg/well), a PPARγ expression plasmid that contains mouse PPARγ2 cDNA (Tontonoz et al., 1994) at the Hind III and XhoI restriction sites in the expression vector pRc/CMV (Invitrogen, Groningen, The Netherlands) (0.1 µg/well), and internal control plasmid pRL-SV40 (Promega, Madison, WI) (0.01 µg/well) using Effectene Transfection Reagent (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. After 24 h at 37°C in 5% CO₂, the transfection mix was replaced by RPMI 1640 containing 1% (v/v) FBS with or without the test drugs. After an additional incubation for 18 h at 37°C in 5% CO₂, luciferase activity was determined using Dual-Luciferase Reporter Assay System (Promega) and TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), according to the manufacturers' instructions. Firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical Analysis. The data are expressed as means ± S.D. IC₅₀ is the concentration that caused a 50% inhibition of cell proliferation or viability. The IC₅₀ was calculated by interpolation. Statistical analysis was done using Dunnett’s test. A least-squares linear regression analysis was used for calculation of the correlation coefficient. p values less than 0.05 were considered significant.

Results

Effects of NSAIDs on Proliferation of Rheumatoid Synovial Cells. Initially, we examined the effects of traditional NSAIDs, indomethacin, diclofenac, ketoprofen, oxaprozin, and zaltoprofen; the selective COX-2 inhibitor NS-398; and a weak COX inhibitor, acetaminophen, on the proliferation (DNA synthesis) of rheumatoid synovial cells by measuring the cellular incorporation of BrdU (Fig. 1). Indomethacin, diclofenac, oxaprozin, zaltoprofen, and NS-398 suppressed the cell proliferation in a concentration-dependent manner, with IC₅₀ values of 50.6 ± 8.2, 48.5 ± 4.3, 72.8 ± 23.4, 48.9 ± 8.4, and 58.7 ± 9.5 µM (n = 3, mean ± S.D.), respectively, whereas ketoprofen and acetaminophen had little or no effect on the cell proliferation at concentrations up to 300 µM.

We further examined the effects of NSAIDs on the proliferation of IL-1β-treated synovial cells. It is known that IL-1β induces COX-2 but not COX-1 expression in synovial cells (Kawai et al., 1998). However, treatment with IL-1β (1 ng/ml) did not influence the effects of NSAIDs on the proliferation of synovial cells (data not shown).

Effects of NSAIDs on Viability of Rheumatoid Synovial Cells. To explore whether cell death is involved in the suppression of cell proliferation caused by NSAIDs, the viability of rheumatoid synovial cells treated with NSAIDs was examined by WST-1 assay. As shown in Fig. 2, the viability of synovial cells was concentration-dependently reduced by in-
dometacin, diclofenac, oxaprozin, and zaltoprofen when the cells were incubated with these drugs for 24 h. In contrast, ketoprofen and acetaminophen, which had little or no effect on cell proliferation, had no effect on cell viability at concentrations up to 300 μM. In addition, NS-398 also had no effect on cell viability, although it suppressed cell proliferation. Prolonged treatment with ketoprofen, acetaminophen, or NS-398 (for 48–96 h) also had no appreciable effect on the cell viability (data not shown).

Furthermore, cell morphology was observed with a light microscope after treatment with NSAIDs (Fig. 3). The synovial cells treated with indometacin (300 μM), diclofenac (100 μM), oxaprozin (300 μM), or zaltoprofen (300 μM) showed distinctive morphological changes, cellular rounding, shrinkage, and membrane blebbing, and separated from neighboring cells. Treatment with NS-398 (300 μM), which suppressed the cell proliferation but had no effect on the cell viability, also revealed pronounced morphological changes, cellular elongation, although the morphological change was apparently different from that of indometacin-, diclofenac-, oxaprozin-, or zaltoprofen-treated cells. In contrast, cells treated with ketoprofen (300 μM) and acetaminophen (300 μM) maintained normal cell morphology.

PPARγ mRNA Expression and PPARγ Ligand-Induced Apoptosis in Rheumatoid Synovial Cells. Recently, it has been reported that activation of PPARγ induces apoptosis in various cell types such as cancer cells (Kitamura et al., 1999; Takahashi et al., 1999). Because some NSAIDs are ligands of PPARγ (Lehmann et al., 1997), we examined whether activation of PPARγ also induces apoptosis in rheumatoid synovial cells using two PPARγ ligands (Forman et al., 1995), the synthetic thiazolidinedione troglitazone, and natural PG 15dPGJ₂.

First, the expression of PPARγ mRNA in three rheumatoid synovial cell lines from three different patients was determined by RT-PCR. As shown in Fig. 4a, all three cell lines expressed PPARγ mRNA detected as a 474-bp RT-PCR product, similar to TPA-treated U937 cells used as a positive control, which have been shown to express PPARγ mRNA at a high level (Marx et al., 1998).

Next, the effects of troglitazone and 15dPGJ₂ on the proliferation of synovial cells were examined by BrdU incorporation assay (Fig. 4b). Both troglitazone and 15dPGJ₂ suppressed the proliferation of synovial cells, with IC₅₀ values of 14.1 ± 2.0 and 1.4 ± 1.4 μM (n = 3, mean ± S.D.), respectively. 15dPGJ₂ was more potent than troglitazone. Furthermore, the effects of the PPARγ ligands on DNA fragmentation, a hallmark of apoptosis, in synovial cells were quantitatively analyzed by fragmented DNA ELISA that specifically detects cytoplasmic histone-associated DNA fragments, and mono- and oligonucleosomes (Fig. 4c). Treatments with troglitazone and 15dPGJ₂ resulted in a 6-fold induction of cellular DNA fragmentation at 30 μM and 4-fold induction at 10 μM, respectively, compared with the untreated condition.

Induction of Apoptosis by NSAIDs in Rheumatoid Synovial Cells. To determine whether the synovial cell death induced by NSAIDs was due to apoptosis, we examined whether NSAIDs could induce DNA fragmentation in rheumatoid synovial cells, similar to PPARγ ligands. Ketoprofen and acetaminophen were not included in this examination because their effects on cell proliferation and viability were only minor or null. As shown in Fig. 5, indometacin, diclofenac, oxaprozin, and zaltoprofen induced DNA fragmentation in a concentration-dependent manner at 30 to 300 μM. These doses corresponded to the doses that induced cell death as previously determined by WST-1 assay (Fig. 2). Treatments with these NSAIDs resulted in a 3- to 4-fold induction of cellular DNA fragmentation at 300 μM compared with the untreated condition. In contrast, NS-398, which had an inhibitory effect on cell proliferation but had no effect on cell viability, did not affect DNA fragmentation at concentrations up to 300 μM.

To confirm that apoptosis was induced by NSAIDs, apoptotic synovial cells were detected by TUNEL assay after treatment with indometacin. As shown in Fig. 6, untreated synovial cells were not stained by TUNEL assay. In contrast, treatment with indometacin (300 μM) as well as troglitazone (30 μM) resulted in a significant number of TUNEL-positive cells.

Effects of NSAIDs on Activation of PPARγ in Rheumatoid Synovial Cells. To explore whether activation of PPARγ is involved in the mechanism by which NSAIDs induce apoptosis, we performed a luciferase reporter assay by cotransfection of rheumatoid synovial cells with a PPRE-driven luciferase reporter plasmid and PPARγ expression plasmid. As shown in Fig. 7a, PPARγ ligands, troglitazone, and 15dPGJ₂ significantly induced PPRE-driven luciferase activity in a concentration-dependent manner in this system. Similarly, indometacin, diclofenac, oxaprozin, and zaltoprofen, which could induce apoptosis,

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**Fig. 3.** Morphology of NSAID-treated rheumatoid synovial cells. Rheumatoid synovial cells were untreated (a) or treated with 300 μM indometacin (b), 100 μM diclofenac (c), 300 μM oxaprozin (d), 300 μM zaltoprofen (e), 300 μM ketoprofen (f), 300 μM acetaminophen (g), or 300 μM NS-398 (h) for 24 h. Cell morphology was observed with a light microscope. Arrows indicate representative morphological changes of synovial cells (magnification, 60×).
significantly induced activation of PPARγ/H9253 in synovial cells in a concentration-dependent manner (Fig. 7b). In contrast, ketoprofen, acetaminophen, and NS-398, which could not induce apoptosis, had little inductive effect on PPARγ/H9253 activation. Furthermore, we examined the relationship between the activation of PPARγ/H9253 and decreased cell viability by NSAIDs and PPARγ ligands. The concentration at which the drugs exhibit a 25-fold induction of the activation of PPARγ/H9253 in the luciferase reporter assay significantly correlated with the concentration at which the drugs reduce the viability of synovial cells by 50% in the WST-1 assay (r = 0.92, p < 0.01). In addition, we examined the relationship between the activation of PPARγ and induction of DNA fragmentation by NSAIDs and PPARγ ligands. The concentration at which the drugs exhibit a 25-fold induction of the activation of PPARγ also significantly correlated with the concentration at which the drugs exhibit 2-fold induction of DNA fragmentation in ELISA (r = 0.97, p < 0.001).

Discussion

Data obtained on the effects of individual NSAIDs on rheumatoid synovial cells are summarized in Table 1 with the inhibitory effects on COX activity, which were reported previously (Cryer and Feldman, 1998; Kawai et al., 1998). There was variation in the effects of different NSAIDs on proliferation, viability, and apoptosis in rheumatoid synovial cells. These effects were not correlated with the activities and specificities of NSAIDs toward COX isozymes. In addition, additional study showed that treatment with IL-1β, which can induce COX-2 (Kawai et al., 1998), did not influence the effects of NSAIDs on the proliferation of synovial cells, and that treatment of synovial cells with PGE2 tended to suppress the proliferation rather than stimulate it (data not shown). Taken together, these results indicate that inhibi-

Fig. 4. PPARγ mRNA expression and PPARγ ligand-induced apoptosis in rheumatoid synovial cells. a, RT-PCR analysis for PPARγ mRNA expression in rheumatoid synovial cells. Total RNA was extracted from rheumatoid synovial cells of three patients (RA1, RA2, and RA3) and TPA-treated U937 cells, and subjected to RT-PCR using specific primers for PPARγ and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as described under Materials and Methods. b, synovial cells were treated with troglitazone (●) or 15dPGJ₂ (○) for 24 h. The proliferation of the cells was assessed from cellular BrdU incorporation and presented as a percentage of the control value. Data are means ± S.D. of triplicate cultures. Results are representative of three independent experiments. *p < 0.05; **p < 0.001 versus untreated control cells. Highest concentration of 15dPGJ₂ is 30 μM. c, Effects of PPARγ ligands on DNA fragmentation in rheumatoid synovial cells. Synovial cells were treated with troglitazone (●) or 15dPGJ₂ (○) for 24 h. The amount of fragmented DNA in the cytoplasm of synovial cells was measured by ELISA using anti-histone and anti-DNA mouse monoclonal antibodies. The fold-induction of DNA fragmentation is given relative to the value for the control culture. Data are means ± S.D. of triplicate cultures. Results are representative of three independent experiments. *p < 0.05 versus untreated control cells.

Fig. 5. Effects of NSAIDs on DNA fragmentation in rheumatoid synovial cells. Synovial cells were treated with indometacin (●), diclofenac (○), oxaprozin (■), zaltoprofen (□), or NS-398 (●) for 24 h. The level of fragmented DNA in the cytoplasm was measured by ELISA using anti-histone and anti-DNA mouse monoclonal antibodies. The fold-induction of DNA fragmentation is given relative to the control value. Data are means ± S.D. of triplicate cultures. Results are representative of three independent experiments. *p < 0.05; **p < 0.001 versus untreated control cells.
tion of COX-1 or COX-2 does not contribute to the inhibition of cell proliferation and induction of apoptosis by NSAIDs. It is possible that NSAIDs induce apoptosis via cellular targets that are not necessarily related to their COX inhibitory activity or specificity. In this respect, the observation that some NSAIDs act as a direct ligand for PPARγ is of interest (Lehmann et al., 1997). Recent reports have indicated that PPARγ is expressed in a variety of cells such as cancer cells (Kitamura et al., 1999; Takahashi et al., 1999), monocyte-derived macrophages (Chinetti et al., 1998), endothelial cells (Bishop-Bailey and Hla, 1999), T lymphocytes (Harris and Phipps, 2001), and synovial cells (Kawahito et al., 2000), and ligands for PPARγ, thiazolidinediones, and 15dPGJ2 induce growth inhibition and apoptosis in these cells. We also demonstrated that rheumatoid synovial cells expressed PPARγ mRNA and that troglitazone and 15dPGJ2 increased the activation of PPARγ, reduced cell proliferation, and induced apoptosis in the synovial cells. Similarly, indometacin, diclofenac, oxaprozin, and zaltoprofen, which could induce apoptosis, also induced activation of PPARγ in rheumatoid synovial cells. The activation of PPARγ induced by these NSAIDs occurred over the concentration ranges that caused the apoptotic cell death. In contrast, ketoprofen, acetaminophen, and NS-398, which could not induce apoptosis, had little inductive effect on the activation of PPARγ. Of note, there is a clear and rigid correlation between the ability of these compounds to induce apoptosis and their ability to stimulate the activation of PPARγ in rheumatoid synovial cells. These results suggest that PPARγ is an attractive tar-
get for induction of apoptosis in rheumatoid synovial cells, and that the activation of PPARγ is associated with the induction of apoptosis by NSAIDs.

One of the pathological features of rheumatoid arthritis is synovial hyperplasia, which leads to the destruction of joints. The proliferation of synovial cells contributes to hyperplasia of the synovium and the formation of inflammatory pannus tissue that exhibits tumor-like proliferation and invades articular cartilage and surrounding tissues (Zvaifler and Fирестейн, 1994). The hyperplasia of invasive synovial cells has been proposed to be due to an imbalance between cell proliferation and apoptotic cell death (Еугучи, 2001). Therefore, the identification of agents that induce apoptosis in rheumatoid synovial cells may be a key step toward the successful treatment of rheumatoid arthritis (Hui et al., 1997; Kawakami et al., 1999). In this study, we demonstrated that some of the NSAIDs induce apoptosis in rheumatoid synovial cells. To our knowledge, this is the first report to document that a class of NSAIDs can induce cell death by apoptosis in synovial cells from a patient with chronic inflammation. However, it remains to be seen whether the results presented herein, obtained from an in vitro study, can be extrapolated to humans. In fact, much higher doses of NSAIDs were required to achieve an induction of apoptosis than to inhibit COX. However, it is possible that the concentrations of oxaprozin used herein can be reached in plasma in vivo. For example, plasma concentrations of oxaprozin are about 240 to 420 μM when oxaprozin is given orally at doses that regress inflammation in patients with rheumatoid arthritis (Todd and Brogden, 1986). Therefore, it is conceivable that the cellular effects we observed in vitro might occur in humans. Recently, we and others reported that several NSAIDs have chondroprotective effects via suppression of the promatrix metalloproteinase production and release of proteoglycans from synovial cells and chondrocytes (Akimoto et al., 2000; Yamazaki et al., 2000) and stimulate the synthesis of matrix in articular cartilage from patients with rheumatoid arthritis and osteoarthritis (Dingle, 1999). We also suggest herein that some of the NSAIDs, which can induce the activation of PPARγ, may suppress pannus formation through the apoptosis of synovial cells, thereby reducing cartilage destruction.

In summary, the present results demonstrated that some NSAIDs as well as PPARγ ligands induced apoptotic cell

**Table 1**

Summary of effects of NSAIDs and PPARγ ligands on cell proliferation, cell viability, apoptosis, and activation of PPARγ in rheumatoid synovial cells

<table>
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<tr>
<th>Drug</th>
<th>Proliferation</th>
<th>Viability</th>
<th>Apoptosis</th>
<th>PPARγ</th>
<th>IC₅₀</th>
<th>COX-1</th>
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<td>↑</td>
<td>54.5</td>
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<tr>
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<td>↑</td>
<td>54.5</td>
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N.T., not tested.

*IC₅₀ values for inhibition of cell viability.

*Concentration at which the drugs inhibit the growth of synovial cells, was observed in this study, is consistent with the results in colon cancer cells. In fact, the effective dose range of indometacin and diclofenac to inhibit the growth of synovial cells is the same as that needed to induce growth inhibition of colon cancer cells (Shiff et al., 1996; Seed et al., 1997). Furthermore, additional study showed that the effects of the seven NSAIDs used herein on proliferation, viability, and DNA fragmentation in HT-29 colon adenocarcinoma cells were very similar to the effects in synovial cells observed in this study (data not shown). Interestingly, recent reports have demonstrated that PPARγ is also expressed in colon cancer cells, including HT-29 cells (Kitamura et al., 1999). This evidence suggests that some NSAIDs likely exert their apoptotic effects in colon cancer cells as well as synovial cells through the activation of PPARγ. Further study of this matter is needed.

Traditional NSAIDs inhibit not only COX-2 but also COX-1 activity, resulting in their most common side effect, gastric damage. To reduce the side effects, selective COX-2 inhibitors have been developed (Jackson and Hawkey, 2000). Therefore, it is of interest whether selective COX-2 inhibitors can induce activation of PPARγ. In this study, a selective COX-2 inhibitor, NS-398, inhibited cell proliferation but did not induce apoptosis in rheumatoid synovial cells. Furthermore, NS-398 had no effect on the activation of PPARγ in synovial cells. It has been reported that selective COX-2 inhibitors, including NS-398, reduce the angiogenesis driven by basic fibroblast growth factor and vascular endothelium growth factor (Masferrer et al., 1999). Basic fibroblast growth factor derived from rheumatoid synovial cells also plays a role in stimulating their proliferation in an autocrine manner (Melnyk et al., 1990). Thus, it is possible that NS-398 suppresses the proliferation of synovial cells through control of the growth factors.

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<tr>
<td>Zaltoprofen</td>
<td>↓</td>
<td>&gt;300</td>
<td>N.T.</td>
<td>&gt;100</td>
<td>42</td>
<td>11</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>↓</td>
<td>&gt;300</td>
<td>N.T.</td>
<td>&gt;100</td>
<td>12</td>
<td>0.0055</td>
<td>1263</td>
<td></td>
</tr>
</tbody>
</table>

N.T., not tested.

*IC₅₀ values for inhibition of COX-1 and COX-2 by acetaminophen were cited from the reference by Cryer et al. (1998).

*IC₅₀ values for inhibition of COX-1 and COX-2 by NSAIDs were cited from the reference by Kawai et al. (1998).

*Concentration at which the drugs exhibit a 25-fold induction of the activation of PPARγ over the untreated condition in the luciferase reporter assay.

*Decrease.

*No change.

*Increase.

One of the pathological features of rheumatoid arthritis is synovial hyperplasia, which leads to the destruction of joints. The proliferation of synovial cells contributes to hyperplasia of the synovium and the formation of inflammatory pannus tissue that exhibits tumor-like proliferation and invades articular cartilage and surrounding tissues (Zvaifler and Fir-esteem, 1994). The hyperplasia of invasive synovial cells has been proposed to be due to an imbalance between cell proliferation and apoptotic cell death (Еугучи, 2001). Therefore, the identification of agents that induce apoptosis in rheumato-oid synovial cells may be a key step toward the successful treatment of rheumatoid arthritis (Hui et al., 1997; Kawakami et al., 1999). In this study, we demonstrated that some of the NSAIDs induce apoptosis in rheumatoid synovial cells. To our knowledge, this is the first report to document that a class of NSAIDs can induce cell death by apoptosis in synovial cells from a patient with chronic inflammation. However, it remains to be seen whether the results presented herein, obtained from an in vitro study, can be extrapolated to humans. In fact, much higher doses of NSAIDs were required to achieve an induction of apoptosis than to inhibit COX. However, it is possible that the concentrations of oxaprozin used herein can be reached in plasma in vivo. For example, plasma concentrations of oxaprozin are about 240 to 420 μM when oxaprozin is given orally at doses that regress inflammation in patients with rheumatoid arthritis (Todd and Brogden, 1986). Therefore, it is conceivable that the cellular effects we observed in vitro might occur in humans. Recently, we and others reported that several NSAIDs have chondroprotective effects via suppression of the promatrix metalloproteinase production and release of proteoglycans from synovial cells and chondrocytes (Akimoto et al., 2000; Yamazaki et al., 2000) and stimulate the synthesis of matrix in articular cartilage from patients with rheumatoid arthritis and osteoarthritis (Dingle, 1999). We also suggest herein that some of the NSAIDs, which can induce the activation of PPARγ, may suppress pannus formation through the apoptosis of synovial cells, thereby reducing cartilage destruction.

In summary, the present results demonstrated that some NSAIDs as well as PPARγ ligands induced apoptotic cell
death in rheumatoid synovial cells. Furthermore, these effects paralleled the increase in activation of PPARγ. Therefore, it is possible that the apoptotic effects of NSAIDs are, at least in part, due to their inductive effects on the activation of PPARγ. These results suggest that the activation of PPARγ caused by some NSAIDs may help to prevent the degradation of articular cartilage in rheumatoid arthritis through the induction of apoptosis in synovial cells, after the inhibition of synovial hyperplasia and pannus formation.

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References


