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)-2H-5-tetrazolio]-1,3-benzene disulfonate (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. Indomethacin, diclofenac, oxaprozin, and zaltoprofen reduced cell proliferation and induced apoptotic cell death in synovial cells, whereas ketoprofen and acetaminophen did not. N-[2-(cyclohexyloxyl)-4-nitrophenyl]-methanesulfonamide (NS-398), a selective cyclooxygenase-2 inhibitor, also inhibited cell proliferation, whereas it did not cause 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 isofoms 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.,

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; PPARγ, peroxisome proliferator-activated receptor γ; 15dPGJ2, 15-deoxy-Δ12,14-PGJ2; TPA, 12-O-tetradecanoylphorbol 13-acetate; FBS, fetal bovine serum; IL-1β, interleukin-1β; BrdU, 5-bromo-2-deoxyuridine; ELISA, enzyme-linked immunosorbent assay; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; bp, base pair(s); PPRE, peroxisome proliferator response element; NS-398, N-[2-(cyclohexyloxyl)-4-nitrophenyl]-methanesulfonamide.
1996; Elder et al., 1997) mechanism in the apoptotic action of NSAIDs has been reported. Therefore, the mechanism by which NSAIDs induce apoptosis is not well defined. Thus, it is possible that, in addition to COX, NSAIDs interact with other cellular targets (Tegeder et al., 2001).

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of transcription factors that mediates ligand-dependent transcriptional activation and repression (Marcus et al., 1993). PPARγ is expressed at high level in adipose tissue (Forman et al., 1995) and monocyte-derived macrophages (Marx et al., 1998), and it plays a pivotal role in adipocyte and macrophage differentiation. Recently, some NSAIDs, including indometacin, have been shown to act as a direct ligand for PPARγ (Lehmann et al., 1997). In addition, ligand activation of PPARγ in monocyte/macrophages has been shown to inhibit inflammatory mediator and cytokine production (Jiang et al., 1998; Ricote et al., 1998), which is regarded as a COX-independent mechanism of anti-inflammatory action of NSAIDs. Moreover, recent reports have indicated that PPARγ is also expressed in a variety of cancer cells such as colon (Kitamura et al., 1999) and gastric (Takahashi et al., 1999) cancer cells, and that specific ligands for PPARγ such as synthetic thiazolidinediones induce growth inhibition and apoptosis in these cells. In addition to the cancer cells, PPARγ activation can induce apoptosis in monocyte-derived macrophages (Chinetti et al., 1998), endothelial cells (Bishop-Bailey and Hla, 1999), and T lymphocytes (Harris and Phipps, 2001). However, it is unclear whether activation of the PPARγ pathway is associated with the apoptotic action of NSAIDs.

Recent evidence also shows that synovial cells from patients with rheumatoid arthritis express PPARγ, and that ligands for PPARγ, a thiazolidinedione, troglitazone, and a natural PG, 15-deoxy-Δ12,14-PGJ2 (15dPGJ2), inhibit the growth of synovial cells through apoptosis (Kawahito et al., 2000). Therefore, we used rheumatoid synovial cells as a model to investigate the possible mechanisms of apoptotic action of NSAIDs, and describe herein the effect of seven NSAIDs, which display differential COX-1 and COX-2 inhibitory activity, on the tumor-like proliferation of synovial cells. In addition, we also analyzed the activation of PPARγ associated with induction of apoptosis by NSAIDs in the rheumatoid synovial cells.

Experimental Procedures

Materials. Indometacin, diclofenac, ketoprofen, acetaminophen, RPMI 1640, and 12-O-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma-Aldrich (St. Louis, MO). N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) and 15dPGJ2 were purchased from Cayman Chemicals (Ann Arbor, MI). Oxaprozin and zaltoprofen were obtained from Wyeth Lederle Japan (Tokyo, Japan) and Zeria (Tokyo, Japan), respectively. Troglitazone was supplied by Sankyo (Tokyo, Japan). Also purchased were fetal bovine serum (FBS) (In Vitrogen, Carlsbad, CA) and interleukin-1β (IL-1β) (Genzyme-Technie, Cambridge, MA).

Cells and Cell Cultures. Rheumatoid synovial cells were prepared from synovial tissues as described previously with slight modification (Kawai et al., 1998; Yamazaki et al., 2000). The synovial tissues were obtained during a total knee replacement from patients with rheumatoid arthritis who fulfilled the revised American Rheumatism Association criteria for the classification of rheumatoid arthritis (Arnett et al., 1988). Experiments were carried out according 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 (In Vitrogen). 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/monocytes (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 to 96 h, cell viability was measured as mitochondrial NADH-dependent dehydrogenase activity with a Cell Counting kit (Dojindo, Kumamoto, Japan) 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 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 monocular 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 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'-TCTCTTCGTAATGGAAGACC-3' (sense) and 5'-GCATTATGAGACATCACC-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'-CCACCATTGCTAAATCTCATGCA-3' (sense) and 5'-TCTGGAGCAGTGGTACGTCACC-3' (antisense), yielding a 598-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 XbaI 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-
duced Apoptosis in Rheumatoid Synovial Cells.

Recently, it has been reported that activation of PPAR

...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 acetonaminophen (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 1.4 ± 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 acetonaminophen 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 expression 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,
significantly induced activation of PPAR\textsubscript{gamma} 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 PPAR\textsubscript{gamma} activation. Furthermore, we examined the relationship between the activation of PPAR\textsubscript{gamma} and decreased cell viability by NSAIDs and PPAR\textsubscript{gamma} ligands. The concentration at which the drugs exhibit a 25-fold induction of the activation of PPAR\textsubscript{gamma} 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 ($r^{2} = 0.92$, $p = 0.01$).

In addition, we examined the relationship between the activation of PPAR\textsubscript{gamma} and induction of DNA fragmentation by NSAIDs and PPAR\textsubscript{gamma} ligands. The concentration at which the drugs exhibit a 25-fold induction of the activation of PPAR\textsubscript{gamma} 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-1\beta, 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 PGE\textsubscript{2} tended to suppress the proliferation rather than stimulate it (data not shown). Taken together, these results indicate that inhibi-
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-

Fig. 6. Detection of apoptotic synovial cells by TUNEL assay. Rheumatoid synovial cells were left untreated (a) or else treated with 300 μM indometacin (b) or 30 μM troglitazone (c) for 24 h, and then apoptotic cells were identified by TUNEL assay as described under Materials and Methods. The synovial cells were also counterstained with methyl green. TUNEL-positive cells (apoptotic cells) are stained brown (magnification, 60×).

Fig. 7. Effects of NSAIDs on the activation of PPARγ in rheumatoid synovial cells. Rheumatoid synovial cells were cotransfected with a PPRE-driven luciferase reporter plasmid, PPARγ expression plasmid, and internal control plasmid. The transfected cells were treated with PPARγ ligands (a) or NSAIDs (b) for 18 h. Luciferase activity in cells was determined as described under Materials and Methods. The fold-induction of luciferase activity is relative to untreated control cells. Data are means ± S.D. of triplicate cultures. Results are representative of three independent experiments. *, p < 0.05 and **, p < 0.001 versus untreated control cells.
get for induction of apoptosis in rheumatoid synovial cells, and that the activation of PPARγ is associated with the induction of apoptosis by NSAIDs.

It has been reported that NSAIDs can inhibit the proliferation of human colon cancer cells (Hanif et al., 1996; Shiff et al., 1996; Elder et al., 1997; Seed et al., 1997; Li et al., 2001). The ability of NSAIDs to inhibit the growth of rheumatoid synovial cells, which was observed in this study, is consistent with the results in colon cancer cells. In fact, the effective dose range of indomethacin 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 (Melnik et al., 1990). Thus, it is possible that NS-398 suppresses the proliferation of synovial cells through control of the growth factors.

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 Firestein, 1994). The hyperplasia of invasive synovial cells has been proposed to be due to an imbalance between cell proliferation and apoptotic cell death (Eguchi, 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. It has been reported that NSAIDs can 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. It has been reported that NSAIDs can induce apoptosis in rheumatoid synovial cells.

**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>
<thead>
<tr>
<th>Drug</th>
<th>Proliferation</th>
<th>Viabilitya</th>
<th>Apoptosis</th>
<th>PPARγb</th>
<th>IC50\textsuperscript{c}</th>
<th>COX-1</th>
<th>COX-2</th>
<th>COX-1/COX-2 Ratio</th>
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</thead>
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<tr>
<td></td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
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<tr>
<td>Troglitazone</td>
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<td>25.3</td>
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<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>15dPGJ\textsubscript{2}</td>
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<td>&lt;0</td>
<td>0.8</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
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</tr>
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<td>&gt;0</td>
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<td>0.044</td>
<td>0.30</td>
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<tr>
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<td>&gt;0</td>
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<td>0.0097</td>
<td>38</td>
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<td>&gt;0</td>
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<td>&gt;0</td>
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<td>0.34</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>&lt;0</td>
<td>&gt;300</td>
<td>N.T.</td>
<td>&gt;100</td>
<td>42\textsuperscript{d}</td>
<td>11\textsuperscript{f}</td>
<td>3.8\textsuperscript{e}</td>
<td></td>
</tr>
<tr>
<td>NS-398</td>
<td>&lt;0</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>

\textsuperscript{a}IC50 values for inhibition of cell viability.
\textsuperscript{b}Concentration at which the drugs exhibit a 25-fold induction of the activation of PPARγ over the untreated condition in the luciferase reporter assay.
\textsuperscript{c}IC50 values for inhibition of COX-1 and COX-2 by NSAIDs were cited from the reference by Kawai et al. (1998).
\textsuperscript{d}IC50 values for inhibition of COX-1 and COX-2 by acetaminophen were cited from the reference by Cryer et al. (1998).
\textsuperscript{e}Decrease.
\textsuperscript{f}No change.
\textsuperscript{g}Increase.

N.T., not tested.

The proliferation of synovial cells can from synovial cells and chondrocytes (Akimoto et al., 2000) and stimulate the synthesis of matrix metalloproteinase production and release of proteoglycan 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 can 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. It has been reported that NSAIDs can induce apoptosis in rheumatoid synovial cells.
death in rheumatoid synovial cells. Furthermore, these effects paralleled the increase in activation of PPARy. Therefore, it is possible that the apoptotic effects of NSAIDs are, at least in part, due to their inductive effects on the activation of PPARy. These results suggest that the activation of PPARy 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