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Comparison of cyclooxygenase-1 and -2 inhibitory activities of various nonsteroidal anti-inflammatory drugs using human platelets and synovial cells

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Abstract

Recent studies have shown that cyclooxygenase exists in two isozyme forms. Since differences in the pharmacological profiles of nonsteroidal anti-inflammatory drugs (NSAIDs) might be accounted for by varying degrees of selectivity for these isozymes, cyclooxygenase-1 and -2, the relative potency of various NSAIDs in inhibiting their activities was examined in intact human cells. We used human platelets cyclooxygenase-1 and interleukin-1β-stimulated human synovial cell cyclooxygenase-2 for measuring cyclooxygenase selectivity. The presence of the enzymes was confirmed by immunoblotting and immunoprecipitation analysis, and by the reverse transcriptase-polymerase chain reaction. Mean IC_{50} values (μM) for human platelet cyclooxygenase-1 and interleukin-1β-stimulated human synovial cell cyclooxygenase-2 and cyclooxygenase-1/-2 IC_{50} ratio of various NSAIDs were as follows: aspirin, 3.2, 26, 0.12; diclofenac, 0.037, 0.00097, 38; etodolac, 122, 0.68, 179; ibuprofen, 3.0, 3.5, 0.86; indomethacin, 0.013, 0.044, 0.30; loxoprofen (active metabolite), 0.38, 0.12, 3.2; NS-398, 12, 0.0095, 1263; oxaprozin, 2.2, 36, 0.061; zaltoprofen, 1.3, 0.34, 3.8; respectively. Our bioassay system employing intact human cells to assess the cyclooxygenase selectivity of NSAIDs may provide clinically useful information.

Keywords: Cyclooxygenase-1; Cyclooxygenase-2; Nonsteroidal anti-inflammatory drug; Platelet; Synovial cell

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) produce their therapeutic and toxic effects by decreasing the biosynthesis of prostaglandins and other proinflammatory agents (Vane, 1971). NSAIDs decrease the production of proinflammatory prostaglandins by the inhibition of cyclooxygenase or prostaglandin G/H synthase (Flower et al., 1972). Recent studies have shown that cyclooxygenase exists in two isozyme forms (Xie et al., 1991; Kujubu et al., 1991) which differ in their basal expression, tissue localization, and induction during inflammation (Vane and Botting, 1996). Cyclooxygenase-1 is constitutively expressed and has been detected in every cell type examined to date (O'Neill and Ford-Hutchinson, 1993). The level of expression of the cyclooxygenase-1 gene, as detected by quantitative cyclooxygenase-1 mRNA studies, shows little change during the inflammatory process (Crofford et al., 1994). In contrast, cyclooxygenase-2 expression is nearly undetectable in unstimulated cells, but is inducible, and its induction coincides with an increase of inflammatory prostaglandins in vivo (Masferrer et al., 1994). The expression of cyclooxygenase-2 varies in magnitude over the course of the inflammatory response (Appleton et al., 1994; Anderson et al., 1996). The existence of these two cyclooxygenase isozymes with different characteristics may help explain differences in the pharmacological profiles of various NSAIDs and may have important clinical consequences. It has been proposed that cyclooxygenase-1 inhibition in gastric mucosal cells and the resultant decreased synthesis of prostaglandin E_2 with loss of a local protective effect accounts for the significant gastric toxicity of NSAIDs (Emery, 1996). Similarly, cyclooxygenase-1 inhibition in renal cells decreases prostaglandin E_2 synthesis and causes loss of the regulation of vital renal functions,

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which may account for the renal toxicity of NSAIDs (Inoue et al., 1994). Cyclooxygenase-2 activity, which parallels the onset of inflammation and produces proinflammatory substances, is clearly a more selective therapeutic target for NSAIDs.

Differences in the pharmacological profiles of various NSAIDs might be accounted for by varying degrees of selectivity for cyclooxygenase-1 and -2. Previous studies have compared the selective inhibition of cyclooxygenase-1 and -2 by NSAIDs using various systems. They were categorized into intact cell systems using murine macrophages (Mitchell et al., 1994) or non-human cell-lines transfected with human cyclooxygenase genes (Laneuville et al., 1994; Riendeau et al., 1997) or human whole blood (Glaser et al., 1995; Brideau et al., 1996; Riendeau et al., 1997) or human mononuclear cells and platelets (Grossman et al., 1995), or enzyme systems using cyclooxygenases derived non-human tissues (Mitchell et al., 1994; Yamazaki et al., 1997) or human recombinant cyclooxygenases (Laneuville et al., 1994; Glaser et al., 1995). The relative potency and selectivity of some NSAIDs in these models appears to correspond with their gastric toxicity, with the more cyclooxygenase-2-selective agents exhibiting lower incidence of gastric injury (Hayllar and Bjarnason, 1995).

Cyclooxygenase-2 in the synovial cells of patients with rheumatoid arthritis plays an important role in the inflammatory process (Sano et al., 1992). Since arthritis is one of the major clinical applications of NSAIDs, the present study was designed to characterize the cyclooxygenase-1 and cyclooxygenase-2 inhibitory activities of various NSAIDs using human platelets and synovial cells.

2. Materials and methods

2.1. Reagents

RPMI-1640, 100 U/ml penicillin with 100 μg/ml streptomycin, fetal calf serum, dialyzed-fetal calf serum, HEPES buffer, and Hank's balanced salt solution (HBSS) were all obtained from Gibco (Gaithersburg, MD, USA). Recombinant human interleukin-1β, tumor necrosis factor α (TNF-α), interleukin-2, interleukin-4, interleukin-6, and interferon γ were purchased from Genzyme (Cambridge, MA, USA). Enzyme-linked immunosorbent assay kits for prostaglandin E_2, and thromboxane B_2 were purchased from Cayman (Ann Arbor, MI, USA). Polyclonal rabbit anti-ovine cyclooxygenase-1, which reacted with human cyclooxygenase-1, and polyclonal rabbit anti-human cyclooxygenase-2 antibodies were obtained from Oxford Biomedical (Oxford, MI, USA). Other reagents were purchased from Wako (Osaka, Japan).

The NSAIDs tested were as follows: aspirin, diclofenac sodium, ibuprofen, and indomethacin (Sigma, St. Louis, MO, USA); etodolac and oxaprozin [Wyeth (Japan), Tokyo, Japan]; (2*S*)-2-[4-*trans*-(1*R*,2*S*)-2-hydroxycyclopentylmethyl)phenyl] propionic acid (loxoprofen-SRS, an active metabolite of loxoprofen sodium, Sankyo, Tokyo, Japan); *N*-[2-cyclohexyloxyl-4-nitrophenyl] methanesulfonamide (NS-398, Taisho, Tokyo, Japan); and zaltoprofen (Zeria, Tokyo, Japan).

2.2. Preparations of human platelets and synovial cells

Platelets were prepared by a modification of a previously described method (Minkes et al., 1977). Peripheral venous blood was obtained from healthy volunteers and mixed with 7.5% (v/v) 77 mM ethylenediaminetetraacetic acid (EDTA) by gentle inversion. The mixture was centrifuged at $150 \times g$ for 10 min to obtain platelet-rich plasma. The platelet-rich plasma was then centrifuged at $800 \times g$ for 15 min to obtain a platelet-rich pellet, which was suspended in HBSS with 10 mM HEPES and 1.54 mM EDTA. Washed platelets were resuspended in HBSS with 10 mM HEPES and 0.5 mM $MgCl_2$, and then used for the following studies.

Human synovial cells were obtained from patients with rheumatoid arthritis at the time of total knee replacement. Synovial tissues were digested with 0.2% collagenase for 90 min and were cultured in RPMI-1640 with penicillin and streptomycin and 10% fetal calf serum at 37°C under 5% CO_2. Synovial cells that adhered to plastic flasks were cultured as described previously (Dayer et al., 1976; Yamazaki et al., 1997). Cells at passage 1 to 3 were resuspended in RPMI-1640 medium and used for following studies.

2.3. Western blotting analysis

Synovial cells were incubated with or without 1 ng/ml of interleukin-1β for 24 h. Washed human platelets and interleukin-1β-treated synovial cells were lysed in 25 mM Tris–HCl (pH 7.5) containing 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 0.1% Tween-20 on ice, and were centrifuged at $10\,000 \times g$ for 10 min at 4°C. The supernatant was collected and the protein content was determined using the DC Protein Assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard. Cell lysates, which were adjusted to 100 μg protein for platelets and 70 μg protein for synovial cells, were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10% (w/v) acrylamide slab gels under reducing conditions. Then the proteins were electro-transferred onto nitrocellulose membranes. After blocking with 5% non-fat powdered milk, the membranes were reacted with an anti-cyclooxygenase-1 antibody (dilution 1:100) or an anti-cyclooxygenase-2 antibody (dilution 1:10). Immunoreactive bands were visualized with biotinylated anti-rabbit immunoglobulin G (IgG), streptavidin–peroxidase conjugate, and

3-amino-9-ethylcarbazole as the substrate (HistoScan, Biomeda, Foster City, CA). Rainbow™ colored protein molecular weight markers (Amersham, Buckinghamshire, UK) were used for detection of approximate molecular weight of the bands.

2.4. Metabolic labeling and immunoprecipitation

Synovial cells (4×10^5 cells/well) were plated into 6-well plates containing RPMI-1640 with 10% fetal calf serum. The cells were grown to confluence and then cultured with or without 1 ng/ml of interleukin-1β in RPMI-1640 with 2% fetal calf serum for 18 h. Next, cells were washed and incubated for 4 h with methionine-free RPMI-1640 containing 2% dialyzed fetal calf serum and 100 μCi of [^{35}S]methionine (Amersham). Labelled cells were washed 3 times with phosphate-buffered saline (PBS) and solubilized in solubilization buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Tween 20, pH 7.6) for 60 min on ice. After centrifuging the cell lysates for 15 min at $18\,000 \times g$, 70 μl of each supernatant adjusted as equal amount of radioactivity was precleaned by incubation with 10 μl of non-immune rabbit serum (Sigma) at 4°C for 60 min, followed by addition of protein G/A-agarose (Oncogene, Uniondale, NY) and incubation for an additional 60 min. After centrifugation for 5 min at $15\,000 \times g$, the precleaned samples were incubated with 2 μl of anti-cyclooxygenase-1 antibody or 10 μl of anti-cyclooxygenase-2 antibody for 60 min at 4°C. After protein G/A-agarose was added for 60 min, immune complexes were collected by centrifugation for 5 min at $15\,000 \times g$. The pellet was washed twice with 50 mM Tris buffer containing 150 mM NaCl, 1 mM EDTA, 10 μg/ml aprotinin, and 0.5% Tween 20 (pH 7.6), and then washed twice with the same Tris buffer without detergent. Then the pellet was suspended in reducing SDS-PAGE sample buffer, boiled for 3 min, and centrifuged for 5 min at $15\,000 \times g$. The supernatant was subjected to 10% SDS-PAGE. The gel was stained with Coomassie brilliant blue, dried, and exposed to Hyperfilm-MP (Amersham) at −70°C.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

To identify mRNA for cyclooxygenase-1 and cyclooxygenase-2 in synovial cells by RT-PCR, we automatically synthesized (DNA Synthesizer Model 380B, Applied Biosystems, Foster City, CA, USA) primers based on the report of O'Neill and Ford-Hutchinson (1993). For cyclooxygenase-1, the primers were 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ (a 24-mer sense oligonucleotide at position 516) and 5′-GTGCATCAACACAGGCGCCTCTTC-3′ (a 24-mer antisense oligonucleotide at position 819), yielding a 303 bp PCR product. For cyclooxygenase-2, the primers were 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ (a 27-mer sense, oligonucleotide at position 573) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (a 24-mer antisense oligonucleotide at position 878), yielding a 305 bp PCR product. Primers were also synthesized to amplify the cDNA encoding human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed gene, as control. The primers for GAPDH were 5′-CCACCCATGGCAAATTCCATGGCA-3′ (a 24-mer sense oligonucleotide at position 216) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (a 24-mer antisense oligonucleotide at position 809), yielding a 593 bp product. After treatment with or without 1 ng/ml interleukin-1β for 4 h in RPMI-1640 with 2% fetal calf serum, total RNA from synovial cells was extracted using an ISOGEN nucleic acid extraction kit (NipponGene, Tokyo, Japan) and the thiocyanic acid–guanidine method (Chomczynski and Sacchi, 1987). Total RNA (0.7 μg) was converted to single-stranded cDNA using an RNA PCR kit (TaKaRa, Ohtsu, Japan) with avian myeloblastosis virus reverse transcriptase XL and 50 pmol/μl of random 9 mer according to the manufacturer's protocol. The cDNA samples were then split into 3 equal aliquots for amplification by the specific primers for cyclooxygenase-1, cyclooxygenase-2, and GAPDH. PCR reactions were carried out in a buffer containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.2 mM deoxynucleotides triphosphates, 0.2 μM primers, and 2.5 U/100 μl *Taq* DNA polymerase (TaKaRa) using a cycling program of 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min in a Perkin Elmer DNA Thermal Cycler PJ2000. After 30 cycles, aliquots of the products were separated by 2.5% agarose gels and visualized by ethidium bromide staining.

2.6. Thromboxane B_2 production by human platelets

The inhibitory effect of NSAIDs on thromboxane B_2 production was evaluated in human platelets. The platelet count was adjusted to 2×10^8 cells/ml, and the suspension was placed into tubes that were incubated at 37°C for 3 min. Then various NSAIDs or vehicle (the final concentration of ethanol was 0.1%) were added to the suspensions and incubation was done at 37°C for 15 min. After incubation, arachidonic acid (final concentration: 3 μM) was added to each platelet suspension to evaluate cyclooxygenase enzyme activity by inducing thromboxane B_2 production. Fifteen min later, ice-cold HBSS was added to stop the reaction. Each reaction solution was then centrifuged at $10\,000 \times g$ for 3 min at 4°C, and the thromboxane B_2 concentration in the supernatant was measured using a thromboxane B_2 assay kit. Each measurement was done in triplicate.

2.7. Prostaglandin E_2 production by human synovial cells

Human synovial cells were plated in 48-well plastic plates (0.7 to 3×10^4 cells/well) and cultured for 24 to 48

h. After washing with RPMI-1640, the cells were incubated with interleukin-1β, TNF-α, interleukin-2, interleukin-6, or interferon γ in RPMI-1640 containing 2% fetal calf serum for 24 h to detect the effects of various cytokines. Then cells were washed with RPMI-1640 without fetal calf serum and 3 μM arachidonic acid was added to each well. After incubation for 30 min, the culture medium was centrifuged at $1000 \times g$ at 4°C for 1 min. For NSAIDs studies, the cells were incubated with 1 ng/ml interleukin-1β in RPMI-1640 containing 2% fetal calf serum for 24 h. Cells were washed with RPMI-1640 without fetal calf serum and then treated with various NSAIDs or the vehicle at 37°C for 30 min (the final concentration of ethanol was 0.1%). After incubation, 3 μM arachidonic acid was added to each well to evaluate cyclooxygenase enzyme activity by inducing prostaglandin E_2 production, and the plate was incubated for an additional 30 min. Then the culture medium was centrifuged at $1000 \times g$ at 4°C for 1 min. In both experiments, the concentration of prostaglandin E_2 in the supernatant was measured using an assay kit and each measurement was done in triplicate.

3. Results

3.1. Effects of cytokines on prostaglandin E_2 production by synovial cells

Fig. 1 shows the effects of various cytokines on the production of prostaglandin E_2 by human synovial cells. There were good dose–response curves for interleukin-1β and TNF-α. However, interleukin-2, interleukin-4, inter-

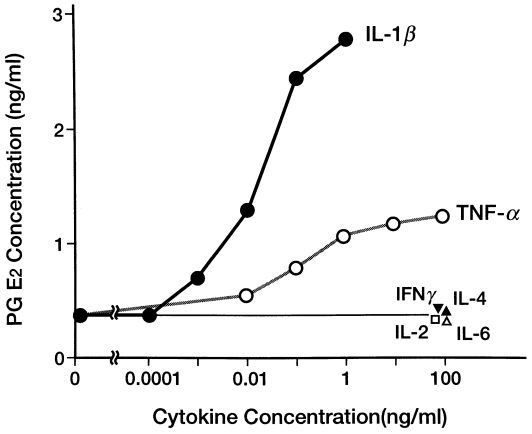

Fig. 1. Effect of various cytokines on the production of prostaglandin E_2 by human synovial cells. Synovial cells were treated with various cytokines at 37°C for 24 h. After washing the cells, 3 μM arachidonic acid was added for 30 min and then the prostaglandin E_2 concentration of the medium was measured. Each point represents mean value of three samples. IL = interleukin; TNF-α = tumor necrosis factor α; IFNγ = interferon γ.

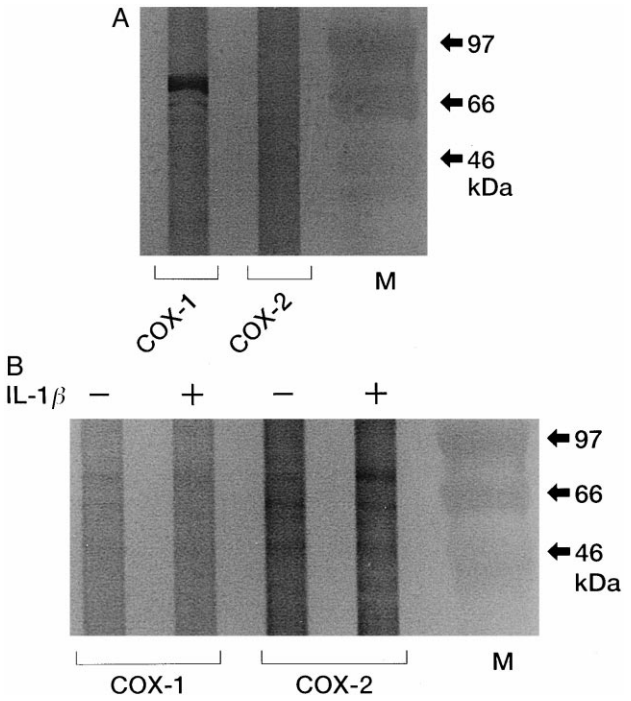

Fig. 2. Western blot analysis of cyclooxygenase-1 and -2 in human platelets (Panel A) and synovial cells (Panel B). Cell lysates (100 μg protein for platelets and 70 μg protein for synovial cells) were subjected to 10% SDS-PAGE. Proteins transferred onto nitrocellulose membrane were treated with specific rabbit antibodies against cyclooxygenase-1 or -2, and then visualized using biotinylated anti-rabbit IgG, streptavidin-peroxidase conjugate, and 3-amino-9-ethylcarbazole as the substrate. COX = cyclooxygenase, IL = interleukin, M = protein molecular mass markers.

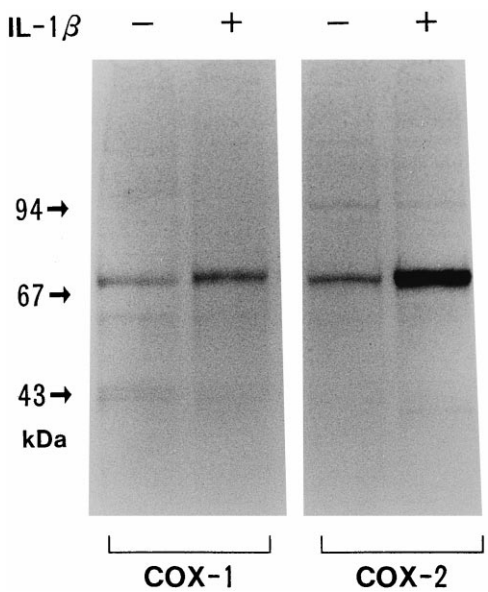

Fig. 3. Immunoprecipitation analysis of cyclooxygenase-1 and -2 proteins in human synovial cells. Solubilized [^{35}S]methionine-labelled proteins were precipitated by specific rabbit antibodies against cyclooxygenase-1 or -2 and incubation with protein G/A-agarose at 4°C for 60 min. After 10% SDS-PAGE, the dried gels were exposed to X-ray film. COX = cyclooxygenase, IL = interleukin.

leukin-6, and interferon γ had no effect on prostaglandin E_2 production by human synovial cells.

3.2. Western blotting for cyclooxygenase-1 and -2

The anti-cyclooxygenase-1 antibody recognized a band of approximately 70 kDa in human platelets, while the anti-cyclooxygenase-2 antibody did not recognize any band in the same sample (Fig. 2, Panel A). In contrast, the anti-cyclooxygenase-1 antibody recognized a weak band in human synovial cells cultured with or without 1 ng/ml of interleukin-1β. The cyclooxygenase-2 band was visible at a position of approximately 70 kDa in human synovial cells without interleukin-1β stimulation, and it was enhanced by the addition of interleukin-1β (Fig. 2, Panel B).

3.3. Immunoprecipitation of cyclooxygenase-1 and -2 in synovial cells

Since the sensitivity of the Western blotting was not satisfactory, as mentioned above, we examined de novo synthesis of cyclooxygenase-1 and -2 in rheumatoid synovial cells by the immunoprecipitation method (Fig. 3). Cells were labelled with [^{35}S]methionine and extracts were immunoprecipitated with anti-cyclooxygenase-1 or -2 antibodies as described in Section 2. Untreated cells synthesized both cyclooxygenase-1 and -2 proteins, however, treatment of the cells with interleukin-1β for 4 h resulted in a little and marked enhancement of cyclooxygenase-1 and -2 syntheses, respectively.

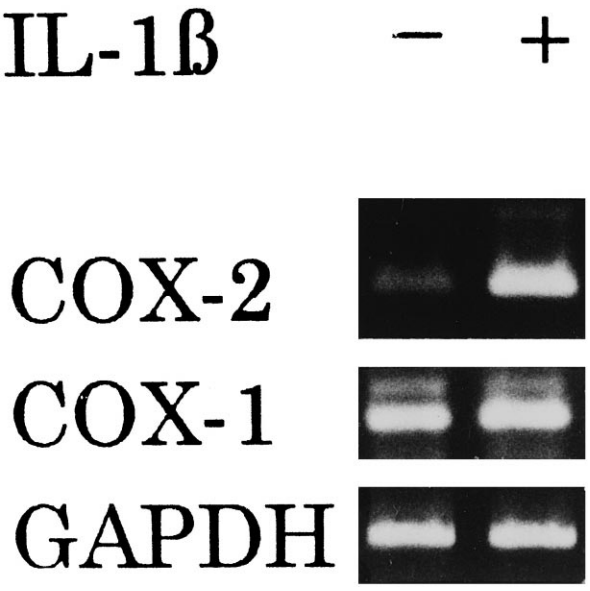

Fig. 4. RT-PCR analysis of cyclooxygenase-1, cyclooxygenase-2, and GAPDH mRNA in synovial cells. Cells with or without interleukin-1β treatment for 4 h were subjected to extraction of total RNA which was converted to cDNA by reverse transcriptase XL. Then amplification by the polymerase chain reaction was done using specific primers for cyclooxygenase-1, cyclooxygenase-2, and GAPDH as described in the text. IL = interleukin, COX = cyclooxygenase, GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

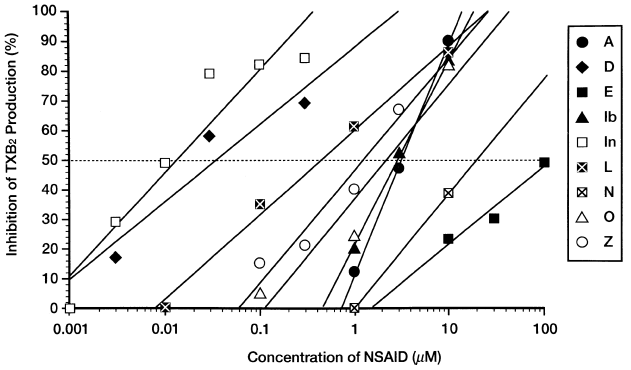

Fig. 5. Inhibitory effects of various NSAIDs on thromboxane B_2 production by human platelets. The platelet suspension was incubated with each NSAID at 37°C for 15 min followed by addition of arachidonic acid. After another 15 min of incubation, the thromboxane B_2 concentration of the supernatant was measured. Results are expressed as the mean percentagerelative to the control without NSAID. Each point shows the mean percent inhibition for each NSAID in all experiments. A = aspirin, D = diclofenac sodium, E = etodolac, Ib = ibuprofen, In = indomethacin, L = loxoprofen-SRS, N = NS-398, O = oxaprozin, and Z = zaltoprofen, TX = thromboxane.

3.4. RT-PCR analysis

The results of RT-PCR analysis of cyclooxygenase-1 and -2, and GAPDH are shown in Fig. 4. Although the cycles and conditions of amplification were the same for these samples, there was a marked difference in the expression of cyclooxygenase-2 mRNA with and without interleukin-1β treatment. However, there were no differences in the expression of cyclooxygenase-1 and GAPDH mRNAs with and without interleukin-1β treatment.

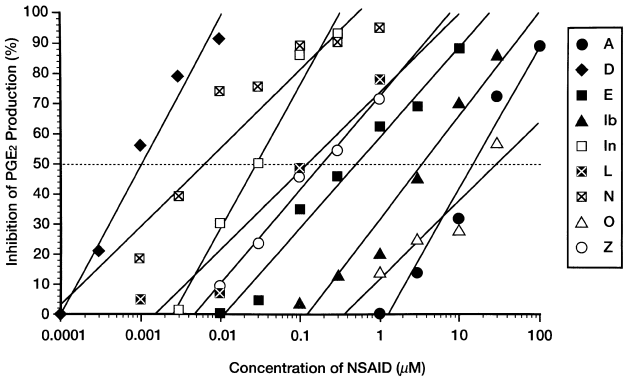

Fig. 6. Inhibitory effects of various NSAIDs on prostaglandin E_2 production by human synovial cells. Interleukin-1β-stimulated synovial cells were treated with each NSAID at 37°C for 30 min followed by addition of arachidonic acid. After another 30 min of incubation, the prostaglandin E_2 concentration of supernatant was measured. Results are expressed as the mean percentage relative to the control without NSAIDs. Each point shows the mean percent inhibition for each NSAID in all experiments. A = aspirin, D = diclofenac sodium, E = etodolac, Ib = ibuprofen, In = indomethacin, L = loxoprofen-SRS, N = NS-398, O = oxaprozin, and Z = zaltoprofen, PG = prostaglandin.

Table 1
Comparison of IC_{50} values of various NSAIDs for human platelet cyclooxygenase-1 and human interleukin-1β-stimulated synovial cell cyclooxygenase-2

	IC_{50} of NSAIDs (μM)		Cyclooxygenase-1/-2 ratio
	Cyclooxygenase-1[a]	Cyclooxygenase-2[b]	
Aspirin	3.2 ± 0.45	26 ± 27	0.12
Diclofenac	0.037 ± 0.012	0.00097 ± 0.00020	38
Etodolac	122 ± 42	0.68 ± 0.87	179
Ibuprofen	3.0 ± 1.3	3.5 ± 0.81	0.86
Indomethacin	0.013 ± 0.004	0.044 ± 0.038	0.30
Loxoprofen-SRS	0.38[c]	0.12[c]	3.2
NS-398	12 ± 5.0	0.0095 ± 0.0073	1263
Oxaprozin	2.2 ± 0.56	36 ± 28	0.061
Zaltoprofen	1.3 ± 0.26	0.34 ± 0.38	3.8

[a] Mean ± S.D. of four experiments except loxoprofen-SRS.
[b] Mean ± S.D. of six experiments except loxoprofen-SRS.
[c] Mean of two experiments.

3.5. Inhibitory effects of various NSAIDs on cyclooxygenase-1 and -2

Fig. 5 shows the mean dose–response curves for the inhibitory effects of various NSAIDs on thromboxane B_2 production in human platelets. This figure shows mean percent inhibition relative to the basal concentration of thromboxane B_2 in the control supernatant with no NSAIDs, which ranged from 137 to 157 ng/ml. The rank order of relative potency of inhibition for cyclooxygenase-1 among NSAIDs was indomethacin, diclofenac, loxoprofen-SRS, zaltoprofen, oxaprozin, ibuprofen, aspirin, NS-398, and etodolac.

Fig. 6 shows the inhibitory effects of various NSAIDs on prostaglandin E_2 production by interleukin-1β-stimulated synovial cells. This figure shows mean percent inhibition relative to the basal concentration of prostaglandin E_2 in the control supernatant with no NSAIDs, which ranged from 4.8 to 16.2 ng/ml. The rank order of relative potency was diclofenac sodium, NS-398, indomethacin, loxoprofen-SRS, zaltoprofen, etodolac, ibuprofen, aspirin, and oxaprozin.

For each NSAID, the concentration–response curve was analyzed by linear regression method to give IC_{50} values. Table 1 summarizes the mean (±S.D.) values of the individual IC_{50} of these NSAIDs for cyclooxygenase-1 and -2. In addition, the ratios of cyclooxygenase-1/-2 inhibitory activities of the NSAIDs calculated from each of mean IC_{50} value are shown.

4. Discussion

In the present study, we established a reliable assay system to measure the inhibitory effects of NSAIDs on cyclooxygenase-1 and -2 for clinical utilization. Previous studies on cyclooxygenase selectivity have been performed using purified (Mitchell et al., 1994; Yamazaki et al., 1997) or recombinant (Laneuville et al., 1994; Glaser et al., 1995) enzymes and thus were likely to be influenced by assay conditions, such as the incubation time (Laneuville et al., 1994). It has been suggested that assays using intact cells are the most inclusive approach to screening for cyclooxygenase selectivity (Laneuville et al., 1994). In addition, Morita et al. (1995) reported that intracellular cyclooxygenase-1 and -2 had different properties in murine 3T3 cells, which meant that NSAID activity detected by enzymatic assays might be different from that detected by cellular assays.

Previous studies employing intact cells for measuring human cyclooxygenase selectivity utilized cos-7 cells (Laneuville et al., 1994) or Chinese hamster ovarian cells (Riendeau et al., 1997) transfected with human cyclooxygenase-1 or -2 and human whole blood cells (Glaser et al., 1995; Brideau et al., 1996; Riendeau et al., 1997) or mononuclear cells and platelets (Grossman et al., 1995). To obtain more relevant information for clinical use, we employed human platelets to assess cyclooxygenase-1 and interleukin-1β-stimulated human synovial cells as cyclooxygenase-2 when measuring the cyclooxygenase selectivity of various NSAIDs.

We first tried to confirm that these cell systems were appropriate for measuring cyclooxygenase-1 or -2 activity. Human platelet lysates entirely contained cyclooxygenase-1 by Western blot analysis, as is generally reported (Vane and Botting, 1996). In human synovial cells, cyclooxygenase activity determined by the prostaglandin E_2 concentration after addition of arachidonic acid was dose-dependently enhanced by interleukin-1β and TNF-α, whereas interleukin-2, interleukin-4, interleukin-6, and interferon γ had no effect. Since interleukin-1 and TNF-α are key cytokines associated with pathophysiology in rheumatoid arthritis (Arend and Dayer, 1995), induction of cyclooxygenase and resultant prostaglandin E_2 overproduction induced by these cytokines may play an important role in synovial inflammation in this disease. We further examined whether cyclooxygenase-1 or -2 was induced in synovial cells by interleukin-1β. From the results of Western

blotting, immunoprecipitation, and RT-PCR, it appeared that interleukin-1β mainly induced cyclooxygenase-2 in cultured human rheumatoid synovial cells as was also reported by Crofford et al. (1994). These results suggested that interleukin-1β-stimulated human synovial cells were suitable for measuring the inhibitory activities of NSAIDs for human cyclooxygenase-2 enzyme.

The ratio of the IC_{50} values of each NSAID for platelet cyclooxygenase-1 assay and interleukin-1β-stimulated synovial cell cyclooxygenase-2 gives an indication of cyclooxygenase-2 selectivity. NS-398 was the most cyclooxygenase-2 selective agent, and it has already been shown to be a highly selective cyclooxygenase-2 inhibitor in animal studies (Futaki et al., 1993) and in vitro experiments (Futaki et al., 1994). Among other drugs which are available for clinical use in Japan, etodolac and diclofenac sodium had a high cyclooxygenase-2 selectivity in our assay. Zaltoprofen, loxoprofen-SRS, and ibuprofen had intermediate cyclooxygenase-2 selectivity, while the lowest cyclooxygenase-2 selectivity was observed for indomethacin, aspirin, and oxaprozin.

Several investigators have suggested that cyclooxygenase-2-selective NSAIDs may have greater clinical utility because of their lesser gastric and renal toxicities and weaker effect on platelet function compared to non-cyclooxygenase-2-selective NSAIDs (Appleton et al., 1994; Vane and Botting, 1996). Thus, the most important information for the clinical application of cyclooxygenase-2-selective inhibitors is whether such agents have any clinical superiority in their risk/benefit ratio. NS-398, the most cyclooxygenase-2-selective agent in our study, has potent analgesic and antipyretic effects with minimal gastric complications in a rat adjuvant arthritis model (Futaki et al., 1993), however, it is not available for clinical use yet. As clinically available NSAIDs, Henry et al. (1996) reported a meta-analysis of the risk of severe gastrointestinal complications with several kinds of NSAIDs in 12 clinical studies. They categorized various NSAIDs into three relative risk groups: the low-risk group comprised ibuprofen and diclofenac; the intermediate-risk group was aspirin, sulindac, naproxen, higher doses of ibuprofen, and indomethacin; and the high-risk group included piroxicam, ketoprofen, tolmetin, and azapropazone. In the present study, diclofenac had high, ibuprofen had intermediate, and indomethacin and aspirin had low cyclooxygenase-2 selectivity. Very recently, Singh et al. (1997) reported a comparative study of serious gastrointestinal toxicity of NSAIDs in patients with rheumatoid arthritis in North America. The rank order of incidence rates of the serious gastrointestinal toxicity of matched NSAIDs with our study was etodolac, diclofenac, ibuprofen, indomethacin, and oxaprozin, which was exactly the same rank order as the cyclooxygenase-2 selectivity in our study. These findings suggest that the cyclooxygenase-2 selectivity of NSAIDs might influence the relative risk of severe gastrointestinal complications.

In the present study, etodolac was one of more cyclooxygenase-2-selective agents among the clinically available NSAIDs in our human platelet and synovial cell assays. Lanza et al. (1987) compared the gastrointestinal mucosa of male volunteers after oral administrations of several NSAIDs for 7 days. Subjects treated with indomethacin, ibuprofen, and naproxen had significantly worse gastric and duodenal endoscopy scores when compared with those given etodolac and placebo. Syntheses of gastric and duodenal mucosal prostaglandin E_2, prostaglandin I_2, and thromboxane B_2 in biopsy specimens from patients with rheumatoid arthritis were significantly suppressed by naproxen treatment for 4 weeks (Laine et al., 1995), while etodolac had almost no effect on these parameters. These results also suggest that higher cyclooxygenase-2 selectivity may result in considerably less gastrointestinal complications for NSAIDs.

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