Available online at www.sciencedirect.com
ScienceDirect
Toxicology 228 (2006) 111–123
TOXICOLOGY
www.elsevier.com/locate/toxicol

Effects of non-steroidal anti-inflammatory drugs on cell proliferation and death in cultured epiphyseal-articular chondrocytes of fetal rats

Je-Ken Chang [a,c,d], Shun-Cheng Wu [b,c], Gwo-Jaw Wang [a,c,d], Ming-Hsuang Cho [b,c], Mei-Ling Ho [b,c,*]

[a] *Department of Orthopaedics, School of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[b] *Department of Physiology, School of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[c] *Orthopaedic Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan*
[d] *Department of Orthopaedics, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*

Received 1 June 2006; received in revised form 14 August 2006; accepted 14 August 2006
Available online 30 August 2006

Abstract

Previous reports indicated that non-steroidal anti-inflammatory drugs (NSAIDs) suppress bone repair. Our previous study further found that ketorolac delayed the endochondral bone formation, and the critical effective timing was at the early stage of repair. Furthermore, we found that NSAIDs suppressed proliferation and induced cell death of cultured osteoblasts. In this study, we hypothesized that chondrocytic proliferation and death, which plays an important role at the early stage of endochondral bone formation, might be affected by NSAIDs. Non-selective NSAIDs, indomethacin, ketorolac, diclofenac and piroxicam; cyclooxygenase-2 (COX-2) selective NSAIDs, celecoxib and DFU (an analog of rofecoxib); prostaglandins (PGs), PGE1, PGE2 and PGF2α; and each NSAID plus each PG were tested. The effects of NSAIDs on proliferation, cell cycle kinetics, cytotoxicity and cell death of epiphyseal-articular chondrocytes of fetal rats were examined. The results showed that all the tested NSAIDs, except DFU, inhibited thymidine incorporation of chondrocytes at a concentration range (10^{-8} to 10^{-4} M) covering the theoretic therapeutic concentrations. Cell cycle was arrested by NSAIDs at the G_0/G_1 phase. Upon a 24 h treatment, LDH leakage and cell death (both apoptosis and necrosis) were significantly induced by the four non-selective NSAIDs in chondrocyte cultures. However, COX-2 inhibitors revealed non-significant effects on cytotoxicity of chondrocytes except higher concentration of celecoxib (10^{-4} M). Replenishments of PGE1, PGE2 or PGF2α could not reverse the effects of NSAIDs on chondrocytic proliferation and cytotoxicity. In this study, we found that therapeutic concentrations of non-selective NSAIDs caused proliferation suppression and cell death of chondrocytes, suggesting these adverse effects may be one of the reasons that NSAIDs delay the endochondral ossification during bone repair found in previous studies. Furthermore, these effects of NSAIDs may act via PG-independent mechanisms. COX-2 selective NSAIDs showed less deleterious effects on chondrocytic proliferation and death.

Keywords: Non-steroidal anti-inflammatory drugs; Cyclooxygenase-2 inhibitors; Chondrocyte; Proliferation; Apoptosis; Necrosis

* Corresponding author at: Department of Physiology, School of Medicine, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan. Tel.: +886 7 3121101x2309/2553; fax: +886 7 3234687/3219452.
E-mail address: homelin@cc.kmu.edu.tw (M.-L. Ho).

0300-483X/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2006.08.028

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for anti-inflammation and analgesia postoperatively in orthopaedic patients. However, several studies demonstrated that anti-inflammatory drugs suppressed bone growth, remodeling and repair (Allen et al., 1980; Ho et al., 1995; Keller et al., 1987; Nilsson et al., 1986; Tornkvist et al., 1985; Tornkvist and Lindholm, 1980). Our previous results indicated that ketorolac, one of the most commonly used NSAIDs prescribed postoperatively (Di Massa et al., 2000), inhibited bone repair in fractured bones and bone remodeling in intact bones (Ho et al., 1995). It was also indicated that the critical effective timing of ketorolac was at the early stage of the bone repair process (Ho et al., 1998). However, the cellular and molecular mechanisms of NSAID effects on bone repair are not well defined.

Fracture healing involves sequential tissue morphogenesis and a cascade of highly coordinated cellular events. Mesenchymal stem cells aggregate to form the initial template of the skeleton which subsequently chondrify and finally ossify (Caplan, 1994; Ferguson et al., 1999; Vortkamp et al., 1998). This endochondral bone formation during fracture healing is generally divided into four temporal stages: inflammation, callus differentiation, ossification and remodeling. Chondrocytes play an important role in the stage of callus differentiation, as do osteoblasts in stages of ossification and remodeling. Our previous studies demonstrated that NSAIDs inhibited proliferation and induced cell death in cultured osteoblasts, suggesting these effects might contribute to the suppressive effect of NSAIDs on bone repair (Chang et al., 2005; Ho et al., 1999). However, the influence of NSAIDs on chondrocytic function during bone repair was rarely investigated.

In this study, we hypothesized that NSAIDs might also suppress proliferation and/or induce cell death of chondrocytes that undergo endochondral bone formation, and thus contribute to the suppressive effects on bone repair. Accordingly, we investigated the effects of four non-selective NSAIDs and two cyclooxygenase-2 (COX-2) selective inhibitors on cell proliferation, cell cycle, cytotoxicity and cell death in cultured chondrocytes obtained from fetal epiphyseal-articular cartilage of knees in rats.

2. Materials and methods

2.1. Chondrocyte cultures

Chondrocytes were isolated from epiphyseal-articular cartilage obtained from knees of fetal Sprague–Dawley rats at the 21st day of gestation (Chang et al., 2002). At this stage of bone development, the secondary ossification centers of the epiphyses of femora and tibiae have not developed. The hyaloid epiphyseal-articular cartilages of the knees were separated from ossified metaphyses. Chondrocytes were released from cartilage by a 2 h collagenase digestion (Worthington Corp., Freehold, NJ) at 37 °C. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 0.01% ascorbic acid, nonessential amino acids and 100 U/ml penicillin/streptomycin (Gibco-BRL, Grand Island, NY). Chondrocytes were cultured in a flask coated with 0.5% agar to avoid attaching onto the soft agar surface, and thus remain suspended. Maintenance of cells in suspension was for removing the contaminated fibroblasts or osteoblasts, which do not survive in suspension for more than 2 or 3 days. Previous reports indicated that chondrocytes remained viable in suspended culture for at least 7 days (Deshmukh and Sawyer, 1977; Horwitz and Dorfman, 1970; Nevo et al., 1972). After a 5-day suspended culture, chondrocytes were collected and grown in monolayer onto a regular 25 cm^2 flask. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Medium was changed every 48 h. Cells within four doublings were used in the experiments. These cells were demonstrated to preserve chondrocytic properties (Chang et al., 2002).

2.2. Drug preparation and treatment

Non-selective and cyclooxygenase-2 (COX-2) selective NSAIDs were tested at a concentration range of 10^{-8} to 10^{-4} M. Prostaglandins (PGs), PGE1, PGE2 and PGF2α (Sigma, St. Louis, MO), were also tested at a concentration range of 10^{-10} to 10^{-7} M. Non-selective NSAIDs were indomethacin, diclofenac, piroxicam (Sigma, St. Louis, MO) and ketorolac (Syntex, Palo Alto, CA). COX-2 selective NSAIDs were celecoxib (Pfizer, New York, NY) and DFU [5,5-dimethyl-3-(3-flurophyenyl)-4-(4-methylsulphonyl)phenyl-2(5*H*)-furanone], an analogue of rofecoxib (Meck Sharp and Dohne Corp., Rahway, NJ). The theoretic therapeutic concentrations of non-selective and COX-2 selective NSAIDs are approximately 10^{-5} and 10^{-6} M, respectively (Blocka et al., 1988; Buckley and Brogden, 1990; Clemett and Goa, 2000; Depre et al., 2000; Oberbauer et al., 1993; Todd and Sorkin, 1988). All the NSAIDs except ketorolac, and PGs were firstly dissolved in DMSO (dimethyl sulfoxide) as stock solutions. The stock solution of ketorolac was a 3% sterile solution. All the agents were diluted with 2% FCS contained medium right before treatment so that the final concentration of DMSO was diluted to 0.1% or less. The 2% FCS contained medium supplemented with 0.1% DMSO was tested in chondrocytes as vehicle controls, revealing no significant different effects on thymidine incorporation and lactate dehydrogenase leakage assay in comparison with those cultured in 2% FCS contained medium only. Accordingly, 2% FCS contained medium (control medium) was used for control cultures. Conditioned media were 2% FCS contained media supplemented with various concentrations of NSAIDs and/or

PGs, which were diluted from the stock solutions as described above. Cells were grown to subconfluence and then changed into the control medium for 24 h. Cultures were then maintained in the conditioned media (the drug-treated cultures) or control medium (the control cultures) for 17 or 24 h prior to the various examinations.

2.3. Cell proliferation assay

2.3.1. [^{3}H]-thymidine incorporation

Cells (2000 cells/well) cultured in 96-well plates were treated with different drugs for 17 or 24 h, and 2 μCi/well of [^{3}H]-thymidine (Amersham, Buckinghamshire, UK) was pulsed 4 h before harvest. Incubations were terminated by washing with phosphate buffered solution (PBS). Cells were detached by using 1% trypsin/EDTA, and collected in a 96-well UniFilter (Packard, Meriden, CT) by using a FilterMate Harvester (Packard, Meriden, CT). The UniFilter was dried by 95% ethanol for 30 min. After being sealed with the adhesive backing tape (Packard, Meriden, CT), liquid scintillatant was added into the sealed UniFilter. The top of the UniFilter was then sealed with TopSeal-A (Packard, Meriden, CT), and counted in a TopCount Microplate Scintillation and Luminescence Counters (Packard, Meriden, CT).

2.3.2. Cell cycle kinetics detected by flow cytometry

After 24 h treatments of drugs, cells were detached and flushed with Hank's buffered solution to prevent any aggregation. After centrifugation, cells were fixed with ice-cold 70% alcohol and incubated at 4 °C for a minimum of 30 min. Cell membranes were permeated with 0.1% TritonX-100, and RNA was digested with 20 μg/ml RNAase at 37 °C for 1 h. Cells were then stained with 50 μg/ml propidium iodide (Sigma, St. Louis, MO) in the dark, and then filtered with a filter with pore size of 41 μm right before analysis. DNA content of an individual cell was measured by using a laser flow cytometer (EPICS Elite, Coulter Hialeah, FL). The cell cycle distribution was analyzed by Wincycle software (EPICS Elite, Coulter Hialeah, FL).

2.4. Cytotoxicity assayed by lactate dehydrogenase (LDH) leakage

Lactate dehydrogenase (LDH) leakage from cells was measured to quantify the cytotoxicity by using the Cytotoxicity Detection kit (Roche, Germany) (Crowston et al., 1998; Mesner and Kaufmann, 1997). Chondrocytes were previously seeded into 24-well plates (5×10^4 cells/well). After drug treatment, the supernatants and cell layers of the cultures were collected for assay. According to the manufacturer's guidelines for the detection kit, cell layers were lysed with 1% TritonX-100, and cell lysates and supernatants were assayed in a 96-well plate, respectively. Briefly, 100 μl of catalyst solution was added in each assay well for 20 min. Absorbance was measured with an ELISA reader with a 490 nm filter. LDH leakage from chondrocytes was calculated as the following formula:

$$\text{LDH leakage} = \frac{\text{supernatant}}{\text{supernatant} + \text{cell}}$$

2.5. Cell death analysis

All the cells growing in monolayer and floating in the culture medium were harvested to analyze the cell death pattern by using the following methods.

2.5.1. TUNEL stain (terminal deoxy-nucleotidyl transferase mediated dUTP nick end labeling stain)

Fragmented DNA of an apoptotic cell was stained by TUNEL (terminal deoxy-nucleotidyl transferase mediated dUTP nick end labeling) staining by using the In Situ Cell Death Detection Kit, TMR red (Roche, Germany). According to the manufacturer's guidelines, cells were fixed with 4% of paraformaldehyde in PBS at a cell density of 1×10^6 ml^{-1}, and incubated at room temperature for 10 min. After centrifugation, cells were fixed in 80% ethanol, and then settled on a slide by centrifugation at a speed of 2000 rpm for 5 min by using a cytospin (Cytospin 3; Shandon, UK). Slides were rinsed twice with PBS, and cells were permeabilized by incubating in permeabilisation solution (0.1% Triton X-100 and 0.1% sodium citrate in PBS) for 2 min on ice. TUNEL reaction mixture, containing terminal deoxy-nucleotidyl transferase and rhodamine (the labeling dye), was added to slides and incubated for 60 min at 37 °C in humidified chamber in the dark. The reaction was stopped by blocking buffer (0.1% TritonX-100 and 0.5% BSA in PBS). Slides were observed on a fluorescence microscope with the excitation wave length of 580 nm. The images of the stained cells were recorded by a digital camera connected to a computer. Apoptotic cells were stained red by rhodamine. Stained cells were counted in five microscopic fields for each slide. Data were analyzed by using Image-Pro® Plus analysis software (Media Cybernetics, Sliver Spring, MD).

2.5.2. Caspase 3 activity assay

Caspase 3 is the ultimate endonuclease to degrade chromatin into DNA fragments during apoptosis process. Caspase 3 activities of chondrocytes were detected by using the Caspase Substract Reagent kit (OncoImmunin, Gaithersburg, MD). Cells were collected from the cell layer and supernatant 24 h after drug treatments. According to the manufacturer's guidelines, cells (1×10^6 cell/ml) were suspended in 50 μl of the Phiphilux-G1D2 substrate solution supplemented with 5% fetal calf serum. Cells were incubated in a 5% CO_2 incubator at 37 °C for 1 h in the dark. The subtract solution was removed, and cells were washed twice and re-suspended in 500 μl of Phiphilux dilution buffer. The fluorescence emission was immediately determined on a flow cytometer (EPICS Elite, Coulter Hialeah, FL) and cells with caspase 3 activities within 10 000 cells were counted. Data were analyzed by Winmidi software (EPICS Elite, Coulter Hialeah, FL).

2.6. Annexin V-FITC and propidium iodide double-stained flow cytometry

Cells were double-stained with 10 μg/ml of Annexin V-FITC (Roche, Germany) and propidium iodide for 20 min at room temperature in the dark immediately before analysis. Cells were then filtered followed by fixation with 4% paraformaldehyde. Cells undergoing apoptosis or necrosis within 10 000 cells were enumerated by flow cytometry. Data were analyzed by Winmidi software (EPICS Elite, Coulter Hialeah, FL).

2.7. Statistic analysis

Data was shown in mean ± S.E.M. from 3 to 8 cultures in each group. All experiments were performed at least three times, and revealed similar results. Statistical significance was evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Statistical difference between two groups was evaluated by Student's *t*-test. $P < 0.05$ was considered significant difference.

3. Results

3.1. Cell proliferation

The [^{3}H]-thymidine incorporation of the control cultures (1183 ± 225 cpm/well) showed no significant differences among the indomethacin-, diclofenac-, ketorolac-, piroxicam-, celecoxib- and DFU-treated groups, revealing each group was at the same baseline condition. In comparison to non-drug-treated control cultures, the [^{3}H]-thymidine incorporation of chondrocytes was significantly decreased after 24 h treatment of four non-selective NSAIDs (indomethacin: 10^{-8} to 10^{-4} M caused a 20–42% decrease, $p < 0.01$; diclofenac: 10^{-8} to 10^{-7} M caused a 13–15% decrease, $p < 0.05$, 10^{-6} to 10^{-4} M caused a 16–63% decrease, $p < 0.01$; ketorolac and piroxicam: 10^{-6} to 10^{-5} M caused a 12–20% decrease, $p < 0.05$, 10^{-4} M caused a 19–28% decrease, $p < 0.01$) (Fig. 1A). The results from COX-2 selective inhibitors (10^{-8} to 10^{-4} M) showed that a 24 h treatment of celecoxib decreased the [^{3}H]-thymidine incorporation of chondrocytes at a concentration range of 10^{-8} to 10^{-4} M (10^{-8} M caused an 11% decrease, $p < 0.05$; 10^{-7} to 10^{-4} M caused a 23–63% decrease, $p < 0.01$), while DFU had no significant effect on [^{3}H]-thymidine incorporation (Fig. 1B). The results from PG-treated cultures showed that PGE1 decreased the [^{3}H]-thymidine incorporation of chondrocytes at concentrations of 10^{-8} M (a 15% decrease, $p < 0.05$) and 10^{-7} M (a 50% decrease, $p < 0.01$), while 10^{-10} to 10^{-9} M of PGE1 and 10^{-10} to 10^{-7} M of PGE2 and PGF2α showed no significant effects (Fig. 2A).

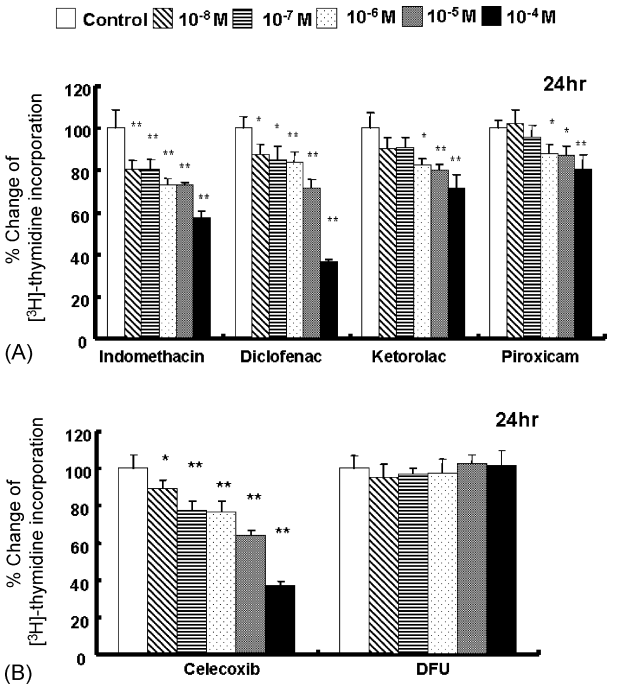


Fig. 1. Concentration–response effects of NSAIDs on thymidine incorporation of chondrocytes. Cultures were treated with non-selective NSAIDs, indomethacin, diclofenac, ketorolac and piroxicam (A), and COX-2 selective NSAIDs, celecoxib and DFU (B), at a concentration range of 10^{-8} to 10^{-4} M for 24 h. Each bar represents the mean ± S.E.M. of eight replicated cultures. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. Experiments were repeated at least three times and revealed similar results. $^{*}p < 0.05$, $^{**}p < 0.01$, in comparison with the control culture.

Furthermore, replenishing either PGE2 or PGF2α (10^{-8} M) in ketorolac (10^{-6} to 10^{-3} M) treated cultures did not reverse the inhibitory effect of ketorolac on [^{3}H]-thymidine incorporation (Fig. 2B).

3.2. Cell cycle kinetics

In comparison to the non-drug-treated control cells, a 24 h treatment of ketorolac (10^{-5} M, $p < 0.05$; 10^{-4} to 10^{-3} M, $p < 0.01$) and indomethacin (10^{-5} to 10^{-3} M, $p < 0.01$) significantly increased the cell distribution in the G_0/G_1 phase (Fig. 3). The result revealed that the cell cycle of chondrocytes was arrested at the G_0/G_1 phase by these NSAIDs.

3.3. Cytotoxicity

In comparison to non-drug-treated control cells, the LDH leakage of chondrocytes with a 17 h treatment of indomethacin, diclofenac or ketorolac significantly elevated by 15–62%, 18–45% or 8–15%, respectively (10^{-6}

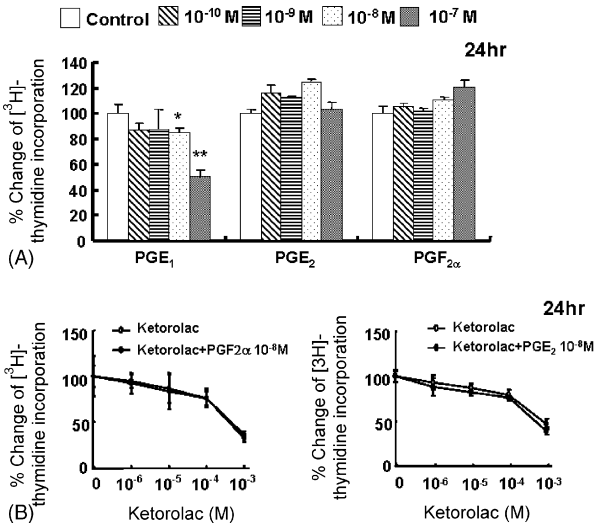


Fig. 2. Effects of prostaglandins on thymidine incorporation of chondrocytes. Cultures were treated with prostaglandins, PGE1, PGE2 and PGF2α, at a concentration range of 10^{-10} to 10^{-7} M for 24 h (A). The thymidine incorporation of cultures treated with ketorolac only and ketorolac plus PGF2α or PGE2 (10^{-8} M) were compared (B). Data shown in mean ± S.E.M. of eight replicated cultures were evaluated by one-way ANOVA and Scheffe's method (A). Comparisons of the data from cultures treated with ketorolac only and ketorolac plus PG were evaluated by Student's *t*-test, and no significant differences were found (B). Experiments were repeated at least three times and revealed similar results. $^{*}p<0.05$, $^{**}p<0.01$, in comparison with the control culture.

to 10^{-5} M, $p<0.05$; 10^{-4} M, $p<0.01$), while piroxicam showed no significant cytotoxic effect on chondrocytes (Fig. 4A). However, upon 24 h treatments of these four NSAIDs, the LDH leakage of chondrocytes was significantly increased at a concentration range of 10^{-6} to 10^{-4} M (indomethacin, a 31–69% increase; diclofenac, a 15–44% increase; ketorolac, a 22–28% increase; piroxicam, a 17–35% increase; $p<0.01$) (Fig. 4B). There was a significant difference between the LDH leakage of the 17 and 24 h drug-treated cultures for each individual drug (indomethacin, 10^{-6} to 10^{-4} M, $p<0.01$; diclofenac, ketorolac, and piroxicam, 10^{-6} to 10^{-4} M, $p<0.05$). This result revealed that the cytotoxic effects of these four NSAIDs were in a time-dependent manner. The results from a 24 h treatment of COX-2 selective inhibitors showed that only higher concentration of 10^{-4} M of celecoxib increased the LDH leakage of chondrocytes ($p<0.01$), while 10^{-6} to 10^{-5} M of celecoxib and DFU (10^{-6} to 10^{-4} M) had no significant cytotoxic effect on chondrocytes (Fig. 4C). The LDH leakage of chondrocytes was not significantly changed upon 24 h treatments of PGE_1, PGE_2 and $PGF_2\alpha$ (Fig. 5A). A replenishment of PGE_1, PGE_2, or $PGF_2\alpha$ (10^{-8} M) in each NSAID-treated culture (10^{-6} to 10^{-4} M) also did not significantly change the LDH leakage of chondrocytes (Fig. 5B).

3.4. Cell death analysis

The tested four non-selective NSAIDs showed significant cytotoxic effects at the concentration range covering the theoretic therapeutic concentration (10^{-5} M) so that their effects on cell death were further tested. On the other hand, COX-2 inhibitors showed no significant cytotoxicity except 10^{-4} M of celecoxib, which is well over the theoretic therapeutic concentration (10^{-6} M), so that their effects on cell death were not further tested.

3.4.1. TUNEL stain

The total cell number (1500–1600) of each sample was counted from five microscopic fields randomly. The apoptotic rate was the percentage of apoptotic cells (fluorescence stained) to total counted cells (Fig. 6C). The result showed that upon a 17 h treatment of these NSAIDs, only 10^{-4} M of indomethacin and diclofenac significantly increased the apoptotic rate of chondrocytes in comparison to the non-drug-treated control cultures ($p<0.01$) (Fig. 6A). However, upon 24 h treatments of these four NSAIDs, the apoptotic rate of all drug-treated cultures was significantly increased (indomethacin and diclofenac, 10^{-5} to 10^{-4} M caused an 11–14% increase, $p<0.01$; ketorolac and piroxicam, 10^{-5} M caused a 6.6–6.8% increase, $p<0.05$, 10^{-4} M caused a 7.9–8.4% increase, $p<0.01$) (Fig. 6B).

3.5. Caspase 3 activity

The caspase 3 activity was measured for further confirming the apoptosis induced by NSAIDs. In each sample, 10 000 cells were analyzed by flow cytomery. The integration of fluorescence intensity was defined caspase 3 activity of each culture (Fig. 7B). The geometric mean of the fluorescence intensity from control and drug-treated cultures were compared (Fig. 7A). The result showed 24 h treatments of the four tested NSAIDs significantly increased the caspase 3 activity of chondrocytes (indomethacin, diclofenac and ketorolac, 10^{-5} to 10^{-4} M caused a 24–94% increase, $p<0.01$; piroxicam, 10^{-5} M caused a 12% increase, $p<0.05$, 10^{-4} M caused a 57% increase, $p<0.01$) (Fig. 7A).

3.6. Flow cytometry

The percentage of cells undergoing apoptosis or necrosis was obtained by flow cytometry followed by

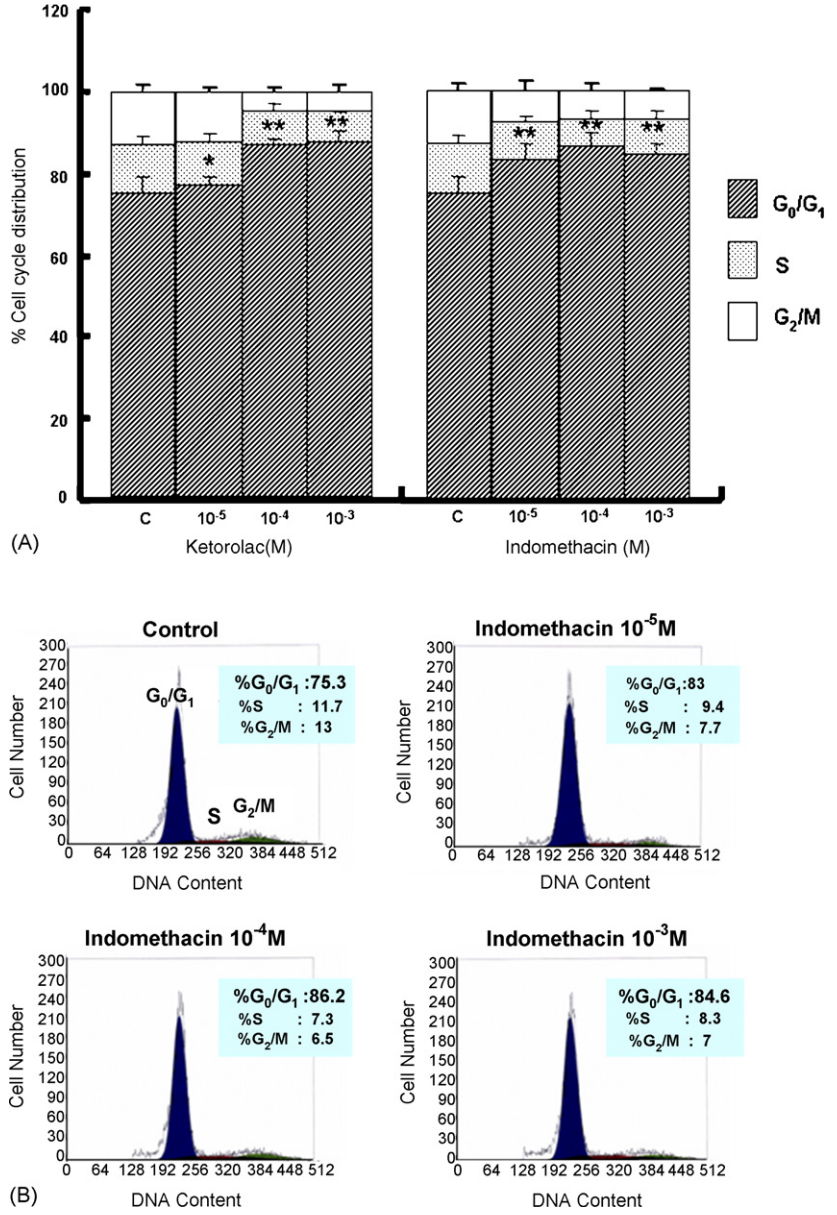


Fig. 3. Effects of NSAIDs on cell cycle kinetics of chondrocytes. Cultures were treated with NSAIDs at a concentration range of 10^{-5} to 10^{-3} M for 24 h. Data shown in mean ± S.E.M. of triplicate cultures were evaluated by one-way ANOVA and Scheffe's method. G_0/G_1, S, or G_2/M represents the percentage of cells distributing in the G_0/G_1, S, or G_2/M phase, respectively (A). The representative profiles of the cell cycle distribution that were measured from flow cytometry after a 24 h treatment of indomethacin are shown (B). Experiments were repeated at least three times and revealed similar results. $^{*}p<0.05$, $^{**}p<0.01$, in comparison with the control culture.

double staining of Annexin V-FITC and propidium iodide. The Annexin-FITC stained cells were defined apoptotic, while the double-stained cells were necrotic (Fig. 8). The results showed that a 24 h treatment of indomethacin, diclofenac, ketorolac, and piroxicam (10^{-5} and 10^{-4} M) significantly induced apoptosis (indomethacin, ketorolac and piroxicam, 10^{-5} M $p<0.05$, 10^{-4} M, $p<0.01$; diclofenac, 10^{-5} to 10^{-4} M, $p<0.01$) in a concentration dependent manner. Simultaneously, chondrocytic necrosis was also significantly induced by the treatments of the four NSAIDs (indomethacin, diclofenac, and ketorolac, 10^{-5} M, $p<0.05$, 10^{-4} M, $p<0.01$; piroxicam, 10^{-5} to 10^{-4} M, $p<0.05$) (Fig. 8).

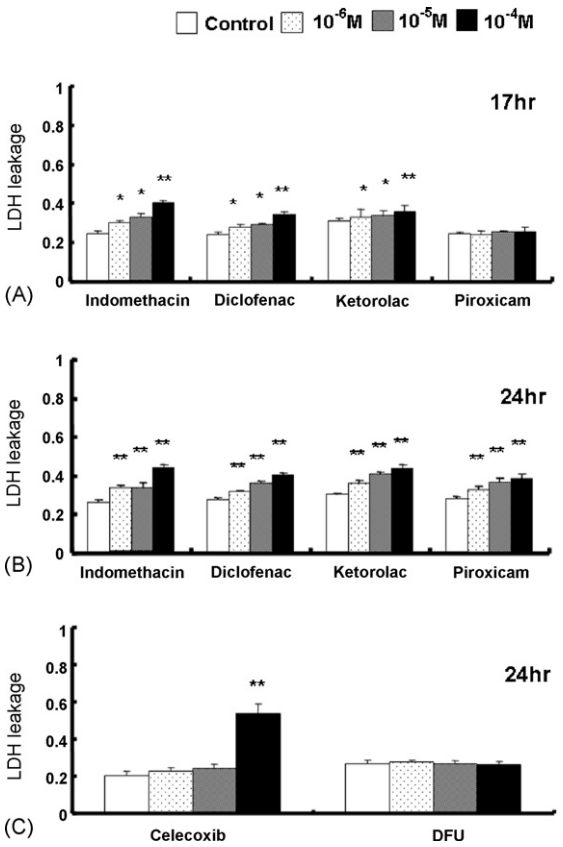


Fig. 4. Concentration–response effects of NSAIDs on cytotoxicity of chondrocytes. Cultures were treated with indomethacin, diclofenac, ketorolac and piroxicam at 10^{-6} to 10^{-4} M for 17 h (A) or 24 h (B); and celecoxib and DFU were treated for 24 h (C). LDH leakage was tested in each culture and calculated as described in Section 2. Each bar represents mean ± S.E.M. of five replicate cultures. Experiments were repeated at least three times and revealed similar results. Data were evaluated by one-way ANOVA and multiple comparisons were performed by Scheffe's methods. $^{*}p<0.05$, $^{**}p<0.01$ in comparison with the control culture.

4. Discussion

It is well known that non-selective NSAIDs suppress bone repair and bone remodeling of normal bones (Allen et al., 1980; Ho et al., 1995; Keller et al., 1987; Nilsson et al., 1986; Tornkvist et al., 1985; Tornkvist and Lindholm, 1980). It has been indicated that COX-2 selective NSAIDs have less gastrointestinal complications, and were suggested for chronic treatment. However, it was reported that COX-2 selective NSAIDs also suppress fracture repair (Brown et al., 2004; Einhorn, 2003). Nevertheless, the mechanisms involved in the NSAID influence on fracture repair are not clearly defined. A previous report indicated that ketorolac, an NSAID, delayed the endochondral ossification during fracture repair (Ho et al., 1998). One of the important events in the processing of endochondral ossification is the coordination of the participated cells, such as stem cells, chondrocytes, osteoblasts and osteoclasts. Accordingly, we investigated the effects of NSAIDs on osteoblastic functions, and found that NSAIDs suppressed proliferation and induced cell death in cultured osteoblasts (Chang et al., 2003, 2005). In this study, we further found that four non-selective NSAIDs and a COX-2 inhibitor, celecoxib, suppressed the proliferation of chondrocytes at a wide concentration range (10^{-8} to 10^{-4} M) covering the theoretic therapeutic concentrations. The theoretic therapeutic concentrations of non-selective NSAIDs and COX-2 inhibitors are approximately 10^{-5} and 10^{-6} M, respectively (Blocka et al., 1988; Buckley and Brogden, 1990; Clemett and Goa, 2000; Depre et al., 2000; Oberbauer et al., 1993; Todd and Sorkin, 1988). Among the four non-selective NSAIDs, indomethacin and diclofenac suppressed chondrocytic proliferation by 13–63% at concentration starting from 10^{-8} to 10^{-4} M, revealing the stronger suppressive effects than the other two NSAIDs (a 12–28% decrease). Furthermore, indomethacin and ketorolac were found to arrest chondrocytic cell cycle at the G_0/G_1 phase. Interestingly, DFU, a highly selective COX-2 inhibitor (Brideau et al., 2001), had no significant effect on chondrocytic proliferation at concentration range of 10^{-8} to 10^{-4} M. It implied that the celecoxib caused proliferation inhibition on chondrocytes might not relate to COX-2 inhibition.

In this study, we further found that the four tested non-selective NSAIDs caused significant cytotoxicity on chondrocytes at a concentration range (10^{-6} to 10^{-4} M) covering the theoretic therapeutic dose (10^{-5} M). These effects of indomethacin, diclofenac and ketorolac could be detected 17 h after drug treatment, and became more severe 24 h after treatment. However, COX-2 selective NSAIDs revealed less cytotoxic on chondrocytes, where celecoxib showed cytotoxic only at 10^{-4} M and DFU showed non-significantly cytotoxic at 10^{-6} to 10^{-4} M. Therefore, the effects of COX-2 selective inhibitors on chondrocyte death were not further investigated. We further found that a 24 h treatment of these non-selective NSAIDs induced cell death of chondrocytes, where almost equal severity of apoptosis and necrosis were detected by flow cytometry. Our results from TUNEL staining revealed that the apoptotic rate of chondrocytes induced by NSAIDs was consistent with the finding from flow cytometry. The specificity of TUNEL staining for detecting apoptotic cells has been doubted since some of the necrotic cells might have been enumerated in this measurement. On the other hand, caspase 3 is the final executive endonuclease to degrade chromatin

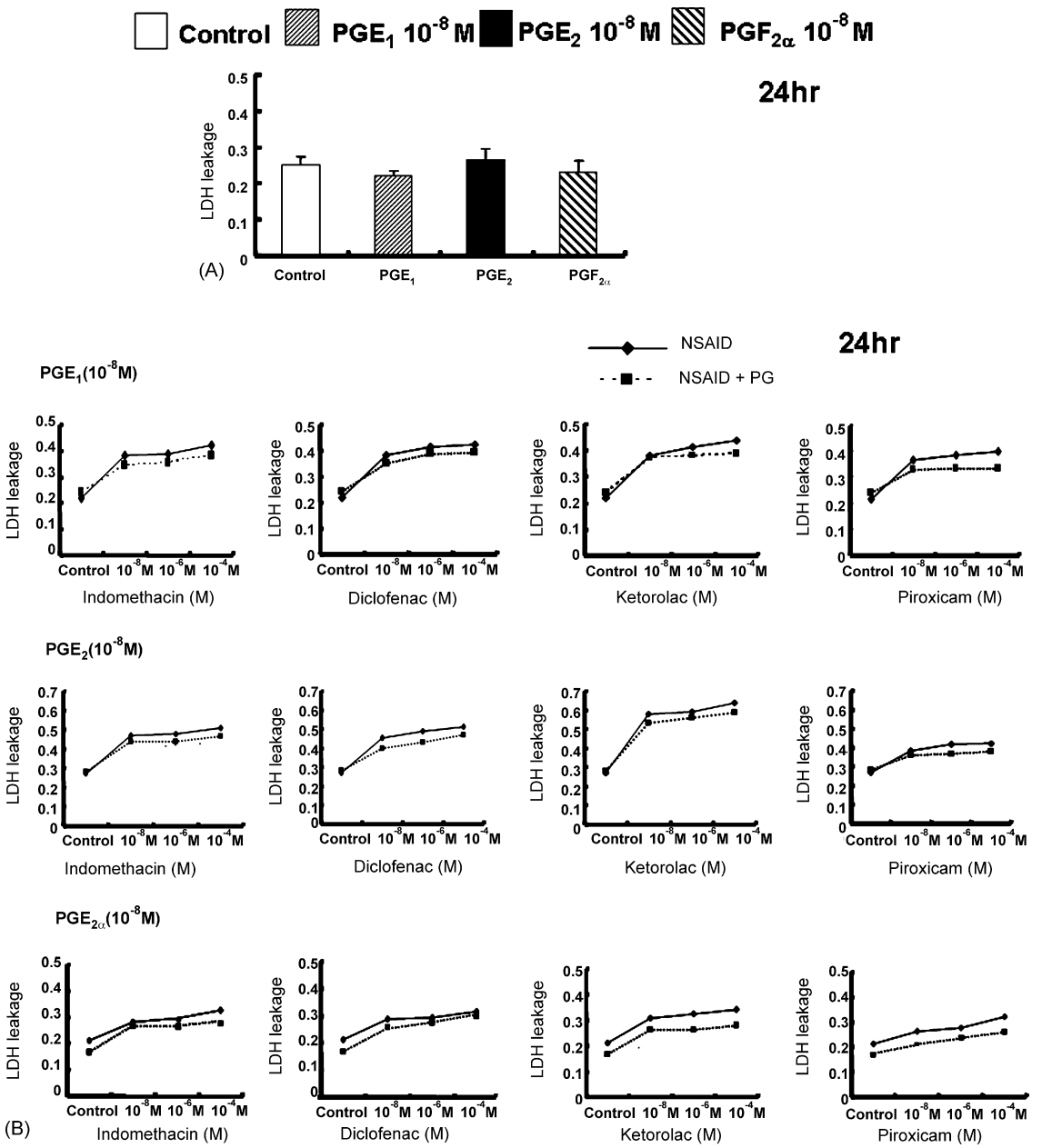


Fig. 5. Effects of prostaglandins on cytotoxicity of chondrocytes. Cultures were treated with PGE_1, PGE_2, or $PGF_2\alpha$, at 10^{-8} M for 24 h (A). LDH leakage was tested in each culture and calculated as described in Section 2. The LDH leakage of cultures treated with NSAID (indomethacin, diclofenac, ketorolac or piroxicam; 10^{-6} to 10^{-4} M) only and NSAID plus PGE1, PGE2 or PGF2α (10^{-8} M) were also compared (B). Data shown in mean ± S.E.M. of six replicated cultures were evaluated by one-way ANOVA, and no significant difference among PG-treated and the non-treated control cultures (A). Comparisons of the data from cultures treated with NSAID only and NSAID plus PG were evaluated by Student's *t*-test, and no significant differences were found in each concentration of individual NSAID. Experiments were repeated at least three times and revealed similar results.

into DNA fragments during apoptosis process, revealing a highly specific marker to indicate cell apoptosis. Accordingly, caspase 3 activity was detected in this study to further confirm these NSAID effects on chondrocytic apoptosis. The results showed that the significance and trend of NSAID effects on chondrocytic apoptosis detected by TUNEL stain and caspase 3 activity from flow cytometry were consistent, but the value detected from the latter was rather low. The different results from both detections could be due to the different sensitivi-

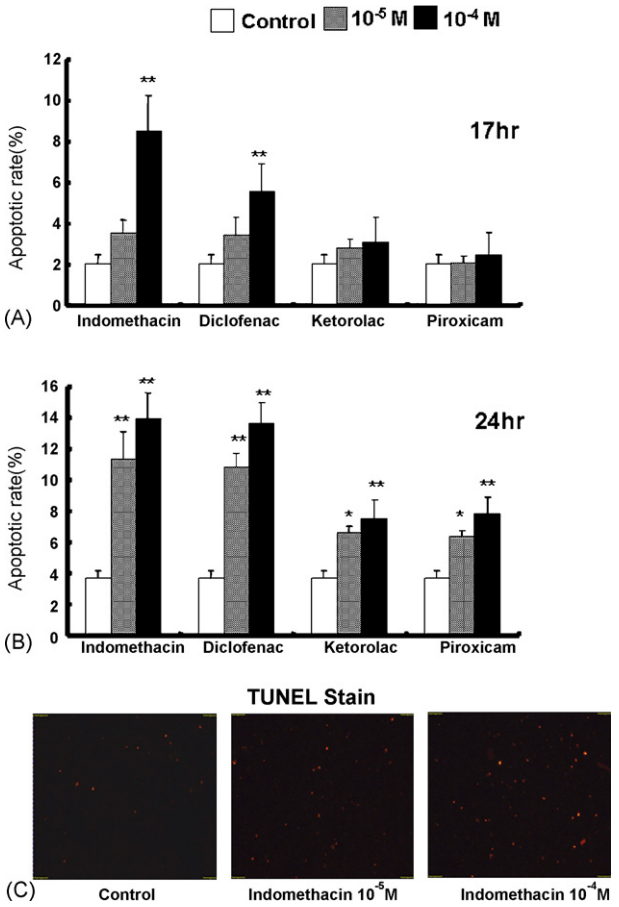


Fig. 6. Effects of NSAIDs on chondrocytic apoptosis measured by TUNEL stain. Representative microphotographs (×100) of TUNEL stained chondrocyte cultures are shown (C). The apoptotic cells were TUNEL stained positive (red fluorescence). Cultures were treated with indomethacin, diclofenac, ketorolac and piroxicam at 10^{-5} and 10^{-4} M for 17 h (A) or 24 h (B). The apoptotic rate was the percentage of apoptotic cells to total counted cells. Each bar represents mean ± S.E.M. of four replicate cultures. Experiments were repeated at least three times and revealed similar results. Data were evaluated by one-way ANOVA and multiple comparisons were performed by Scheffe's methods. $^{*}p<0.05$, $^{**}p<0.01$ in comparison with the control culture. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ties of both detections and/or recognizing the caspase 3 active cells as apoptotic cells revealing higher specificity. Taken together, the results of LDH leakage and cell death from this study indicated that a therapeutic concentration range (10^{-6} to 10^{-4} M) of these tested non-selective NSAIDs might cause cytotoxic effects on epiphyseal-articular chondrocytes through both inducing necrosis and apoptosis.

For the purpose of observing drug effects on cell proliferation and death, especially the drugs that have suppressive effects on bone repair, the stimulatory factors, such as growth factors, existing in serum was a concern. Accordingly, in this study we used 2% FCS instead of 10% FCS in culture medium prior to testing drug effects on cell cultures. LDH leakage and cell cycle kinetics of chondrocytes cultured in 10 and 2% FCS contained media were compared prior to the drug effect experiments, and no significant differences were found. Therefore, the drug effects on cell cycle kinetics and cell death found in this study were not a consequence of decreasing FCS in medium.

The results of this study indicated that non-selective NSAIDs had more deteriorative effects on proliferation and cell death in fetal rat epiphyseal-articular chondrocytes than COX-2 selective NSAIDs did. It has been reported that the selectivity ratio for COX-2 inhibition (IC50 value of COX-1/COX-2) by rofecoxib in human whole blood assay was 35, while celecoxib was 7.6 (Riendeau et al., 2001). DFU has also been reported to possess high COX-2 selectivity in equine, canine and feline blood (COX-1/COX-2 ratio: 775, 75 and 69, respectively) (Brideau et al., 2001). The selectivity of DFU on COX-2 inhibition is much higher than that of celecoxib, while the proliferation inhibition and cytotoxicity could be induced by celecoxib but not DFU. It seems to imply that these negative effects of NSAIDs on chondrocytic functions are due to the COX-1 inhibition and thus decreasing PG synthesis. Previous reports demonstrated that PG stimulates bone formation in vivo (Furuta and Jee, 1986; Jee et al., 1985, 1987; Kawaguchi et al., 1995; Mori et al., 1990; Shih and Norrdin, 1986a,b, 1987; Ueno et al., 1985), suggesting the PG inhibition by NSAIDs might be the reason that NSAIDs suppress bone repair (Norrdin et al., 1990). On the other hand, previous in vitro studies indicated that PGEs inhibited, but not stimulated, osteoblastic proliferation (Centrella et al., 1994; Ho et al., 1999; Yamaguchi et al., 1989). Our previous studies further demonstrated that non-selective NSAIDs inhibit proliferation and induce apoptosis of osteoblasts through pathways not related to PGs (Chang et al., 2005; Ho et al., 1999). Several studies from different sources of cells, such as colon cancer cells, vascular smooth muscle cells, tendon cells, etc., indicated that NSAIDs suppress proliferation and induce apoptosis through affecting the expressions of cell cycle regulators and/or proapoptotic proteins, but not PG related mechanisms (Hanif et al., 1996; Kazanov et al., 2004; Kucab et al., 2005; Marra et al., 2000; Tsai et al., 2004). In this study, our results showed that both 6 and 24 h treatments of PGE2 and PGF2α had no significant effects at a concentration range of 10^{-10} to 10^{-7} M, but a 24 h treatment of PGE1 (10^{-8} to 10^{-7} M) had inhibitory effects. These results revealed that PGE1 has either no effect (10^{-10} to

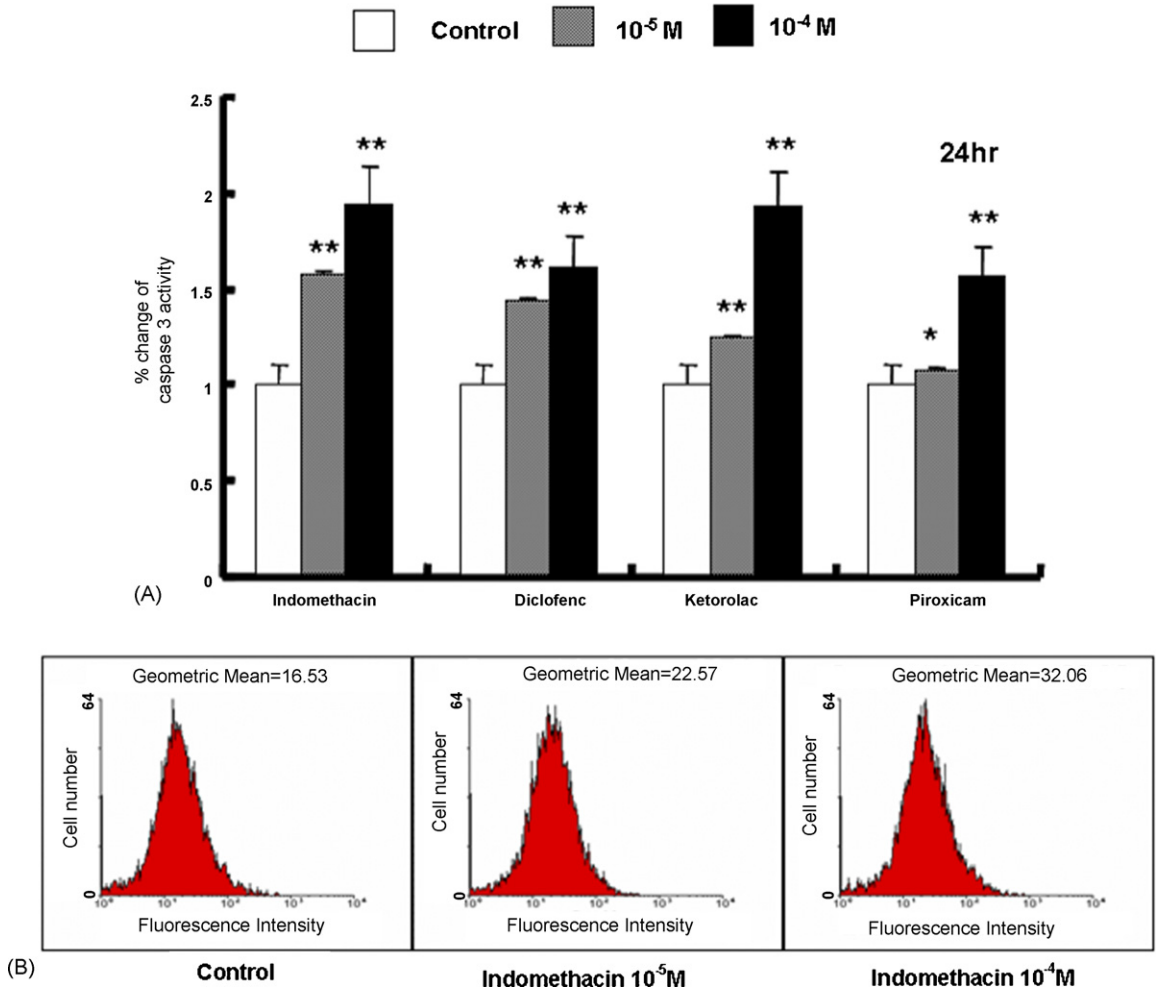
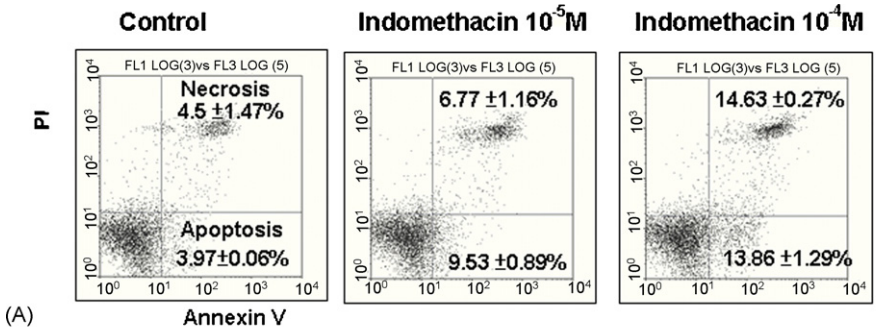


Fig. 7. Effects of NSAIDs on caspase 3 activity of chondrocytes measured by flow cytometry. Cultures were treated with indomethacin, diclofenac, ketorolac and piroxicam at concentrations of 10^{-5} and 10^{-4} M for 24 h. Caspase 3 activity was measured by flow cytometry. Each bar represents mean ± S.E.M. of three replicate cultures (A). Data were evaluated by one-way ANOVA and multiple comparisons were performed by Scheffe's methods. The representative flow cytometry profiles that were measured after a 24 h treatment of indomethacin are shown (B). Experiments were repeated at least three times and revealed similar results. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison with the control culture.

10^{-9} M) or inhibitory effects (10^{-8} to 10^{-7} M) on chondrocytic proliferation, suggesting NSAIDs might not suppress chondrocytic proliferation by blocking PGE1 synthesis. Excluding PGE2 and PGF2α may play a physiologic role in maintaining chondrocytic proliferation, we further replenished PGE2 or PGF2α (10^{-8} M) in ketorolac treated cultures. The results showed that neither PGE2 nor PGF2α could reverse the suppressive effects of ketorolac. Furthermore, we found that PGE1, PGE2 and PGF2α (10^{-8} M) had no significant effect on chondrocytic cytotoxicity. Replenishments of each PG in the NSAID-treated cultures also could not reverse the NSAID effects on LDH leakage. Therefore, we suggest that the inhibitory effects of NSAIDs on chondrocytic proliferation and cytotoxicity found in this study may be through pathways not related to PG synthesis.

Together with our previous finding of non-selective NSAID effects on osteoblasts and those on chondrocyts found in this study, we suggest that the NSAIDs caused proliferation inhibition and cell death of osteoblasts and chondrocytes may be the important factors contributing to the non-selective NSAID effects on delaying the fracture repair found in previous studies. In addition, this finding also implies that these NSAIDs are also possibly affect normal endochondral ossification in epiphyseal growth plate and/or the homeostatic functions of articular chondrocytes. On the other hand, although previous reports indicated that both celecoxib and rofecoxib suppressed bone repair in animal studies, our results showed



(B)

	%APOPTOSIS	%NECROSIS
Control	3.97±0.06	4.5±1.47
Indomethacin 10^{-5}M	9.53±0.89*	6.77±1.16*
10^{-4}M	13.86±1.29**	14.63±0.27**
Diclofenac 10^{-5}M	7.74±0.64**	8.27±1.58*
10^{-4}M	10.4±0.53**	9.6±0.44**
Ketorolac 10^{-5}M	6.67±0.70*	5.53±0.61*
10^{-4}M	7.35±0.40**	9.45±1.60**
Piroxicam 10^{-5}M	6.04±0.44*	6.07±0.49*
10^{-4}M	7.32±0.35**	7.54±1.50*

Fig. 8. Effects of NSAIDs on cell death (apoptosis and necrosis) of chondrocytes measured by double-stained flow cytometry. Cultures were treated with indomethacin diclofenac, ketorolac and piroxicam at concentrations of 10^{-5} and 10^{-4} M for 24 h. Cell apoptosis and necrosis were measured by Annexin V-FITC and PI double-stained flow cytometry. The annexin V-FITC stained cells were defined apoptotic, while the double-stained cells were necrotic (A). The representative flow cytometry profiles that were measured after a 24 h treatment of indomethacin are shown (A). Percentages of apoptotic and necrotic chondrocytes to total counted chondrocytes from the control and drug-treated cultures are shown (B). Data are shown in mean ± S.E.M. of four replicate cultures. Experiments were repeated at least three times and revealed similar results. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison with the control culture.

that only celecoxib suppressed chondrocytic proliferation, but not DFU. Interestingly, our recent unpublished data showed that both COX-2 inhibitors showed very strong suppressive effects on proliferation of osteoblasts and stem cells. We suggest that the adverse effects of these drugs on the functions of the different cells involved in bone repair may result in impairing the process. The molecular mechanisms of the COX-2 selective and/or non-selective NSAIDs that affect the regulation of cell cycle and death in different bone cells may be complicated and need to be further investigated.

Acknowledgements

This study was supported by grants from the National Science Council (NSC 90-2314-B-037-053-).

References

Allen, H.L., Wase, A., Bear, W.T., 1980. Indomethacin and aspirin: effect of nonsteroidal anti-inflammatory agents on the rate of fracture repair in the rat. Acta Orthop. Scand. 51, 595–600.

Blocka, K.L., Richardson, C.J., Wallace, S.M., Ross, S.G., Verbeeck, R.K., 1988. The effect of age on piroxicam disposition in rheumatoid arthritis. J. Rheumatol. 15, 757–763.

Brideau, C., Van Staden, C., Chan, C.C., 2001. In vitro effects of cyclooxygenase inhibitors in whole blood of horses, dogs, and cats. Am. J. Vet. Res. 62, 1755–1760.

Brown, K.M., Saunders, M.M., Kirsch, T., Donahue, H.J., Reid, J.S., 2004. Effect of COX-2-specific inhibition on fracture-healing in the rat femur. J. Bone Joint Surg. Am. 86A, 116–123.

Buckley, M.M., Brogden, R.N., 1990. Ketorolac. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential. Drugs 39, 86–109.

Caplan, A.I., 1994. The mesengenic process. Clin. Plast. Surg. 21, 429–435.

Centrella, M., Casinghino, S., McCarthy, T.L., 1994. Differential actions of prostaglandins in separate cell populations from fetal rat bone. Endocrinology 135, 1611–1620.

Chang, J.K., Cho, M.H., Wang, G.J., Ho, M.L., 2002. Pre-cultivation in suspension obtained chondrocyte-enriched cultures from articular-epiphyseal cartilage in fetal rats. Kaohsiung J. Med. Sci. 18, 106–112.

Chang, J.K., Chuang, L.Y., Ho, M.L., Wang, G.J., 2003. Effects of non-steroidal anti-inflammatory drugs on transforming growth factor-beta expression and bioactivity in rat osteoblast-enriched culture. Kaohsiung J. Med. Sci. 19, 278–288.

Chang, J.K., Wang, G.J., Tsai, S.T., Ho, M.L., 2005. Nonsteroidal anti-inflammatory drug effects on osteoblastic cell cycle. Cytotoxicity and cell death. Connect. Tiss. Res. 46, 200–210.

Clemett, D., Goa, K.L., 2000. Celecoxib: a review of its use in osteoarthritis, rheumatoid arthritis and acute pain. Drugs 59, 957–980.

Crowston, J.G., Akbar, A.N., Constable, P.H., Occleston, N.L., Daniels, J.T., Khaw, P.T., 1998. Antimetabolite-induced apoptosis in Tenon's capsule fibroblasts. Invest. Ophthalmol. Vis. Sci. 39, 449–454.

Depre, M., Ehrich, E., Van Hecken, A., De Lepeleire, I., Dallob, A., Wong, P., Porras, A., Gertz, B.J., De Schepper, P.J., 2000. Pharmacokinetics, COX-2 specificity, and tolerability of supratherapeutic doses of rofecoxib in humans. Eur. J. Clin. Pharmacol. 56, 167–174.

Deshmukh, K., Sawyer, B.D., 1977. Synthesis of collagen by chondrocytes in suspension culture: modulation by calcium, 3′:5′-cyclic AMP, and prostaglandins. Proc. Natl. Acad. Sci. U.S.A. 74, 3864–3868.

Di Massa, A., Scardigli, M., Bruni, L., Valentino, L., 2000. Ketorolac for paediatric postoperative pain. A review. Minerva Anestesiol. 66, 749–756.

Einhorn, T.A., 2003. Cox-2: where are we in 2003?—the role of cyclooxygenase-2 in bone repair. Arthritis Res. Ther. 5, 5–7.

Ferguson, C., Alpern, E., Miclau, T., Helms, J.A., 1999. Does adult fracture repair recapitulate embryonic skeletal formation? Mech. Dev. 87, 57–66.

Furuta, Y., Jee, W.S., 1986. Effect of 16,16-dimethyl prostaglandin E2 methyl ester on weanling rat skeleton: daily and systemic administration. Anat. Rec. 215, 305–316.

Hanif, R., Pittas, A., Feng, Y., Koutsos, M.I., Qiao, L., Staiano-Coico, L., Shiff, S.I., Rigas, B., 1996. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. Biochem. Pharmacol. 52, 237–245.

Ho, M.L., Chang, J.K., Wang, G.J., 1995. Antiinflammatory drug effects on bone repair and remodeling in rabbits. Clin. Orthop., 270–278.

Ho, M.L., Chang, J.K., Wang, G.J., 1998. Effects of ketorolac on bone repair: a radiographic study in modeled demineralized bone matrix grafted rabbits. Pharmacology 57, 148–159.

Ho, M.L., Chang, J.K., Chuang, L.Y., Hsu, H.K., Wang, G.J., 1999. Effects of nonsteroidal anti-inflammatory drugs and prostaglandins on osteoblastic functions. Biochem. Pharmacol. 58, 983–990.

Horwitz, A.L., Dorfman, A., 1970. The growth of cartilage cells in soft agar and liquid suspension. J. Cell Biol. 45, 434–438.

Jee, W.S., Ueno, K., Deng, Y.P., Woodbury, D.M., 1985. The effects of prostaglandin E2 in growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. Calcif. Tiss. Int. 37, 148–157.

Jee, W.S., Ueno, K., Kimmel, D.B., Woodbury, D.M., Price, P., Woodbury, L.A., 1987. The role of bone cells in increasing metaphyseal hard tissue in rapidly growing rats treated with prostaglandin E2. Bone 8, 171–178.

Kawaguchi, H., Pilbeam, C.C., Harrison, J.R., Raisz, L.G., 1995. The role of prostaglandins in the regulation of bone metabolism. Clin. Orthop. Relat. Res., 36–46.

Kazanov, D., Dvory-Sobol, H., Pick, M., Liberman, E., Strier, L., Choen-Noyman, E., Deutsch, V., Kunik, T., Arber, N., 2004. Celecoxib but not rofecoxib inhibits the growth of transformed cells in vitro. Clin. Cancer Res. 10, 267–271.

Keller, J., Bunger, C., Andreassen, T.T., Bak, B., Lucht, U., 1987. Bone repair inhibited by indomethacin. Effects on bone metabolism and strength of rabbit osteotomies. Acta Orthop. Scand. 58, 379–383.

Kucab, J.E., Lee, C., Chen, C.S., Zhu, J., Gilks, C.B., Cheang, M., Huntsman, D., Yorida, E., Emerman, J., Pollak, M., Dunn, S.E., 2005. Celecoxib analogues disrupt Akt signaling, which is commonly activated in primary breast tumours. Breast Cancer Res. 7, R796–R807.

Marra, D.E., Simoncini, T., Liao, J.K., 2000. Inhibition of vascular smooth muscle cell proliferation by sodium salicylate mediated by upregulation of p21(Waf1) and p27(Kip1). Circulation 102, 2124–2130.

Mesner Jr., P.W., Kaufmann, S.H., 1997. Methods utilized in the study of apoptosis. Adv. Pharmacol. 41, 57–87.

Mori, S., Jee, W.S., Li, X.J., Chan, S., Kimmel, D.B., 1990. Effects of prostaglandin E2 on production of new cancellous bone in the axial skeleton of ovariectomized rats. Bone 11, 103–113.

Nevo, Z., Horwitz, A.L., Dorfmann, A., 1972. Synthesis of chondromucoprotein by chondrocytes in suspension culture. Dev. Biol. 28, 219–228.

Nilsson, O.S., Bauer, H.C., Brosjo, O., Tornkvist, H., 1986. Influence of indomethacin on induced heterotopic bone formation in rats. Importance of length of treatment and of age. Clin. Orthop. Relat. Res., 239–245.

Norrdin, R.W., Jee, W.S., High, W.B., 1990. The role of prostaglandins in bone in vivo. Prostaglandins Leukot Essent Fatty Acids 41, 139–149.

Oberbauer, R., Krivanek, P., Turnheim, K., 1993. Pharmacokinetics of indomethacin in the elderly. Clin. Pharmacokinet. 24, 428–434.

Riendeau, D., Percival, M.D., Brideau, C., Charleson, S., Dube, D., Ethier, D., Falgueyret, J.P., Friesen, R.W., Gordon, R., Greig, G., Guay, J., Mancini, J., Ouellet, M., Wong, E., Xu, L., Boyce, S., Visco, D., Girard, Y., Prasit, P., Zamboni, R., Rodger, I.W., Gresser, M., Ford-Hutchinson, A.W., Young, R.N., Chan, C.C., 2001. Etoricoxib (MK-0663): preclinical profile and comparison with other agents that selectively inhibit cyclooxygenase-2. J. Pharmacol. Exp. Ther. 296, 558–566.

Shih, M.S., Norrdin, R.W., 1986a. Effect of prostaglandin E2 on rib fracture healing in beagles: histomorphometric study on periosteum adjacent to the fracture site. Am. J. Vet. Res. 47, 1561–1564.

Shih, M.S., Norrdin, R.W., 1986b. PGE2 induces regional remodeling changes in haversian envelope: a histomorphometric study of fractured ribs in beagles. Bone Miner. 1, 227–234.

Shih, M.S., Norrdin, R.W., 1987. Effect of PGE2 on regional cortico-endosteal remodeling in beagles with fractured ribs: a histomorphometric study. Bone Miner. 3, 27–34.

Todd, P.A., Sorkin, E.M., 1988. Diclofenac sodium. A reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. Drugs 35, 244–285.

Tornkvist, H., Lindholm, T.S., 1980. Effect of ibuprofen on mass and composition of fracture callus and bone. An experimental study on adult rat. Scand. J. Rheumatol. 9, 167–171.

Tornkvist, H., Bauer, F.C., Nilsson, O.S., 1985. Influence of indomethacin on experimental bone metabolism in rats. Clin. Orthop., 264–270.

Tsai, W.C., Tang, F.T., Hsu, C.C., Hsu, Y.H., Pang, J.H., Shiue, C.C., 2004. Ibuprofen inhibition of tendon cell proliferation and upregulation of the cyclin kinase inhibitor p21CIP1. J. Orthop. Res. 22, 586–591.

Ueno, K., Haba, T., Woodbury, D., Price, P., Anderson, R., Jee, W.S., 1985. The effects of prostaglandin E2 in rapidly growing rats: depressed longitudinal and radial growth and increased metaphyseal hard tissue mass. Bone 6, 79–86.

Vortkamp, A., Pathi, S., Peretti, G.M., Caruso, E.M., Zaleske, D.J., Tabin, C.J., 1998. Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair. Mech. Dev. 71, 65–76.

Yamaguchi, D.T., Green, J., Merritt, B.S., Kleeman, C.R., Muallem, S., 1989. Modulation of osteoblast function by prostaglandins. Am. J. Physiol. 257, F755–F761.