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Effects of anti-inflammatory drugs on proliferation, cytotoxicity and osteogenesis in bone marrow mesenchymal stem cells

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) were found to suppress proliferation and induce cell death in cultured osteoblasts, and steroids were found to decrease the osteogenesis potential of mesenchymal stem cells. In this study, we further tested the effects of anti-inflammatory drugs (AIDs) on the functions of bone marrow mesenchymal stem cells (BMSCs). The BMSCs from mice (D1-cells) and humans (hBMSCs) were treated with dexamethasone (10^{-7} to 10^{-6} M), cyclooxygenase-2 (COX-2) selective NSAIDs (10^{-6} to 10^{-5} M) and non-selective NSAIDs (10^{-5} to 10^{-4} M). Drug effects on proliferation, cell cycle kinetics, cytotoxicity and mRNA and protein expressions of cell cycle regulators were tested. The osteogenesis potential of D1-cells were evaluated by testing mRNA expressions of type Iα collagen and osteocalcin 2–8 days after treatments, and testing mineralization 1–3 weeks after treatments. The results showed that all the tested drugs suppressed proliferation and arrested cell cycle of D1-cells, but no significant cytotoxic effects was found. Prostaglandin E1, E2 and F2α couldn't rescue the effects of AIDs on proliferation. The $p27^{kip1}$ expression was up-regulated by indomethacin, celecoxib and dexamethasone in both D1-cells and hBMSCs. Higher concentrations of indomethacin and dexamethasone also up-regulated $p21^{Cip1/Waf1}$ expression in hBMSCs, and so did celecoxib on D1-cells. Expressions of cyclin E1 and E2 were down-regulated by these AIDs in D-cells, while only cyclin E2 was down-regulated by dexamethasone in hBMSCs. All the tested NSAIDs revealed no obvious detrimental effects on osteogenic differentiation of D1-cells. These results suggest that the proliferation suppression of AIDs on BMSCs may act via affecting expressions of cell cycle regulators, but not prostaglandin-related mechanisms.

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1. Introduction

Anti-inflammatory drugs (AIDs) are useful agents for orthopaedic patients who suffer from pain and inflammation after surgery or chronic inflammation. However, steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) have been indicated to suppress bone repair, formation and remodeling in vivo [1–6]. Our previous in vivo study also demonstrated that methylprednisolone and ketorolac suppressed bone repair in fractured bones and inhibited bone remodeling in intact bones [7]. Furthermore, we found that the effective timing of the suppressive effects of ketorolac was at the early stage of the fracture repair [8]. Our in vitro studies further demonstrated that NSAIDs suppressed proliferation, arrested cell cycle and induced cell death in cultured osteoblasts [9–11]. At the same time, we also found that NSAIDs mildly increased alkaline phosphatase activity and type I collagen formation at the early stage of differentiation of cultured osteoblasts, but could not affect the osteoblastic differentiation at mid or late stage [9]. These findings suggest that the proliferation suppression and cell death of osteoblasts caused by NSAIDs may contribute to the inhibitory effects of NSAIDs on bone remodeling in vivo. However, the effects of NSAIDs on the precursor cells of osteoblasts or mesenchymal stem cells are rarely investigated.

Long-term administration of corticosteroids may decrease bone mass and result in osteoporosis. Another complication of the corticosteroid treatment is osteonecrosis, the death of portions of bones and the subsequent collapse of joints. Our previous studies found that glucocorticoid treatment induced fatty marrow change in experiment animals and humans, and also demonstrated steroid decreased osteogenesis but increased adipogenesis in a mesenchymal stem cell line, D1-cells [12]. Dexamethasone was also found to suppress the expressions of osteogenic genes, bone morphogenetic protein 2 (BMP2), and osteocalcin in human bone marrow mesenchymal cells; this effect was more pronounced in osteonecrosis patients [13]. These findings suggested that the shift of osteogenesis to adipogenesis in mesenchymal stem cells might be one of the important mechanisms involved in the pathogenesis of steroid-induced osteonecrosis and osteoporosis. Other reports indicated that glucocorticoid induced apoptosis of osteoblasts may also play an important role in steroid induced osteonecrosis and osteoporosis [14,15]. In this study, we further tested the effect of dexamethasone on DNA synthesis, cell cycle proceeding and cytotoxicity of bone marrow derived mesenchymal stem cells.

The bone cells originate from the mesenchymal stem cells, which develop into osteoblasts, and eventually osteocytes. Cell proliferation and apoptosis are finely tuned in order to balance the cell number that is needed during stem cells differentiation into mature osteoblasts [16]. In this period of time, the important osteogenic markers are core binding factor a1 (cbfa1/RUNX2), bone morphogenetic proteins (BMPs), collagen type I, osteocalcin, alkaline phosphatase, etc. [17]. Accordingly, in order to understand the influences of AIDs on osteogenesis, the cell fate of osteogenic cells such as proliferation, cell death and differentiation needs to be investigated. However, the NSAID effects on the proliferation, osteogenesis and cytotoxicity of mesenchymal stem cells are rarely elucidated. In this study, we tested the NSAID influences on the DNA synthesis, cell cycle kinetics, cytotoxicity and the mRNA expressions of cell cycle regulators and marker genes of osteogenesis in bone marrow mesenchymal stem cells.

2. Materials and methods

2.1. Cell culture

2.1.1. D1-cell

D1-cell, which is a mesenchymal stem cell line cloned from bone marrow cells of Balb/c mice [18], purchased from ATCC (American Type Culture Collection). D1-cells can be induced into osteoblasts, adipocytes and chondrocytes, therein they exhibit osteogenic properties in Dulbecco modified Eagle medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 50 μg/ml sodium ascorbate in a humidified atmosphere of 5% CO_2 at 37 °C [18–20]. For all experiments, cells were cultivated under this condition within 5–6 subcultures, and the medium was changed every 2 days. The doubling time of D1-cells is 14–16 h under the experiment condition.

2.1.2. Human bone marrow mesenchymal stem cells (hBMSCs)

Human bone marrow mesenchymal stem cells (hBMSCs) were isolated from bone marrow aspirated from iliac crest of a 46-year-old female donor who had hip surgery caused by trauma. The nucleated stromal cells were separated from bone marrow fluid by 70% Percoll™ (Amersham Biosciences, Uppsala, Sweden) gradient. The hBMSCs were selected by the Keratinocyte-SFM (Gibco BRL, Rockville, MD) supplemented with the EGF-BPE (Gibco BRL, Rockville, MD), N-acetyl-L-cysteine and L-ascorbic acid 2-phosphate sesquimagnesium salt (Sigma, St. Louis, MO) [21]. After selection, the medium and condition for cultivating BMSCs were the same as those for D1-cells. For all experiments, cells were cultivated under this condition within 3–4 subcultures, and the medium was changed every 2 days. The doubling time of hBMSCs is 36–38 h under the experiment condition.

2.2. Drug treatment

The drugs and concentrations treated upon the cell cultures were as follows—steroidal anti-inflammatory drug (10^{-8} to 10^{-7} M): dexamethasone; NSAIDs (10^{-5} to 10^{-4} M): indomethacin, ketorolac, diclofenac and piroxicam; COX-2 selective NSAIDs (10^{-6} to 10^{-5} M): celecoxib (Pfizer, New York, NY) and DFU [5,5-dimethyl-3-(3-flurophyenyl)-4-(4-methylsulphonyl) phenyl-2(5H)-furanone], an analogue of rofecoxib (Meck Sharp & 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 [22–27], and dexamethasone is 10^{-7} M [28]. Indomethacin, diclofenac, piroxicam, dexamethasone, prostaglandin (PG) E1, PGE2 and PGF2α (Sigma, St. Louis, MO) were dissolved in DMSO as stock

solutions. Ketorolac tromethamine (Syntex, Palo Alto, CA) is a 3% sterile solution. All the agents were diluted with culture medium immediately before drug treatments so that the final concentration of DMSO was diluted to 0.1% or less to eliminate the influence of DMSO. For the experiments of osteogenesis, cultures were treated with drugs in differentiation medium that supplementing with beta-glycerophosphate for 2, 4, 6 or 8 days starting 2 days after confluence. For the experiments of proliferation and cytotoxicity, the culture medium was changed into a medium containing 2% serum when cells reached sub-confluence. After 24 h, cultures were maintained in drug-contained media or non-drug contained medium (the control cultures).

2.3. Thymidine incorporation

Cells (2000 cells/well) cultured in 96-well plates were treated with different drugs for 24 h, and 2 μCi/well of [4] thymidine 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 sealed with a TopSeal-A (Packard, Meriden, CT), liquid scintillant was added into the sealed UniFilter and counted in a TopCount Microplate Scintillation and Luminescence Counters (Packard, Meriden, CT).

2.4. Prostaglandin E2 detection by ELISA

The PGE2 production of D1-cells was measured in NSAID treated and non-treated control cultures. D1-cells were seeded in a six-well plate (5×10^4/well) and cultured to sub-confluence. After treatments of indomethacin (10^{-5} and 10^{-4} M) and celecoxib (10^{-6} and 10^{-5} M) for 24 h, culture media of each well were collected for PGE2 assay. The concentration of PGE2 was determined by using a PGE2 ELISA kit purchased from Cayman Chemical Company (Ann Arbor, MI, USA), which was based on the competition between PGE2 and PGE2-acetylcholinesterase (AchE). Briefly, samples or standard PGE2 were loaded into wells and incubated with PGE2-AchE conjugate and PGE2 monoclonal antibody at 4 °C for 18 h. Wells were then washed with the Wash Buffer, and developed by the Ellman's Reagent. Finally, plates were read by an ELISA reader at 420 nm. All the assays were performed in duplicate. PGE2 concentrations of samples were calculated from the standard curve.

2.5. 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).

2.6. Cytotoxicity assayed by lactate dehydrogenase (LDH) leakage

Lactate dehydrogenase (LDH) leakage from cells was measured to quantify the cytotoxicity by using a cytotoxicity detection kit (Roche, Germany)[29,30]. D1-cells 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 D1-cells was calculated as the following formula:

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

2.7. Real-time PCR

The first strand cDNA was converted by adding 1 μg of RNA, Moloney murine leukemia virus RT, and random hexamer primers. The quantitative real-time PCR was performed in the Bio-Rad iQ5 real-time PCR detection system (Bio-Rad Laboratories Inc, Hercules, CA) using the iQ™ SYBR® green supermix (Bio-Rad Laboratories Inc, Hercules, CA). Reactions were performed in a 25-μl mixture containing cDNA, specific primers of each gene and the iQ™ SYBR® green supermix. The cycling conditions and primer sequences are shown in Tables 1 and 2. The specific PCR products were detected by the fluorescence of SYBR Green, the double stranded DNA binding dye [31]. The relative mRNA expression level was calculated from the threshold cycle (Ct) value of each PCR product and normalized with that of GAPDH by using comparative Ct method [32]. The relative quantity of the expression of each gene from the control cells (of the 2-day treatment group in osteogenic experiment) was set to 1, and all the others were transformed to a relative fold to it. After PCR reaction, a dissociation (melting) curve was generated to check the specificity of PCR reaction. All the PCR amplifications were performed in triplicate, and experiments were repeated at least three times.

2.8. Western blot analysis

Cells were lysed in the PhosphoSafe™ Reagent (Novagen, Darmstadt, Germany). Protein concentrations were determined by the BioRad protein assay (Bio-Rad Laboratories Inc, Hercules, CA). The cell lysate containing 50 μg protein was applied and analyzed by 10% SDS-PAGE. Transferred membranes were blocked by a 5% skim milk and incubated overnight with antibodies of $p27^{kip}$ (BD Biosciences, San Jose, CA) and cyclin E (Cell Signaling Technology, Inc., Danvers, MA). The same

Table 1 – Primer sequences and cycling conditions for real-time PCR

Genes	Mouse	Human
p53	Forward: CAC AGC GTG GTG GTA CCTTA Reverse: GCA CAA ACA CGA ACC TCA AA	Forward: GTT CCG AGA GCT GAA TGA GG Reverse: TGA GTC AGG CCC TTC TGT CT
p21	Forward: AGG CCC AGT ACT TCC TCT GC Reverse: TCT GCG CTT GGA GTG ATA GA	Forward: GAC ACC ACT GGA GGG TGA CT Reverse: CAG GTC CAC ATG GTC TTC CT
p27	Forward: CAG AAT CAT AAG CCC CTG GA Reverse: GGT CCT CAG AGT TTG CCT GA	Forward: ATG TCA AAC GTG CGA GTG TC Reverse: TCT CTG CAG TGC TTC TCC AA
Cyclin D1	Forward: GCG TAC CCT GAC ACC AAT CT Reverse: CTC TTC GCA CTT CTG CTC CT	Forward: CGT GGC CTC TAA GAT GAA GG Reverse: CTG GCA TTT TGG AGA GGA AG
Cyclin D2	Forward: ACC TGT TGA CCA TCG AGG AG Reverse: CCA AGA AAC GGT CCA GGT AA	Forward: TGG GGA AGT TGA AGT GGA AC Reverse: ATC ATC GAC GGT GGG TAC AT
Cyclin E1	Forward: GGA AAA TCA GAC CAC CCA GA Reverse: AGA CTT CGC ACA CCT CCA TT	Forward: CTG GAT GTT GAC TGC CTT GA Reverse: TCC CCG TCT CCC TTA TAA CC
Cyclin E2	Forward: TCT CAG GAG ACG TTC ATC CA Reverse: ACA AAA GGC ACC ATC CAG TC	Forward: CAG GTT TGG AGT GGG ACA GT Reverse: CTT CCT CCA GCA TAG CCA AA
GAPDH	Forward: ATC ACT GCC ACC CAG AAG AC Reverse: CAC ATT GGG GGT AGG AAC AC	Forward: CAA TGA CCC CTT CAT TGA CC Reverse: TTG ATT TTG GAG GGA TCT CG
Cycling conditions	95 °C for 30 s, 95 °C for 4 min, followed by 32 cycles of 95 °C for 10 s, 59.1 °C for 15 s and 72 °C for 15 s.	95 °C for 30 s, 95 °C for 4 min, followed by 35 cycles of 95 °C for 10 s, 61.5 °C for 15 s and 72 °C for 15 s.

membrane was probed with antibody of actin (Sigma, Saint Louis, Missouri), which was provided as a loading control. Membranes were developed by the Western Lighting™ Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA). Blotted bands were digitally detected by using the UVP AutoChemi™ Image and Analysis System (UVP, Upland, CA).

Table 2 – Primer sequences and cycling conditions for real-time PCR

Gene	Mouse
RUNX2	Forward: ACG ACA ACC GCA CCA TGG T Reverse: CGG CTC TCA GTG AGG GAT G
BMP2	Forward: TCC TCA GCG AAT TTG AGT TGA G Reverse: CAA GTT GGC TGC TGC AGG CTT T
Type 1 collagen	Forward: TCT CCA CTC TTC TAG TTC CT Reverse: TTG GGT CAT TTC CAC ATG C
Osteocalcin	Forward: CTT GGT GCA CAC CTA GCA GA Reverse: CTC CCT CAT CGTG TTG TCC CT
18S	Forward: CCG CAG CTA GGA ATA ATG GAA TAG GAC Reverse: ACG ACG GTA TCT GAT CGT CTT CG
Cycling conditions	95 °C for 30 s, 95 °C for 4 min, followed by 32 cycles of 95 °C for 10 s, 59.1 °C for 15 s and 72 °C for 15 s.

2.9. *Alkaline phosphatase (ALP) activity*

D1-cells were cultured in 6-well plates to reach confluence, and were treated with NSAIDs (10^{-5} M) and COX-2 inhibitors (10^{-6} M) for 1–2 weeks. Cells were harvested and washed twice with Ca^{2+}, Mg^{2+} and $NaHCO_3$ free-PBS. A 100 μl of cell lysate was assayed for ALP activity by using the chemiluminescent method (Tropix, Applied Biosystems, Bedford, MA) as previously reported [33]. Total protein was determined as described in Section 2.8. The specific activity of ALP was shown as light unit/mg protein.

2.10. *von Kossa staining and quantification*

Mineralization representing the final step of osteogenesis of D1-cells was examined by von Kossa staining. Cells were cultured in 6-well plates to reach confluence, and were treated with NSAIDs (10^{-5} M) and COX-2 inhibitors (10^{-6} M) for 1–3 weeks. Cultures were fixed with 10% paraformaldehyde in buffered phosphate solution for 30 min, stained with 5% silver nitrate, and then exposed to UV at 254 nm for 60 min. After washing, wells were fixed with 5% sodium thiosulfate for 2 min. Mineral deposits were stained black. The mineral deposit area of each well was calculated from the positive stained areas measured randomly from 20 microscopic fields (96467.83 μm^2/field) by using Image-Pro® Plus analysis software (Media Cybernetics, Sliver Spring MD).

2.11. *Statistic analysis*

In each study group, data were shown in mean and standard error calculating from the results of 4–8 samples. All experiments were repeated at least three times. Statistical significance was evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method, $p < 0.05$ was considered significant.

3. Results

3.1. Thymidine incorporation and prostaglandin E2 concentration

After 24 h treatments of indomethacin and celecoxib, PGE2 secretion was significantly decreased in D1-cell cultures ($p < 0.01$) (Fig. 1A). The PGE2 concentration in the culture media of control cultures is around 1.7×10^{-8} M (17 ± 2 nM) (Fig. 1A); however, treatments of PGE1, PGE2 and PGF2α (10^{-9} to 10^{-8} M) for 24 h significantly decreased thymidine incorporation of D1-cells ($p < 0.01$) (Fig. 1B). On the other hand, the results of anti-inflammatory drug effects showed that dexamethasone (10^{-7} to 10^{-6} M); celecoxib and DFU (10^{-6} to 10^{-5} M); and indomethacin, ketorolac, piroxicam and diclofenac (10^{-5} to 10^{-4} M) significantly inhibited thymidine incorporation of D1-cells ($p < 0.01$) (Fig. 1C). A replenishment of PGE1, PGE2 or PGF2α (10^{-8} M) in dexamethasone and NSAID treated cultures couldn't reverse the suppressive effects of these drugs, yet it further augmented

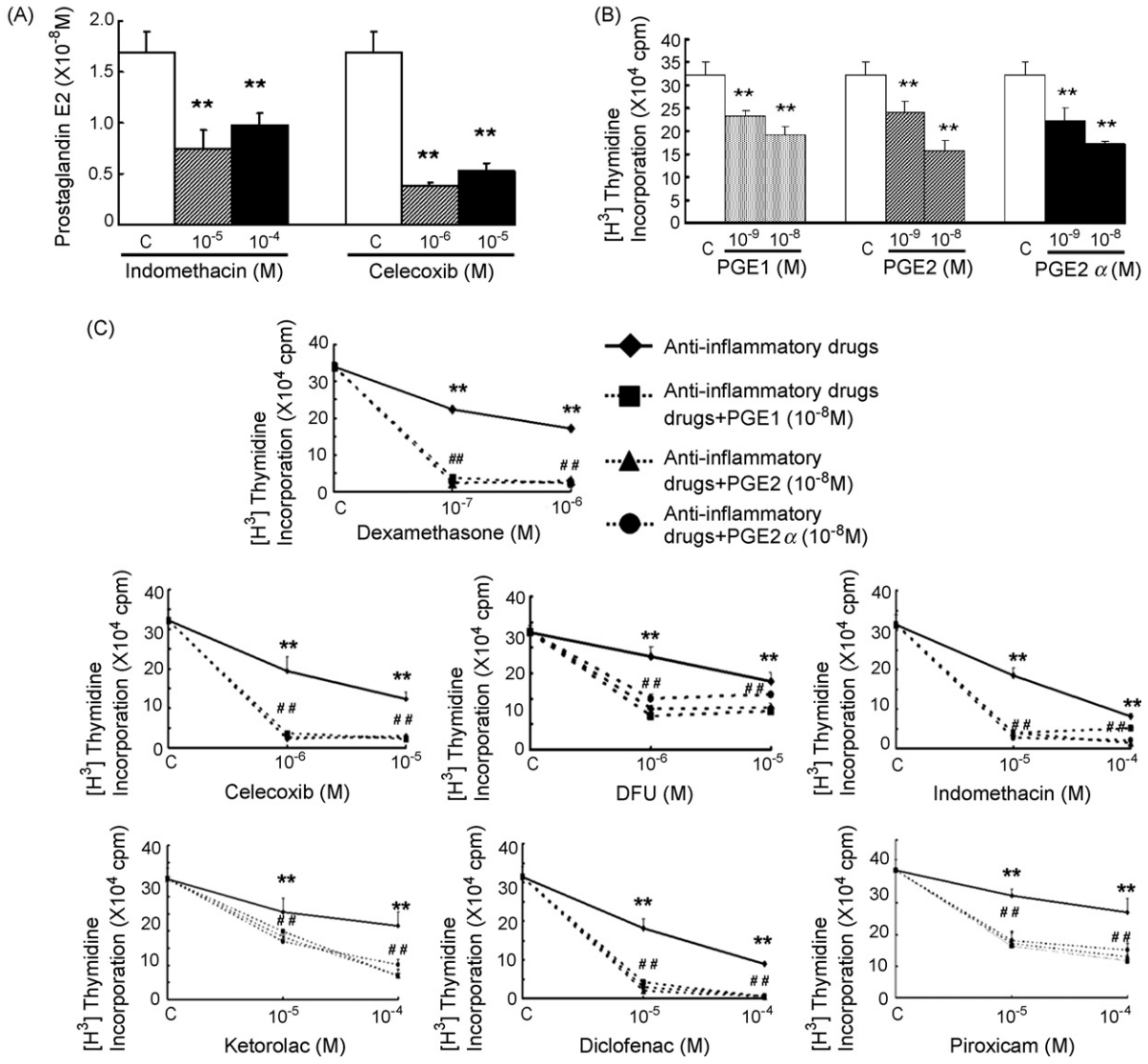

Fig. 1 – NSAID Effects on prostaglandin E2 (PGE2) production of D1-cells (A); and effects of prostaglandins (B), anti-inflammatory drugs and each drug plus PGE1, PGE2 or PGF2α (C) on thymidine incorporation of D1-cells. (A) PGE2 concentration in culture media was measured after 24 h treatments of indomethacin (10^{-5} and 10^{-4} M) and celecoxib (10^{-6} and 10^{-5} M). (B) Thymidine incorporation of cultures were detected after 24 h treatments of PGE1, PGE2 and PGF2α (10^{-9} and 10^{-8} M). (C) Thymidine incorporation of cultures were detected after 24 h treatments of dexamethasone (10^{-7} and 10^{-6} M) and NSAIDs (non-selective NSAIDs: 10^{-5} and 10^{-4} M; COX-2 selective NSAIDs: 10^{-6} and 10^{-5} M). The thymidine incorporation of anti-inflammatory drug treated cultures were further compared with those treated with each anti-inflammatory drug plus PGE1, PGE2 or PGF2α (10^{-8} M). Data are shown in the mean ± S.E.M. of 6–8 replicated cultures. The comparison of data from the control and agent treated cultures were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. The comparison of data from each anti-inflammatory drug treated with drug plus PG treated cultures were evaluated by Student's t-test. *Key*: $^{**}p < 0.01$, in comparison with the control culture. $^{\#\#}p < 0.01$, anti-inflammatory drug plus PG treated cultures compared to anti-inflammatory drug treated cultures.

the suppressive effects of anti-inflammatory drugs ($p < 0.01$) (Fig. 1C).

3.2. Cell cycle kinetics

The representative profile of the cell cycle kinetics from flow cytometry is shown in Fig. 2. In comparison with the non-agent treated control cultures, the percentage of cell population in G0/G1 phase was significantly higher upon treatment with indomethacin (10^{-5} M, $p < 0.05$; 10^{-4} M, $p < 0.01$), ketorolac (10^{-5} and 10^{-4} M, $p < 0.05$), diclofenac (10^{-5} M, $p < 0.05$; 10^{-4} M, $p < 0.01$), piroxicam (10^{-4} M $p < 0.01$), dexamethasone (10^{-7} and 10^{-6} M, $p < 0.01$), celecoxib (10^{-6} and 10^{-5} M, $p < 0.01$) and DFU (10^{-6} and 10^{-5} M, $p < 0.01$) (Fig. 2); and the percentage of cell population in S phase was significantly lower in the cultures that cells in G0/G1 phase increased (Fig. 2). These results showed that all the tested non-selective and COX-2 selective NSAIDs and dexamethasone significantly arrested the cell cycle of D1-cells in the G0/G1 phase.

3.3. Lactate dehydrogenase leakage

There was no significant cytotoxic effect caused by a 24 h treatment of dexamethasone (10^{-8} to 10^{-6} M), non-selective NSAIDs (indomethacin, ketorolac, diclofenac and piroxicam at 10^{-6} to 10^{-4} M), and COX-2 selective NSAIDs (celecoxib at 10^{-6} to 10^{-5} M and DFU at 10^{-6} to 10^{-4} M). Only 10^{-4} M of celecoxib caused LDH leakage in D1-cells. ($p < 0.01$) (Fig. 3).

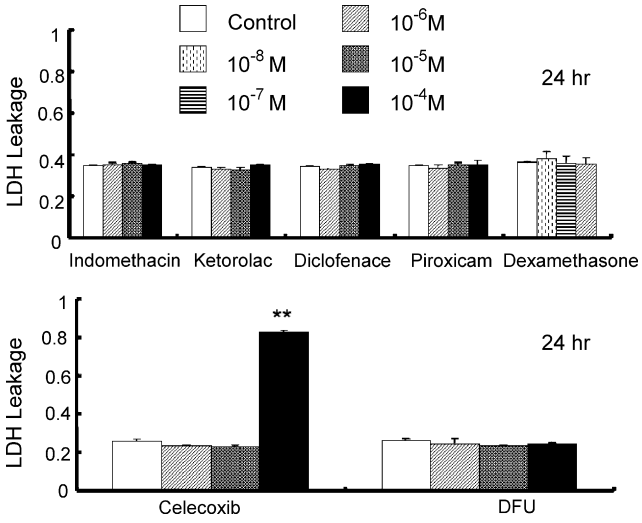

Fig. 3 – Anti-inflammatory drug effects on LDH leakage of D1-cells. Cultures were treated with dexamethasone (10^{-8} and 10^{-6} M) or each of the NSAIDs (10^{-6}, 10^{-5} and 10^{-4} M) for 24 h. There were no significant changes of LDH leakage in cultures after 24 h of drug treatments except 10^{-4} M of celecoxib. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. *Key:* $^{**}p < 0.01$, in comparison with the control culture.

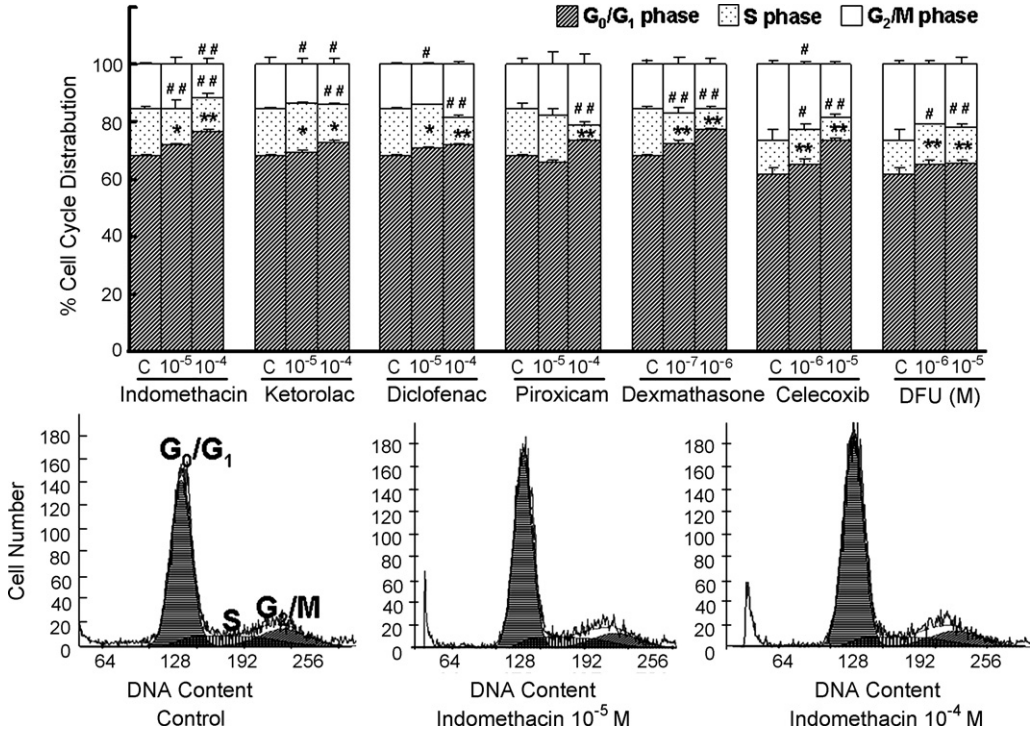

Fig. 2 – Anti-inflammatory drug effects on cell cycle kinetics in D1-cells analyzed by flow cytometry. The representative cell distribution profiles from flow cytometry were shown in low panel. D1-cell cultures were treated with dexamethasone (10^{-7} and 10^{-6} M) or each of the NSAIDs (non-selective NSAIDs: 10^{-5} and 10^{-4} M; COX-2 selective NSAIDs: 10^{-6} and 10^{-5} M) for 24 h. The percentage of cell population distributing in the G0/G1, S or G2/M phase was changed after drug treatments (upper panel). Each bar represents the mean ± S.E.M. of four replicated cultures. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. *Key:* $^{*}p < 0.05$, $^{**}p < 0.01$, in comparison with the control culture for the data of G0/G1 phase. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, in comparison with the control culture for the data of S phase.

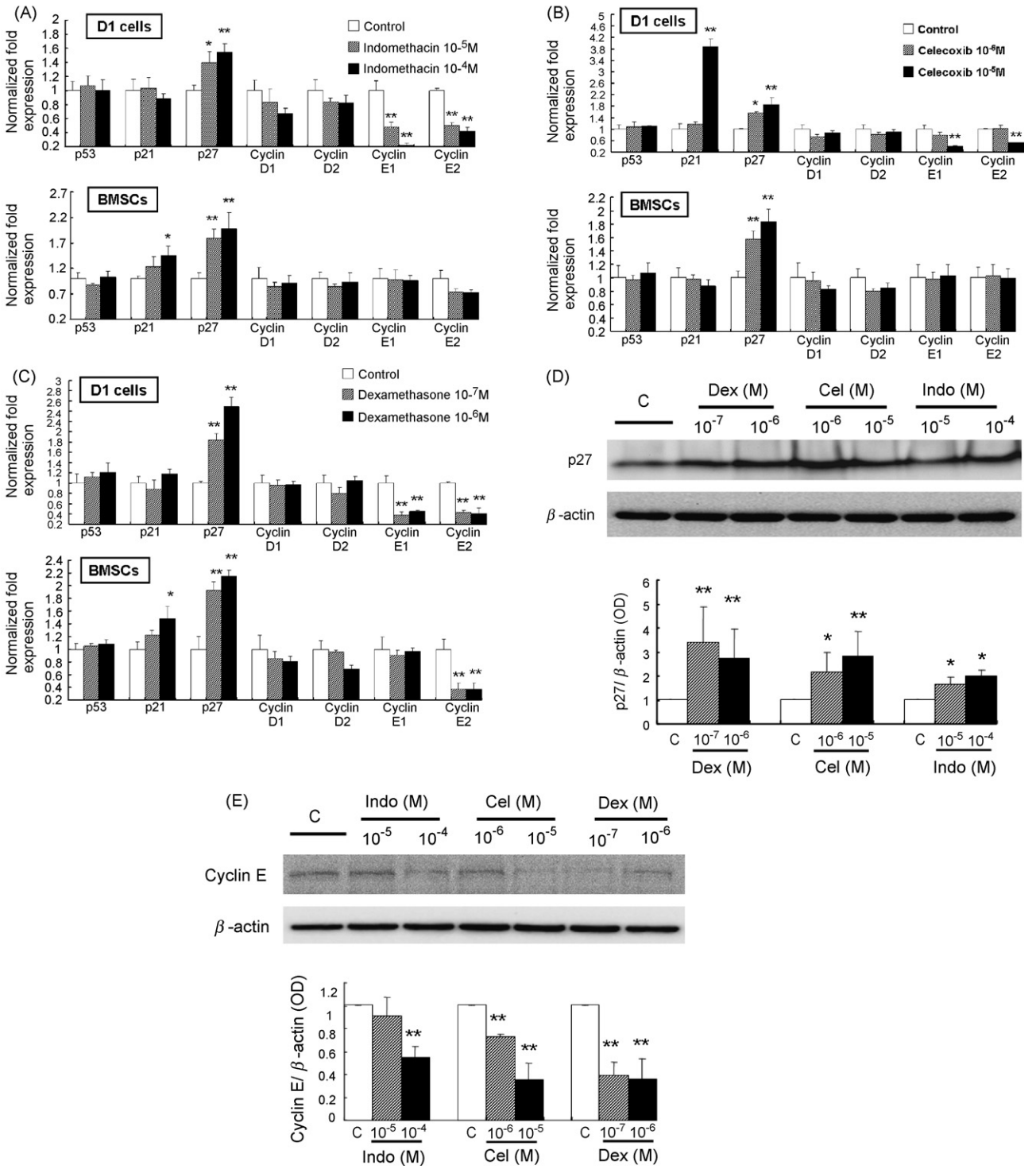

Fig. 4 – Anti-inflammatory drug effects on mRNA and protein expressions of cell cycle regulators in D1-cells and hBMSCs. Cultures were treated with indomethacin (10^{-5} and 10^{-4} M), celecoxib (10^{-6} and 10^{-5} M) and dexamethasone (10^{-7} and 10^{-6} M) for 24 h. The mRNA expressions and protein levels of cell cycle regulators were detected by real-time PCR and Western blot analysis, respectively. The relative mRNA expression level was calculated from the threshold cycle (Ct) value of each PCR product and normalized with that of GAPDH by using comparative Ct method. The relative quantity of the expression of each gene from the control cells was set to 1, and all the others were transformed to a relative fold to it (A–C). The bands of $p27^{kip}$ and cyclin E from Western blot analysis were digitally detected and normalized with that of β-actin (D and E). Each bar represents the mean ± S.E.M. of four replicated cultures. Data were evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. *Key*: $^{*}p < 0.05$, $^{**}p < 0.01$, in comparison with the control culture.

3.4. The mRNA and protein expression of cell cycle regulators

Indomethacin significantly elevated the mRNA expression of $p27^{kip1}$ (10^{-5} M, $p < 0.05$; 10^{-4} M, $p < 0.01$), but decreased the expressions of cyclin E1 and cyclin E2 (10^{-5} and 10^{-4}M, $p < 0.01$) in D1-cells (Fig. 4A). In hBMSCs, indomethacin increased the expressions of $p27^{kip1}$ (10^{-5} and 10^{-4} M, $p < 0.01$) and $p21^{Cip1/Waf1}$ (10^{-4} M, $p < 0.05$), while no significant effect on the expressions of cyclin E1 and cyclin E2 was found (Fig. 4A). Celecoxib not only significantly elevated the mRNA expressions of $p27^{kip1}$ (10^{-6} M, $p < 0.05$; 10^{-5} M, $p < 0.01$) but also that of $p21^{Cip1/Waf1}$ (10^{-5} M, $p < 0.01$), as well as decreased the expressions of cyclin E1 and cyclin E2 (10^{-5} M,

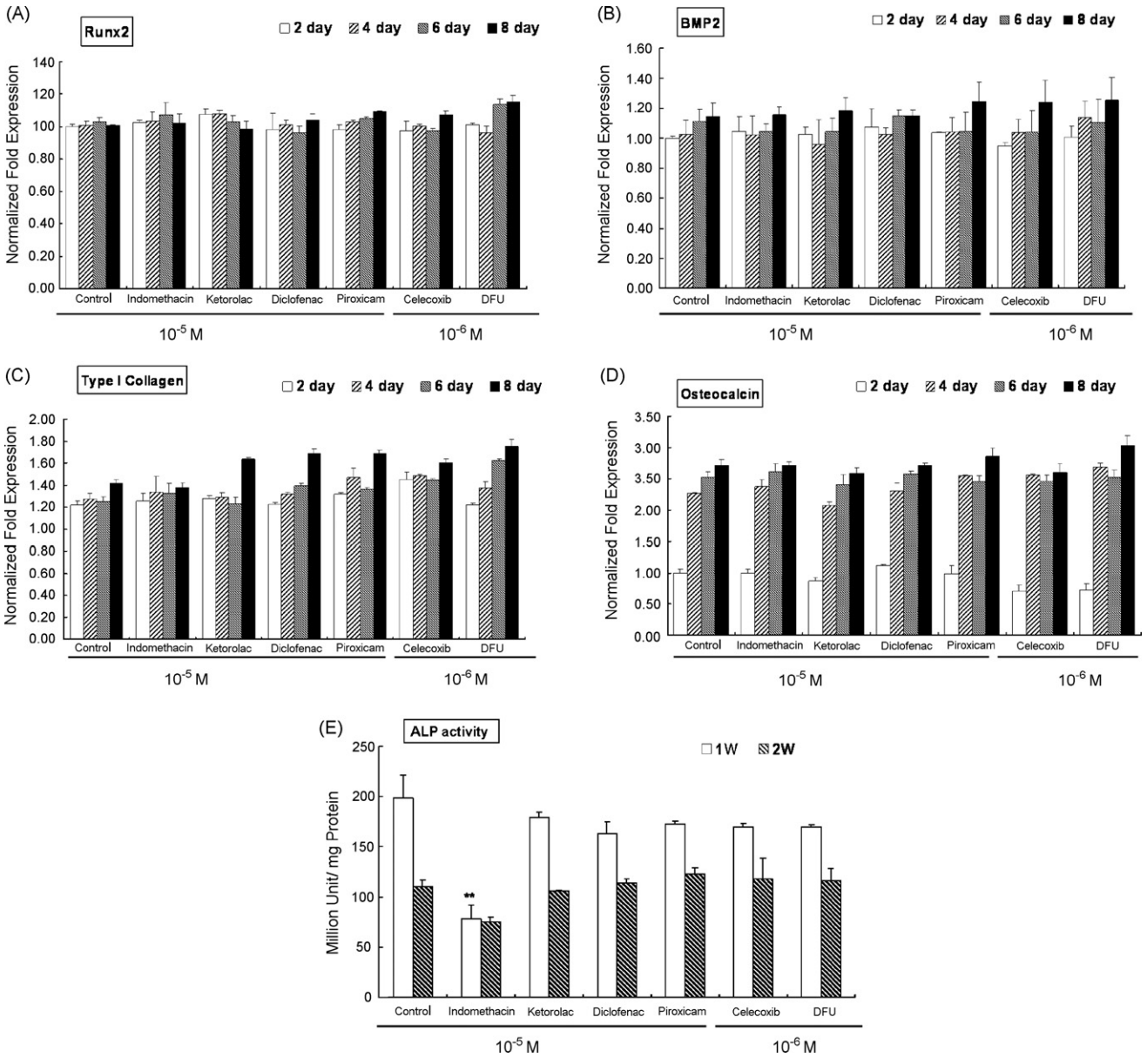

Fig. 5 – Anti-inflammatory drug effects on mRNA expressions of osteogenic genes and ALP activity of D1-cells. For mRNA expression, cultures were treated with 10^{-5} M of indomethacin, diclofenac, ketorolac, piroxicam; and 10^{-6} M of celecoxib and DFU for 2, 4, 6 or 8 days; for testing ALP activity, NSAID treatments were for 1 and 2 weeks. The mRNA expressions of RUNX2, BMP2, type I collagen and osteocalcin were detected by real-time PCR. The relative mRNA expression level was calculated from the threshold cycle (Ct) value of each PCR product and normalized with that of 18S by using comparative Ct method. The relative quantity of the expression of each gene from the control cells of the 2-day treatment group was set to 1, and all the others were transformed to a relative fold to it (A–D). ALP activity was shown as specific activity (million unit/mg protein) (E). Each bar represents the mean ± S.E.M. of four replicated cultures. Data from cultures treated with different drugs at the same time duration were compared and evaluated by one-way ANOVA and multiple comparisons were performed by Scheffe's method. Results of gene expression showed no significant changes by NSAIDs after evaluated by one-way ANOVA (A–D). *Key*: $^{**}p < 0.01$, in comparison with the control culture.

$p < 0.01$) in D1-cells (Fig. 4B). In hBMSCs, celecoxib only increased the expression of $p27^{kip1}$, but did not alter the expressions of the other genes (Fig. 4B). Dexamethasone also increased $p27^{kip1}$espression, but decreased cyclin E1 and cyclin E2 expressions (10^{-7} and 10^{-6} M, $p < 0.01$) in D1-cells (Fig. 4C). In hBMSCs, expressions of $p21^{Cip1/Waf1}$ (10^{-6} M, $p < 0.05$) and $p27^{kip1}$ (10^{-7} and 10^{-6} M, $p < 0.01$) were increased, but cyclin E2 expression was decreased by dexamethasone (10^{-7} and 10^{-6} M, $p < 0.01$) (Fig. 4C). The protein level of $p27^{kip1}$ was also significantly increased by indomethacin (10^{-5} and 10^{-4} M, $p < 0.05$), celecoxib (10^{-6} M, $p < 0.05$; 10^{-5} M, $p < 0.01$) and dexamethasone (10^{-7} and 10^{-6} M, $p < 0.01$) in D1-cells; while cyclin E was decreased by indomethacin (10^{-4} M, $p < 0.05$), celecoxib (10^{-6} M, $p < 0.05$; 10^{-5} M, $p < 0.01$) and dexa-methasone (10^{-7} and 10^{-6} M, $p < 0.01$) in D1-cells (Fig. 4D and E).

3.5. The mRNA expression of osteogenic markers

Treatments of indomethacin, ketorolac, diclofenac and piroxicam at 10^{-5} M, as well as celecoxib and DFU at 10^{-6} M for 2, 4, 6 or 8 days showed no significant effect on mRNA expressions of osteogenic markers, RUNX2, BMP2, type I collagen and osteocalcin, in D1-cells (Fig. 5A–D).

3.6. Alkaline phosphatase activity

In the control cultures, the ALP activity of D1-cells at the first week of experiment was significantly higher than that at the second week of experiment. This result showed that cells had entered the mineralization stage and the ALP activity had declined at the second week of experiment. The results from the first week experiment were compared among groups, the ALP activity of D1-cells could only be decreased by indomethacin (10^{-5} M) treatment, the other treatments (ketorolac, diclofenac, piroxicam at 10^{-5} M; celecoxib and DFU at 10^{-6} M) showed no significant effect (Fig. 5E).

3.7. Mineralization

The results of von Kossa staining showed that the area of mineral deposit in the control cultures significantly increased with the time of cultivation (1-week versus 2-week and 1-week

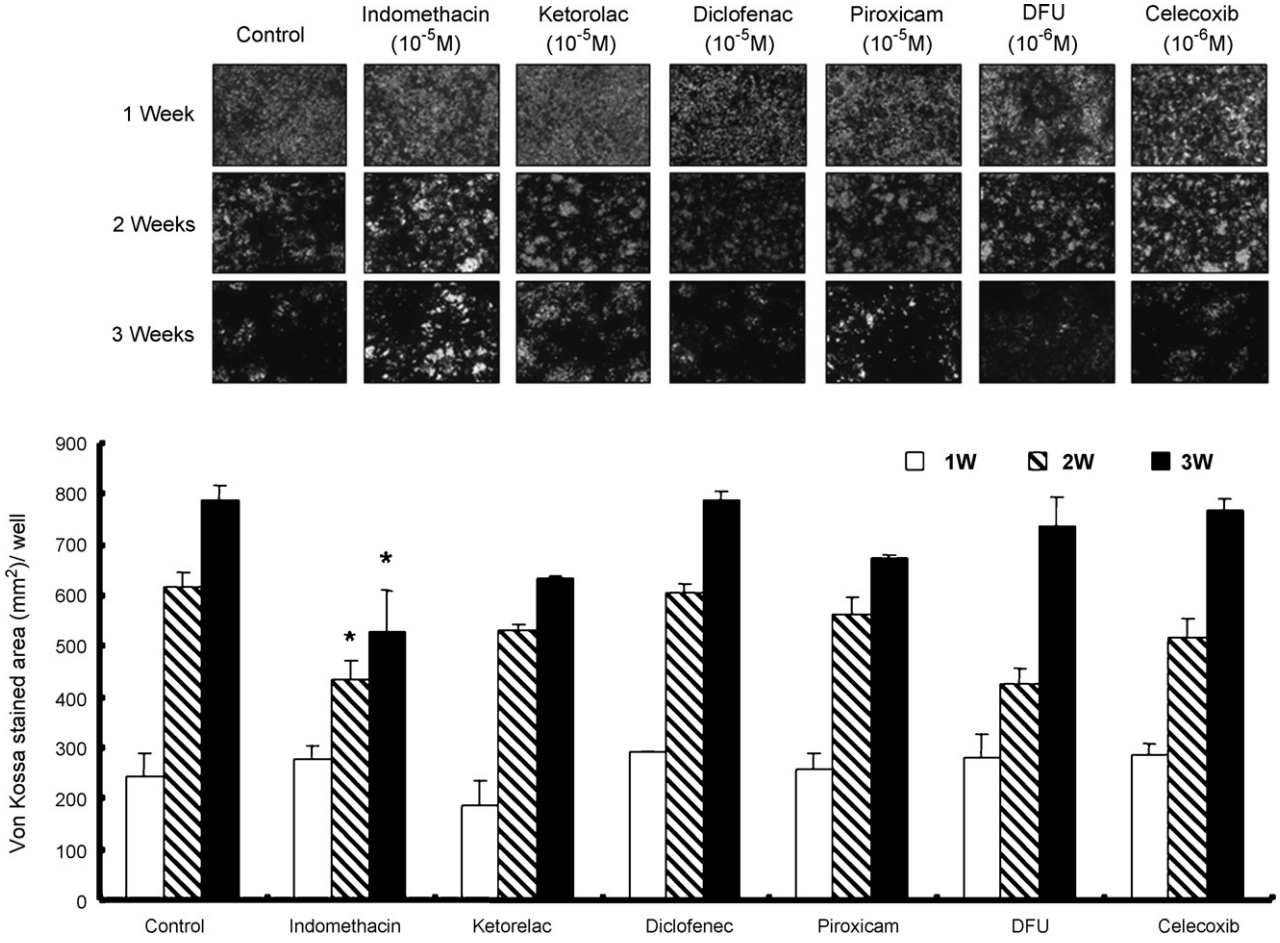

Fig. 6 – Anti-inflammatory drug effects on mineralization of D1-cells. Cultures were treated with non-selective NSAIDs (10^{-5} M), indomethacin, diclofenac, ketorolac and piroxicam; and COX-2 selective NSAIDs (10^{-6} M), celecoxib and DFU, for 1, 2 or 3 weeks. Representative images of von Kossa stained cultures are shown. The mineral deposit area of each well was calculated from 20 microscopic fields randomly. Each bar represents the mean ± S.E.M. of four replicated cultures. Data from cultures treated with different drugs at the same time duration were compared and evaluated by one-way ANOVA, and multiple comparisons were performed by Scheffe's method. *Key*: $^{*}p < 0.05$, in comparison with the control culture at the same time duration.

versus 3-week, $p < 0.01$). Only 2- and 3-week treatments of indomethacin and a 3-week treatment of ketorolac significantly decreased the mineral deposition in D1-cell cultures ($p < 0.05$) (Fig. 6). However, treatments of diclofenac, piroxicam, celecoxib and DFU for either 1, 2 or 3 weeks did not affect the mineralization of D1-cell cultures (Fig. 6).

4. Discussion

Pain and inflammation are the significant concerns for the patients who suffer from the bone or joint diseases. Understanding the influences of anti-inflammatory drugs on the functions of bone cells are definitely an important issue for the orthopaedic researchers. Corticosteroids have been reported to suppress bone formation through suppressing the expressions of osteogenic genes, such as RUNX2, BMP2, osteocalcin, etc. [12,34–36]. Another possible factor for the decrease of bone formation by corticosteroid was indicated to be the induction of osteoblast apoptosis [14,15]. In addition to these effects, the increase of adipogenesis of mesenchymal stem cells was also suggested to be an important factor for steroid induced osteonecrosis and osteoporosis [12,19,20]. In this study, we further identified that the therapeutic concentration of dexamethasone (10^{-7} M) suppressed the proliferation and arrested cell cycle at G0/G1 phase of mesenchymal stem cells, while no significant cytotoxicity was found upon a 24-h treatment. These results suggest that the inhibitory effect of dexamethasone on proliferation of mesenchymal stem cells may be an important factor contributing to the steroid induced osteoporosis and osteonecrosis other than their influences on cell death, osteogenesis and adipogenesis in osteoblast linage.

It has been indicated that several non-selective NSAIDs and COX-2 selective inhibitors suppress bone remodeling and repair in vivo [1–7,37–40]. Our previous study demonstrated that NSAIDs suppressed proliferation and induced cell death, mainly apoptosis, in cultured osteoblasts [9–11]. Some of the researchers hypothesized that NSAID effects on bone cells might be mediated by the inhibition of PG synthesis since PGE_2 stimulates bone formation in vivo [41,42] and enhances osteogenic differentiation in cultured osteoblasts [43]. However, the role of PG in osteoblastic proliferation is controversial; PG stimulated proliferation of some osteoblastic cell lines [44], but inhibited that of primary cultured osteoblasts [9,45]. More importantly, we found that although NSAIDs suppressed PG synthesis in osteoblast cultures, they inhibited osteoblastic proliferation independently from the PG pathway [9]. The important PGs reported to affect bone formation and/or proliferation of osteoblastic cells are PGE1, PGE2 and PGF2α [41,42]. In this study, PGE1, PGE2 and PGF2α were also found to suppress proliferation of cultured mesenchymal stem cells, D1-cells. Interestingly, although PGE2 secreted in culture medium was found at around 10^{-8} M, a 24 h treatment of PGE2 at 10^{-9} to 10^{-8} M still significantly suppressed proliferation of D1-cells. This effect may be because of the auto-amplification of PG production in D1-cells, which has also been observed in cultured osteoblasts [9,46]. More importantly, in this study we further demonstrated that NSAIDs inhibited proliferation of D1-cells and arrested cell cycle at the G0/G1 phase. Although NSAIDs suppressed PG production in D1-cells, replenishing PGE1, PGE2 or PGF2α couldn't reverse the proliferation suppressive effects of anti-inflammatory drugs. Moreover, combined treatments of PG and NSAID further augmented the suppressive effects of both agents. This result suggests that the inhibitory effects of anti-inflammatory drugs on proliferation of D1-cells might be also through PG independent mechanisms. On the other hand, recent reports indicated that COX-2 may play a role in regulating mesenchymal stem cell differentiation and the fracture-healing at early stage of bone repair [40,47]. Whether this influence of COX-2 on bone is independent of the function of PGs has not been elucidated.

In this study, we found that NSAIDs revealed no significant cytotoxic effects on D1-cells, while the proliferation suppressive effects occurred at concentrations covering the therapeutic concentrations (non-selective NSAIDs 10^{-5} M and COX-2 inhibitors 10^{-6} M) [22–27]. These results suggest that the deteriorated effects of NSAIDs on mesenchymal stem cells are more likely due to the inhibition of cell proliferation than induction of cell death. Studies from other laboratories also indicated that NSAIDs suppressed proliferation, arrested cell cycle and/or induced apoptosis in vascular smooth muscle cells [48], tendon cells [49], colon cancer cells [50,51] and promyelocytic leukemia cell lines [52] through affecting cell cycle regulators, but not through the PG pathway. In this study, based on the finding that all the tested AIDs arrested cell cycle in G0/G1 phase, we tested the effects of AIDs on the mRNA expressions of the important cell cycle regulators in G1 phase. The results indicated that indomethacin, celecoxib and dexamethasone enhanced mRNA expression of $p27^{kip1}$ in both D1-cells and hBMSCs. $P27^{kip1}$ is a cyclin-dependent kinase inhibitor (CDKI) that can inhibit the activity of cyclin-dependent kinase (CDK) and subsequently suppresses cell cycle proceeding [53]. The expression of another CDKI, $p21^{Cip1/Waf1}$, was also elevated by higher concentrations of these drugs in D1-cells and hBMSCs. Cyclins are the regulators for proceeding the cell cycle via activating CDKs [53]. Expressions of cyclin E1 and cyclin E2 were found to be decreased by the three classes of AIDs in D1-cells; however, only dexamethasone significantly suppressed cyclin E2 expression in hBMSCs. On the other hand, expressions of p53, cyclin D1 and cyclin D2 were not altered by these AIDs in both D1-cells and hBMSCs. Cyclin D and cyclin E function at different stages of the cell cycle in G1 phase; cyclin D works at whole G1 phase, but cyclin E functions at the second half of the G1 phase. The regulations of their expressions are from different signal transduction pathways [54–56]. The findings in this study provide us a clue that AIDs could be involved in the regulatory mechanisms of cyclin E expression in D1-cells. The effect of AIDs on the transcription regulation and/or intracellular signal transduction pathway of cyclin E will worth further investigation. More importantly, The results indicated that an increase of $p27^{kip1}$ following AID treatments is the common effect of the three classes of AIDs on both murine and human bone marrow mesenchymal stem cells. This result suggests that the enhancement of $p27^{kip1}$ expression by AIDs may be one of the key factors to suppress the proliferation of BMSCs. In addition, up-regulation of $p21^{Cip1/Waf1}$ expression may also involve in arresting cell cycle in BMSCs upon AID treatments at higher concentrations. On

the contrary, down-regulations of cyclin E1 and cyclin E2 by AID treatments were more pronounced in D1-cells than in hBMSCs. The detail mechanisms to elucidate these different effects among AIDs on BMSCs, or the different responses of BMSCs from different species to AID treatments would be complicated and need to be further investigated.

The role of bone marrow mesenchymal stem cells in bone formation involves not only the balance of cell proliferation and apoptosis, but also the potential of osteogenesis. In our previous study, we found that NSAIDs showed no obvious influences on the osteogensis in cultured osteoblasts [9–11]. In this study, we further found that an 8-day treatment of NSAIDs had no significant effects on the mRNA expressions of the important osteogenic genes, RUNX2, BMP2, type I collagen and osteocalcin in D1-cells. However, the ALP activity of D1-cells was suppressed by indomethacin after 1 week of treatment. Furthermore, the mineralization property of D1-cells was not suppressed by the tested NSAIDs, except the continuous treatment with indomethacin for 2–3 weeks or ketorolac for 3 weeks. A long-term and higher concentration treatment of indomethacin and ketorolac may also affect osteogenesis of mesenchymal stem cells, but the therapeutic concentration of NSAIDs showed very mild effect on osteogenesis of D1-cells in this study. This effect is different from that of glucocorticoid on D1-cells and other osteoblastic linage [12,34–36]. It implies that steroid and non-steroid anti-inflammatory drugs may affect mesenchymal stem cell functions through different mechanisms. On the other hand, a previous report indicated that the osteoblastic differentiation of a clonal line of bone marrow derived preadipocytes (ST2 cells) were accelerated by nitric oxide (NO), but reversed by indomethacin [57]. It was suggested that this NO effect was mediated by PG production [57]. This previous study together with our present finding in D1-cells indicated that although PG may play some roles in stem cell functions, the blockage of PG synthesis by therapeutic concentration of different NSAIDs did not always affect the osteogenesis in bone marrow mesenchymal stem cells. The underlying mechanisms could be complicated and worth to be further investigated.

In conclusion, the proliferation inhibition of dexamethasone on mesenchymal stem cells, other than differentiation [12], may result in suppression of bone formation. Moreover, together with these results from D1-cells and hBMSCs, as well as our previous findings from osteoblasts [9], we suggest that the NSAID effects on proliferation suppression in both stem cells and osteoblasts, as well as the induction of apoptosis in osteoblasts, but not stem cells, may contribute to their suppressive effects on bone formation. We also suggest that the suppressive effect of NSAIDs on bone formation might not be due to the inhibitory effect of these drugs on osteogenesis.

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