The effect of COX-2 inhibitor on osteogenesis in human bone marrow-derived mesenchymal stem cells

Je Hyun Yoo

Department of Medicine

The Graduate School, Yonsei University

The effect of COX-2 inhibitor on osteogenesis in human bone marrow-derived mesenchymal stem cells

Directed by Professor Chang Dong Han

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Je Hyun Yoo

December 2008

This certifies that the Doctoral Dissertation of Je Hyun Yoo is approved.

Thesis Supervisor : Chang Dong Han

[Ick Hwan Yang: Thesis Committee Member #1]

[Jin Woo Lee: Thesis Committee Member #2]

[Sahng Wook Park: Thesis Committee Member #3]

[Seung Tae Lee: Thesis Committee Member #4]

The Graduate School
Yonsei University

December 2008

ACKNOWLEDGEMENTS

In spite of 8 years of time that have elapsed after I was introduced to the way of orthopedic surgeon, I yet felt myself as an orthopedic surgeon inadequate in many ways, and I started the doctorate program as another learning way. Now, this paper may be another footstep to further achievements. However, even the smallest achievement could not be realized without encouragement and supports of professors, colleagues, and more.

I'd like to say thank to Prof. Chang Dong Han for this support and teachings, as well as to Prof. Ick Hwan Yang, Prof. Jin Woo Lee, Prof. Seung Tae Lee of the department of Internal Medicine, and Prof. Sahng Wook Park of the department of Biochemistry and Molecular Biology who all generously share their knowledge and support on this paper. Moreover, I do appreciate Prof. Eung Sik Kang, Prof. Soo

Bong Han, and all professors in school of orthopedics who all made myself as a real orthopedic surgeon.

I truly thank to my mother, parents in-law as well. Also I never thank enough to my loving wife and proud two sons (Ho Jun and Ho Sung). I love you all.

<TABLE OF CONTENTS>

ABSTRACT ··· 1

I. INTRODUCTION ··· 4

II. MATERIALS AND METHODS ··· 7

1. Preparation of hMSCs and cell culture ··· 7
2. Osteogenic differentiation and drugs ··· 8
3. Determination of IL-1β concentration for COX-2 expression in hMSCs ··· 9
   A. Western blot analysis ··· 9
4. Determination of cytotoxicity of Celecoxib and Naproxen using MTT assay ··· 10
5. Drug treatment during osteogenesis from hMSCs ··· 11
6. Evaluation for the effects of Celecoxib and Naproxen on the synthesis of PGE_2 in hMSCs using PGE_2 ELISA ··· 11
7. Evaluation for the effects of NSAIDs on osteogenesis from hMSCs ··· 12
   A. ALP staining ··· 12
   B. Von Kossa staining ··· 13
   C. Calcium contents assay ··· 13
   D. RNA isolation and real time RT-PCR ··· 13
   E. Semiquantitative RT-PCR ··· 15
8. Statistical analysis ··· 17

III. RESULTS ········· 17
1. Determination of IL-1β concentration for the maximal induction of COX-2 expression in hMSCs ········· 17
2. Determination of Celecoxib and Naproxen concentrations for COX-2 inhibition in hMSCs as determined by the MTT assay ········· 19
3. Effects of NSAIDs on the mRNAs levels of inflammation-related enzymes in the undifferentiated hMSCs ········· 21
4. Effects of IL-1β and NSAIDs on PGE_2 synthesis in the undifferentiated hMSCs ········· 23
5. Effects of NSAIDs on ALP expression during osteogenesis ··· 25
6. Calcium quantification and mineralization ········· 26
7. Effects of NSAIDs on gene expression of inflammation-related enzymes, transcription factors, and osteogenic markers during osteogenic differentiation ········· 32

IV. DISCUSSION ········· 37
V. CONCLUSION ········· 44

REFERENCES ········· 46
ABSTRACT(IN KOREAN) ········· 51

LIST OF FIGURES

Figure 1. Western blot analyses of COX-2 expression for 24 hours by IL-1β in hMSCs························18
Figure 2. Cytotoxicity of NSAIDs in hMSCs·························20
Figure 3. Real-time RT-PCR analyses for the effects of NSAIDs on expression of inflammation-related genes in the undifferentiated hMSCs ·················22
Figure 4. Effects of Celecoxib and Naproxen on PGE_2 determined by ELISA·······································24
Figure 5. ALP staining of hMSCs and hMSCs pretreated with IL-1β··26
Figure 6. Effects of Celecoxib and Naproxen on the calcium accumulation on day 7 of osteogenesis in hMSCs with or without IL-1β pretreatment ·······················27
Figure 7. Effects of NSAIDs on calcium accumulation during osteogenic differentiation···························30
Figure 8. Von Kossa staining performed on day 14 of osteogenesis to evaluate the effects of the two NSAIDs on mineralization································31
Figure 9. Real-time RT-PCR analyses for the effects of the two NSAIDs on the mRNA expression of inflammation-related enzymes on day 14 of osteogenesis···33

Figure 10. Semiquantitative RT-PCR analysis of mRNA expression for transcription factors required for osteogenesis······35
Figure 11. Effect of IL-1β and NSAIDs on the expression of osteocalcin and osteopontin······37

LIST OF TABLES

Table 1. Primer sequences of real time RT-PCR······················15
Table 2. Primer sequences of semiquantitative RT-PCR··········16
Table 3. Semiquantitative RT-PCR conditions·························16

<ABSTRACT>

The effect of COX-2 inhibitor on osteogenesis in human bone marrow-derived mesenchymal stem cells

Je Hyun Yoo

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Chang Dong Han)

Non-steroidal anti-inflammatory drugs (NSAIDs) exert their effects by suppressing cyclooxygenase (COX) enzymes, which convert arachidonic acid to prostaglandins (PGs). COX enzymes and PGs have been shown to play important roles in bone metabolism. It is generally accepted that prostaglandin E_2 (PGE_2) is the most important PG in bone formation and resorption. In addition to bone resorption, COX-2 has also been shown to have a role in bone formation by increasing the expression of core-binding factor a1 (*Runx2/Cbfa1*) and osterix, the two genes required for bone formation. However, until now, no *in vitro* study has been conducted on whether COX inhibitors such as NSAIDs suppress the osteogenic differentiation of human bone marrow cells. The purpose of this study is to identify whether the osteogenesis of human bone marrow-derived mesenchymal stem cells (hMSCs) is inhibited by

NSAIDs such as the COX-2 inhibitor, Celecoxib, and the non-selective COX inhibitor, Naproxen.

The mRNA levels of membrane-associated PGE synthase (mPGES) were reduced in the Celecoxib-treated cells, suggesting that mPGES, a downstream molecule of COX-2, was selectively inhibited by Celecoxib. In contrast, both cytosolic PGES (cPGES) and mPGES were reduced in a dose-dependent manner in the cells treated with Naproxen. The expression of COX-1 and COX-2 was not changed in the cells treated with Celecoxib and Naproxen. Human recombinant IL-1β (1 ng/ml) induced the expression of COX-2 and mPGES and osteogenesis in hMSCs, whereas a combination of IL-1β and either Celecoxib or Naproxen decreased osteogenesis. The inhibitory effects of the two NSAIDs on osteogenesis were dose-dependent, and alkaline phosphatase (ALP) expression and calcium mineral deposition were significantly inhibited in the cells treated with high-dose NSAIDs (40 μM Celecoxib and 300 μM Naproxen). The inhibition of osteogenesis in hMSCs by high doses of NSAIDs may be correlated with decreased PGES, suggesting that cPGES and/or mPGES are involved in bone formation. The osteogenesis of hMSCs was not affected by therapeutic doses of 10

μM Celecoxib and 100 μM Naproxen, of which the concentration is generally used in *in vitro* experiments. However, 20 and 40 μM Celecoxib and 200 and 300 μM Naproxen (over-therapeutic doses) significantly inhibited osteogenic differentiation.

The expression of *Runx2/Cbfa1* was increased by pretreatment of IL-1β during the early stage of osteogenesis in inflammatory-conditioned hMSCs, and the expression of osterix was increased during the late stage. The expression of ALP increased during the early stage of osteogenesis, and the increased expression of ALP was reduced in a dose-dependent manner by the two NSAIDs. These results suggest that bone healing under inflammatory conditions after fracture is achieved by different pathways than the one taken during normal bone formation. NSAID treatments at over-therapeutic doses in inflammatory-conditioned osteogenic hMSCs may have serious inhibitory effects on bone healing by suppressing the gene expression of transcription factors essential for osteogenesis. Therefore, the negative effects of NSAIDs on osteogenesis in hMSCs must be considered when prescribing high or greater than therapeutic doses of NSAIDs.

Key words : mesenchymal stem cell, osteogenesis, NSAID, COX-2 inhibitor

The effect of COX-2 inhibitor on osteogenesis in human bone marrow-derived mesenchymal stem cells

Je Hyun Yoo

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Chang Dong Han)

I. INTRODUCTION

In general, analgesics are classified into narcotics and non-narcotics, and non-narcotic analgesics are classified into steroidal and non-steroidal anti-inflammatory drugs (NSAIDs)[1,2]. Steroidal anti-inflammatory drugs exert anti-inflammatory and analgesic effects by interfering with the process of arachidonic acid release from membrane phospholipids, but their use may also result in serious side effects due to the suppression of immunologic functions[3]. NSAIDs exert their effects by suppressing the cyclooxygenase (COX) enzymes that catalyze the conversion of arachidonic acid to prostaglandin H_2 (PGH_2)[4]. COX has been shown to exist in three isomers, namely COX-1, COX-2, and COX-3[5-7]. COX-1 has been found in most mammalian tissues and has a role in platelet aggregation and cytoprotection against stomach injury[8,9]. COX-3 was recently identified as an alternatively spliced form of COX-1 in dogs. It retains the in-frame intron 1, but is

otherwise identical to the full-length form of COX-1[10]. COX-3 is expressed most abundantly in brain and heart, and it is considered to be involved in fever and pain. In contrast to the role of COX-1 as a housekeeping gene, COX-2 is an inducible enzyme of which the expression is rapidly increased by inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), or by a growth factor as basic-fibroblast growth factor (bFGF)[12-14]. These differential patterns of COX expression strongly suggest that COX-2 is a key modulator of inflammation.

COX enzymes have also been shown to play an important role in bone metabolism[15,16]. Sato et al[17] demonstrated that the COX-2 induced by IL-1 in stromal cells promoted osteoclastogenesis. Moreover, studies with COX-2 knockout mice have demonstrated its role on bone resorption and formation[18]. COX-2 is responsible for the bulk of PGE_2 production in osteoblasts[19]. Systemic or local injection of PGE_2 stimulates bone formation as well as bone resorption[20]. Zhang et al[21] have reported that inducible COX-2 increases the expression of core-binding factor a1 (*Runx2/Cbfa1*) and osterix, the two genes required for bone formation.

Meanwhile, it has been reported that COX-2 selective inhibitors inhibit bone repair and bone formation[22-24] resulting from the suppression of angiogenesis[24,25] as well as the potential interference with osteoblast and osteoclast functions. These reports suggest that bone formation is suppressed

or delayed by COX-2 inhibitors because the synthesis of PGs, which play an important role in osteogenesis and bone healing, is suppressed[26,27]. However, there is a controversy over the effects of COX-2 inhibitors on bone healing processes *in vivo*.

It has been hypothesized that COX-2 participates in the initial step of osteogenesis and the latter step of osteoblast maturation, and that it controls genes such as *cbfa1* and osterix that are related to bone formation using bone marrow-derived stromal cells from *COX-2* knockout mice[21]. Studies using rabbits showed that non-selective NSAIDs also delay bone healing[23,28]. However, the other study using rabbit demonstrated that COX-2 inhibitors showed no apparent suppressive effects on spinal fusion, and that non-selective NSAIDs have greater suppressive effects on bone healing than COX-2 selective inhibitors[28]. Human studies have shown no inhibitory effect on fracture healing by COX-2 inhibitors[29], otherwise showed inhibition of bone healing by other causes such as smoking[30,31].

It has been widely used for postoperative pain control since it was reported that Celecoxib, a COX-2 inhibitor, significantly reduced the use of opioid drugs[2]. However, concerns regarding a possible inhibitory effects of COX-2 inhibitors on bone healing have limited their routine use after operation for fracture, spinal fusion, and total joint arthroplasty despite of its well-reported safety. Although there are arguments regarding the use of NSAIDs, they are

currently widely prescribed. Many studies have shown negative effects with regard to the use of COX-2 inhibitors. However, these results are difficult to apply to humans because important parameters, including the dosage and period of administration, were not yet established, and the advantages of COX-2 inhibitors cannot be overlooked.

To date, no *in vitro* study has been conducted to determine whether COX inhibitors such as NSAIDs suppress the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs). Therefore, the purpose of this study is to verify whether the osteogenic differentiation of hMSCs is inhibited by non-selective and COX-2 selective NSAIDs.

II. MATERIALS AND METHODS

1. Preparation of hMSCs and cell culture

Bone marrow aspirates were obtained from 15 donors ranging from 20 to 60 years of age, after approval from the Institutional Review Board. hMSCs were isolated from human bone marrow aspirates (2-3 ml) by mixing with 10 ml of growth media [Dulbecco's Modified Eagle's Medium supplemented with 5 mM glucose (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, WelGene, Daegu, South Korea)] and cultured for 7 days in growth media at 37℃ in a humidified atmosphere

under 5% CO_2. On day 7 from primary culture, non-adherent cells were removed by washing the cells twice with phosphate buffered saline (PBS, Invitrogen) and then adherent cells were detached using 0.05% trypsin/EDTA (Invitrogen).

2. Osteogenic differentiation and drugs

hMSCs subcultured up to 4 passages were used for differentiation analysis. For osteogenesis, cells were seeded at 8 x 10^4 or 1.6 x 10^5 cells in 12-well culture plate or 6-well culture plate respectively and maintained for 14 days in osteogenic media [DMEM supplemented with 10 mM β-glycerophosphate (Sigma, St Louis, MO, USA), 100 nM dexamethasone (Sigma), and 50 μg/mL ascorbic acid-2-phosphate (Invitrogen)]. These successive procedures were performed very gently to exclude the mechanical stimulation for osteogenesis of hMSCs.

The subjects were divided into the control group and 3 experimental groups. For the control group, isolated hMSCs were cultured in osteogenic media. For experimental group 1, hMSCs were cultured in osteogenic media containing IL-1β (R&D, Minneapolis, MN, USA). For experimental group 2, hMSCs were cultured in osteogenic media containing NSAIDs to investigate the effect of NSAIDs on osteogenesis under normal condition. For experimental group 3, hMSCs were cultured in osteogenic media containing

both NSAIDs and IL-1β to investigate the effect of NSAIDs on osteogenesis under inflammatory condition *in vitro*. Naproxen (Myungmoon Pharmaceuticals, Seoul, South Korea) was used as a non-selective COX inhibitor, and Celecoxib (Pfizer, New York, NY, USA) as a COX-2 selective inhibitor.

3. Determination of IL-1β concentration for COX-2 expression in hMSCs

To optimize the concentration of IL-1β for COX-2 induction in hMSCs *in vitro*, the study groups were organized as a control group (no treatment) and 3 experimental groups (IL-1β treatment; 0.1, 1, 10 ng/ml). The cells were cultured in cell culture plates for 24 hours in serum free condition and the extent of COX-1 and COX-2 expressions were measured at 1 hour and at every 4 hours thereafter, total seven times by western blotting.

A. Western Blot analysis

Cells were lysed in the Passive lysis buffer (Promega, Madison, Wl, USA). Protein concentrations were determined by the BioRad protein assay (Bio-Rad Laboratories Inc, Hercules, CA, USA). The cell lysate containing 30 μg protein was applied and analyzed by 10% SDS-PAGE (Sigma). Transferred membranes were blocked with PBS with 0.05% NP-40 and 5% skim milk (BD, Sparks, MD, USA) and then incubated for 4 hours with

antibodies against COX-2 (BD Biosciences, San Jose, CA, USA) and COX-1 (abCAM, Cambridge, UK). The same membrane was reprobed with antibody against β-actin (Santacruz Biotechnology, Santa cruz, CA, USA), which was provided as a loading control.

4. Determination of cytotoxicity of Celecoxib and Naproxen using methylthiazoletetrazolium (MTT) assay

To determine the optimal concentrations of Celecoxib and Naproxen for COX-2 inhibition in hMSCs, the study groups were organized as a control group (no treatment) and experimental groups (Celecoxib treatment: 10, 40, 80, 120, & 150 μM ; Naproxen treatment: 100, 200, & 300 μM). The cells were cultured in 48-well cell culture plates for 24 hours. To identify the cytotoxity of Celecoxib or Naproxen in hMSCs, the cell viability of hMSCs treated with each concentration of NSAIDs was determined by MTT assay kit (Sigma). Briefly, hMSCs were seeded at a density of 3×10^4 cells/well in a 300 μl volume of medium in 48-well plate and allowed to attach overnight. The cells were then treated with various concentrations of NSAIDs for 24 hours, while the control group was treated with 0.1% dimethyl sulphoxide (DMSO, Sigma) only. After incubation, culture medium was removed and 100 μl of fresh medium and MTT solution (0.5 mg/ml) were added to each well to identify the cytotoxicity of the two NSAIDs (Celecoxib and

Naproxen) and incubated at 37°C for 3 hours. The upper medium was then carefully removed, and the intracellular formazan was solublized by adding 800 μl of DMSO into each well. Absorbance was measured at 570 nm by a 96-well spectrophotometric microplate reader. All samples were tested in triplicate.

5. Drug treatment during osteogenesis from hMSCs

Two NSAIDs were dissolved in DMSO as stock solution and diluted with culture medium immediately before drug treatment. The final concentration of DMSO was adjusted to 0.1% for all the cell groups. To examine the effect of NSAIDs on osteogenesis of hMSCs, cells were treated with NSAIDs in different concentrations (Celecoxib: 10 μM-therapeutic dose, 20 μM and 40 μM; Naproxen: 100 μM-therapeutic dose, 200 μM and 300 μM). NSAIDs-treated media were replaced every 2 days during osteogenic differentiation.

6. Evaluation for the effect of Celecoxib and Naproxen on the synthesis of PGE_2 in hMSCs using PGE_2 enzyme-linked immunosorbent assay (ELISA)

Intracellular lysate and supernatant conditioned for 24 hours were prepared in a lysis buffer. Lysates were clarified by centrifugation at 13,000 rpm at 4°C for 10minutes. PGE_2 concentration in the supernatant fraction was

measured by PGE_2 ELISA system (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. Briefly, IL-1β alone or both IL-1β and NSAID-treated cell lysates and PGE_2 conjugates were loaded into wells coating with sheep anti-mouse IgG, and the wells were incubated at room temperature for 1 hour on a microplate shaker. After washing with wash buffer provided, all wells were incubated with enzyme substrate, and mixed on a microplate shaker for exactly 30 minutes at room temperature. Finally, plates were read by a 96-well spectrophotometric microplate reader at 450nm. All the assays were performed in duplicate. PGE_2 concentrations were calculated from the standard curve.

7. Evaluation for the effects of NSAIDs on osteogenesis from hMSCs

A. Alkaline phosphatase (ALP) staining

The hMSCs were cultured in 12-well plates to reach semi-confluence, and treated with COX-2 inhibitor (Celecoxib) and non-selective COX inhibitor (Naproxen) for 14 days. Cells were washed twice with PBS, and then fixed with citrate and acetone (2:3) solution for 90 seconds. After washing it with PBS twice, the cells were stained with alkaline dye mixture (diazonium salt solution to naphthol AS-MX phosphatate alkaline solution, Sigma) for 30 minutes at room temperature in shaded condition.

B. Von Kossa Staining

To detect mineral deposition representing the final step of osteogenesis using von Kossa stain, samples were rinsed with distilled water, 1 ml of freshly prepared 3% silver nitrate (wt/vol) (Sigma) was added, and the slides were incubated in the dark for 30 minutes. They were then rinsed with distilled water and exposed to bright light for 30 minutes while covered with water. Mineral deposits were stained black.

C. Calcium contents assay

To detect calcium contents, samples were washed with PBS twice, 800 μl of 0.5 N acetic acid added, and incubated for 24 hours at room temperature. After incubation, 300 μl of Fresh reagent (O-Cresolphthalein Complexon, ethanolamine/boric acid, hydroxyquinol, Sigma) was added to 50 μl of sample supernatants. And then absorbance was measured at 560 nm. Standard were prepared from a $CaCl_2$ solution, and the results expressed as mg/ml calcium equivalents per microgram of total protein.

D. RNA isolation and Real-time reverse transcription-polymerase chain reaction (Real time RT-PCR)

Real-time RT-PCR was performed to determine changes in mRNA

expression of prostaglandin synthesis-related enzymes (COX-1, COX-2, cytosolic prostaglandin E synthase (cPGES), and membrane-associated prostaglandin E synthase (mPGES)), osteoblast markers (osteocalcin and osteopontin), and transcription factors associated with osteogenesis (*Runx2/Cbfa1*, osterix, *Dlx5*, and *Msx2*). Total RNA was isolated from cells using RNeasy kit (Qiagen, Valencia, CA, USA). The purity and amount of isolated RNA were assessed by spectrophotometric measurements at 260 and 280 nm. One microgram of total RNA was reverse-transcribed using an Omniscript kit (Qiagen). The PCR reactions contained a final concentration of 1X SYBR Green PCR PreMix (Bioneer, Daejon, South Korea), 10 pM gene-specific primers, and 1 μl of cDNA. The primers used in real time RT-PCR were summarized in Table 1. The cycling conditions were as follows: 40 cycles of denaturing at 94℃ for 10 seconds and annealing at 58℃ for 30 seconds, and then extension at 72℃ for 1 minute.

The mean cycle threshold (CT) values from duplicate measurements were used to calculate the gene expression, with normalization to β-actin as an internal control. Bioneer Exicycler Real Time PCR system (Bioneer) was used for PCR reaction.

Table 1. Primer sequences of real time RT-PCR

Genes		Primer Sequences	Size (bp)
mPGES	(S)	5′ - GCG TGC TGC GTG TGA TGG - 3′	148
	(AS)	5′ - TGC CTT CCC TCT GCT CTG C - 3′	
cPGES	(S)	5′ - GAA CAA ATT GGC TGA CAC CTT ACT G - 3′	146
	(AS)	5′ - AAG ATA CAC CTG GAA CAC TGA CAA G - 3′	
COX-2	(S)	5′ - GGT GCC TGG TCT GAT GAT GTA TG - 3′	126
	(AS)	5′ - AGT ATT AGC CTG CTT GTC TGG AAC - 3′	
COX-1	(S)	5′ - CTT GAC CGC TAC CAG TGT GAC - 3′	124
	(AS)	5′ - GCA GGA AGT GGG TGA AAG AGG - 3′	
β-actin	(S)	5′ - GTC CTC TCC CAA GTC CAC ACA G - 3′	124
	(AS)	5′ - GGG CAC GAA GGC TCA TCA TTC - 3′	

Size represents the size of the amplified product for each gene. S: sense primer, AS: antisense primer, bp: base pair

E. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

As described above, cDNA was amplified in a total volume of 50 μl containing 1X PCR buffer, 0.4 μM of each primer, 0.2 mM dNTP mix, and 1 U of Taq DNA polymerase (Qiagen) at optimal temperature. The primers used in semiquantitative RT-PCR were summarized in Table 3, and the cycling conditions for each gene were described in Table 4, respectively.

PCR products were analyzed in a 1.5% agarose gel at 100 V/cm in 1X Tris-Borate-EDTA (TBE) buffer, followed by staining with ethidium bromide. The density values for PCR products were normalized to β-actin values to yield a semiquantitative assessment.

Table 2. Primer sequences of semiquantitative RT-PCR

Genes		Primer Sequences	Size (bp)
Osteocalcin	(S)	5' - ATG AGA GCC CTC ACA CTC CT - 3'	197
	(AS)	5' - GCC GTA GAA GCG CCG ATA GG - 3'	
Osteopontin	(S)	5' - CCA AGT AAG TCC AAC GAA A - 3'	347
	(AS)	5' - GGT GAT GTC CTC TCT CCT CTG - 3'	
Dlx5	(S)	5' - GAA TGG TGA ATG GCA AAC CAA AG - 3'	400
	(AS)	5' - GAA TTG ATT GAG CTG GCT GCA CT - 3'	
Msx2	(S)	5' - GCC AAG ACA TAT GAG CCC TAC CAC CT - 3'	400
	(AS)	5' - GGA CAG GTG GTA CAT GCC ATA TCC CA - 3'	
Osterix	(S)	5' - CAT TGC TTT CCA TTC TTC AGA AC - 3'	402
	(AS)	5' - ATT ACA AGA GAA ACC CTA TCA AC - 3'	
Runx2/Cbfa1	(S)	5' - CCA CCT CTG ACT TCT GCC TC - 3'	172
	(AS)	5' - GAC TGG CGG GGT GTA AGT AA - 3'	
β-actin	(S)	5' - GTC CTC TCC CAA GTC CAC ACA G - 3'	132
	(AS)	5' - GGG CAC GAA GGC TCA TCA TTC - 3'	

S: sense primer, AS: antisense primer, bp: base pair

Table 3. Semiquantitative RT-PCR conditions

Genes	Condition	Cycle
Osteocalcin	94℃ 15 min 94℃ 1 min → 56℃ 30 sec → 72℃ 1 min 72℃ 7 min	30
Osteopontin	94℃ 5 min 94℃ 30 sec → 55℃ 30 sec → 72℃ 30 sec 72℃ 7 min	32
Dlx5	94℃ 5 min 94℃ 30 sec → 58℃ 30 sec → 72℃ 30 sec 72℃ 7 min	32
Msx2	94℃ 5 min 94℃ 30 sec → 62℃ 30 sec → 72℃ 30 sec 72℃ 7 min	33
Osterix	94℃ 5 min 94℃ 30 sec → 55℃ 30 sec → 72℃ 30 sec 72℃ 7 min	35
Runx2/Cbfa1	94℃ 2 min 95℃ 30 sec → 55℃ 30 sec → 72℃ 1 min 72℃ 10 min	33
β-actin	94℃ 5 min 94℃ 30 sec → 57℃ 30 sec → 72℃ 30 sec 72℃ 7 min	22

8. Statistic analysis

The statistical analysis for the results of staining test and gene expression pattern obtained from hMSCs was carried out using Student's t-test, and the data were expressed as the mean±SEM.

III. RESULTS

1. Determination of IL-1β concentration for the maximal induction of COX-2 expression in hMSCs

Treatment of hMSCs with 1 or 10 ng/ml of IL-1β significantly increased COX-2 levels at 4 to 8 hours, as determined by western blot analysis, whereas COX-1 remained constant. The induced COX-2 expression decreased to basal levels within 24 hours. In contrast, IL-1β at a concentration of 0.1 ng/ml had no significant effect on COX-1 or COX-2 expression (Figure 1). Therefore, 1 ng/ml of IL-1β was determined to be an appropriate dose to enhance the expression of COX-2 and provoke inflammatory conditions *in vitro* similar to those with bone fractures.

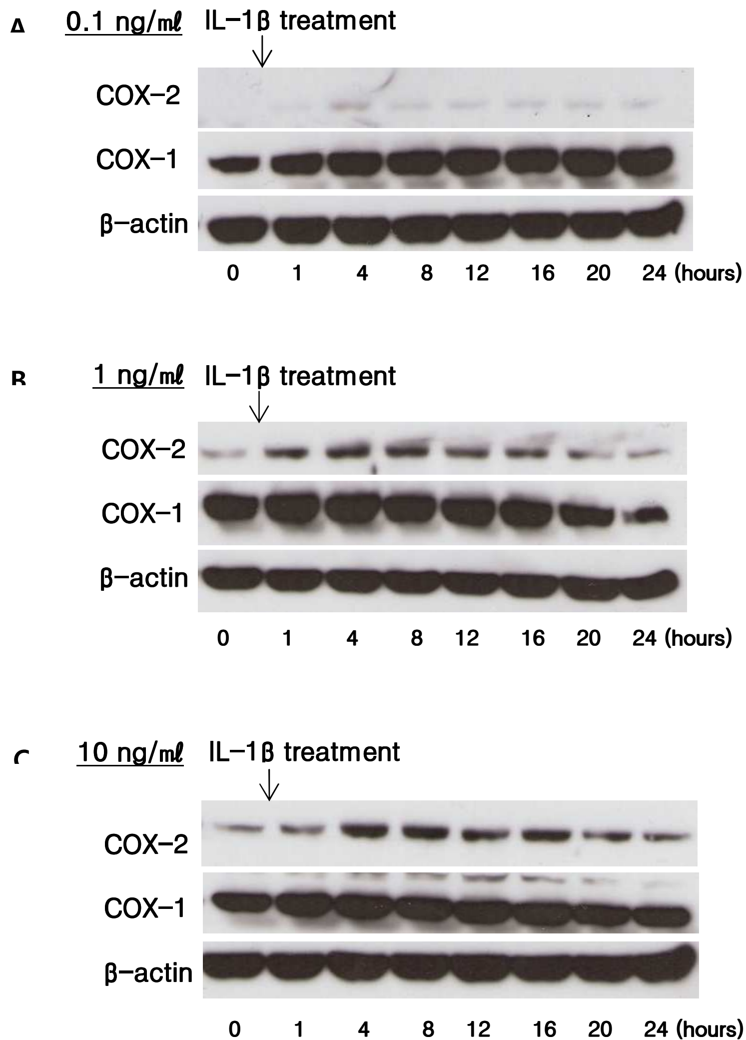
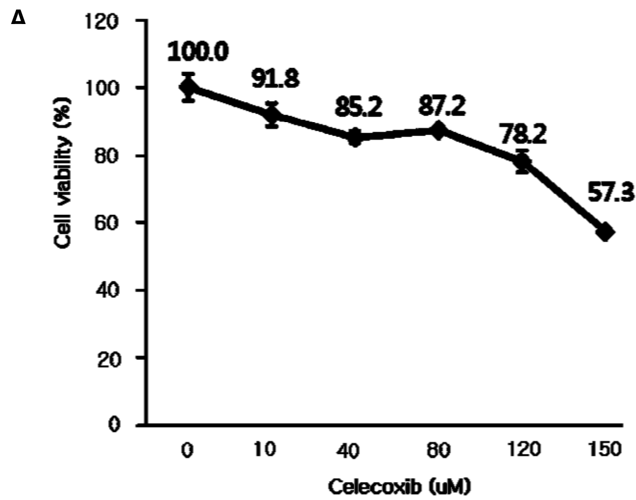


Figure 1. Western blot analysis of COX-2 expression for 24 hours by IL-1β in hMSCs. (A) The COX-2 expression was not affected by 0.1 ng/ml IL-1β. (B) and (C) It was enhanced at 4 to 8 hours and slowly decreased over 12 hours by 1 ng/ml and 10 ng/ml IL-1β. However, the COX-1 was expressed

constantly regardless of IL-1β concentration.

2. Determination of Celecoxib and Naproxen concentrations for COX-2 inhibition in hMSCs as determined by the MTT assay

Treatment with Celecoxib showed a dose-dependent reduction in cell viability. At 24 hours after treatment with Celecoxib in 10% serum, we found decreases in cell viability to 91.8% at 10 μM, 85.2% at 40 μM, 78.2% at 120 μM, and 57.3% at 150 μM (Figure 2A). Meanwhile, no marked effect was seen in cells treated with Naproxen and the cell viability for all doses of Naproxen was over 85% (Figure 2B). In this study, the doses for Celecoxib and Naproxen were determined based on cell viabilities over 85% for the standards. For Celecoxib, the therapeutic dose (10 μM) and two over-doses (40 μM, 80 μM) were used according to this standard. For Naproxen, the therapeutic dose (100 μM) and two over-doses (200 μM, 300 μM) were used as well.



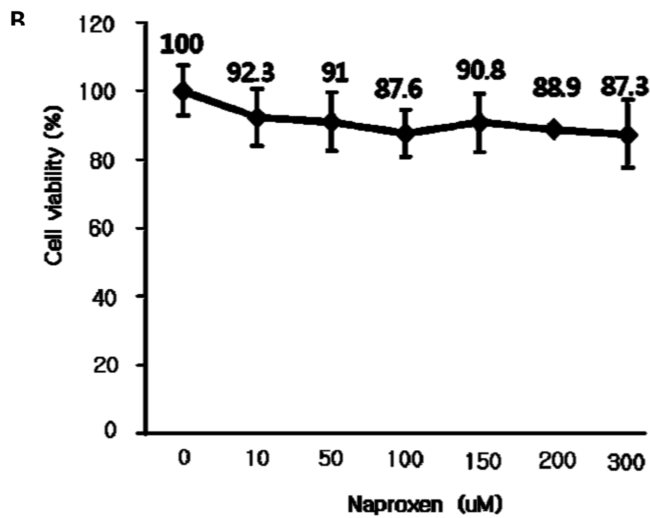


Figure 2. Cytotoxicity of NSAIDs in hMSCs. (A) Cell viability, determined by MTT assay for 24 hours after treatment of Celecoxib, was decreased in a dose-dependent manner. (B) Naproxen had no marked effect on cell viability, based on MTT assay for 24 hours.

3. Effects of NSAIDs on the mRNA levels of inflammation-related enzymes in the undifferentiated hMSCs

The hMSCs were treated with 1 ng/mL of IL-1β and Celecoxib or Naproxen for 24 hours. The study groups were organized as two control groups (no treatment) and six experimental groups (Celecoxib treatment: 10 μM (therapeutic concentration) and 20 & 40 μM (over-therapeutic concentrations); Naproxen treatment: 100 μM (therapeutic concentration) and 200 & 300 μM (over-therapeutic concentrations)). The expression of prostaglandin synthesis-related enzymes (COX-1, COX-2, cPGES, mPGES) was identified by real-time RT-PCR. Among these enzymes, only the mRNA level of mPGES was reduced in the Celecoxib-treated hMSCs, suggesting that mPGES, an inducible enzyme that is coordinately induced with COX-2 on the peri-nuclear membrane, was selectively inhibited by Celecoxib (Figure 3A). In contrast, both cPGES, a promoter of COX-1-mediated PGE_2 production, and mPGES were reduced in a concentration-dependent manner in hMSCs treated with Naproxen (Figure 3B). However, the mRNA expression of COX-1 and COX-2 was not consistently changed in the hMSCs treated with Celecoxib or Naproxen.

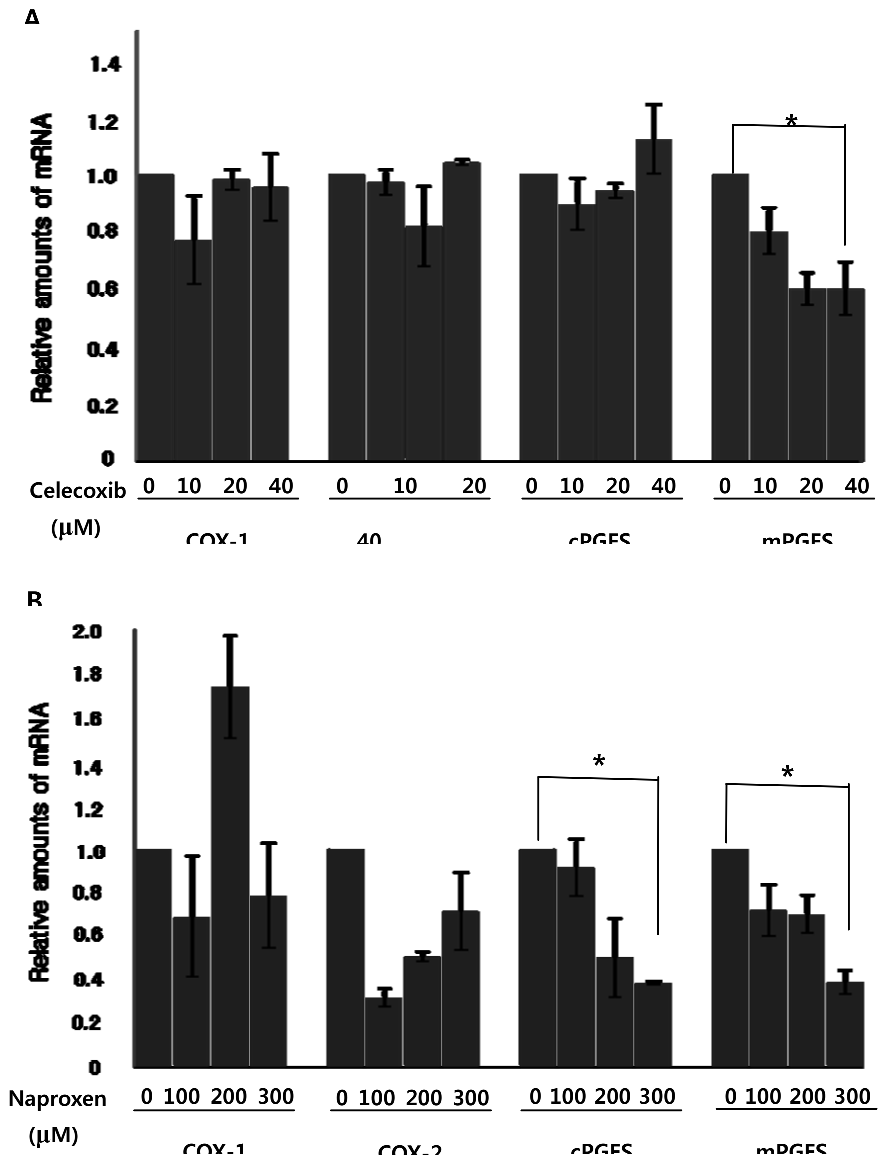


Figure 3. Real-time RT-PCR analysis for the effects of NSAIDs on expression of inflammation-related genes in the undifferentiated hMSCs. (A) Only the mRNA level of mPGES was reduced in the Celecoxib-treated

hMSCs, (B) cPGES and mPGES were reduced in a concentration-dependent manner in the Naproxen- treated hMSCs (*$p < 0.05$). Relative amounts of mRNA expression of the target gene were determined by comparing the β-actin mRNA level, which was set at 1.

4. Effect of IL-1β and NSAIDs on PGE_2 synthesis in the undifferentiated hMSCs

To evaluate the extent of the inhibition of PGE_2 by NSAIDs in undifferentiated hMSCs, ELISAs were performed on hMSC supernatants that were collected 24 hours after treatment with either IL-1β or IL-1β and NSAIDs. PGE_2 was secreted in the non-inflammatory conditioned-hMSCs (not pretreated with IL-1β of 1ng/ml) as well as in the pretreated, inflammatory-conditioned hMSCs. This study revealed that the synthesis of PGE_2 in hMSCs was significantly inhibited by the two NSAIDs at all of their concentrations. However, the concentrations had no correlation with the extent of PGE_2 synthesis inhibition, and there was no significant difference in the extent of inhibition between hMSCs and hMSCs pretreated with IL-1β (Figure 4).

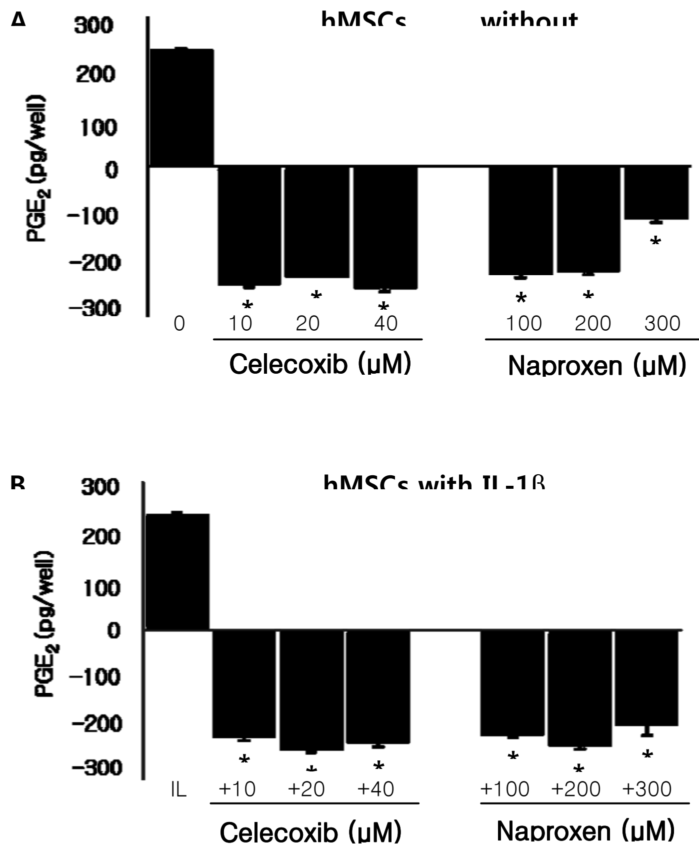


Figure 4. Effects of Celecoxib and Naproxen on PGE_2 determined by ELISA. The synthesis of PGE_2 was significantly reduced in all concentrations regardless of concentrations of the two NSAIDs in both (A) hMSCs without pretreatment with 1 ng/ml IL-1β and (B) hMSCs with it (*$p < 0.05$).

5. Effect of NSAIDs on ALP expression during osteogenesis

On day 7 after inducing osteogenesis, ALP, an earlier marker of osteoblast differentiation, was expressed very weakly in the non-inflammatory conditioned-hMSCs without IL-1β, and there was no significant differences in the extent of ALP staining between the doses of the two NSAIDs, Celecoxib and Naproxen, respectively (Figure 5A).

However, in the inflammatory-conditioned hMSCs pretreated with IL-1β, ALP staining was dose-dependently reduced by the over-therapeutic concentrations of NSAIDs (20 and 40 μM of Celecoxib and 200 and 300 μM of Naproxen), compared to the control group or the therapeutic concentration group (10 μM of Celecoxib and 100 μM of Naproxen) (Figure 5B). The therapeutic concentration treatments showed no significant decrease in ALP expression during the osteogenic differentiation. This finding suggests that high concentration of NSAIDs may inhibit osteogenic differentiation in hMSCs.

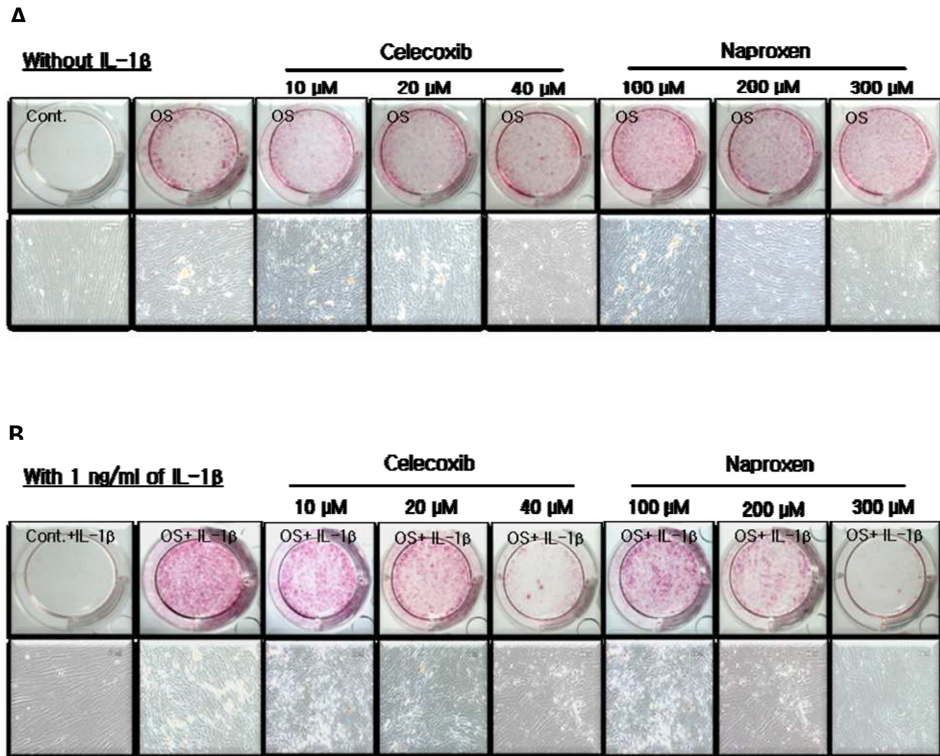


Figure 5. ALP staining of hMSCs and hMSCs pretreated with IL-1β. On day 7 of osteogenic differentiation in hMSCs (A) without pretreatment with 1 ng/ml IL-1β and hMSCs (B) with it. ALP expression was inhibited at over-therapeutic dose of each NSAID only in inflammatory-conditioned-hMSCs after pretreatment with IL-1β.

6. Calcium quantification and mineralization

To determine whether the effects of Celecoxib and Naproxen on the calcium accumulation in the osteogenic hMSCs is affected by pretreatment with IL-1β, the calcium content assay was carried out on day 7 of osteogenesis in Celecoxib-treated hMSCs and Naproxen-treated hMSCs under inflammatory condition with IL-1β pretreatment and normal condition without it, respectively. The calcium accumulation was not significantly reduced at all the concentrations of the two NSAIDs in the differentiated hMSCs without pretreatment with IL-1β.

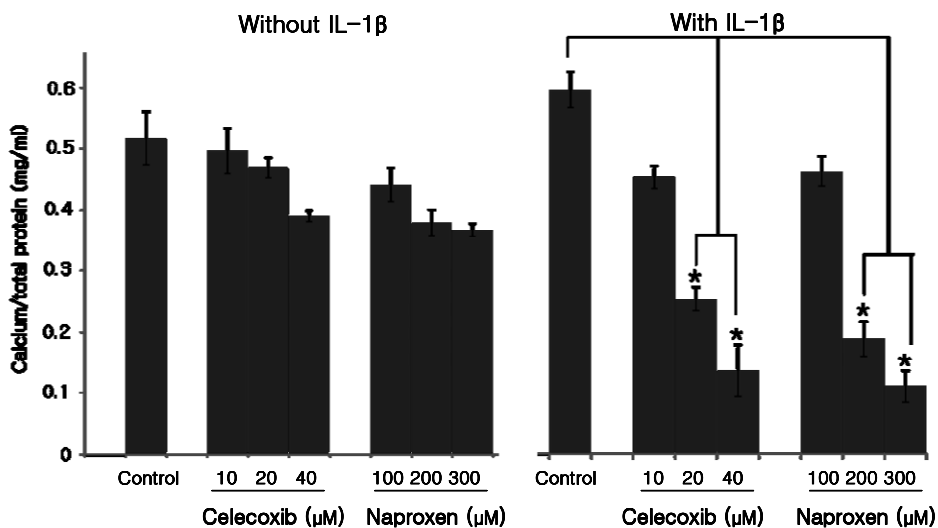


Figure 6. Effects of Celecoxib and Naproxen on the calcium accumulation on day 7 of osteogenesis in hMSCs with or without IL-1β pretreatment. The calcium accumulation was significantly reduced at over-doses of the two

NSAIDs only in the differentiated hMSCs after pretreatment with IL-1β (* $p < 0.05$).

However, it was dose-dependently reduced at all the concentrations and significantly reduced at over-therapeutic concentrations of the two NSAIDs in the differentiated hMSCs after pretreatment with IL-1β (Figure 6). This finding suggests that the osteogenesis of hMSCs was not affected by NSAIDs under normal condition without IL-1β pretreatment.

So, I proceeded the calcium content assay and Von Kossa staining only under inflammatory condition provoked by IL-1β pretreatment thereafter.

The calcium contents in the NSAID-treated osteogenic hMSCs were significantly decreased in a dose-dependent manner compared to the control osteogenic hMSCs on day 10. On day 10 of osteogenic differentiation, Celecoxib decreased calcium contents to 1.411 mg/ml at 10 μM, 0.723 mg/ml at 20 μM, and 0.449 mg/ml at 40 μM, in comparison to a 2.714 mg/ml of calcium content in control osteogenic hMSCs. Naproxen also decreased calcium contents to 1.089 mg/ml at 100 μM, 0.849 mg/ml at 200 μM, and 0.196 mg/ml at 300 μM (Figure 7A).

Treatments with therapeutic doses of NSAIDs (10 μM Celecoxib & 100 μM Naproxen) for 2 weeks did not affect calcium contents during the osteogenesis of the hMSCs. However, 2-week treatments with

over-therapeutic doses of NSAIDs (40 μM Celecoxib; 300 μM Naproxen) caused significant decreases in calcium content during the osteogenesis of the hMSCs. After *in vitro* osteogenic differentiation for 14 days, Celecoxib decreased the calcium contents to 2.825 mg/ml at 20 μM and 2.266 mg/ml at 40μM (over-therapeutic concentrations). Naproxen also decreased calcium contents to 0.715 mg/ml at 300 μM (over-therapeutic concentration), in comparison to a 3.411 mg/ml calcium content for the control osteogenic hMSCs (Figure 7B). This finding suggests that NSAIDs at high concentrations inhibit the osteogenic differentiation of hMSCs.

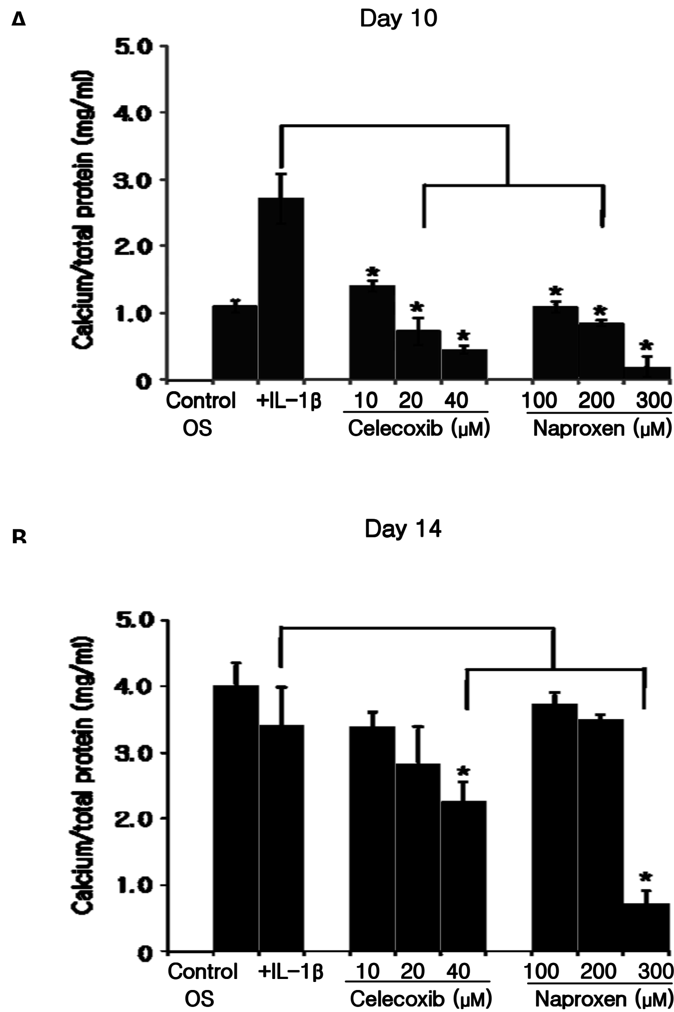


Figure 7. Effects of NSAIDs on calcium accumulation during osteogenic differentiation. Calcium contents assay showed the inhibitory effects of Celecoxib and Naproxen on calcium accumulation during osteogenic differentiation at day 10 and 14 under inflammatory condition by IL-1β. (A) On day 10, (B) On day 14 (*$p < 0.05$).

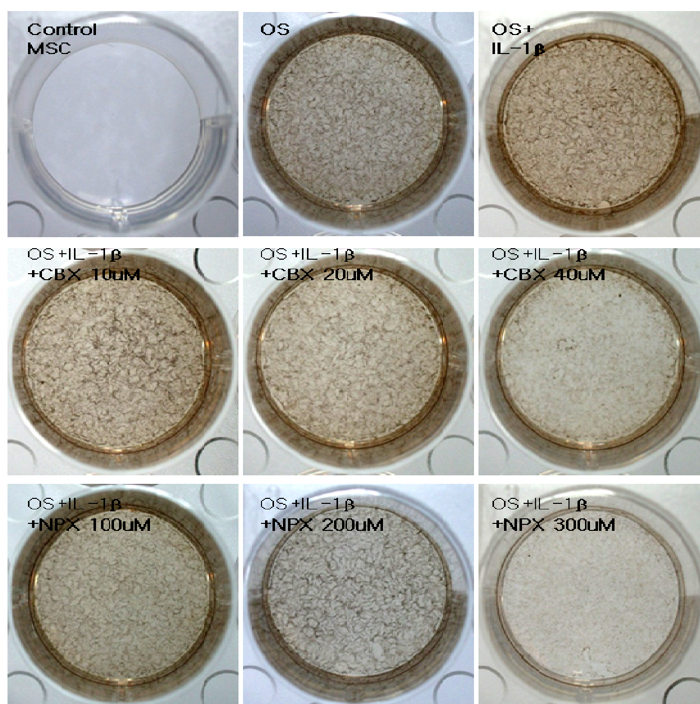


Figure 8. Von Kossa staining performed on day 14 of osteogenesis to evaluate the effects of the two NSAIDs on mineralization. The treatments of the two NSAIDs at over-doses for 2 weeks decreased mineral deposition by the differentiated hMSCs. This finding coincided with it on the calcium contents assay.

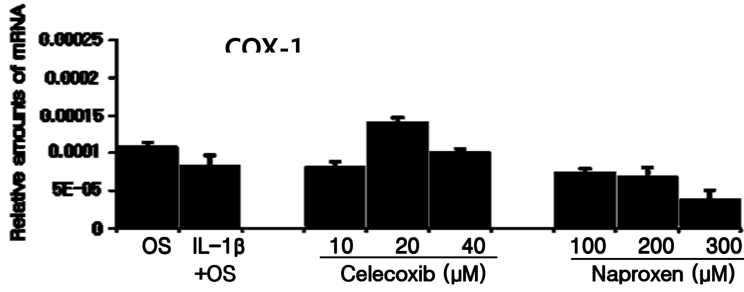
The results of Von Kossa staining showed that treatments of the two NSAIDs at over-therapeutic doses for 2 weeks (20 and 40 μM Celecoxib; 200 and 300 μM Naproxen) significantly decreased mineral deposition by

osteogenic hMSCs. However, treatments of NSAIDs at therapeutic doses (10 μM Celecoxib and 100 μM Naproxen) did not affect the mineralization in osteogenic hMSCs (Figure 8).

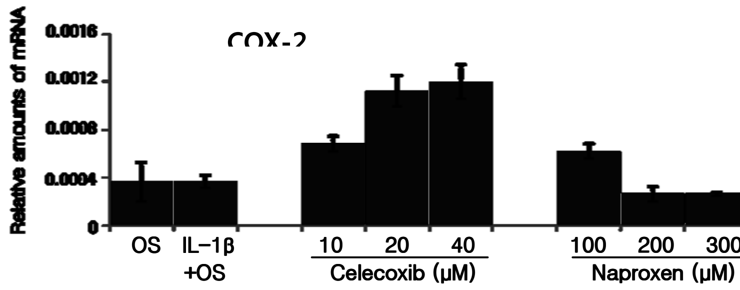
7. Effects of NSAIDs on the gene expression of inflammation-related enzymes, transcription factors, and osteogenic markers during osteogenic differentiation

Based on the results of real-time RT-PCR analyses performed after the analysis of Von Kossa staining for osteogenic differentiation on day 14, Celecoxib increased the mRNA expression of COX-2 and mPGES in a dose-dependent manner. In contrast, Naproxen induced a relatively dose-dependent decrease of mRNA expression of all inflammation-related enzymes (Figure 9). These findings suggest that the inhibitory effects of the two NSAIDs on osteogenesis of hMSCs are not shown by directly suppressing the mRNA expression of inflammation-related enzymes during osteogenic differentiation.

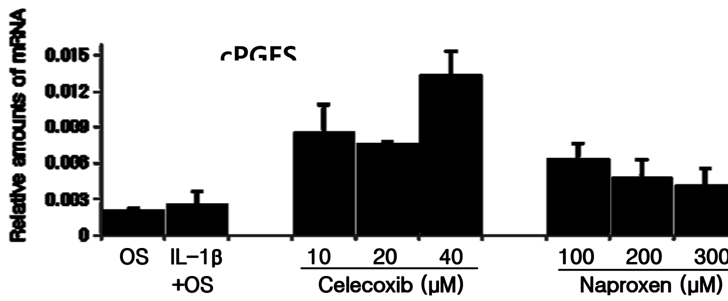
A



B



C



D

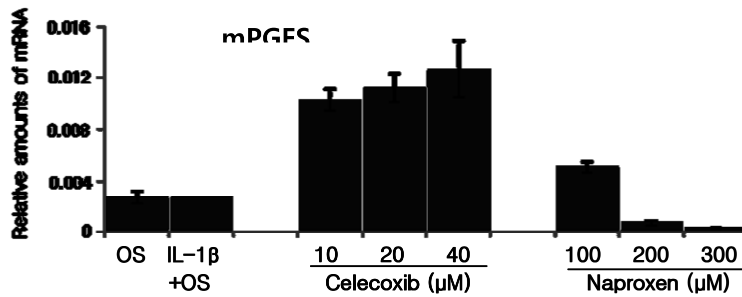


Figure 9. Real-time RT-PCR analyses for the effects of the two NSAIDs on the mRNA expression of inflammation-related enzymes on day 14 of osteogenesis. Only Naproxen induced a relatively dose-dependent decrease of mRNA expression of all inflammation-related enzymes in the differentiated hMSCs under the inflammatory condition after pretreatment with IL-1β. (A) COX-1, (B) COX-2, (C) cPGES, and (D) mPGES.

The expression of *Runx2/Cbfa1*, necessary for osteoblastogenesis, was increased on day 4 and day 7 by pretreatment with IL-1β. The increased expression of *Runx2/Cbfa1* by IL-1β was decreased in a dose-dependent manner by both Celecoxib and Naproxen and showed an especially serious decrease for the over-therapeutic doses. However, it was not affected by the two NSAIDs in the non-inflammatory conditioned-osteogenic hMSCs (that were not pretreated with IL-1β) (Figure 10A).

The expression of *Dlx5* was also decreased by NSAIDs on day 4 of osteogenesis, like *Runx2/Cbfa1*, but was not increased by IL-1β. Also, the inhibitory effect of NSAIDs on the gene expression of *Dlx5* was not shown in non-inflammatory conditioned-osteogenic hMSCs (Figure 10A).

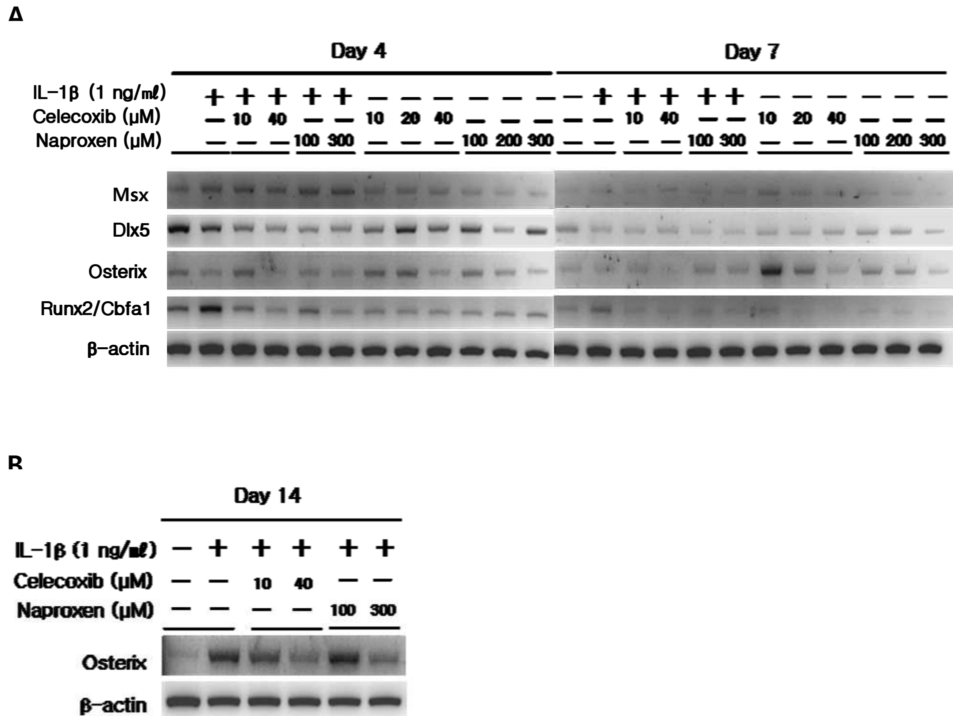


Figure 10. Semiquantitative RT-PCR analysis of mRNA expression for transcription factors required for osteogenesis. (A) The expression of *Runx2/Cbfa1* was enhanced by IL-1β on day 4 and 7 of osteogenesis. They were inhibited by NSAIDs in a dose-dependent manner on day 4 and 7. (B) The expression of osterix was enhanced by IL-1β and inhibited at over-therapeutic concentrations of the two NSAIDs on day 14.

The expression of osterix was not affected by IL-1β or NSAIDs during the

early stage of osteogenesis, but it was increased during the late stage of osteogenesis. On the other hand, it was decreased by NSAIDs in a dose-dependent manner and was seriously decreased at over-therapeutic concentrations (Figure 10B).

The expression of *Msx2* showed no consistent change by NSAIDs during osteogenesis, and the gene expression of transcription factors except osterix was not consistently affected by NSAIDs on day 14 of osteogenesis (data not shown).

The gene expression of osteocalcin, an osteoblast marker, was dose-dependently inhibited by the two NSAIDs only in hMSCs under inflammatory condition provoked by pretreatment with IL-1β on day 4 and 7 of osteogenesis, but the expression was not affected by NSAIDs in hMSCs under normal condition without pretreatment with IL-1β. The expression of osteopontin was not affected by the two NSAIDs (Figure 11).

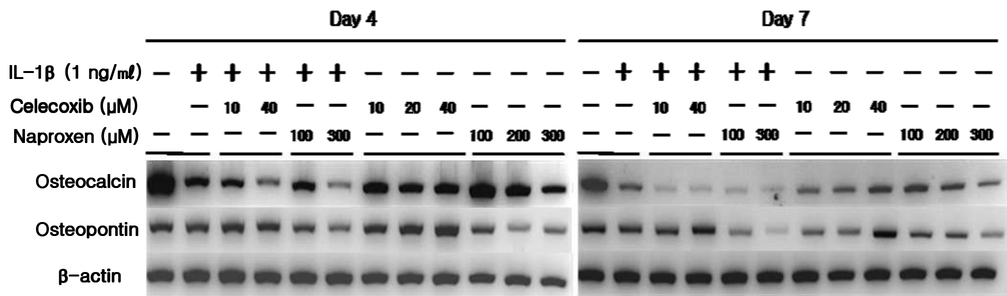


Figure 11. Effect of IL-1β and NSAIDs on the expression of osteocalcin and osteopontin. On day 4 and 7 of osteogenesis, osteogenic differentiated hMSCs were lysed and total RNA was subjected to semiquantitative RT-PCR analysis for osteocalcin and osteopontin. The gene expression of osteocalcin, an osteoblast marker, was dose-dependently inhibited by the two NSAIDs on day 4 and 7 of osteogenesis, but the expression of osteopontin gene was not consistently affected by the two NSAIDs.

IV. DISCUSSION

COX enzymes and PGs have been shown to play important roles in bone metabolism[15,16]. Moreover, it is generally accepted that, among various PGs, PGE_2 is the most important in bone formation and resorption[32]. COX-2 has also been shown to have a role in bone formation and to increase the expression of core-binding factor a1 (*Runx2/Cbfa1*) and osterix, two genes

required for bone formation[21]. Several studies reporting the reduction or suppression of bone fusion and/or bone formation by COX-2 inhibitors show that they may have negative effects on bone healing and/or bone formation[22-24]. The studies using rabbits showed that non-selective NSAIDs also delay bone healing[23.28]. These reports suggest that bone formation may be suppressed or delayed because COX-2 and the synthesis of PGE_2, which play important roles in osteogenesis and bone healing, are suppressed. In contrast, Gerstenfeld et al[33] reported that stable bony union occurred in spite of the administration of a non-selective COX inhibitor, ketorolac, and a COX-2 selective inhibitor, parecoxib, in a rat model. However, such reports have many possible problems because they were conducted using only animals.

Meanwhile, human studies showed no inhibitory effects on bone fracture healing by COX-2 inhibitors[29]. There is still a controversy over the negative effects of NSAIDs on osteogenesis in animals and humans in *in vivo* studies like this. Therefore, because no *in vitro* study of COX inhibitors on osteogenesis has been conducted to date, this study was designed and performed to identify whether the osteogenesis of hMSCs is inhibited by a non-selective COX inhibitor, Naproxen, and a COX-2 selective inhibitor, Celecoxib.

Several previous studies reported that the suppression of PGE_2 synthesis by NSAIDs inhibits osteogenic differentiation and that both *in vivo* bone

formation and *in vitro* osteogenic differentiation are enhanced by PGE_2 synthesis[35-37]. Our results showed that high-dose NSAIDs decreased the osteogenic differentiation of hMSCs *in vitro* via the same mechanism described above. In contrast, the therapeutic doses of NSAIDs showed no inhibitory effects on the osteogenic differentiation of hMSCs, although the synthesis of PGE_2 was suppressed by all doses of the two NSAIDs, including the therapeutic doses. These results suggest that a mechanism other than the suppression of PGE_2 synthesis may also be involved in the inhibitory effect of NSAIDs on the osteogenesis of hMSCs.

Murakami et al[37] reported that cPGES is functionally coupled with COX-1, and mPGES is functionally coupled with COX-2. This data support the importance of the coupling between COX enzymes and PGES during *in vitro* osteogenesis. However, I found that the NSAIDs (Celecoxib and Naproxen) did not directly suppress the mRNA expression of COX-1 or COX-2 in the hMSCs, while they actually inhibited the mRNA expression of PGES. In other words, Celecoxib, a COX-2 selective inhibitor, suppressed only mPGES expression, whereas Naproxen, a non-selective NSAID, suppressed both cPGES and mPGES in a dose-dependent manner. These results are similar to those reported by Arikawa et al[32], which demonstrated that the mRNA expression of COX-1, COX-2, and cPGES was not decreased by NS-398, a COX-2 selective inhibitor, whereas the mRNA of only mPGES was decreased.

This suppressive effect of NSAIDs on PGES expression might be correlated with the reduced osteogenesis of hMSCs at over-therapeutic doses of NSAIDs. This is based on the finding that high-dose NSAID treatment during *in vitro* osteogenesis resulted in significant dose-dependent reductions in the expression of ALP and the deposition of calcium phosphate-containing minerals. Zhang et al[21] reported that PGE_2 is necessary for mesenchymal cell differentiation into the osteoblast lineage in COX-2 knockout mice. They also demonstrated that induced COX-2 and exogenous PGE_2 induce the expression of *Runx2/Cbfa1* and osterix, which are transcription factors essential for osteogenesis. These reports have a thread of connection with this data, which revealed that the inhibitory effect of NSAIDs on COX-2 suppressed the expression of transcription factors essential for osteogenesis, as well as suppressing the production of PGE_2, which resulted in the decrease of osteogenesis in the hMSCs.

However, no author has reported the differences in osteogenesis of hMSCs under both inflammatory conditions and normal conditions, and the effect of NSAIDs thereon. Therefore, in this study, we investigated the effect of NSAIDs on osteogenesis of inflammatory-conditioned-hMSCs and non-inflammatory conditioned-hMSCs. This study showed that ALP was expressed rather rapidly during the early stage of osteogenesis (day 4 & day 7) in differentiated hMSCs pretreated with IL-1β in comparision with hMSCs not

pretreated with IL-1β, and the suppression of ALP by NSAIDs showed a consistent pattern in a dose-dependent manner. However, the suppression of ALP by NSAIDs in differentiated hMSCs not pretreated with IL-1β showed no differences among the doses of the NSAIDs. This suggests that the inhibitoty effect of NSAIDs on ALP expression during osteogenesis in inflammatory-conditioned-hMSCs may proceed via pathways different from those in normal osteogenesis.

Mengshol et al[38] reported that *Runx2* enhanced the IL-1 induction of matrix metalloproteinase-13 (MMP-13) transcription. It is known that MMP-13 is expressed by hypertrophic chondrocytes and osteoblasts in the fracture callus[39]. In addition, it has been reported that MMP-13 is required for the resorption of hypertrophic cartilage and for normal bone remodeling during non-stabilized fracture healing that occurs via endochondral ossification[40]. These studies suggest that MMP-13 may be associated with the osteogenic differentiation of inflammatory-conditioned-hMSCs pretreated with IL-1β.

This data showed that the expression of *Runx2/Cbfa1* and ALP increased during the early stage of osteogenesis after pretreatment with IL-1β, and that the increased expression of them was reduced in a dose-dependent manner by two NSAIDs (Celecoxib and Naproxen). I think that these results may be associated with the extent of expression of *Runx2/Cbfa1* in inflammatory-conditioned-hMSCs. However, non-inflammatory-conditioned-

hMSCs (not pretreated with IL-1β) showed no marked changes in the expression of *Runx2/Cbfa1* and ALP during the early stage of osteogenesis, and the expression of them was also not affected by the two NSAIDs. On the other hand, the expression of *Dlx5* and osteocalcin, which have been identified as important transcription factors and osteogenic markers, respectively, in osteogenic differentiation, was decreased in a dose-dependent manner by NSAIDs during the early stage of osteogenesis from inflammatory-conditioned -hMSCs. These results suggest that the osteogenesis from inflammatory-conditioned-hMSCs and non-inflammatory-conditioned-hMSCs may proceed via different pathways during the early stage of osteogenesis, and the finding that NSAIDs had no consistent effect on the expression of *Runx2/Cbfa1* and ALP in the non-inflammatory conditioned- hMSCs supports my hypothesis.

It has been reported that osterix is a transcription factor essential for the osteogenesis of hMSCs that acts downstream of *Runx2/Cbfa1* and is deeply associated with the mineralization of endochondral ossification[41]. In this data, osterix mRNA expression showed a consistent pattern during the early stage of osteogenesis from inflammtory-conditioned-hMSCs and was markedly expressed during the late stage of osteogenesis (day 14). The increased expression of osterix was decreased in a dose-dependent manner by NSAIDs. These results were consistent with those from the calcium content assay during the late stages of osteogenesis. Such a pattern of osterix expression

suggests that it may have an important role in the late stages of osteogenic differentiation of inflammatory-conditioned-hMSCs and that it may especially be associated with mineralization.

In other words, I propose that the suppressed expression of COX-2 and PGE_2 synthesis by NSAIDs under inflammatory conditions inhibits the expression of *Runx2/Cbfa1* and *Dlx5* during the early stage of osteogenesis, via direct or indirect pathways. Therefore, they have an inhibitory effect on the expression of ALP, an early marker of osteogenic differentiation. Also, during late osteogenesis, the persistent suppression of *Runx2/Cbfa1* expression may have an inhibitory effect on osterix expression, which decreases calcium accumulation and mineralization. This pathway may be very important during bone healing under inflammatory conditions, such as fracture, where there is enhanced COX-2 expression. However, this pathway would not be important during normal osteogenesis when the expression of COX-2 is limited and not enhanced. The finding that the two NSAIDs used in this study had no effects on the expression of ALP and osteogenesis-related genes under the non-inflammatory condition without IL-1β pretreatment supports these opinions.

V. CONCLUSION

This study was performed to determine whether the osteogenic differentiation of hMSCs is inhibited by a non-selective COX inhibitor, Naproxen and a COX-2 selective inhibitor, Celecoxib. The conclusions are, as follows: the therapeutic doses of Celecoxib (10 μM) and Naproxen (100 μM) had no significant inhibitory effect on the osteogenesis of hMSCs, but over-therapeutic doses of both NSAIDs inhibited osteogenesis. However, these results were demonstrated only in inflammatory-conditioned, osteogenic hMSCs that were pretreated with IL-1β. The osteogenesis of hMSCs under normal conditions without IL-1β pretreatment was not affected by either Celecoxib or Naproxen. These results suggest that bone healing under the inflammatory conditions after fracture may proceed via a pathway different from the pathway that occurs during normal bone formation.

NSAID treatments of over-therapeutic doses in inflammatory-conditioned, osteogenic hMSCs inhibited the osteogenesis of the hMSCs by suppressing the gene expression of the transcription factors *cbfa1*, *Dlx5* and osterix, and the osteogenic marker, osteocalcin.

Therefore, precautions must be taken to minimize the negative effects on the osteogenic differentiation of hMSCs when considering the administration of

high or greater than therapeutic doses of NSAIDs. However, these results are difficult to apply directly to human since important parameters, including dosage and the period of administration, are not yet established. Therefore, an *in vivo* study will be required in the future for clinical applications.

REFERENCES

1. Kaplan-Machlis B, Klostermeyer BS. The Cyclooxygenase-2 inhibitors: safety and effectiveness. Ann Pharmacother 1999;33:979-88.

2. Clemett D, Goa KL. Celecoxib: a review of its use in osteoarthritis, rheumatoid arthritis and acute pain. Drugs 2000;59:957-80.

3. Bang UC, Semb S, Nojgaard C, Bendtsen F. Pharmacological approach to acute pancreatitis. World J Gastroenterol 2008; 21:2968-76.

4. Gray PA, Warner TD, Vojnovic I, Del Soldato P, Parikh A, Scadding GK, et al. Effects of non-steroidal anti-inflammatory drugs on cyclo-oxygenase and lipoxygenase activity in whole blood from aspirin-sensitive asthmatics vs healthy donors. Br J Pharmacol 2002;137:1031-8.

5. Botting RM. Mechanism of action of acetaminophen: Is there a cyclooxygenase 3 ? Clin Infect Dis 2000;31:S202–10.

6. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998;38:97-120.

7. Warner TD, Mitchell JA. Cyclooxygenase-3 (COX-3): Filling in the gaps toward a COX continuum? Proc Natl Acad Sci USA 2002;99:13371-3.

8. Ferraz JGP, Sharkey KA, Reuter BK, Asfama S, Tigley AW, Brown ML, et al.

Induction of cyclooxygenase 1 and 2 in the rat stomach during endotoxemia: Role in resistance to damage. Gastroenterology 1997;113:195-204.

9. Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. FASEB J 1991;5:2304-12.

10. Chandrasekharan NV, Dai H, Roos KLT, Evanson NK, Tomsik J, Elton TS, et al. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. Proc Natl Acad Sci USA 2002;99:13926-31.

11. Qin N, Zhang SP, Reitz TL, Mei JM, Flores CM. Cloning, expression, and functional characterization of human cyclooxygenase-1 splicing variants: evidence for intron 1 retention. J Pharmacol Exp Ther 2005;315:1298-305.

12. Chen Q, Miyamura C, Higashi S, Murakami M, Kudo I, Saito S, et al. Activation of cytosolic phospholipase A_2 by platelet-derived growth factor is essential for cyclooxygenase-2-dependent prostaglandin E_2 synthesis in mouse osteoblasts cultured with interleukin-1. J Biol Chem 1997;272:5952-8.

13. Kawaguchi H, Pilbeam CC, Gronowicz G, Abreu C, Fletcher BS, Herschman HR, et al. Transcriptional induction of prostaglandin G/H synthase-2 by basic fibroblast growth factor. J Clin Invest 1995;96:923-30.

14. Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor kB and nuclear factor-interleukin-6 in the tumor necrosis factor

α-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. J Biol Chem 1995;270:31315-20.

15. Forwood MR. Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading *in vivo*. J Bone Miner Res 1996;11:1688-93.

16. Okada Y, Lorenzo JA, Freeman AM, Tomita M, Norham SG, Raisz LG, et al. Prostaglandin G/H synthase-2 is required for maximal formation of osteoclast-like cells in culture. J Clin Invest 2000;105:823-32.

17. Sato T, Morita I, Sakaguchi K, Nakahama K, Smith WL, Dewitt DL, et al. Involvement of prostaglandin endoperoxide H synthase-2 in osteoclast-like cell formation induced by interleukin-1β. J Bone Miner Res 1996;11:392-400.

18. Zhang X, Morham SG, Langenbach R, Young DA, Xing L, Boyce BF, et al. Evidence for a direct role of cyclo-oxygenase 2 in implant wear debris-induced osteolysis. J Bone Miner Res 2001;16:660-70.

19. Tai H, Miyamura C, Pilbeam CC, Tamura T, Ohsugi Y, Koishihara Y, et al. Transcriptional induction of cyclooxygenase-2 in osteoblast is involved in interleukin-6 induced osteoclast formation. Endocrinology 1997;138:2372-9.

20. Suponitzky I, Weinreb M. Differential effects of systemic prostaglandin E2 on bone mass in rat long bones and calvariae. J Endocrinol 1998;156:51-7.

21. Zhang X, Schwarz EM, Young DA, Puzas JE, Rosier RN, O'Keefe RJ.

Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. J Clin Invest 2002;109:1405-15.

22. Simon AM, Manigrasso MB, O'Connor JP. Cyclooxygenase 2 function is essential for bone fracture healing. J Bone Miner Res 2002;17:963-76.

23. Goodman S, Ma T, Trindade M, Ikenoue T, Matsuura I, Wong N, et al. COX-2 selective NSAID decreases bone ingrowth *in vivo*. J Orthop Res 2002;20:1164-9.

24. Murnaghan M, Li G, Marsh DR. Nonsteroidal anti-inflammatory drug-induced fracture nonunion: an inhibition of angiogenesis? J Bone Joint Surg 2006;88-A:140-7.

25. Katori M, Majima M, Harada Y. Possible background mechanisms of the effectiveness of cyclooxygenase-2 inhibitors in the treatment of rheumatoid arthritis. Inflamm Res 1998;47(suppl 2):S107-11.

26. Miller SC, Marks SC Jr. Effects of prostaglandins on the skeleton. Clin Plast Surg 1994;21:393-400.

27. Norrdin RW, Jee WS, High WB. The role of prostaglandins in bone *in vivo*. Prostaglandins Leukot Essent Fatty Acids 1990;41:139-49.

28. Long J, Lewis S, Kuklo T, Zhu Y, Riew KD. The effect of cyclooxygenase-2 inhibitors on spinal fusion. J Bone Joint Surg 2002;84-A:1763-8.

29. Reuben SS, Ekman EF. The effect of cyclooxygenase-2 inhibition on

analgesia and spinal fusion. J Bone Joint Surg 2005;87-A:536-42.

30. Andersen T, Christensen FB, Laursen M, Høy K, Hansen ES, Bünger C. Smoking as a predictor of negative outcome in lumbar spinal fusion. Spine 2001;26:2623-8.

31. Glassman SD, Rose SM, Diamr JR, Puno RM, Campbell MJ, Johnson JR. The effect of post operative nonsteroidal anti-inflammatory drug administration on spinal fusion. Spine 1998;23:834-8.

32. Arikawa T, Omura K, Morita I. Regulation of bone morphogenetic protein-2 expression by endogenous prostaglandin E2 in human mesenchymal stem cells. J Cell Physiol 2004;200:400-6.

33. Gerstenfeld LC, Thiede M, Seibert K, Mielke C, Phippard D, Svagr B, et al. Differentiation inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs. J Orthop Res 2003;21:670-5.

34. Chang JK, Li CJ, Wu SC, Yeh CH, Chen CH, Fu YC, et al. Effects of anti-inflammatory drugs on proliferation, cytotoxicity and osteogenesis in bone marrow mesenchymal stem cells. Biochem Pharmacol 2007;74:1371-82.

35. Weinreb M, Shamir D, Machwate M, Rodan GA, Harada S, Keila S. Prostaglandin E2 (PGE2) increases the number of rat bone marrow osteogenic stromal cells (BMSC) via binding the EP4 receptor, activating sphingosine kinase and inhibiting caspase activity. Prostaglandins Leukot Essent Fatty

Acids 2006;75:81-90.

36. Weinreb M, Suponitzky I, Keila S. Systemic administration of an anabolic dose of PGE2 in young rats increases the osteogenic capacity of bone marrow. Bone 1997;20:521-6.

37. Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, et al. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 2000;275:32783-92.

38. Mengshol JA, Vincenti MP, Brinckerhoff CE. IL-1 induces collagenase-3 (MMP-13) promoter activity in stably transfected chondrocytic cells: requirement for Runx-2 and activation by p38 MAPK and JNK pathways. Nucleic Acids Res 2001;29:4361-72.

39. Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C, et al. Critical roles for collagenase-3(Mmp13) in development of growth plate cartilage and in endochondral ossification. Proc Natl Acad Sci USA 2004;101:17192-7.

40. Behonick DJ, Xing Z, Lieu S, Buckley JM, Lotz JC, Marcucio RS, et al. Role of matrix metalloproteinase 13 in both endochondral and intramembranous ossification during skeletal regeneration. PLoS ONE 2007;2(e1150):1-10.

41. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al.

The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002;108:17-29.

인간 골수 유래 중간엽 줄기세포의 골형성 분화에 대한 COX-2 억제제의 영향

< 지도교수 한 창 동 >

연세대학교 대학원 의학과

유 제 현

비스테로이드성 소염진통제는 arachidonic acid를 prostaglandin (PG)로 전환시키는 cyclooxygenase (COX)를 억제시켜 그 효과를 나타낸다. 이러한 COX 와 PG는 골대사에 중요한 역할을 하며, 특히 PGE_2가 가장 중요한 역할을 하는 것으로 알려져 있다. COX-2 는 골형성에 필요한 유전인자인 *Runx2/Cbfa1*와 osterix의 발현을 유도한다. 하지만, 지금까지 비스테로이드성 소염진통제와 같은 COX-2 억제제가 인간 골수 유래 중간엽 줄기세포의 골형성 분화를 억제하는 지에 대한 실험실내 연구는 없었다.

본 연구에서는 COX-2 선택적 억제제인 Celecoxib와 비선택적 COX 억제제인 Naproxen과 같은 비스테로이드성

소염진통제에 의해 인간 골수 유래 중간엽 줄기세포의 골형성 분화가 억제되는 지에 대해 분석해 보았다.

실시간 역전사 중합효소 연쇄반응 (real-time RT-PCR)에서 Celecoxib가 처리된 세포에서 단지 mPGES의 mRNA 발현이 감소된 반면 Naproxen이 처리된 세포에서는 농도에 비례하여 cPGES와 mPGES의 발현이 감소되었다. 그러나, COX-1과 COX-2의 발현에는 영향이 없었다. IL-1β (1 ng/ml)은 인간 골수 유래 중간엽 줄기세포에서 COX-2와 mPGES의 발현과 골형성 분화를 유도하였다. 반면 세포에 IL-1β와 Celecoxib 혹은 Naproxen을 처리한 경우 농도-의존적으로 골형성 분화를 감소시켰다. 고농도의 비스테로이드성 소염진통제 (40 μM Celecoxib and 300 μM Naproxen)로 처리된 세포에서는 alkaline phosphatase (ALP)의 발현과 칼슘 침착 정도가 의미있게 저해되었다. 고농도의 비스테로이드성 소염진통제로 처리된 세포에서 골형성 분화의 저해는 감소된 cPGES 혹은 mPGES와 관련이 있을 것이다. 일반적으로 치료 적정 농도라고 알려진 10 μM Celecoxib와 100 μM Naproxen에서는 인간 골수

유래 중간엽 줄기세포의 골형성 분화의 억제 효과가 없었다. 하지만, 치료 적정 농도보다 높은 고농도의 20, 40 μM의 Celecoxib와 200, 300 μM의 Naproxen 골형성 분화를 심각하게 억제하였다.

IL-1β가 처리된 염증성 조건의 중간엽 줄기 세포의 골형성 분화 초기에 *Runx2/Cbfa1* 발현이 증가되었고, osterix 발현은 분화 후기에 증가하였다. 그리하여 분화 초기에 ALP의 발현이 증가되었고, Celecoxib와 Naproxen에 의해 농도 의존적으로 감소하였다. 이러한 결과들은 골절과 같은 염증성 환경에서의 골 치유가 정상 골형성시 일어나는 기전과는 다른 기전에 의해 일어난다고 할 수 있다. 따라서, 염증성 조건하의 중간엽 줄기세포에서 의 처리는 골형성 분화에 필요한 유전인자의 발현을 억제시켜 골 치유에 심각한 억제 효과를 나타낼 수 있다. 그러므로 이러한 고농도 비스테로이드성 소염진통제의 골형성 분화에 대한 억제 효과로 인해, 고용량의 비스테로이드성 소염진통제를 복용시에는 주의를 요한다.

핵심되는 말 : 인간 골수 유래 중간엽 줄기 세포, 골 형성, 비스테로이드성 소염진통제, COX-2 억제제