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**A STUDY ON EFFECTS OF
NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs) ON
DIFFERENTIATION CAPACITY OF CANINE OSTEOGENIC CELLS**

(イヌ骨芽細胞の分化に対する非ステロイド性抗炎症薬の影響に関する研究)

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ABBREVIATIONS

ALP: alkaline phosphatase

BMSC: bone marrow derived mesenchymal stem cell

cDNA: complementary deoxyribonucleic acid

COX: cyclooxygenase

cPGES: cytosolic prostaglandin E synthase

DEPC: diethyl pyrocarbonate

DMEM: Dulbecco's Modified Eagle Medium

EP: prostaglandin E₂ receptor

FBS: fetal bovine serum

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

KO: knockout

M-MLV: Moloney Murine Leukemia Virus

mPGES: microsomal prostaglandin E synthase

mRNA: messenger ribonucleic acid

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NSAID: non-steroidal anti-inflammatory drug

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PG: prostaglandin

qRT-PCR: quantitative real-time polymerase chain reaction

rhIL-1 β : recombinant human interleukin-1 β

RPMI 1640: Roswell Park Memorial Institute 1640

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1. General introduction

Benefits and risks on the use of non-steroidal anti-inflammatory drugs (NSAIDs) for pain management in fracture patients

Pain management is important for specific orthopedic patients with osteoarthritis (Sanderson *et al.*, 2009) or fracture (Gruet *et al.*, 2011) in animals as well as human beings. Prostaglandins (PGs) participate in inflammatory nociceptive processing in acute and chronic pain responses (Ito *et al.*, 2001). Studies on pharmacological inhibition of prostanoid synthesis have elucidated the insight of physiological and pathophysiological roles of PGs. Major PGs inducing pain would be prostaglandin (PG) E₂ (Kidd and Urban, 2001; Ricciotti and FitzGerald, 2011). During inflammatory phase of fracture healing process, PGE₂ is markedly released from various types of cells in surrounding damaged tissue (Dekel *et al.*, 1981). Peripheral nociceptive response is enhanced by edema resulted from increased vascular permeability, increased excitability of peripheral nociceptors and facilitated propagation of nociceptive signals along the peripheral nerves, which are mediated by a variety of PGE₂-induced signals. Furthermore, PGE₂ signaling could alter synaptic transmission within dorsal horn of spinal cord that is first site of synaptic integration in the pain pathway to convey nociceptive information to higher areas of central nervous system. Peripheral nociception would be more amplified in dorsal horn of spinal cord because PGE₂ could modulate both excitatory and inhibitory neurotransmission within synaptic contacts with local excitatory and inhibitory interneurons and central projection neuron (Schäfers and Sorkin, 2008). Based on those key roles of PGE₂ in development of inflammatory pain, inhibitory effects of NSAIDs on PGE₂ synthesis could be effective agents for pain management of orthopedic patients

during perioperative period or conservative management.

Despite usefulness of NSAIDs for pain-control, there has been controversy in literature between its benefits and potential risks in fracture patients. For prophylactic purpose, NSAIDs have also been prescribed to the human patients who to reduce the risk of heterotopic bone formation after hip arthroplasty or reduction of spinal cord injury (Banovac *et al.*, 2001; Barthel *et al.*, 2002; Neal *et al.*, 2000). Conversely, this inhibitory effects of NSAIDs on bone formation has been a concern for fracture patients regarding the possibility of delaying fracture healing (Burd *et al.*, 2003). Animal studies suggest that NSAIDs could delay repair of fracture (Dimmen *et al.*, 2009; Gerstenfeld *et al.*, 2003; Gerstenfeld *et al.*, 2007; Karachalios *et al.*, 2007; Ochi *et al.*, 2011; Simon and O'Connor, 2007; Sudmann *et al.*, 1979; Zhang *et al.*, 2002). In most of these experiments, delay of fracture healing occurred in a dose- and time-dependant manner (Dimmen *et al.*, 2009; Gerstenfeld *et al.*, 2007; Karachalios *et al.*, 2007; Simon and O'Connor, 2007; Sudmann *et al.*, 1979). It is important to note that data from various animal studies have limitations when extrapolating to clinical use because of substantial differences among various species in metabolic profiles or difficulty to convert the dosage for human beings into the animal studies. One study used clinical dose of carprofen in dogs for unrealistic long-term treatment (120 days) showed clear evidence that NSAIDs had negative effects on osteogenesis (Ochi *et al.*, 2011). Nevertheless, fracture patients in animals, as well as those in human beings, are still being prescribed with NSAIDs. No clear clinical report has shown direct correlation between delayed fracture healing and NSAIDs, which has been used for pain control during perioperative periods. Negative effects of NSAIDs on bone healing, which were shown by using NSAIDs with high-dose and long-time, have limitations to explain safety of NSAIDs in clinical use.

Role of PGE₂ and its receptors in osteogenesis

Exogenous administration of PGE₂ stimulates bone growth in rats (Keila *et al.*, 2001). Osteoblastic differentiation was induced by PGE₂ in cultured mesenchymal stem cells and primary calvarial cells (Flanagan and Chambers, 1992; Scutt and Bertram, 1995). Osteoblastic differentiation was decreased in mesenchymal stem cells or primary calvarial cells cultured from cyclooxygenase (COX) -2 deficient mice, compared to cells from wild type mice (Zhang *et al.*, 2002). In the same study, osteogenesis was also suppressed by treatment with NSAIDs in wild type cells. Conversely, osteoblastic differentiation in COX-2 deficient cells was increased by exogenous supply of PGE₂. One of the most consistent anabolic effects of PGE₂ in osteogenesis would be to promote osteoblastic differentiation.

Receptors for PGE₂, such as EP2 and EP4, have been implicated in anabolic effects of bone (Alander and Raisz, 2006; Minamizaki *et al.*, 2009; Raisz and Woodiel, 2003). Exogenous PGE₂ or EP2 and EP4 agonists could be an osteogenic stimulator for patients with osteogenic disorders, while EP4 agonist would be more likely to be efficacious than EP2 agonist on osteogenic cells (Xie *et al.*, 2009). Synthesized PGE₂ can induce COX-2 expression through PG-mediated auto-amplification via EP2 and EP4 receptors (Pilbeam *et al.*, 1995; Sakuma *et al.*, 2004), suggesting that endogenous PGE₂ acts as an autocrine stimulus for osteogenic differentiation.

Suppressive effects of PGE₂ on osteoblastic differentiation were also observed with high concentration of PGE₂ (Ramirez-Yanez and Symons, 2012), suggesting dose-independent effects of PGE₂ on osteoblastic differentiation. In fracture healing, under chronic inflammatory condition, such as rheumatoid arthritis, NSAIDs may have positive effects on osteogenic cells by controlling undesirable high concentration of PGE₂.

Regulation of biosynthesis of PGE₂ in osteogenic cells

Biosynthesis of PGE₂ from arachidonic acid is initiated by two types of prostaglandin H₂ synthases, such as COX-1 and COX-2, which are expressed from distinct genes located on distinct chromosomes (Kraemer *et al.*, 1992). As both enzymes are membrane-bound homodimers, COX-1 is more located in the endoplasmic reticulum than in perinuclear membrane, and COX-2 is found predominantly in perinuclear membrane (Morita *et al.*, 1995). Generally, COX-1 is described as constitutively expressed in most tissue, while COX-2 is normally undetectable in most cell types and is highly regulated at physiologically relevant levels by a variety of inflammatory cytokines and/or growth factors (Vane *et al.*, 1998).

Recently, three different terminal enzymes for PGE₂ synthesis, such as prostaglandin E synthases, have been identified, which were named as microsomal prostaglandin E synthase (mPGES)-1, cytosolic PGES (cPGES), and mPGES-2. As the origin of their names, cPGES is located in the cytosol whereas mPGES-1 and mPGES-2 are detected in microsomes. Studies using co-transfection and anti-sense technique in primary cells and tumor cell lines have shown that cPGES would be constitutively expressed in most cells and could be functionally linked to COX-1 to promote immediate PGE₂ (Tanioka *et al.*, 2000). Whereas mPGES-1 is a perinuclear protein, which is induced by pro-inflammatory stimuli and preferentially coupled to COX-2 (Murakami *et al.*, 2000), resulting in a response of delayed PGE₂ release. As cPGES, mPGES-2 is constitutively expressed, but can be coupled with either COX-1 or COX-2 to function as both tissue homeostasis and disease processes (Murakami *et al.*, 2003). Collectively, inflammation within surrounding tissues at fracture site induces expressions of COX-2 and mPGES-1 that produce significant amount of PGE₂, resulting in promotion of efficient osteogenesis.

Aims of this study

Differentiated osteogenic cells, such as mature osteoblasts, are also well-known to synthesize abundant PGE₂ in osteogenic environment, which is also regulated by systemic regulators of bone metabolism. In early stage of bone healing, osteoprogenitor cells, as well as mature osteoblasts, migrate from bone marrow or surrounding tissue into bone defects (Shapiro, 2008). These cells proliferate and differentiate into functional osteoblasts to fill the bone defects with calcified matrix. Therefore, to investigate whether the differentiation capacity of osteogenic cells is affected by NSAIDs during their osteogenic differentiation would answer a primary question for using NSAIDs in fracture patients. A study noted that the amount of PGE₂ production in fibroblast cells from COX-1 or COX-2 deficient mice is compensated by counterpart COX (Kirtikara *et al.*, 1998). Furthermore, the level of PGE₂ production was higher in the COX-2 null cells than the wild type cells, although it was less than that in COX-1 null cells. This study implies the existence of cellular potentials to respond to PGE₂ deficiency.

Thereby, the purpose of this study was to evaluate the effects of NSAIDs on differentiation capacity of osteogenic cells. To demonstrate cellular responses against NSAIDs to maintain osteogenic differentiation, following experiments were performed; 1) evaluation of reversibility of osteogenic differentiation after short-term treatment with NSAIDs, such as carprofen and meloxicam, in different stage of osteoblastic differentiation using canine osteosarcoma cell line (POS); 2) Determination of compensatory responses of osteogenic cells against various classes of NSAIDs, including carprofen, meloxicam, indomethacin and robenacoxib, during osteogenic differentiation of canine bone marrow-derived mesenchymal stem cells (BMSCs).

2. Limited inhibitory effects of NSAIDs on in vitro osteogenic differentiation in canine cells

2.1. Introduction

Deterrent effects of NSAIDs on inflammation and pain are obtained by suppressing the action of COX, which catalyzes the conversion of arachidonic acids into PGs (Simmons *et al.*, 2004). Over the last decades, with development of new generation of NSAIDs that specifically inhibit inducible COX-2, incidence of major adverse effects of conventional NSAIDs, including gastro-intestinal tract bleeding, are dramatically reduced (Wallace, 2008). While COX-1, a housekeeping enzyme, is constitutively expressed in almost all types of cells to maintain homeostatic levels of PGs (Vane *et al.*, 1998), COX-2 is rapidly induced to produce inflammatory PGs by various stress factors related to structural damage, including physical trauma, mechanical stimulation, and by numerous inflammatory mediators (Chen *et al.*, 1997; Muscará *et al.*, 2000; Topper *et al.*, 1996). High selectivity of NSAIDs for COX-2 inhibition could control pain with benefits from COX-1 sparing effects. However, COX-2 selective NSAIDs also seem to have risks of some inhibitory effects on essential physiological phenomenon.

The most abundant PG should be PGE₂ that plays a critical role in bone repair (Elves *et al.*, 1982; Sudmann *et al.*, 1979). The concentration of PGE₂ reached at the highest level around the fracture site within the day after fracture, and after then the level of PGE₂ diminished steadily as bone was healing in experimental fractures in rats (Gerstenfeld *et al.*, 2007). In the same study, administration of selective COX-2 inhibitors resulted in lower levels of PGE₂ production at fracture sites and delayed fracture union, compared with non-selective NSAIDs treated rats (Gerstenfeld *et al.*, 2007). In COX-2 knockout (KO) mice, far less fracture callus formation was identified than that in wild type mice, and the osteoblastogenesis of

mesenchymal stem cells that were cultured from COX-2 KO mice was rescued by PGE₂ supplementation (Zhang *et al.*, 2002). Results of these studies suggest that COX-2 participates in bone healing through mediation of PGE₂ production.

Controversy regarding clinical use of COX-2 inhibitors in fracture patients still exists in the literature (Brown *et al.*, 2004; Gerstenfeld *et al.*, 2007; Simon and O'Connor, 2007). Although animal studies showed that high dose and long-term treatment with COX-2 inhibitors delayed fracture repair, inhibitory dose and duration in those experiments could not be converted to clinical use directly. On the contrary to the COX-2 KO animals, wild-type animals could express COX-2 gene (Chikazu *et al.*, 2002), which may also partially act to restore deficient PGE₂, leading to keep osteogenic cells to proceeding bone forming process. Thereby, it is assumed that COX-2 inhibitor would temporarily affect bone healing within the duration of its use.

The present study was aimed to elucidate the cellular behavior of canine osteogenic cells after exposure to the preferential COX-2 inhibitors, such as carprofen and meloxicam, which are among the most available NSAIDs in veterinary medicine. The time course of genetic, biochemical and morphological changes during osteoblastic differentiation were determined by using canine POS osteosarcoma cell line that could show the same characteristics over a series of osteogenic differentiation.

2.2. Materials and methods

2.2.1. Culture of canine osteoblast-like cells

Canine POS cells (Kadosawa *et al.*, 1994; Nozaki *et al.*, 1995) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 25 mM NaHCO₃, 100 U/ml penicillin and 100 µg/ml streptomycin. To induce osteogenic differentiation, 10 nM 1,25-dihydroxyvitamin D₃ (calcitriol; Sigma-Aldrich, St. Louis, MO, USA) was also added into the medium (Barroga *et al.*, 2000; Majeska and Rodan, 1982; Manolagas *et al.*, 1983). POS cells were seeded at a density of 4.5×10^4 cells/well in 12-well, flat-bottomed culture plates (Costar 3513; Corning Inc., Corning, NY, USA). Media were changed every other day. All cultures were maintained in an incubator at 37°C with humidified 5% CO₂ atmosphere.

2.2.2. Cell viability assay

Concentrations of carprofen (LKT Laboratories, Inc., St. Paul, MN, USA) and meloxicam (Wako, Pure Chemical Ind., Osaka, Japan) that had no cytotoxicity on the bioavailability of POS cells were determined by following colorimetric assay. The cells were seeded at a density of 5×10^3 cells in 96-well plate (Costar 3595; Corning Inc.) with 100 µl of RPMI-1640 culture medium. Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. After 24 hours of incubation for cell adaptation to the culture condition, cells were treated with various concentrations (0.01, 0.10, 1.00, 10.00 and 100.00 µM) of drugs for 48 hours of exponential growth phase. The cell viability was measured by the colorimetric assay based on the conversion of 3-(4,5-dimethyl-2-thiazol) 2,5-diphenyl-2H-tetrazolium bromide (MTT; Wako) to a formazan pigment by mitochondrial enzymes in viable cells during four

hours of incubation period (Mosmann, 1983). Colorimetric measurement was performed using a plate reader (MPT-120; Corona Electric, Ibaraki, Japan) at wave length of 570 nm. All samples evaluated by MTT assay were measured in triplicates.

2.2.3. Periods of COX-2 inhibition during osteogenic differentiation

Results from our preliminary experiments revealed that the entire period for osteogenic differentiation in POS cells was 14 days (Fig. 2-1A). Stages of osteoblast differentiation divided into pre-osteoblastic stage, transitional stage and mature osteoblastic stage by morphologic changes during osteogenic differentiation of the cells, which were characterized by proliferation with no nodule formation from day 0, non-calcified nodule formation from day 4 and calcified nodule formation from day 8, respectively. Carprofen (1 μ M) and meloxicam (1 μ M) were added to culture media for 72 hours during each osteogenic stage (Fig. 2-1B). Samples for the analyses of gene and protein expressions were harvested on days 3, 7 and 11.

2.2.4. Analysis of gene expression

To perform relative quantification of gene expression, total RNA was extracted using TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instruction. Reverse transcription was performed with Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen) with oligo (dT)₁₅ as a primer. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany) with KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems, Boston, MA, USA). Primers used in this study were purchased from the external manufacturer (Hokkaido System Science Co.; Sapporo, Hokkaido, Japan). The acquired data were analyzed with normalization to

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. The nucleotide sequences of the primers that were used in this experiment were as follows: 5'-CCAAGCTCAACAGACCCTGA-3' (forward) and 5'-GAGACACCCATCCCATCTCC-3' (reverse) for alkaline phosphatase (ALP) (GenBank accession no., NM_001197137.1; product size, 102 bp); 5'-AGGAAGCTTACCAGCGCTTC-3' (forward) and 5'-TGACAAGGACCCCACACTTG-3' (reverse) for osteocalcin (GenBank accession no., XM_547536.4; product size, 138 bp); 5'-AAGGTCATCCCTGAGCTGAA-3' (forward); 5'-GACCACCTGGTCCTCAGTGT-3' (reverse) for GAPDH (GenBank accession no., NM_001003142.1; product size, 192 bp).

2.2.5. Intracellular ALP activity

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with vigorous shaking and sonication for 30 minutes in 10 mM Tris-HCl solution (pH 7.4) containing 0.05% non-ionic surfactant, Triton X-100 (ICN Biomedicals Inc., Aurora, OH, USA). The supernatant of the cell lysates was separated by centrifugation at 20,000 x g for 30 minutes. The supernatants and *p*-nitrophenyl phosphate (Sigma-Aldrich) were reacted for 10 minutes at 37°C in substrate solution (pH 10.5) containing 1 mg/ml *p*-nitrophenyl phosphate, 100 mM glycine, 1 mM MgCl₂ and 1mM ZnCl₂. Released *p*-nitrophenol was measured using a plate reader (MPT-120) at wave length of 405 nm. Activity of ALP was determined after normalization by the protein concentration measured by modified Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA, USA) (Bradford, 1976)

2.2.6. Morphological evaluation

Visual observation of effects of COX-2 inhibitors on non-calcified nodule formation of POS cells was performed using ALP staining after each 72 hours of the drug treatment on days 3, 7 and 11. Briefly, cells were washed with PBS and fixed with 4%

paraformaldehyde for 2 minutes. After washing, cells were incubated in 0.1 M Tris-HCl buffer (pH 9.3) containing 0.25% (w/v) naphthol-AS-BI-phosphoric acid sodium salt (Sigma-Aldrich) and 0.75% (w/v) fast blue RR salt (Sigma-Aldrich) at 37°C for 30 minutes.

To observe the effect of COX-2 inhibitors on calcium deposition in post-medication period, von Kossa staining was performed on day 14. Briefly, after fixation with 4% paraformaldehyde for 2 minutes, POS cells were treated with 2% silver nitrate (Wako) solution and placed under ultraviolet light at room temperature for 1 hour. After rinsing with distilled water, cells were bleached in 5% sodium thiosulfate (Wako) for 2 minutes. Results were observed using optical microscope after cells were rinsed with distilled water.

2.2.7. Statistics

The statistical data analysis was performed by one-way analysis of variance, followed by Tukey's Honestly Significant Difference test, using commercial software (SPSS, version 12.0.1; SPSS Inc.). All data were expressed as mean \pm standard deviation (SD). Values of $p < 0.05$ were considered to be statistically significant.

2.3. Results

2.3.1. Non-cytotoxic concentrations of carprofen and meloxicam

At 48 hours after carprofen or meloxicam treatment, cell viability showed more than 90% at 0.01, 0.10 and 1.00 μM of each drug concentration (Fig. 2-2). Definitive cytotoxic effects of these drugs were observed at concentrations $\geq 10 \mu\text{M}$ ($p < 0.05$). Concentrations of carprofen and meloxicam used in this experiment were at the maximum concentration (1 μM each) that had no effect on viability of POS cells. Maximum plasma concentrations of these drugs in dogs presented by previous studies were 8.43 $\mu\text{g/ml}$ (30.77 μM) (Lascelles *et al.*, 1998) and 0.78 $\mu\text{g/ml}$ (2.09 μM) (Yuan *et al.*, 2009) after injection of recommended clinical doses of carprofen (4.00 mg/kg/dose) and meloxicam (0.20 mg/kg/dose), respectively. These data indicated that doses of these drugs supplemented in this experiment were within the plasma concentration in dogs treated with these drugs at clinical doses.

2.3.2. Short-term effects of NSAIDs on mRNA and protein expressions of ALP in osteogenic cells

Gene expression of ALP was continuously increased with maintaining ALP activity during osteoblast differentiation in control (Fig. 2-3 A and B). Considering ALP is a marker of pre-osteoblast cells in early period of osteoblast differentiation, those results indicated that POS cells constantly differentiated into pre-osteoblast-like cells during almost entire differentiation period. Expression of ALP mRNA (Fig. 2-3A) and that of ALP activity (Fig. 2-3B) showed no significant difference between control and drug-treated groups in all three periods examined.

2.3.3. Short-term effects of NSAIDs on mRNA expression of osteocalcin in osteogenic cells

Osteocalcin, a marker of mature osteoblast in late period of osteoblast differentiation, showed increase in its mRNA expression from the period between days 4 and 7 (Fig. 2-4). Morphologically, non-calcified nodule formation started with expression of osteocalcin mRNA, indicating this period was transitional stage for POS cells to mature into functional osteoblast-like cells. Expression of osteocalcin mRNA was significantly decreased by carprofen and meloxicam in transitional stage ($p < 0.001$) (Fig. 2-4), but no significant suppression was observed in mature osteoblastic stage.

2.3.4. Short-term effects of NSAIDs on morphologic changes during osteogenesis of osteogenic cells

The results of ALP staining revealed delay in start of non-calcified nodule formation by carprofen and meloxicam from day 4 to 7 (Fig 2-5). However, from day 8 to 11, nodule formation showed no suppression by the COX-2 inhibitors. These results were consistent with the differentiation stage-dependent suppression in expression of osteocalcin which occurred by effects of the COX-2 inhibitors. On day 14, no different level of calcification was observed between control and groups of drug treatment by von Kossa staining regardless of treatment period (Fig. 2-6). The retarded nodule formations during transitional stage were also recovered with further differentiation after the discontinuation of the drugs. These results suggested that the transitional stage-limited suppressive effects of carprofen and meloxicam were reversible.

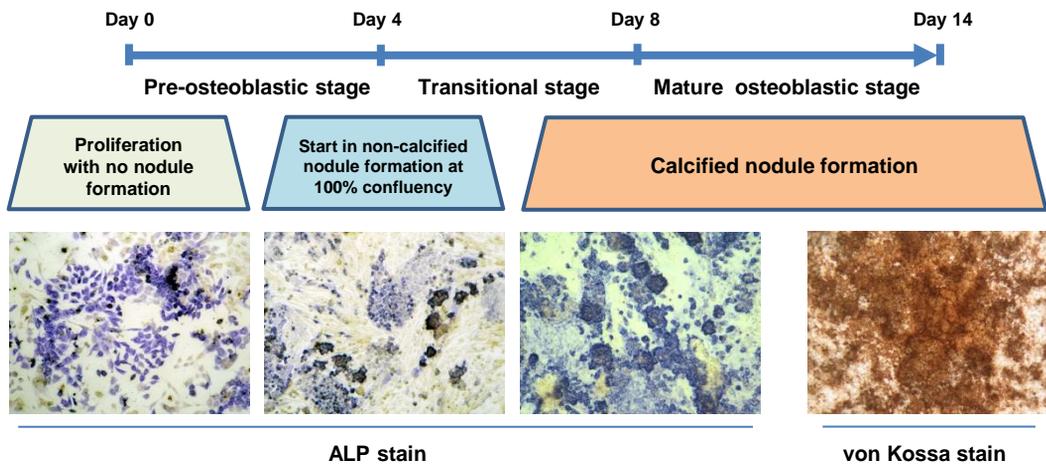
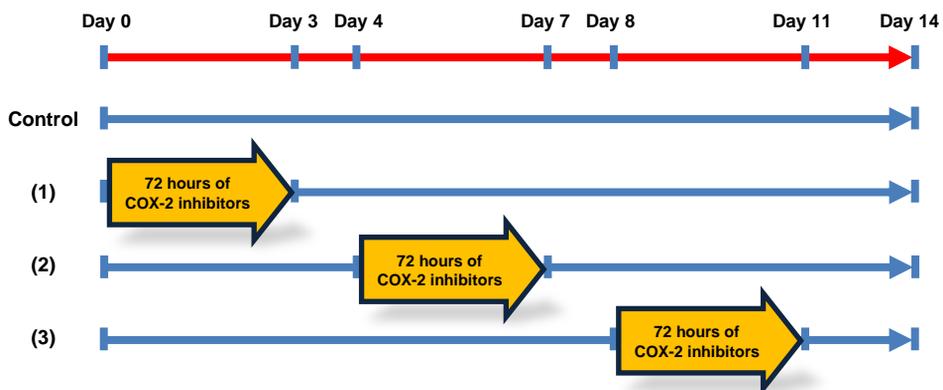
A**Cellular events over osteogenic differentiation****B****Periods of COX-2 inhibitors supplementation**

Fig. 2-1. Schematic representation of cellular events over osteogenic differentiation of canine POS osteosarcoma cell line (A) and periods for supplementation of COX-2 inhibitors (B). The observation of morphological changes of POS cells during osteogenesis was performed with conventional light microscope after ALP staining or von Kossa staining at each time point. The periods of drugs supplementation were designed for this study according to the three characteristic changes of morphology in each stage, including proliferation at 80% confluency from day 0 to 3 (1), non-calcified nodule formation from day 4 to 7 (2) and calcified matrix formation from day 8 to 11 (3).

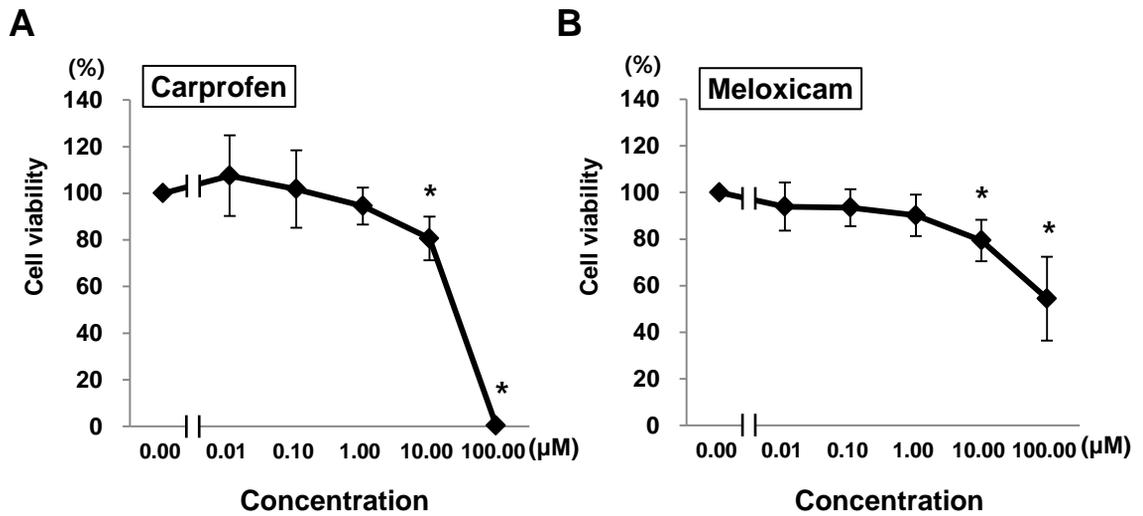


Fig. 2-2. Effects of carprofen and meloxicam on viability of canine POS osteosarcoma cell line. The cell viability determined by MTT assay after 48 hours of drug treatment. Carprofen (A) and meloxicam (B) decreased cell viability in a concentration-dependent manner. Data were expressed as mean \pm SD from three independent experiments performed in triplicate. * $p < 0.05$.

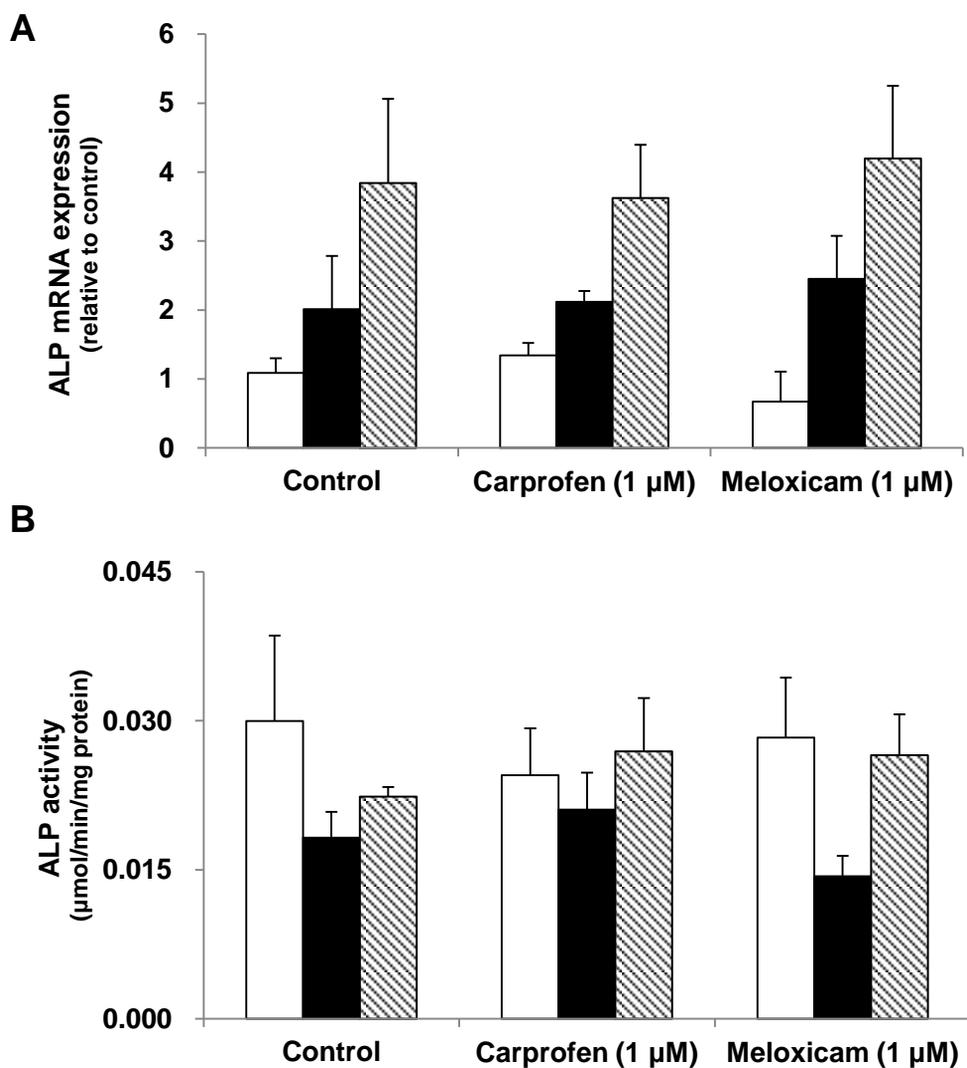


Fig. 2-3. Effects of carprofen and meloxicam on alkaline phosphatase (ALP) gene expression and ALP activity of canine POS osteosarcoma cell line in various stages of osteogenic differentiation. The level of mRNA expression of ALP was measured by quantitative real-time PCR. ALP activity was measured by colorimetric assay using *p*-nitrophenyl phosphate. No significant difference was observed between control and experimental group in ALP gene expression (A) and ALP activity (B) over all stages. Data were expressed as mean \pm SD (n=3). (□, Day 0 to 3; ■, Day 4 to 7; ▨, Day 8 to 11)

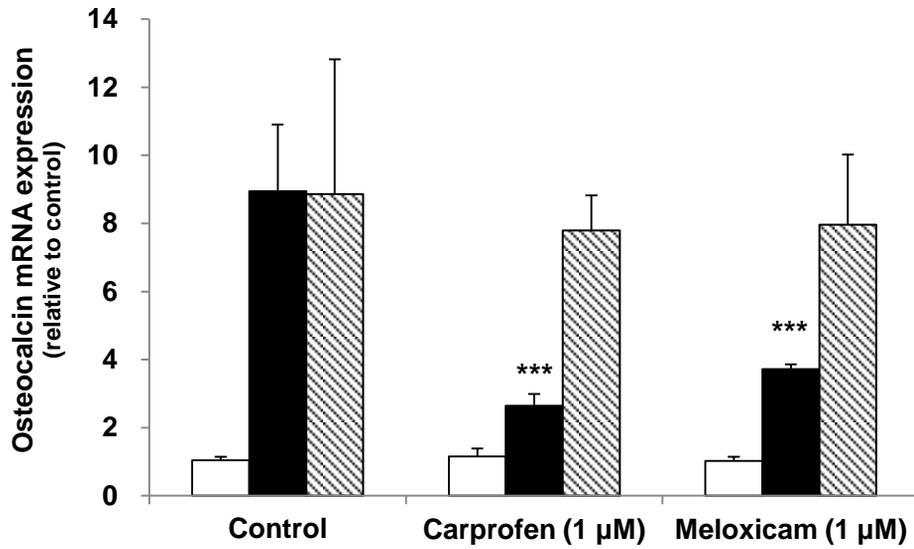


Fig. 2-4. Effects of carprofen and meloxicam on osteocalcin gene expression of canine POS osteosarcoma cell line in various stages of osteogenic differentiation. The level of mRNA expression of osteocalcin was measured by quantitative real-time PCR. Osteocalcin gene expression was significantly decreased in transitional stage by carprofen (1 μM) and meloxicam (1 μM). Data were expressed as mean ± SD (***) $p < 0.001$; relative to control; n=3). (□, Day 0 to 3; ■, Day 4 to 7; ▨, Day 8 to 11)

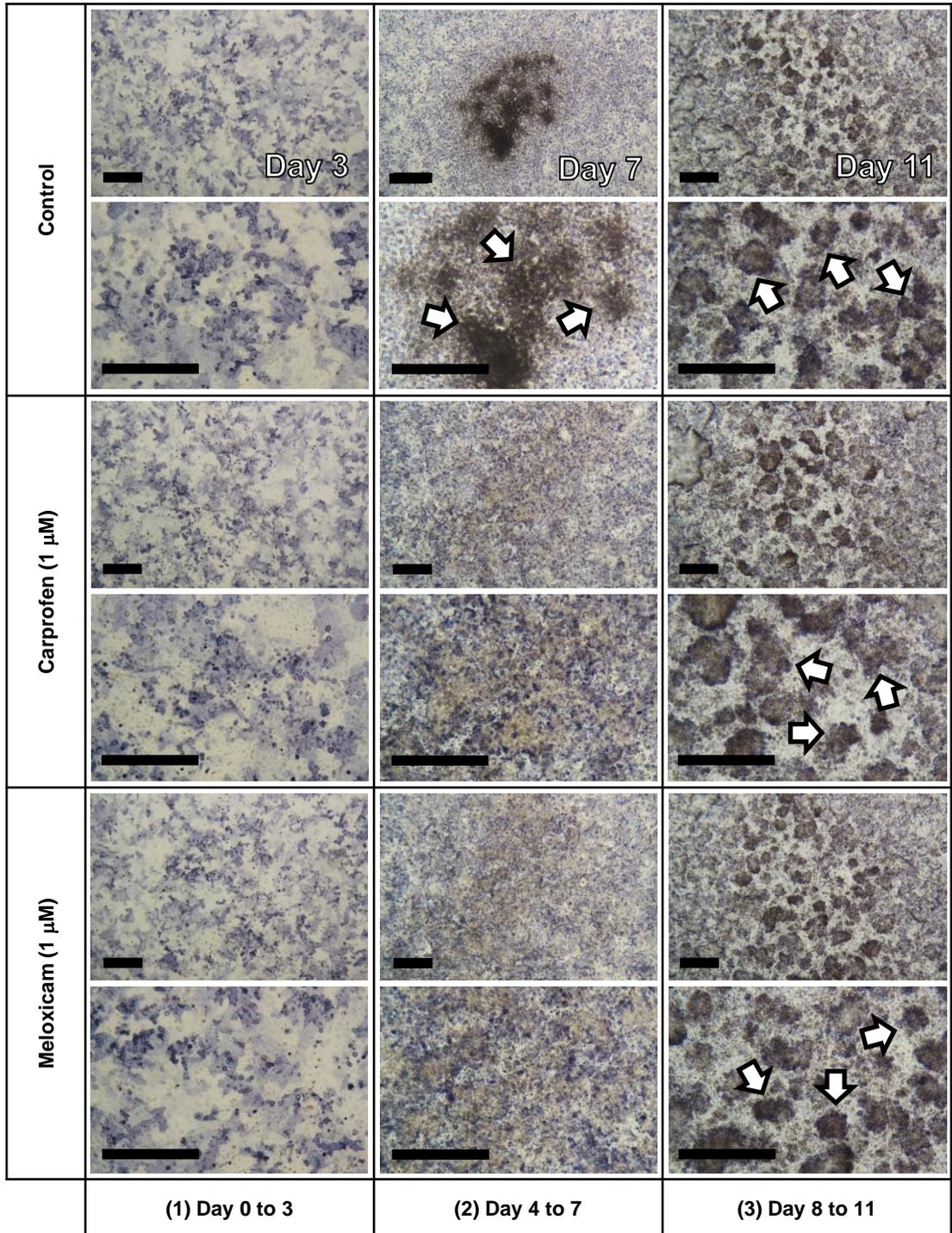


Fig. 2-5. Morphological analysis of effect of carprofen and meloxicam on canine POS osteosarcoma cell line by alkaline phosphatase (ALP) staining in various stages of osteoblast differentiation. Non-calcified nodule formation (white arrow) was delayed by carprofen (1 μ M) and meloxicam (1 μ M) during transitional stage. Each bar represents 1 μ m.

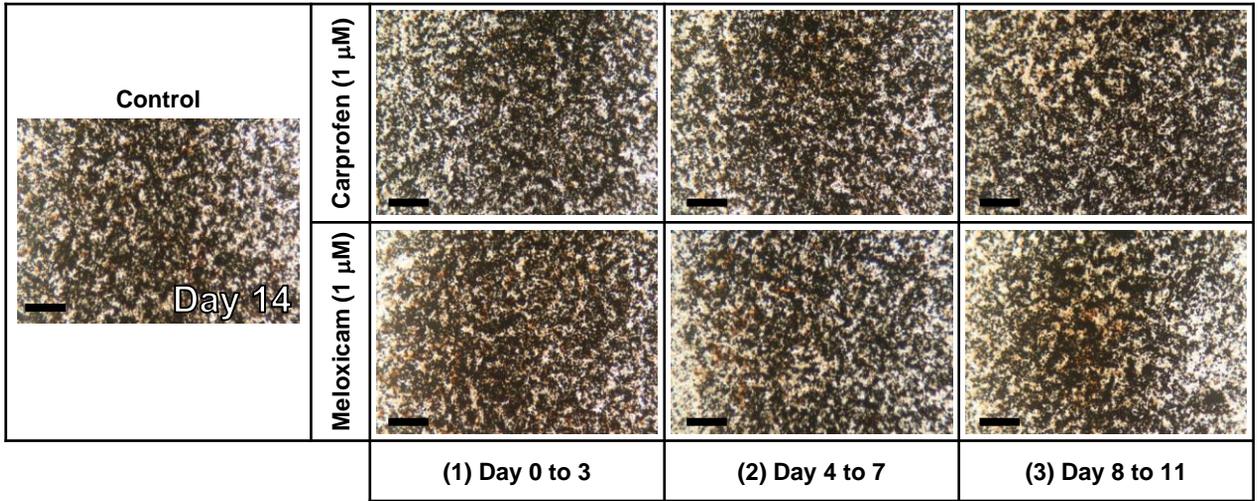


Fig. 2-6. Morphological analysis of effect of carprofen and meloxicam on calcified matrix formation in canine POS osteosarcoma cell line by von Kossa staining on day 14 after further osteogenic induction with withdrawal of drug treatments. No significant difference was observed between control and experimental group in calcification level. Each bar represents 1 μ m.

2.4. Discussion

In this Chapter, POS cells showed continuous proliferation of ALP-positive cells, nodule formation and matrix calcification as other osteosarcoma cell lines (Rodan *et al.*, 1987; Siggelkow *et al.*, 1998) and osteoblasts from mesenchymal stem cells (Malaval *et al.*, 1999) or calvarial cells (Nefussi *et al.*, 1997). Short-term treatment with COX-2 inhibitors, such as carprofen (Ricketts *et al.*, 1998) and meloxicam (Engelhardt, 1996), limitedly inhibited osteoblast-like differentiation of POS cells at the transitional stage. However, no suppressive effect was observed in fully mature osteoblast-like cells by the COX-2 inhibitors. Delay of non-calcified nodule formation during transitional stage was recovered after withdrawal of the drugs. These data represented that, while transitional stage of osteoblastic differentiation was the most vulnerable to COX-2 inhibitors, short-term use of COX-2 inhibitor did not affect calcified matrix formation.

Recently, effects of carprofen on fracture healing have been reported in dog fracture model (Ochi *et al.*, 2011), which suggested long-term treatment (120 days) with carprofen could delay bone healing with clinical dosage. Effects of meloxicam on bone healing in rabbit fracture model (Karachalios *et al.*, 2007) showed that low therapeutic dose for short-term administration (5 days) of meloxicam had no major complications in bone healing. These results imply that retardation of bone healing, which may occur with use of COX-2 inhibitors with very long-term and high dose, would not be permanent, but could be reversible by withdrawal of medication.

Basically PGE₂ increases ALP activity of osteoblastic cells in a differentiating stage-dependent manner (Ho *et al.*, 1999; Xu *et al.*, 2007). Our preliminary experiment showed that ALP activity of POS cells reached highest level on day 4 when cell density became 100% confluency, then it decreased as cells mature. This pattern of expression of ALP was consistent with the data from other studies (Aubin *et al.*, 1995; Bonewald *et al.*, 1992;

Mulkins *et al.*, 1983). However, during all stages of osteoblast-like differentiation including on day 4 when ALP activity was highest, no significant decrease was observed in expression of ALP by carprofen or meloxicam genetically, biochemically and morphologically. There would be a possibility of PGE₂ restoration under NSAID treatment in POS cells, which resulted in the pre-osteoblast-like cells to be normally induced under COX-2 inhibitions in each stage of osteoblast-like differentiation.

As osteoblast-like cells continued to mature, level of mRNA expression of osteocalcin on day 7 increased eight times higher than that on day 3, and this high expression level was continuously observed until day 11. Osteocalcin is a matrix protein that is secreted by mature osteoblasts (Carpenter *et al.*, 1998; Lian *et al.*, 1989). In this study, not only gene expression of osteocalcin, but also non-calcified nodule formation was reduced by carprofen and meloxicam in transitional stage. Other studies using bone marrow cells in mice also demonstrated down-regulation of osteocalcin expression by COX-2 inhibitors in mature osteoblasts (Nakai *et al.*, 2006). However, in POS cells, mRNA expression of osteocalcin as well as nodule formation was not suppressed by the drugs during mature osteoblastic stage. It is speculated that compensatory capacity to restore PGE₂ would increase with differentiation of the cell.

Reversible suppressive effect of COX-2 inhibitors on osteogenic differentiation was confirmed by calcification levels that showed no difference from control at the end of osteogenesis. Several long-term treatment study in rat fracture-healing models histologically demonstrated suppressive effects of selective COX-2 inhibitors on bone union (Brown *et al.*, 2004; Gerstenfeld *et al.*, 2007; Simon and O'Connor, 2007; Simon *et al.*, 2002). Expression of COX-2 mRNA was up-regulated in the callus during first two weeks of fracture healing relative to unfractured bone (Gerstenfeld *et al.*, 2003). Moreover, PGE₂ level was highly detected from the day of fracture and gradually decreased until thirty-five days (Gerstenfeld

et al., 2007). The functions of COX-2 on bone healing via deriving PGE₂ in this period would be important for differentiation of osteogenic cells. This might also explain why long-term use of COX-2 inhibitor from early stage of fracture healing could be one of the reasons for delaying the bone healing *in vivo*. However, one of these studies demonstrated that decreased PGE₂ production by COX-2 selective or non-selective inhibitor within the fracture site was rebounded after a week of drug withdrawal (Gerstenfeld *et al.*, 2007). Response from the body under PGE₂ deficiency would also assist osteogenic cells in continuing bone healing. These results imply that the ideal level of PGE₂ is required in certain period of bone healing to induce differentiation of osteoprogenitor cells, but short-term use of COX-2 inhibitor minimally affect bone healing because of their reversible effect on COX-2 inhibition.

Canine POS osteosarcoma cell line used in the present study was not pure osteoblast cells and had somewhat different life cycle due to its origin of tumors. Although there are limitations to extrapolate these data directly to the normal physiological phenomena in bone metabolism, it is assumed that short-term treatment with COX-2 inhibitors, such as carprofen and meloxicam, for pain control in fracture patients would be more beneficial to patient because of its minor effects on osteogenesis. Further studies will be needed, using normal osteogenic cell source, to clearly determine the effects of various classes of NSAIDs on osteogenic differentiation.

In conclusion, short-term treatment with carprofen and meloxicam would reversibly suppress osteogenic differentiation, suggesting that short-term treatment with carprofen and meloxicam in bone fracture patients should be allowed.

2.5. Summary

The aim of this Chapter was to assess reversibility of osteogenic potential of osteogenic cells after short-term treatment with NSAIDs during different stages of osteogenic differentiation. By using COX-2 preferential inhibitors, such as carprofen and meloxicam, which have pharmacological action to inhibit PGE₂ synthesis of osteogenic cells, effects on osteogenic differentiation were analyzed.

Canine POS osteosarcoma cell line that spontaneously differentiates into osteoblastic cells was exposed to carprofen (1 μM) or meloxicam (1 μM) for 72 hours during three different stages of osteoblast differentiation, including day 0 to 3 (pre-osteoblastic stage), day 4 to 7 (transitional stage) and day 8 to 11 (mature osteoblastic stage). As osteogenic markers, expression of ALP was estimated by analysis of mRNA expression, enzymatic activity and ALP staining. Expression of osteocalcin was estimated by analysis of mRNA expression. Finally, calcified matrix formation was observed by von Kossa staining after further osteogenic differentiation with drug withdrawal on day 14.

Expression of ALP showed no significant suppression by carprofen and meloxicam during all three stages. However, expression of osteocalcin mRNA and non-calcified nodule formations were delayed by carprofen and meloxicam during transitional stage. Nevertheless, fully calcified nodule formation was observed in all experimental groups during post-medication period. These results indicated that short-term treatment with carprofen and meloxicam would reversibly suppress osteogenic differentiation of osteoblasts.

3. Compensatory cellular reactions to NSAIDs on osteogenic differentiation in canine bone marrow-derived mesenchymal stem cells

3.1. Introduction

In the previous Chapter, effects of NSAIDs on osteogenic differentiation showed reversibility after their short-term treatment, which indicated possibility to have compensatory responses to PGE₂ insufficiency in osteogenic cells. In Chapter 3, intrinsic potential of osteogenic cells to maintain osteogenic differentiation under inhibition of PGE₂ synthesis by NSAID treatment was analyzed by using normal osteogenic cells, such as canine BMSCs.

Therapeutic effects of NSAIDs can be evoked by inhibiting the enzyme activity of COX, resulting in decreased synthesis of inducible PGE₂, which is one of key mediators of inflammation and a stimulator of pain-sensitizing neurons (Kidd and Urban, 2001; Ricciotti and FitzGerald, 2011). To date, effects of NSAIDs on bone healing process have remained controversial (Barry, 2010; Cottrell and O'Connor, 2010; Einhorn, 2003) because detrimental effects of NSAIDs on fracture healing are premised on the fact that one of the most consistent anabolic effects of PGE₂ in osteogenesis is promotion of the differentiation of osteoblasts (Flanagan and Chambers, 1992; Scutt and Bertram, 1995).

Synthesis of PGE₂ is a harmonized process of several enzymes such as prostaglandin H₂ synthases, including COX-1 and COX-2, and prostaglandin E synthases, consisting of cytosolic prostaglandin synthase (cPGES), microsomal prostaglandin synthase (mPGES)-1 and mPGES-2. In general, the constitutively expressing enzyme, COX-1 is ubiquitous in most of cells for maintaining homeostasis, but COX-2 is an inducible enzyme that can be upregulated by various proinflammatory stimuli (Vane *et al.*, 1998). Downstream of COX enzymes, cPGES is also constitutively expressed and mainly coupled with the COX-1 enzyme (Tanioka *et al.*, 2000). In contrast, mPGES-1 is induced coordinately with the COX-2 enzyme, and it has preference

for COX-2 over COX-1 (Murakami *et al.*, 2000). mPGES-2 can couple with either of the COX enzymes and is readily detected as COX-1 and cPGES, suggesting it is also a constitutive enzyme for maintenance of cell homeostasis (Murakami *et al.*, 2003).

Animal studies performed with COX-2 KO mice revealed that the activity of the COX-2 enzyme has an important role in osteogenesis (Simon *et al.*, 2002; Zhang *et al.*, 2002). Fracture sites in wild-type animals showed delayed bone healing when they were treated with COX-2 inhibitors for very long-term with high dose (Gerstenfeld *et al.*, 2007; Gregory and Forwood, 2007; Ochi *et al.*, 2011; Simon and O'Connor, 2007; Simon *et al.*, 2002). Although these data could explain that PGE₂ is an essential molecule in fracture healing, the question still remains concerning why there is no clear evidence for delayed fracture healing as a result of COX-2 inhibitor treatment in clinical use. Furthermore, use of NSAIDs would be beneficial in some cases of bone healing because the biology of osteoblasts is likely to be negatively derived when PGE₂ exceeds certain levels (Ramirez-Yanez and Symons, 2012); that is, overflow of PGE₂ is required to be controlled in chronic inflammatory disease such as rheumatoid arthritis (Redlich and Smolen, 2012).

An experimental study using fibroblasts from wild-type or COX KO mice demonstrated over-production of PGE₂ by up-regulated counterpart COX enzymes in COX-1- or COX-2-deficient cells when compared with intact cells (Kirtikara *et al.*, 1998). Bone is one of the few organs that can be fully repaired under the best osteogenic milieu (Shapiro, 2008). Based on these findings, it was hypothesized that differentiation of osteogenic cells can be maintained under NSAID treatment by utilizing other channels for PGE₂ synthesis to restore the level of PGE₂ during shortages caused by the differences in selectivity of COX-1/COX-2 in each type of NSAID. The purpose of this study was to confirm the cellular compensatory responses to NSAIDs such as carprofen, meloxicam, indomethacin and robenacoxib during osteogenic differentiation in canine BMSCs.

3.2. Materials and methods

3.2.1. Collection and culture of canine BMSCs

All experimental protocols were reviewed and approved by the Animal Care and Use Committee of Hokkaido University. Canine bone marrow cells were harvested from the proximal humeri of three 1-year-old female beagle dogs. Approximately 2 ml of bone marrow was aspirated into a heparinized syringe and was immediately suspended in Dulbecco's Modified Eagle Medium (DMEM; GIBCO). The cells in the medium were promptly centrifuged at 800 x g for 20 minutes. Mononuclear cell layers were transferred into a plastic culture dish (100 mm x 20 mm 430167; Corning) containing DMEM with 10% heat-inactivated FBS and maintained in an incubator at 37°C with a humidified 5% CO₂ atmosphere. From the day when outgrowing cell colonies were observed, the medium was replaced every 3 days with elimination of the non-adherent cell fraction until adherent cells proliferated and became sub-confluent, which took about 10 days. Cells from the second passage were used in this experiment.

3.2.2. Cell viability assay

Carprofen, meloxicam, indomethacin (Wako) or robenacoxib (Novartis Animal Health US, Inc., Greensboro, NC, USA) were prepared with different concentrations to achieve final concentrations of 0.01, 0.10, 1.00, 10.00 and 100.00 µM in culture medium. Canine BMSCs were placed at a density of 5×10^3 cells in each well of 96-well plate (Costar 3595) with 100 µl of culture medium. After 24 hours of incubation for cell adaptation to the culture conditions, each concentration of drugs was treated for 48 hours of the exponential growth phase. Cell viability was determined by a colorimetric assay based on the conversion of MTT to a formazan pigment by mitochondrial enzymes in surviving cells during four

hours of incubation period (Mosmann, 1983). Colorimetric measurement was performed using a microplate photometer (Multiskan FC; Thermo Fisher Scientific Inc., Waltham, MA, USA) at 570 nm. All samples were evaluated in triplicates.

3.2.3. Establishment of inflammatory condition

The optimal concentration of recombinant human interleukin-1 β (rhIL-1 β ; Wako) to trigger mRNA expression of inflammatory enzymes for PGE₂ synthesis such as COX-2 and mPGES-1 was confirmed. Briefly, after canine BMSCs were cultured under serum-free conditions for 24 hours, 0.1, 1.0 and 10.0 μ M of rhIL-1 β were supplemented into the medium. Gene expressions were analyzed at 2 hours after each rhIL-1 β stimulus using qRT-PCR as described in the section concerning analysis of gene expression.

3.2.4. Induction of osteogenic differentiation

Osteogenic differentiation was performed using canine BMSCs at the second passage. As a negative control, canine BMSCs were cultured in basal medium, DMEM, and, as positive control, osteogenic differentiation of the cells was induced by using conventional osteogenic medium, DMEM containing 0.1 μ M dexamethasone (Sigma-Aldrich), 10 mM β -glycerophosphate (Sigma-Aldrich) and 50 mg/ml L-ascorbic acid (Wako). To observe effects of NSAIDs on the colony formation of ALP-positive cells, canine BMSCs were seeded in a multiple micro-mass manner at a density of 2×10^4 cells per drop of 5 μ l basal medium in 12-well, flat-bottomed culture plates (Costar 3513). After the cells adhered to the plate, the media were changed every other day with or without NSAIDs for 20 days.

3.2.5. Quantification of PGE₂ synthesis

The level of endogenous PGE₂ in culture supernatant was measured using

Prostaglandin E2 Parameter Assay Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. Briefly, after the cells were cultured in serum-free medium for 24 hours, PGE₂ production was measured at 0, 24 and 48 hours under inflammatory conditions with 1 ng/ml of rhIL-1 β . Effects of NSAIDs on PGE₂ synthesis were evaluated by supplementation with 10 μ M of carprofen, meloxicam, indomethacin or robenacoxib.

3.2.6. Analysis of gene expression

Extraction of total cellular RNA was performed by disruption of cultured BMSCs with TRizol reagent. Total RNA was precipitated by centrifugation (15000 x g, 20 min, 4°C) of aqueous phase with isopropanol. Pellets were washed with 70% ethanol and dissolved in 0.1% v/v DEPC-treated water after air drying. The revers-transcription reaction performed using M-MLV Reverse Transcriptase. The Levels of gene expression were detected by using qRT-PCR in Rotor-Gene Q thermal cyler with KAPA SYBR FAST qPCR Master Mix. The acquired data were analyzed by normalization to GAPDH as a reference gene. All sequences of primers used in this experiment are listed in Table 3-1.

3.2.7. Intracellular ALP activity

Cell layers were washed twice with ice-cold PBS and lysed in 10 mM Tris-HCl solution (pH 7.4) containing 0.05% (v/v) Triton X-100. After vigorous shaking and 30 minutes of sonication, supernatants of the cell lysates were separated by centrifugation at 20,000 x g for 30 minutes and were stored at -80°C until analysis. To access ALP activity of the cells, samples were thawed and reacted with *p*-nitrophenyl phosphate for 10 minutes at 37°C in substrate solution (pH 10.5), containing 1 mg/ml *p*-nitrophenyl phosphate, 100 mM glycine, 1 mM MgCl₂ and 1 mM ZnCl₂. Released *p*-nitrophenol were measured by a using microplate photometer (Multiskan FC) at 405 nm and normalized using the total protein concentration

measured by modified Bradford method (Bradford, 1976). The ALP activity was expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein.

3.2.8. Quantification of osteocalcin production

Osteocalcin secreted by mature osteoblasts was evaluated using Intact Human Osteocalcin EIA Kit (Biomedical Technologies Inc., Stoughton, MA, USA) according to the manufacturer's instruction. Culture supernatants were collected during medium change and were stored at -80°C until the day they were quantified.

3.2.9. Quantification of extracellular calcium deposition

The amounts of extracellular matrix mineralized during osteoblast differentiation were compared by colorimetric quantification method using Alizarin red S (ARS; Sigma-Aldrich)-based assay (Gregory *et al.*, 2004). Briefly, the cell layers in the well was washed with PBS and fixed in 10% (v/v) formaldehyde (Wako) at room temperature for 15 minutes. After washing twice with distilled water, the plate was incubated with 40 nM ARS (pH 4.1) per well for 20 minutes. Unincorporated dye was washed with distilled water and aspirated. The dye was extracted by detachment of cell layers by 10% (v/v) acetic acid and transferred to microcentrifuge tube. The slurry was centrifuged at $20,000 \times g$ for 15 minutes after dissolution by heating at 85°C for 10 minutes. The optical density of the supernatant was read by a microplate photometer (Multiskan FC) at a wavelength of 405 nm.

3.2.10. Morphological evaluation

The early period of differentiation of osteoblasts from canine BMSCs was observed using ALP staining. Briefly, the cells were washed with PBS and fixed with 4% paraformaldehyde for 2 minutes. After rinsing, the cells were incubated in 0.1 M Tris-HCl

buffer (pH 9.3) containing 0.25% (w/v) naphthol-AS-BI-phosphoric acid sodium salt and 0.75% (w/v) fast blue RR salt at 37°C for 30 minutes.

Calcium deposition in the matrix of differentiated osteoblasts was confirmed using von Kossa staining. Briefly, the cells fixed with 4% paraformaldehyde for 2 minutes were treated with 2% silver nitrate solution and placed under ultraviolet light at room temperature for 1 hour. After rinsing, the cells were bleached in 5% sodium thiosulfate for 2 minutes.

3.2.11. Statistics

Data were statistically analyzed by performing the nonparametric Mann-Whitney U test using commercial software (SPSS, version 12.0.1). All data were expressed as mean \pm standard deviation (SD). Values of $p < 0.05$ were considered to be statistically significant.

3.3. Results

3.3.1. Doses of NSAIDs inhibiting PGE₂ synthesis without effects on cell viability

Effects of NSAIDs on viability of canine BMSCs were assessed quantitatively by MTT assay. Cell culture for 48 hours resulted in no significant effects on cell viability for all NSAIDs at concentrations up to 10 μ M (Fig. 3-1A to D). The optimal concentrations of rhIL-1 β to induce enzymes responsible for inflammatory PGE₂ synthesis were assessed by confirmation of the expression of genes such as COX-2 and mPGES-1. At 2 hours after stimulation with 0.1, 1.0 and 10.0 ng/ml of rhIL-1 β , the expression levels of COX-1 and COX-2 mRNA were increased (Fig. 3-2). The gene expression of mPGES-1 was increased by 1.0 and 10.0 ng/ml of rhIL-1 β , indicating that inflammatory conditions could be triggered by rhIL-1 β at concentration \geq 1.0 ng/ml. In the present study, 1.0 ng/ml of rhIL-1 β was supplemented into osteogenic medium to induce the expression of COX-2 and mPGES-1. At 24 hours during cell culture under inflammatory conditions, PGE₂ synthesis was dose-dependently suppressed by the NSAIDs, except for robenacoxib, at concentrations \leq 10 μ M, which were shown to not interfere with cell viability (Fig. 3-1A to D). The dose for each NSAID in this experiment was 10 μ M and was selected to inhibit COX activity.

3.3.2. Differentiation of osteoblasts from canine BMSCs

The time-course expression levels of markers for osteoblasts changed during osteogenic differentiation. The levels of ALP expression fluctuated after the highest expression was observed on day 4. Osteocalcin expression gradually increased after the peak of ALP expression, which was followed by an increase in calcium deposition. Calcification in the cellular matrix reached the maximum level on day 20 (Fig. 3-3C), at

which point osteocalcin secretion was detected at its highest level. Differentiation of osteoblasts in canine BMSCs was also supported by morphologic analysis (Fig. 3-3D), including colony formation of ALP-positive cells on day 4 and calcified matrix formation on day 20.

3.3.3. Expression of osteoblastic markers during NSAID treatment

Effects of NSAIDs on differentiation of osteoblasts were evaluated based on the expression levels of ALP, osteocalcin and calcification on the day when each osteoblastic marker was expressed at the highest level. On day 4, the expression level of ALP mRNA (Fig. 3-4A), ALP activity (Fig. 3-4B) and colony formation of ALP-positive cells (Fig. 3-4C) were decreased by all the NSAIDs. Suppressed differentiation of ALP-positive cells by NSAIDs was restored by supplementation with PGE₂ (Fig. 3-4C). The expression level of osteocalcin mRNA showed no decrease due to treatment with NSAIDs on day 20 (Fig. 3-4D). Synthesis of protein of osteocalcin also showed no significant difference between control and groups receiving NSAID treatment (data not shown). Level of calcification was decreased by NSAIDs on day 20, which was restored by continuous supplementation with PGE₂ (Fig. 3-4E).

3.3.4. Expressions of PGE₂-related genes and synthesis of PGE₂ under NSAID treatment

Continuous treatment with NSAIDs induced up-regulation of genes for PGE₂-related receptors (Fig. 3-5A) and enzymes (Fig. 3-5B). Gene expression levels of PGE₂ receptors, such as EP2 and EP4, were up-regulated on day 4 by NSAIDs, except in the case of meloxicam and indomethacin for EP2 mRNA expression. Expression of COX-2 mRNA was also up-regulated by NSAIDs on day 4 and was more increased by carprofen than by the other NSAIDs. The rates of PGE₂ synthesis by NSAIDs were suppressed for

24 hours (Fig. 3-5C) and were negatively correlated with the up-regulated expression patterns of COX-2 mRNA. Suppressed PGE₂ synthesis was restored in each group at 48 hours, at which time the amount of PGE₂ synthesized was about 2-fold higher in the robenacoxib group than in the other groups.

Table 3-1. Sequences of primers used for real-time PCR

Gene	Primer sequence (5' to 3')	Product size (bp)	GenBank accession no.
<i>COX-1</i>	(Forward) GGC AGT TCA GAA TGT TGT GC (Reverse) GCA ATG CAC TCT GGT TAG GC	162	NM_001003023.2
<i>COX-2</i>	(Forward) GCG AGG AAC CAA CAG CTT AC (Reverse) TGA CAC GGG TTA CGT CAT GT	171	NM_001003354.1
<i>cPGES</i>	(Forward) AAA AGG TGA ATC TGG CCA GTC ATG G (Reverse) ATC CTC ATC ACC ACC CAT GTT GTT C	172	XM_005625521.1
<i>mPGES-1</i>	(Forward) ACT GGC CAT GAG CCG CTG TG (Reverse) TCC TGT GTT CAG CAC GCT GCC	104	NM_001122854.1
<i>EP2</i>	(Forward) AAA TGG GAC CTC CAA GCT CT (Reverse) ATG AAA CCC GAC AAC AGA GG	124	NM_001003170.1
<i>EP4</i>	(Forward) GTG TTT GGC TGT GCT CAG AA (Reverse) CAT GGG TTC CCG TAT GAA TC	110	NM_001003054.1
<i>ALP</i>	(Forward) CCA AGC TCA ACA GAC CCT GA (Reverse) GAG ACA CCC ATC CCA TCT CC	102	NM_001197137.1
<i>Osteocalcin</i>	(Forward) AGG AAG CTT ACC AGC GCT TC (Reverse) TGA CAA GGA CCC CAC ACT TG	138	XM_547536.4
<i>GAPDH</i>	(Forward) CTG AAC GGG AAG CTC ACT GG (Reverse) CGA TGC CTG CTT CAC TAC CT	129	NM_001003142.1

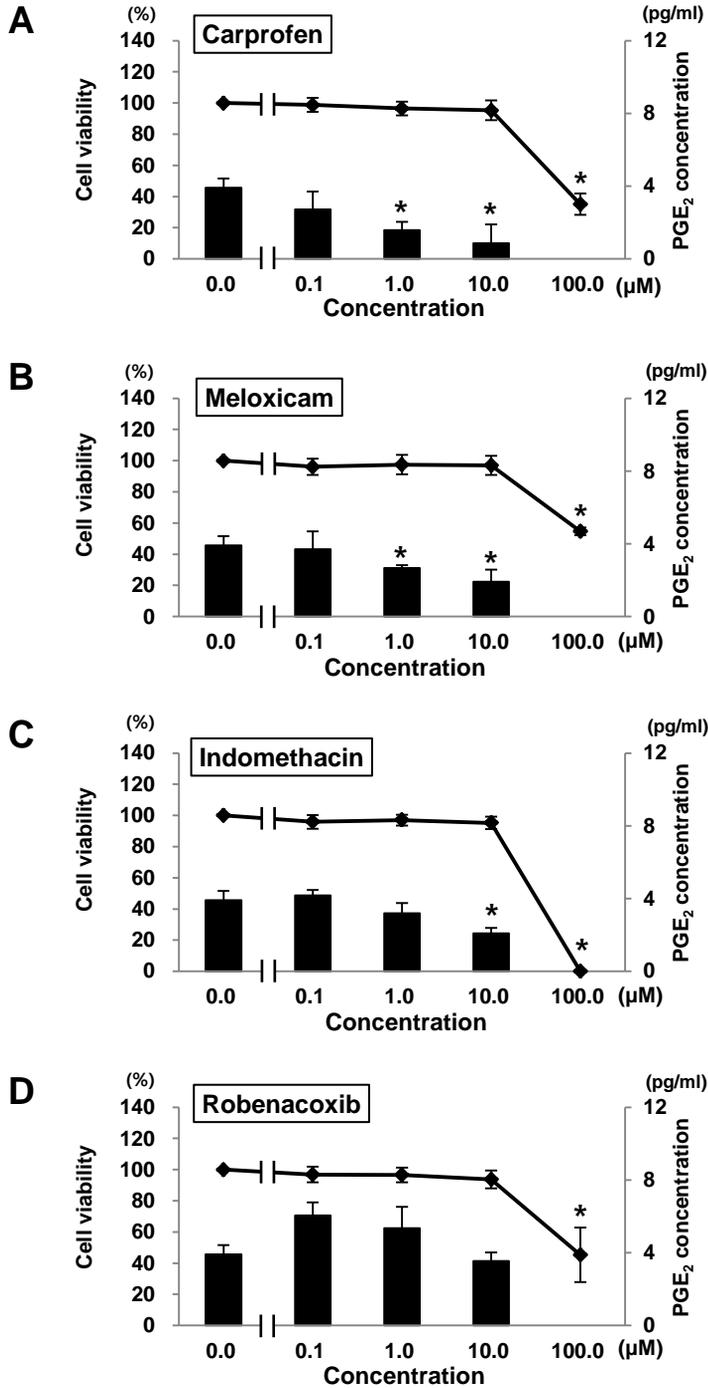


Fig. 3-1. Effects of carprofen (A), meloxicam (B), indomethacin (C) and robenacoxib (D) on viability (◆) and PGE₂ synthesis (■) in canine bone marrow-derived mesenchymal stem cells. Cell viability was determined using MTT assay after drug treatment for 48 hours. Production of PGE₂ (C) was quantified by ELISA after 24 hours of cell culture with 0.1, 1.0 and 10.0 μM of NSAIDs. Data were expressed as mean ± SD from three independent experiments that were performed in triplicate. * *p* < 0.05.

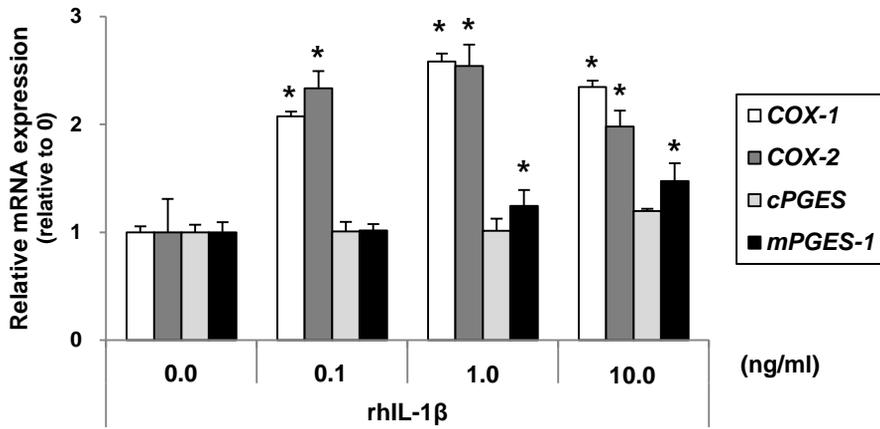


Fig. 3-2. Effects of recombinant human interleukin-1β (rhIL-1β) on mRNA expression of enzymes for PGE₂ synthesis. Canine bone marrow-derived mesenchymal stem cells were stimulated with each concentration of rhIL-1β (0.1, 1.0 and 10.0 ng/ml) after 24 hours of incubation in serum-free medium. Gene expression at 2 hours was analyzed using quantitative real-time polymerase chain reaction. Data were expressed as mean ± SD (n=3). * $p < 0.05$.

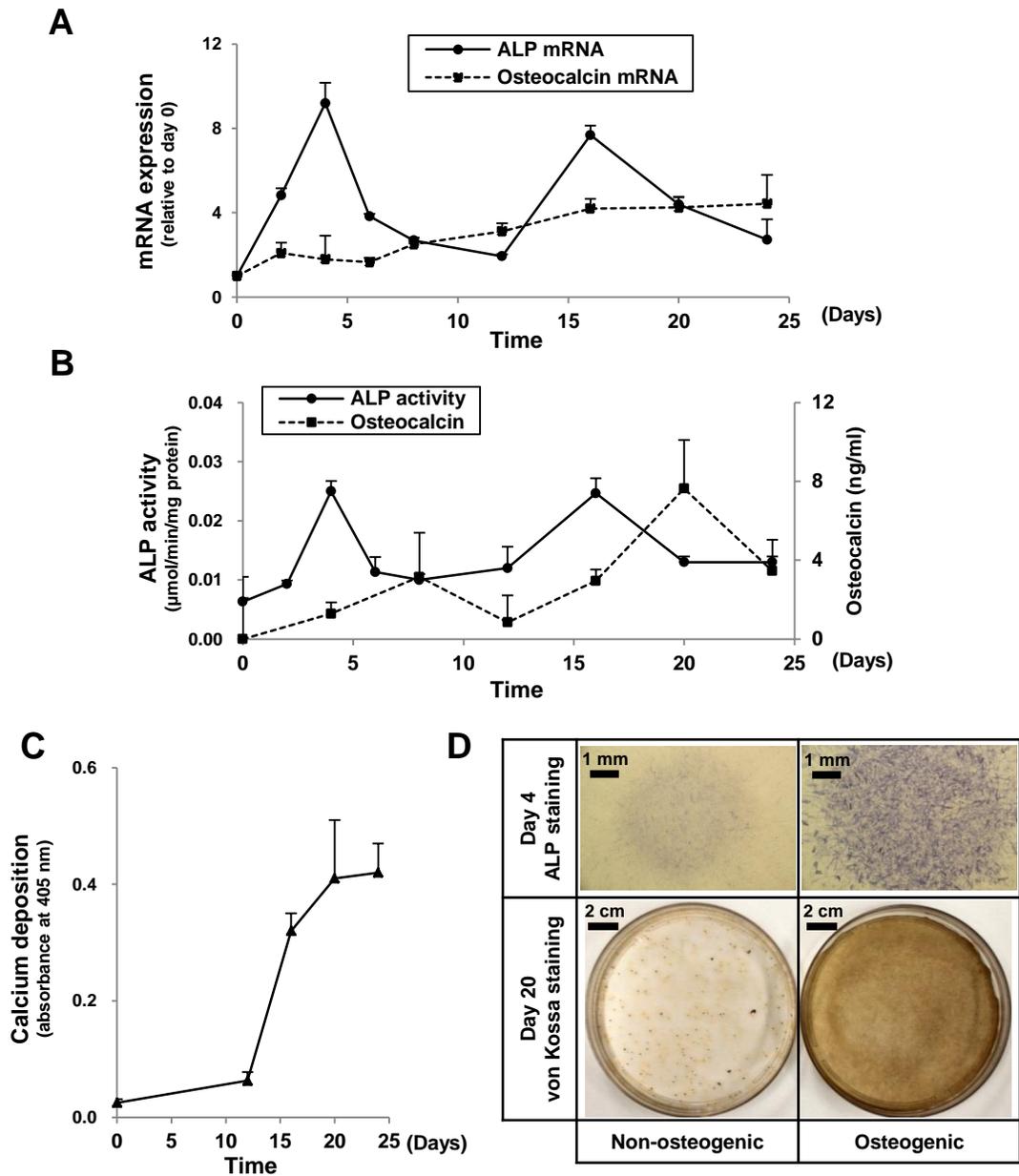


Fig. 3-3. Differentiation of osteoblasts in canine bone marrow-derived mesenchymal stem cells. Gene expression (A) was analyzed using quantitative real-time polymerase chain reaction. Enzyme activity of ALP (B) was measured by colorimetric assay using *p*-nitrophenyl phosphate. Osteocalcin (B) was quantified by ELISA. Level of calcification (C) was quantified by Alizarin red S assay. Morphological analysis (D) was performed using ALP staining and von Kossa staining. Data were expressed as mean \pm SD (n=3).

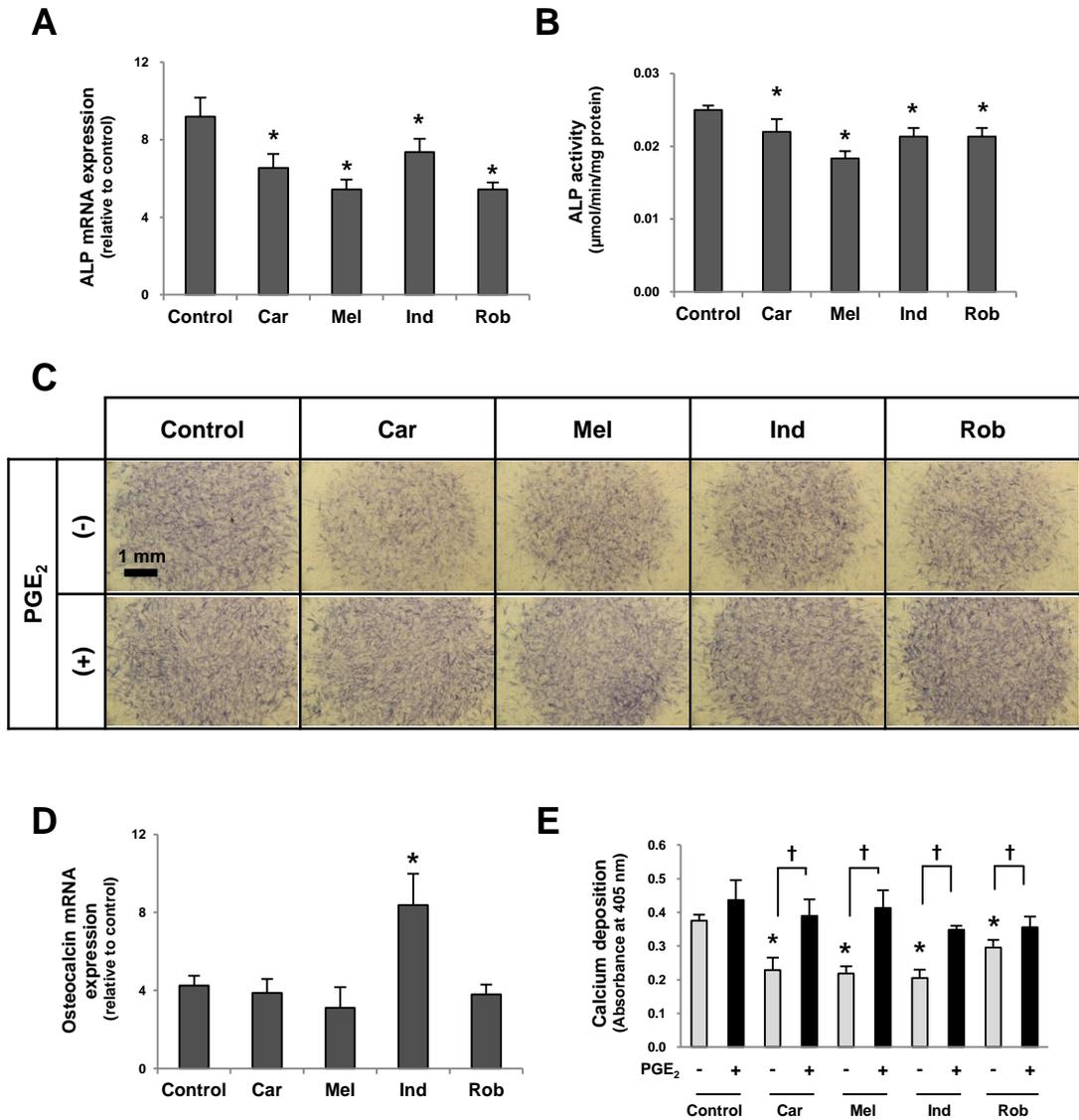


Fig. 3-4. Effects of NSAIDs on differentiation of osteoblasts in canine bone marrow-derived mesenchymal stem cells. Gene expression (A, D) was analyzed using quantitative real-time polymerase chain reaction. Enzyme activity of ALP (B) was measured by colorimetric assay using *p*-nitrophenyl phosphate. Morphological analysis (C) was performed using ALP staining. Level of calcification (E) was quantified by Alizarin red S assay on day 20. Expression levels of ALP (A, B and C) were analyzed on day 4. In groups for recovery, 10 nM of PGE₂ was added (C and E). Car, carprofen (10 µM); Mel, meloxicam (10 µM); Ind, indomethacin (10 µM); Rob, robenacoxib (10 µM). Data were expressed as mean ± SD (n=3). * *p* < 0.05; † *p* < 0.05 between two columns.

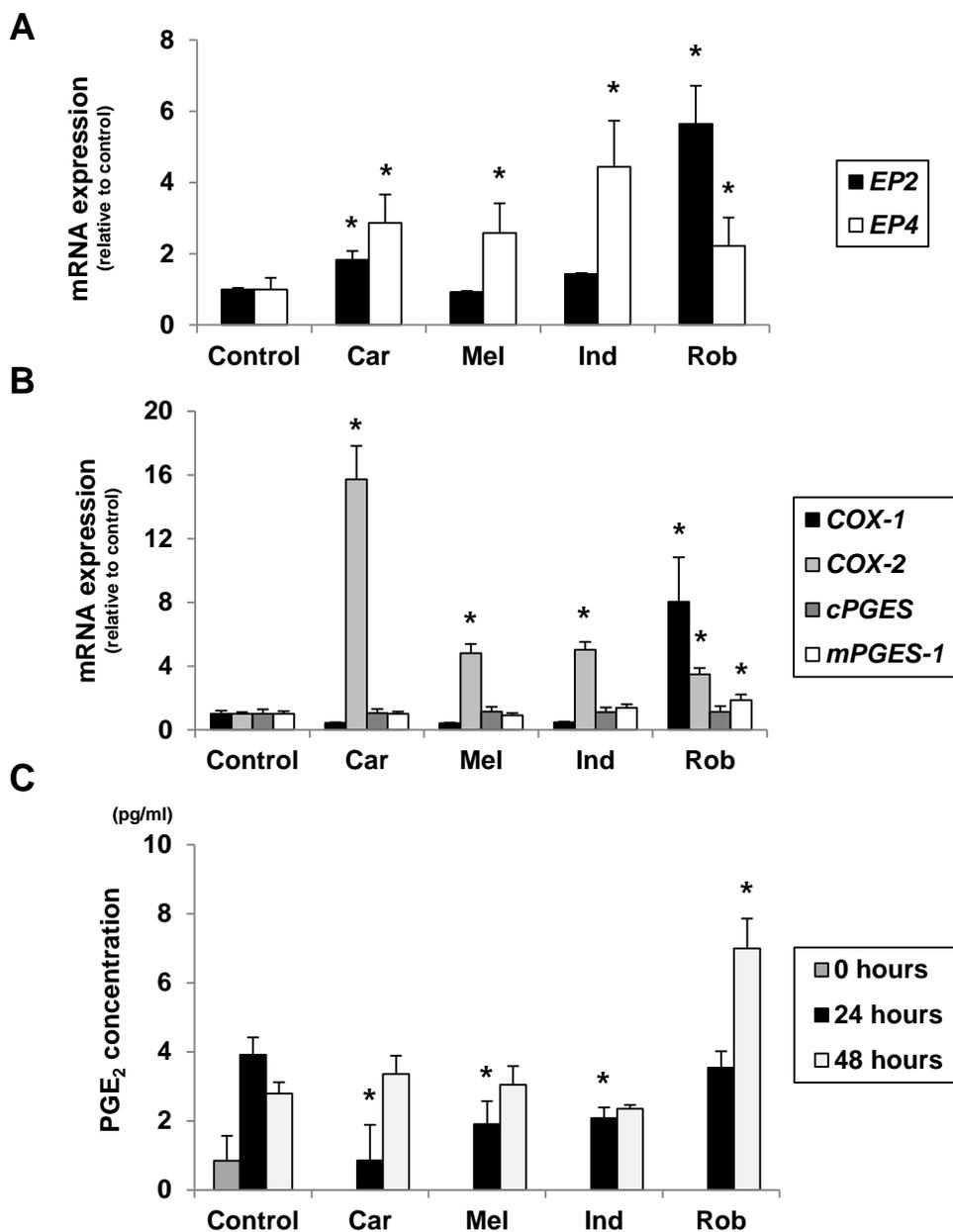


Fig. 3-5. Compensatory response under NSAID treatment during osteoblastic differentiation in canine bone marrow-derived mesenchymal stem cells. Gene expression was analyzed on day 4 using quantitative real-time polymerase chain reaction (A and B). Production of PGE₂ (C) was quantified by ELISA. Car, carprofen (10 μM); Mel, meloxicam (10 μM); Ind, indomethacin (10 μM); Rob, robenacoxib (10 μM). Data were expressed as mean ± SD (n=3). * $p < 0.05$.

3.4. Discussion

In this Chapter, canine BMSCs differentiated into osteoblasts under inflammatory conditions of COX-2-induced PGE₂ synthesis. Serial changes in the expressions of osteoblastic markers during the differentiation were confirmed by up-regulation of ALP activity in pre-osteoblasts, followed by an increase in osteocalcin synthesis in mature osteoblasts and by calcification of matrix, which was previously shown to be correlated with osteoblastic differentiation of mesenchymal stem cells in humans (Kulterer *et al.*, 2007) and rats (Nakamura *et al.*, 2009).

Continuous treatment with NSAIDs partially suppressed the osteogenic process in canine BMSCs, such as ALP expression and calcification. While expression of ALP was decreased by NSAIDs, the level of expression could have gradually increased with differentiation into mature osteoblasts, resulting in unsuppressed expression of osteocalcin on day 20. Expression of osteocalcin mRNA decreased by NSAIDs during differentiation of osteoblasts in human BMSCs on day 4 (Yoon *et al.*, 2010). However, we detected uninhibited expression of mRNA and protein production of osteocalcin when its expression was maximal, suggesting that NSAIDs would only temporarily suppress expression of osteocalcin. The level of calcification on day 20 was somewhat suppressed, which would be related to delayed ALP expression resulted from insufficiency of PGE₂ synthesis. It is important to note that the deficiency in PGE₂ in the osteogenic milieu under treatment with NSAIDs could be corrected by the osteogenic cells, leading to prevention of markedly derailed osteoblast differentiation.

Inhibitory effects of NSAIDs on PGE₂ synthesis in canine BMSCs are caused by partial blockage of the COX-1 or COX-2 pathway, depending on the selectivity of the drugs for COXs. Expressions of genes for inflammatory PGE₂ synthesis, such as COX-2 and mPGES-1 mRNA, were induced by 1 ng/ml of rhIL-1 β , which was also shown to be the

concentration that stimulates synthesis of protein of COX-2 in human BMSCs (Yoon *et al.*, 2010). In this condition, carprofen, meloxicam and indomethacin showed significant suppression of PGE₂ synthesis at 24 hours, supporting the expected pharmacological inhibitory effects on inflammatory PGE₂ synthesis at the dose of the respective drugs used in the present study. However, restoration of the level of PGE₂ during shortage was observed in every NSAID group, even when over-synthesized by robenacoxib, at 48 hours, indicating that there would be some intrinsic mechanisms to restore shortages of PGE₂ in canine BMSCs.

Up-regulated expressions of receptors for PGE₂, such as EP2 and EP4, could be an effective way to increase sensitivity to PGE₂. It has been demonstrated that endogenous PGE₂ could induce COX-2 expression via a PG-mediated auto-amplification loop by stimulating EP2 and EP4 in osteoblasts (Pilbeam *et al.*, 1995; Sakuma *et al.*, 2004). These results imply that increased expression of EP2 or EP4 acts not only as a part of the corrective mechanism for PGE₂ shortage, but also as an enhancer for osteogenic differentiation. Furthermore, EP2 and EP4 have been implicated in the bone anabolic effect of PGE₂ (Alander and Raisz, 2006; Minamizaki *et al.*, 2009; Raisz and Woodiel, 2003). A study performed with selective agonists for PGE₂ receptors and cells cultured from COX-2 KO mice demonstrated that signaling via EP4 was more likely to play an important role in fracture repair than that via EP2 (Xie *et al.*, 2009). It is probable that increased expression of EP4 contributes to the up-regulated expression of osteocalcin mRNA during indomethacin treatment (Fig. 3-4D).

Significantly increased expression of COX-2 mRNA was also detected and was negatively correlated with suppression of PGE₂ synthesis. Furthermore, expression of COX-1 mRNA was up-regulated by robenacoxib, while it is generally known to be expressed continuously. The rapid rate of restoration via the up-regulated COX-1 channel would be

the reason why synthesis of PGE₂ was not suppressed at 24 hours of treatment with robenacoxib. These results indicate that both COX-1 and COX-2 could be induced by PGE₂ shortage to play physiological roles in compensation of PGE₂ in canine BMSCs. Therefore, differentiation of osteoblasts would be partially affected by NSAIDs, depending on the net level of PGE₂ that would be maintained by compensatory up-regulation of COX-1/COX-2 with or without EP2/EP4, while the efficiency of synthesis of PGE₂ is further affected by COX-2 than by COX-1 (Kirtikara *et al.*, 1998).

Suppressive effects of NSAIDs on osteogenesis would be minimal in clinical short-term use for analgesic purposes. The maximum plasma concentrations of carprofen (Lascelles *et al.*, 1998), meloxicam (Yuan *et al.*, 2009), indomethacin (Hucker *et al.*, 1966) and robenacoxib (Jung *et al.*, 2009) in dogs are have been determined in kinetic studies: 76.29 µM (20.60 µg/ml at 4.00 mg/kg/dose s.c.), 2.09 µM (0.78 µg/ml at 0.20 mg/kg/dose s.c.), 58.38 µM (20.9 ± 4.5 µg/ml at 10.00 mg/kg/dose p.o.) and 2.01 µM (0.66 µg/ml at 1.00 mg/kg/dose s.c.), respectively, indicating that the doses of meloxicam and robenacoxib used in this experiment were over their maximum plasma concentrations but that of carprofen was not. Because analgesic effects of NSAIDs are attained not only by peripheral inhibition of synthesis of PGE₂ but also by an increase in the threshold in nociceptive neurons at the central site (Cashman, 1996) and the concentration of NSAIDs in peripheral tissue is lower than in plasma (Grace *et al.*, 2000), analgesic doses of NSAIDs would hardly affect osteogenic differentiation at the site of fracture. However, fracture patients who need analgesic treatment with NSAIDs for a longer term are recommended to be prescribed with NSAIDs that offer a more secure compensatory response to shortage of PGE₂ in osteogenic cells, since long-term treatment with some NSAIDs has been shown to have effects that reduce heterotopic bone formation after arthroplasty (Vasileiadis *et al.*, 2011).

In conclusion, canine BMSCs have a compensatory mechanism that protects them against NSAIDs, which would be a reversible switch that regulates the level of PGE₂ for maintenance of osteogenesis. These data could be used to explain the discrepancy between suppressive effect of NSAIDs on osteogenesis *in vitro* and the rarely reported deterioration of bone healing during clinical use of NSAIDs as pain-killers.

3.5. Summary

The purpose of this Chapter was to demonstrate that osteogenic cells have compensatory response to PGE₂ deficiency caused by NSAIDs. Several classes of NSAIDs, such as carprofen, meloxicam, indomethacin and robenacoxib, were used to inhibit COX activities in canine BMSCs. To confirm the effects of long-term treatment with NSAIDs on osteogenic differentiation, non-cytotoxic concentration of each NSAIDs (10 μM) were used during 20 days of osteogenic differentiation with human recombinant IL-1β (1.0 ng/ml) as an inflammatory stimulator.

Expression of ALP was suppressed by all NSAIDs, leading to decrease of calcium deposition. However, protein production of osteocalcin showed no significant suppression by NSAIDs. Gene expression levels of PGE₂ related receptors (EP2 and EP4) and enzymes (COX-1, COX-2 and mPGES-1) were up-regulated during continuous treatment with NSAIDs, while certain channels for PGE₂ synthesis were utilized differently depending on the kind of NSAIDs.

These data suggested that canine BMSCs have compensatory mechanism to restore PGE₂ synthesis, which would be an intrinsic regulator to maintain differentiation of osteoblasts under NSAID treatment.

4. General conclusion

Bone could be repaired after injury by migrated osteogenic cells, which would be one of the most important rescue units to be more efficiently differentiated into bone forming cells by inflammation-mediating factors such as PGE₂. Because rare clinical evidences regarding delayed bone healing by clinical use of NSAIDs have been reported for several decades, the author hypothesized that there would be a reason why NSAIDs could limitedly inhibit osteogenic differentiation of bone forming cells. At this point, the purpose of present study was to evaluate effects of NSAIDs on differentiation capacity of canine osteogenic cells.

In Chapter 1, effects of short-term treatment with NSAIDs, such as carprofen and meloxicam, on osteogenic differentiation were estimated. Suppressive effects of NSAIDs were shown by delayed non-calcified nodule formation and with decreased expression level of osteocalcin mRNA in transitional stage between pre-osteoblastic and mature osteoblastic stages. However, fully calcified nodule formation was observed in all experimental groups during post-medication period, indicating NSAIDs reversibly suppress differentiation of osteoblasts. Short-term treatment with NSAIDs during each of main differentional stages, including pre- and mature osteoblastic stage, showed no deteriorative effects on expression of osteoblastic markers. These results indicated short-term treatment with NSAIDs reversibly inhibits osteogenesis, which led present author to suspect the possibility that osteogenic cells would have intrinsic potentials to restore insufficient PGE₂ under NSAID treatment. The results of this Chapter had a limitation in that the source of the cells used for osteogenic model was canine POS osteosarcoma cell line, which presented marked morphological changes good for observation, but it was re-evaluated in next experiment with normal osteogenic cells.

In Chapter 2, the present author focused on the cellular compensatory responses to PGE₂ deficiency under NSAID treatment. To understand cellular reactions under inhibition of COX-1/-2 activities by NSAIDs in inflammatory condition, canine BMSCs were differentiated into osteoblasts in osteogenic medium supplemented with human recombinant IL-1 β (1 ng/ml) as an inflammatory stimulator. In this Chapter, effects of various classes of NSAIDs, including carprofen, meloxicam, indomethacin and robenacoxib were evaluated during osteogenic differentiation. To estimate reversibility of osteogenic capacity of canine BMSCs after long-term treatment with NSAIDs, each of the NSAIDs was treated during 20 days of osteogenic period that matrix could fully calcified. Decreased ALP expression and delayed differentiation into ALP-positive cells by each of NSAIDs were detected on day 4 when its expression was highest. Level of calcium deposition was somewhat suppressed by NSAIDs on day 20, while osteocalcin production showed no significant suppression. However, gene expression levels of PGE₂ related receptors and enzymes were up-regulated on day 4, suggesting intracellular compensatory responses occurred under PGE₂ deficiency derived by treatment with NSAIDs. These responses would indicate that derailed osteogenic differentiation could be prevented by compensatory potentials of osteogenic cells after long-term treatment with NSAIDs. Channels for PGE₂ synthesis were utilized differently depending on the classes of NSAIDs, supported by differences in expression levels of EP2/EP4 and COX-1/COX-2 mRNA and in rates of PGE₂ restoration among the groups, which suggested that inhibition of osteogenic differentiation would be more limited by using specific classes of NSAIDs.

In conclusion, canine osteogenic cells were demonstrated to have compensatory potentials to restore PGE₂ deficiency derived by NSAIDs. These intra-cellular phenomena would be the intrinsic compensatory mechanism presenting reversibility or minimal inhibitory effects on osteogenic differentiation after short-term or long-term of the drug

treatments, respectively. Considering the doses of NSAIDs used in this study were non-cytotoxic and higher than plasma concentration in dogs, differentiation of osteoblasts would be more limitedly affected by clinical doses of NSAIDs.

Because pharmacological potency of drugs sometimes has tissue-specific differences, tissue-targeted profiling on specific drugs has clinical value. Contributions of present study can be introduced into clinical use of NSAIDs for fracture patients.

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6. Japanese summary

非ステロイド性抗炎症剤 (NSAIDs) は、主にシクロオキシゲナーゼ (COX) の活性を阻害することでエイコサノイドの発現を抑制して鎮痛効果などを発現させる。COXはさまざまな組織・細胞で発現・誘導され、NSAIDsによって生理的にCOXを必要とする器官や組織で副作用が引き起こされる。しかし、炎症刺激によってCOX-2が過剰発現することによりプロスタグランジン (PG) E₂が大量に産生されることは、痛みを誘発する主原因であるが、一方でこの過剰に生成されたPGE₂は骨芽細胞の増殖と分化を促進させる重要な因子でもあることが示されている。骨折治癒の初期には、骨折部に各種の骨前駆細胞が集簇し、それらがPGE₂などの炎症因子の刺激を受けて骨へと分化することにより骨折の修復が促進される。鎮痛を目的としたNSAIDsの使用は骨折部位のPGE₂濃度を減少させ、骨折治癒を遅延させる可能性がある。一方で、生体の過剰な恒常性維持機構の活性化を誘導する疼痛は、骨折治癒の際に、生体にとって大きな負担となる。これらの相反する事象について、臨床的なNSAIDs使用が決して骨折治療が行われている動物にとってネガティブな因子ではなく、総体として有効であると考えられる獣医師も少なくない。さらに、医学領域でも、NSAIDsの臨床使用による骨折治癒遅延は喫煙によるそれよりも小さいと考えられているが、科学的な解析は十分に行われているわけではない。本研究では、イヌ骨形成細胞の分化能力に対するNSAIDsの影響を検討し、NSAIDsの存在下での骨折治癒機転初期の細胞反応を解析することを目的とした。

第1章では、骨芽細胞の骨分化におけるNSAIDs短期間作用による影響を

検討した。骨芽細胞の骨分化モデルとしてイヌ骨肉腫由来細胞株であるPOS細胞を用いた。この細胞株は *in vitro* で自律的な骨芽細胞様細胞へ分化が可能であり、基質を石灰化させるまで前骨芽細胞期、移行期および成熟骨芽細胞期の段階的分化を示すことが証明されている。本細胞の各骨分化段階にカルプロフェン、またはメロキシカムを72時間作用させ、定量的real-time polymerase chain reaction (qRT-PCR)を用いて骨芽細胞分化マーカーであるアルカリホスファターゼ (ALP) およびオステオカルシンの遺伝子発現を評価するとともにALPの酵素活性と染色性、さらにvon Kossa染色を用いて細胞集塊の石灰化を観察した。その結果、骨分化移行期においてオステオカルシンの遺伝子発現が抑制され、非石灰化細胞集塊の形成が遅延することが示された。しかし、各薬剤について作用期間に関係なく、薬剤の作用が中止された後に骨分化が進行し、石灰化細胞集塊の形成時期に差はみられなかった。この結果は、これらの薬剤が骨芽細胞に可逆的に作用すること、および薬剤により骨分化が抑制されていた細胞は、薬剤の中止後に分化がより促進され、石灰化する際にはそれらの影響はほとんどなくなることを示唆している。

第2章では、NSAIDsによるPGE₂生成抑制に対する骨芽細胞の反応について検討した。POS細胞より正常な骨分化細胞としてイヌ骨髄由来間葉系幹細胞を骨分化させて用い、それらに炎症刺激 (1 ng/mlのヒトリコンビナント (rh) IL-1 β) を作用させた。カルプロフェン、メロキシカム、インドメタシンおよびロベナコキシブを20日間作用させた。第1章と同様にALP、オステオカルシンおよびCOX-1、COX-2、cPGES、mPGES-1、さらにPGE₂受容体であるEP2およびEP4遺伝子発現量の変動をqRT-PCRを用いて

評価した。また、ALP、オステオカルシンおよびPGE₂の培養液中濃度、ALP染色によるALP陽性細胞のコロニー形成状況およびアリザリンレッド染色による石灰化の観察を実施した。細胞におけるALP遺伝子発現および培養液中ALP活性は、培養開始から4日目に最大に達したが、薬剤の作用によってその発現が有意に抑制された。ALP陽性細胞のコロニー形成能も同様に抑制されたが、PGE₂の添加により、対照細胞と同様のレベルに回復することが確認された。培養開始20日目には、NSAIDsによってオステオカルシン蛋白の産生量が抑制されていなかったにも関わらず、石灰化の程度は対照細胞より有意に低下し、長期間のNSAIDs投与によるPGE₂産生の絶対量の減少がALPの発現を抑制していることが示された。なお、培養開始4日目にPGE₂産生関連酵素およびその受容体の遺伝子発現が増加しており、骨芽細胞では細胞周囲のPGE₂濃度低下に対応する代償的な反応が存在することが示唆された。一方、各NSAIDsにおいてPGE₂濃度に対する反応、およびその関連酵素と受容体の遺伝子発現量様式は異なると考えられた。

以上の結果から、NSAIDsの作用に対する骨分化細胞の反応としてPGE₂低下を補完する代償機構が存在し、薬剤（カルプロフェンおよびロベナコキシブ）によっては、骨分化に与える影響が限定的であることが示唆され、骨分化過程におけるNSAIDs使用による影響は最小限である可能性があると考えられた。

以上のように、本研究結果から、骨折発症動物に対するNSAIDsによる骨分化遅延は、その臨床的な使用様式ではかなり軽微であると考えられた。

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I hope this place will continuously remain as an academia for fostering not only intellectual, but also responsible, risk-taking and humble leaders. Because scientists sometimes forget that they also exist for human beings.