The Effect of Δ^{12}PGJ2 and PPAR γ Agonist on the Proliferation and Differentiation of Osteoblast

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I. Introduction

Inflammatory periodontal disease is the most important cause of tooth loss in adults. Although multifactorial, the pathogenesis of periodontitis involves the presence of the plaque-retentive factors that initiates local inflammatory reaction in a predisposed host, thus provoking edema, cell influx, and release of inflammatory mediators. Among these, eicosanoids, mainly prostaglandins, seem to be important candidates in causing tissue destruction and ultimately, alveolar bone loss. Prostaglandins (PG) are 20-carbon essential fatty acids, a family of biologically active molecules and synthesized in most tissues.

Early studies showed that PGs are important mediators of the inflammatory process and bone resorption, especially PGE_2 is the most potent agent. PGF_2 are detected in significantly high levels in inflammed gingival tissues, and up-regulate the production of the inflammatory cytokine, MMP-1.

However, increased bone formation was also confirmed in animal studies by both systemic and local injection of PGE_2, and it is through the divergent actions on growth and differentiation of oteoblastic cells.

PGD_2 is a early anti-inflammatory signal in experimental colitis, and stimulates calcification of human osteoblastic cells. PGJ_2 is formed from PGD_2 in vivo and PGJ_2 can be rapidly converted to $\Delta^{12}PGJ_2$ in the presence of plasma.

Unlike other PGs which act through plasma membrane receptors, $\Delta^{12}PGJ_2$ is a high affinity ligand to peroxisome proliferator-activated receptor gamma (PPAR γ). PPARs are ligand-activated transcription factors belonging to the nuclear receptor family. Although adipose tissue has been recognized as a principle site of expression of PPAR γ, it is expressed at lower levels in many other tissues and cell types, including cells of the monocyte/macrophage lineage, neutrophils, T lymphocyte, and chondrocytes. Recent studies in different cell types suggest that PPAR ligands not only regulate lipid and glucose homeostasis, but may also mitigate

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the inflammatory process.

The purpose of this study is to examine the expression of PPAR γ and the effect of Δ^{12} PGJ_2 on the proliferation and differentiation of human osteoblastic Saos-2 cell line and mouse osteoblastic MC3T3-E1 cells in vitro to investigate the function of $\Delta^{12}PGJ_2$ on ossification.

II. Review of Literature

1. Prostaglandins (PG)

1) Biosynthesis

Prostaglandins (PG) are 20-carbon essential fatty acids that contain three, four, or five double bonds with a cyclopentane ring, a family of biologically active molecules and synthesized in most tissues. The predominant precursor is arachidonic acid, cleaved from phospholipids in the lipid bilayer of cells. The first step in the formation of PGs involves intracellular release of arachidonic acid from plasma membrane phospholipids via the action of phospholipase A_2. The key step in the formation of PGs is the convertion of arachidonic acid by the cyclooxygenase and peroxidase activities of a single enzyme, PGH synthase (also called PG synthase or simply cyclooxygenase). One enzymatic action of COX is to convert arachidonic acid to PGG_2, and the other action is to reduce PGG_2 to PGH_2 by its peroxidase activity[1-2]. PGH_2 is converted to PGE_2, PGD_2, PGF2α, prostacyclin (PGI_2), and thromboxane by tissue-specific isomerase and reductase.

There are two forms of COX, constitutive form (COX-1) and inducible form (COX-2). 2 isozymes are encoded by different genes[3], and expression of COX-2 is induced by proinflammatory cytokines, mitogens, lipopolysaccharides [4-6] and mechanical tension force[7].

2) PG receptors

PGs have complex actions on bone metabolism that depend on interactions with different types and subtypes of receptors. Physiological actions of E,D, and F series prostaglandins are mediated by binding to specific high affinity trans-membrane G-protein coupled prostanoid receptors. For PGE_2, there are four classes of receptors: EP1, which increases the intracellular Ca 2+ concentration ; EP2 and EP4, which increases the intracellular cAMP levels ; and EP3, which mainly decreases the intracellular cAMP ceoncentration[8]. Suda *et al.* reported that EP1, EP2, and EP4 are present in MC3T3-E1 cells, EP1 promotes cell growth, and EP2 and EP4 mediate differentiation of osteoblast[9]. In human osteoblasts, the presence of DP,EP4, IP, FP and TP receptor mRNA was revealed in primary culture experiment[10].

On the other hand, the actions of cyclopentenone prostaglandins PGA_2,PGA_1, and PGJ_2 result from their interaction with other cellular target proteins. $\Delta^{12}PGJ_2$ is a high affinity ligand for the nuclear recepter PPAR γ [11].

3) Role of PGs on bone resorption

Early in vitro work showed that PGs are important mediators of the inflammatory process and bone resorption[12], prostaglandins of the E series are the most potent, while PGFs are somewhat less potent.

Akatsu *et al.* showed in their in vitro assay systems that PGE_2 is involved in the mechanism of IL-1 mediated osteoclast-like cell formation, and its direct interaction between osteoclast progenitors and osteoblastic cells is required in the osteoclast recruitment induced by PGE_2 and IL-1. [13] Gardner *et al.* reported IL-6 as another agonist to PG induced bone resorption in their neonatal mouse parietal bone culture study[14].

This led to clinical investigations of their possible role in in vivo conditions in which there was local-

ized bone resorption associated inflammation like periodontal disease. Elevated level of PG are detected in the crevicular fluid of periodontitis patients, and it has been associated with increased severity of the disease[15], and COX inhibition prevented alveolar bone loss in experimental periodontal disease model[16].

However, when comparison were made in some studies with prostanoid levels in gingival crevicular fluid, there was poor correlation between PG concentration and the stage of disease, indicating other resorbing factors, not sensitive to the antiprostaglandins were involved[17-18].

4) Role of PGs on bone formation

Many studies indicate PG is also a powerful bone forming agent. Yoshiyuki *et al.* first reported stimulatory effect of PGs on the differentiation of osteoblastic clone MC3T3-E1 cells ; PGE_2 stimulated ALP activity in the cells in a dose-depentednt fashion and PGE_2 also increased cAMP content with the maximal effective concentration of 100 ng/ml[19]. In fetal rat calvarial cell culture study, 3 X 10^{-8} M PGE_2 induced a 2-fold increase in mineralized bone nodule formation and a 1.5-fold increase in alkaline phosphatase activity without affecting cell growth, suggesting that PGE_2 may increase the proportion of functional osteoblasts able to produce mineralized bone nodules in the population by stimulating differentiation during the post-confluent stage of rat calvarial cell culture. [20] In assay system using a cultured cell from human femur explant, Koshihara *et al.* showed PGD_2 and its derivatives stimulate ALP activity and calcification unexpectedly, with potencies nearly equal to 1,25(OH)$2D_3$ at 10^{-8} M and suggested that the effective form is probably a metabolite, $\Delta^{12}PGJ_2$[21]. Later, Tasaki *et al.* reported that PGD_2 metabolite, $\Delta^{12}PGJ_2$ enhanced transcription of type I collagen mRNA synthesis at 10^{-5} M using the same cultural system[22].

Many in vivo studies also have shown that PGs are involved with increases in bone formation. In their series of extensive in vivo studies in rats, Jee *et al.* reported increased metaphyseal hard tissue and cortico-endosteal bone formation in growing rats,[23] production of new cancellous bone in the axial skeleton of ovariectomized rats[24], and increase of bone mass and activity of intracortical bone remodeling in intact and ovariectomized female rats[25]. While relatively high doses of PGE_2 were administrated in Jee *et al.*'s experiments, Yang *et al.* showed that a lower dose of PGE_2, as low as 1 pmol/day for 2 weeks induced a greater response in bone formation without significant changes of metaphyseal cancellous bone tissue in the contralateral control limb[26]. Takagi *et al.* reported that subcutaneous administration of a slow-release preparation of PGD_2 not only prevented the ovariectomy-induced suppression of bone mineral density, but also augmented the steady increase in bone mineral density of the sham-operated rat[27].

2. PGJ_2 : a member of the cyclopentenone prostaglandins

1) Biosynthesis and cellular target

The cyclopentenone prostaglandins PGA_2, PGA_1, and PGJ_2 are formed by dehydration within the cyclopentane ring of PGE_2, PGE_1, and PGD_2, respectively. PGJ_2 was discovered by Fukushima and coworkers in the course of studies of antitumor activity of PGD_2[28]. These investigators discovered that PGD_2 undergoes spontaneous dehydration in aqueous solutions to yield PGJ_2, a compound known previously only as a synthetic prostanoid. In aqueous solutions containing serum albumin, PGJ_2 isomerizes to yield $\Delta^{12}PGJ_2$[29]. Further study demonstrated that $\Delta^{12}PGJ_2$ had considerably more potent anti-tumor activity than the parent compound, PGD_2, but had

very low activity or was inactive in bioassay for PGD2 such as inhibition of platelet aggregation and relaxation of rabbit stomach strip[30]. These results suggested that the effect of $\Delta^{12}PGJ_2$ was not mediated by binding to high affinity DP receptors, but by an unidentified cellular targets. Much excitement was generated in 1995 when this compound was found to be a high affinity ligand for the peroxisome proliferator-activated receptor (PPAR γ)[31].

2) Influence on inflammation

It has been suggested that the locally produced 15 d-PGJ_2 may function as a negative feedback regulator of inflammation by the inhibition of pro-inflammatory genes in activated macrophages[32]. On the basis of transfection studies in macrophage-like cell lines, antagonism of the transcription factors AP-1, NF-κB, and STAT was implicated as the mechanism of the observed anti-implammatory effects[33].

Azuma *et al.* reported that $dPGJ_2$ inhibits LPS-induced IL-10 and IL-12 production by macrophages and the inhibition may be through PPAR γ[34].

However, growing body of evidence has emerged that PGJ_2 metabolites, in addition to the PPAR γ activation, exerts also PPAR γ-independent effects. Inhibition of inducible nitric-oxide synthase[35], TNF-α, IL-12[36] production by PGJ_2 in microglial cell and macrophage[37] does not appear to involve PPAR γ because these actions were not mimicked by other specific PPAR γ agonist. Boyault *et al.* reported that 15 d-PGJ_2 was highly potent to counteract IL-1β effects in human chondrocytes by inhibiting NF-κB and AP-1 (activator protein-1) activation pathway through PPAR γ-independent action, and suggested the possibility of 15 d-PGJ_2 as a modulater of inflammatory disease, osteoarthritis[38]. Some studies, however, indicated that 15d-PGJ_2 can be a mediator of inflammatory response by PPAR γ dependent[39], and independent pathway[40-41].

3) Influence on cell cycle

15d-PGJ_2 is recognized as a potent apoptotic and growth inhibitory factor. $\Delta^{12}PGJ_2$ effectively inhibited cell growth, caused a cell cycle arrest in G1 through a non-cAMP mediated mechanism[42]. 15 d-PGJ_2 is a potent apoptotic factor for human hepatic myofibroblasts, and 15 d-PGJ_2-induced cell death is independent of PPAR γ activation because PPAR γ is not expressed in these cells. [43] Kondo *et al.* showed that cyclopentanone prostaglandins are potential inducers of intracellular oxidative stress in human neuroblastoma cells (SH-SY5Y) and suggested that it may serves as second messenger of the apoptotic effect[44]. Details regarding the mechanism for anti-neoplastic activity remain to be determined.

3. PPAR γ

1) Classification and tissue distribution

The peroxisome proliferator-activatied receptors (PPAR γ) are a family of transcriptional factors belong to the nuclear receptor superfamily that includes the estrogen receptors, thyroid hormone receptors, and glucocorticoid receptors. So far, three distinct PPAR γ, termed α, δ (also called β, NUC-1 or FAAR) and γ, have been identified, each encoded by a separate gene and showing a distinct tissue distrubution[45].

The PPAR γ gene is transcribed into three PPAR γ messenger RNA (mRNA) speicies, that is PPAR γ1, PPAR γ2, and PPAR γ3, which are derived from alternative splicing and promoter usage[46]. Although adipose tissue has been recognized as a principal site of expression of PPAR γ2, PPAR γ1 is expressed in many other tissue and cell types, including hepatocyte, fibroblasts, myocytes, breast and colony epithelial cells, human marrow precursors, monocyte/machrophage lineage, neutrophils, and T lymphocytes[47-50]. PPAR γ can be activated by naturally

occuring arachidonic acid metaboletes derived from the cyclooxygenase pathway, such as $\Delta^{12}PGJ_2$, 15 d-PGJ_2[51], but also by synthetic ligands such as thiazolidinediones, which are insulin sensitizers used as orally active antidiabetic agents[52], or certain nonsteroidal antiinflammatory drugs (NSAIDs)[53].

2) Function of PPAR γ

PPAR γ was originally characterized as a regulator of adipocyte differentiation and lipid metabolism[54]. But, recent studies in different cell types suggest that PPAR γ ligands may be important antiinflammatory agents[55]. Fahmi *et al.* reported that PPAR γ is present and functionally active in human chondrocytes, and PPAR γ ligands inhibit IL-1 β induced production of nitric-oxide and MMP-13 at the transcriptional level, probably through repression of NF-κB and AP-1 signaling[56].

Few researches were undertaken about the role of PPAR γs in osteoblastic differentiation and proliferation. Czernik *et al.* recently reported that PPAR γ2 stimulate adipocyte differentiation, suppress osteoblast differentiation in U-33/γ2 cells, a model bipotential mesenchymal progenitor cell line[57]. On the other hand, Jackson *et al.* demonstrated that activators of PPAR α, δ, and γ induced alkaline phosphatase activity, matrix calcification and the expression of osteoblast genes as determined by reverse transcriptase-polymerase chain reaction[58]. The precise role of PPAR γ on osteoblastic cells is therefore not fully elucidated.

III. Materials and methods

1. Cell culture and incubation with $\Delta^{12}PGJ_2$ and ciglitazone

A human osteosarcoma cell line Saos-2 (ATCC. HTB 85) obtained from the American Type culture collection, and MC3T3-E1 osteoblasts (Riken, Japan) derived from a mouse calvaria, were grown to confluence in culture flask of 75 cm^2 surface (Falcon, Oxnard, CA). Cell lines were incubated at 37℃ in a humidified atmosphere containing 5 % CO_2 in air with 20 ml of minimum essential medium (α-MEM ; Gibco, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS ; Gibco NY) and 1 % penicillin- streptomycin solution (Gibco, NY).

When confluent monolayer was reached, the cells were enzymatically lifted from the dishes using 0.25 % trypsin and 4 mM EDTA (Gibco, Grand Island, NY). Aliquots of 20 μl of cell suspension were seeded into 24-well flat-bottomed tissue culture plates (Corning, New York, NY, USA) or 96-well multiplates at a density of 5 x 10^4 cells/well. The test groups were cultured with medium described above, except it contained 10^{-5} - 10^{-9} M of $\Delta^{12}PGJ_2$ (Cayman Chemicals, Ann Arber, MI) or 10^{-5} - 10^{-9} M of ciglitazone (Biomol, Plymouth Meeting, MA). The medium was changed every 2-3 days. For mineralization assay, cell lines were cultured in 6-well multiplates with αMEM supplemented with 50 μg/ml acsorbic acid (Sigma, St Louis, MO) and 10 mM β-glycerophosphate (Sigma, MO).

2. Cell proliferation assay

Cell proliferation was measured at 1, 2, and 3 days. For measurement of cell proliferation, Saos-2 and MC3T3-E1 cells were cultured in 96-well multiplates. The effects of $\Delta^{12}PGJ_2$, ciglitazone on the proliferation of these cells were determined by the tetrazorazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay. Briefly, 50 μl of 0.2% MTT in phospate buffered saline (PBS) was added to 200 ul of medium of each culture. After incubation for 4 h at 37℃, the medium was removed and the cells were dissolved in

DMSO. Then, the absorbance of lysates was measured at 540 nm.

3. Alkaline phosphatase activity

Production of alkaline phosphatase (ALPase) was measured spectroscopically at 2 days. After incubation, the adherent cells were removed from the wells by incubation of 1.0 ml of 0.25 % trypsin in 4 mM EDTA for 30 minutes at 37℃, washed with 0.5 ml of PBS, and centrifuged for 5 minute at 6.5 X 10 g.

The cell pellet was homogenized with 0.5 ml of double distilled water (DDW) and sonicated for 1 minute in ice. 0.1 ml of cell lysate were mixed with 0.1 ml of 0.1 M glycin-NaOH buffer, 0.1 ml of 15 mM *para*-nitrophenol phosphate (*p*NPP), 0.1 % triton X-100/saline and 0.1 ml of DDW. Each aliquots was incubated at 37℃ for 30 minutes. After incubation, each tube was added 2.5 ml of 0.1 N NaOH and placed on ice. The production of *para*-nitrophenol (PNP) in the presence of ALPase was measured by monitoring light absorbance by the solution at 405 nm. The slope of absorbance versus time plot was used to calculate the ALPase activity.

4. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

1) RT-PCR for expression of PPAR γ

Total RNA from cultured osteoblasts was isolated with the use of high pure RNA Isolation Kit (Roche Molecular Biochemicals, Manheim, Germany) according to manufacturer's instruction. RT-PCR assays were carried out with the thermal cycler (Effendorf).

Two micrograms of total RNA were reverse transcribed into complementary DNA (cDNA) with 2 units/㎕ RT (AMV reverse transcriptase, Roche Molecular Biochemicals), 2.0 ㎕ of 1 reaction buffer (100 mM Tris, 500 mM KCl ; pH 8.3), 4.0 ㎕ of 5 mM $MgCl_2$, 2.0 ㎕ of deoxynucleotide mix primer, 1.0 ㎕ of RNAase inhibitor at 25℃ for 10 minutes for annealing and then at 42℃ for 60 minutes for reverse transcription resulting in cDNA synthesis. Following the 42℃ incubation, the AMV reverse transcriptase is denaturated by incubating the reaction at 99℃ for 5 minutes and then cooling to 4℃ for 5 minutes.

The resulting single stranded DNA is amplified using the reverse transcribed mixture containing 250 ㎛M dNTP, 2 mM $MgCl_2$, 1 X volume of reaction buffer, and 0.5 unit of Taq polymerase (Roche) as a

Table 1. Nucleotide sequences of the primers used for RT-PCR

	Sequence	Expected size of PCR product (bp)
Human PPAR γ	(s) 5' -TCTCTCCGTAATGGAAGACC-3' (as) 5' -GCATTATGAGACGTCCCCAC-3'	474
Human type I collagen	(s) 5' -TATGGCGGCCAGGGCTCCGACCCTG-3' (as) 5' -CCAAGGGGGCCACATCGATGATGGG-3'	325
Human alkaline phosphatase	(s)5' -ACGTGGCTAAGAATGTCATC-3' (as) 5' -CTGGTAGGCGATGTCCTTA-3'	475
Human osteopontin	(s) 5' -CCAAGTAAGTCCAACGAAAG-3' (as) 5' -GGTGATGTCCTCGTCTGTA-3'	347
Human osteocalcin	(s) 5' -CATGAGAGCCCTCACA-3' (as) 5' -AGAGCGACACCCTAGAC-3'	310
18S ribosomal RNA	(s) 5' -GCGAATTCCTGCCAGTAGCATATGCTTG-3' (as) 3' -GGAAGCTTAGAGGAGCGAGCGACCAAGG-3'	126

template with the specific oligonucleotide primers for human PPAR γ [55] which were derived from known sequences (Table 1).

The PCR mixtures were incubated at 95℃ for 1 minute, followed by 35 cycles each at 94℃ for 30 seconds and 60℃ for 1 minute, with a final elongation step at 60℃ for 8 minutes. PCR product (10 μl/50 μl) reactions were separated on a 1.8 % agarose gel and stained with ethidium bromide.

2) Semi-quantitative RT-PCR for osteoblastic differentiation markers.

For quantitation of mRNA expression, primers for collagen type I (COL I), alkaline phosphatase (ALPase), osteopontin (OPN), osteocalcin (OCN), and 18s ribosomal RNA (rRNA) were used for reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from cultures of Saos-2 cells and reverse transcribed to cDNA as described above. Then the cDNA was amplified with gene-specific primers, which were derived from known sequences (Table 1) by using AMV reverse transcriptase mixture[59-60].

The mRNA level of 18s ribosomal RNA (rRNA) was analyzed in the same samples as an amplification control. The amplification condition was 95℃ (1 min) - 60℃ (2 min) for 23 cycles. PCR products were electrophorased onto a 2 % agarose gel containing ethidium bromide and visualized under UV light. The intensity of the bands was quantified by gel doc 2000 (Bio-Rad). The amount of RT-PCR products were compared to the amount of 18s rRNA, and relative expression ratios were obtained.

5. Mineralization assay

MC3T3-E1 cells were cultured in 12-well multiplates with α-MEM containing 50 ul/ml ascorbic acid, 10 mM β-glycerophoxphate (β-GP), and $\Delta^{12}PGJ_2$ or ciglitazone for 20 days. The medium was aspirated and the cells were fixed in 10% neutral formalin for 20 min at 4℃, washed with PBS, and stained with 2.5 % silver nitrate for 30 min.

For alizarin Red S staining, washed monolayers were fixed for 30 minuutes at room temperature in 10 % buffered formaline, washed 2 times with dH_2O, and stained for 10 minutes at room temperature with 2 % Alizarin Red S (Sigma, Aldrich) (pH 4.1-4.3). Monolayers were washed extensively with dH_2O. Alizarin Red S and von Kossa staining were visualized using light microscopy.

6. Statistical analysis

All measurements were collected in more than triplicate and expressed as means ± standard deviations. Analysis of differences were performed with one-way ANOVA with Fisher LSD test using SPSS version 11.0 program (SPSS, Chicago, IL). $p < 0.05$ and $p < 0.01$ were considered significant.

IV. RESULTS

1. PPAR γ mRNA expressed by osteoblastic cells

To investigate the expression of PPAR γ in osteoblastic cells, we performed an RC-PCR analysis using specific primers on RNA from Saos-2 cells. PPAR γ mRNA was detected as a single band with the predicted size (474 bp) in all 3 groups of cells ; control, $\Delta^{12}PGJ_2$, and ciglitazone treated cells (Figure 1).

2. Effect on the proleferation of Saos-2 cells

Because RT-PCR analysis indicated that the Saos-2

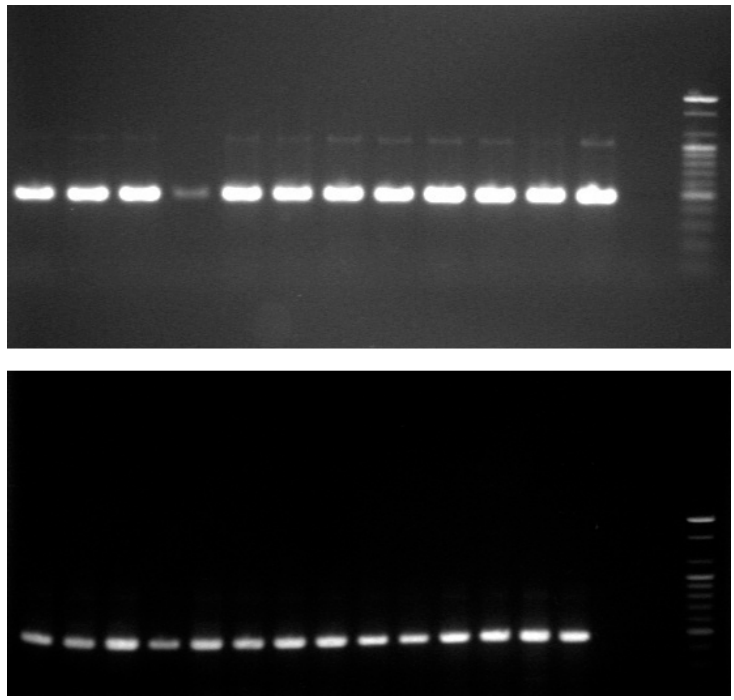


Figure 1. PPAR γexpression in Saos-2 cell

Table 2. Cell proliferation by MTT assay

		1 day	2 day	3 day
Control		0.584 ±0.039	0.604 ±0.069	0.743 ±0.058
Δ12PGJ$_2$ (M)	10^{-9}	0.582 ±0.315	0.637 ±0.085	0.762 ±0.044
	10^{-8}	0.604 ±0.042	0.647 ±0.035	0.832 ±0.065
	10^{-7}	0.584 ±0.037	0.615 ±0.040	0.715 ±0.063
	10^{-6}	0.583 ±0.024	0.598 ±0.053	0.730 ±0.047
	10^{-5}	0.494 ±0.043	0.374 ±0.082	0.333 ±0.036
Ciglitazone (M)	10^{-9}	0.596 ±0.055	0.595 ±0.070	0.740 ±0.034
	10^{-8}	0.562 ±0.034	0.615 ±0.079	0.724 ±0.027
	10^{-7}	0.587 ±0.055	0.634 ±0.098	0.731 ±0.061
	10^{-6}	0.557 ±0.043	0.655 ±0.093	0.772 ±0.067
	10^{-5}	0.506 ±0.062	0.628 ±0.055	0.751 ±0.061

cells have PPARγmRNA, we next investigated the biological activity of Δ^{12}PGJ$_2$ and selective PPARγ agonist, ciglitazone, on the cells. First we studied the effect on the proliferation of the cells. As shown in Table 2, Δ^{12}PGJ$_2$ stimulated the proliferation of Saos-2 cells inversely proportional to concentration in 10^{-5} - 10^{-8} M range. Ciglitazone also stimulated proliferation, but the effect was modest. Both agent indicated inhibitory effect at 10^{-5} M (Figure 2). When 10^{-8} M of Δ^{12}PGJ$_2$ and 10^{-6} M of ciglitazone are compared to control cells, they indicated higher proliferation rate in a time-dependent manner and their

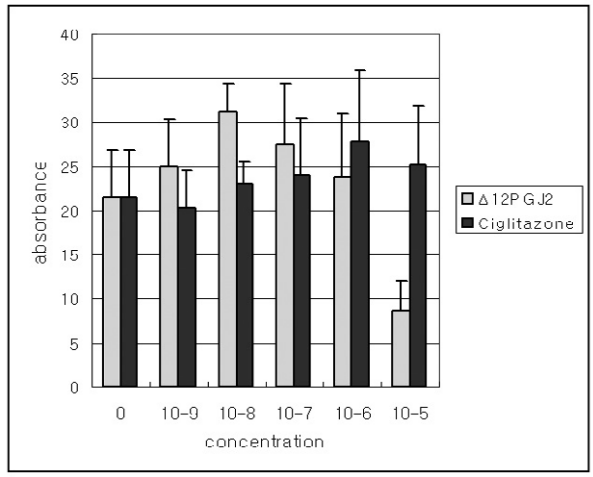


Figure 2. Effect of the concentration of $\Delta^{12}PGJ_2$ and ciglitazone on the proliferation of Saos-2 cells at day 2

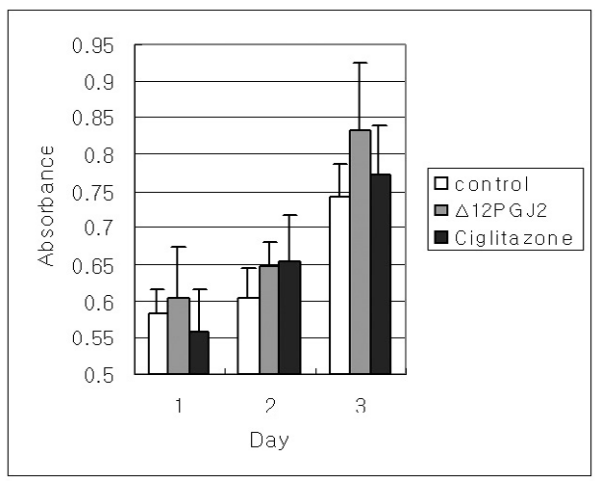


Figure 3. Effect of the $\Delta^{12}PGJ_2$ and ciglitazone on time-course proliferation

Table 3. ALPase activity (nM/㎍/30 min)

	control	10-9	10-8	10-7	10-6	10-5
Δ12PGJ2	21.42 ±5.44	24.94 ±5.33	31.18 ±3.22	27.48 ±6.78	23.82 ±7.15	8.68 ±3.36
Ciglitazone	21.42 ±5.44	20.36 ±4.09	22.94 ±2.53	23.94 ±6.59	27.78 ±8.01	25.14 ±6.70

effect were observable after 1day (Figure 3). Here, the effect of ciglitazone was more modest than $\Delta^{12}PGJ_2$.

3. ALPase activity

The effect on ALPase was investigated at day 2 in 10^{-5} - 10^{-9} M concentration range. The stimulation pattern was similar to that of proliferation ; $\Delta^{12}PGJ_2$ stimulated the ALPase activity of Saos-2 cells mostly at 10^{-8} M and ciglitazone modestly at 10^{-6} M. (Table 3, Figure 4). So, there seems to be appropriate maximum effective concentration in both agents.

4. Effects of $\Delta^{12}PGJ_2$ and ciglitazone on the expression of mRNA of osteoblastic- differentiation in Saos-2 cells.

Based upon the results of MTT assay and ALPase activity test, the appropriate concentrations for $\Delta^{12}PGJ_2$ and ciglitazone were determined as 10^{-8} M and 10^{-6} M for Saos-2 cells, respectively. To investigate the effect on the expression of the differentiation markers of osteoblast, we next performed semi-quantitative RT-PCR using specific primer sets at day 1 and day 7. The relative mRNA levels of COLI and ALPase in the $\Delta^{12}PGJ_2$ treated cells were significantly higher than those in ciglitazone treated cells or control cells (Figure 5). The relative mRNA levels of OPN were also higher in $\Delta^{12}PGJ_2$ treated cells (Figure 6).

5. Effect on the mineralization of MC3T3-E1 cells

To investigate whether $\Delta^{12}PGJ_2$ and ciglitazone induced formation of mineralized bone nodules by MC3T3-E1 cells, these cells treated with 10^{-5} - 10^{-9} M of $\Delta^{12}PGJ_2$ or ciglitazone were cultured in the presence of 10 mM β-GP and 50 μg/ml ascorbic acid for 30 days. MC3T3-E1 cells treated with 10^{-6} M of $\Delta^{12}PGJ_2$ and 10^{-5} M of ciglitazone showed marked mineralization whereas untreated control cells showed only slight mineralization.

V. Discussion

Prostaglandins are lipid regulators of a number of

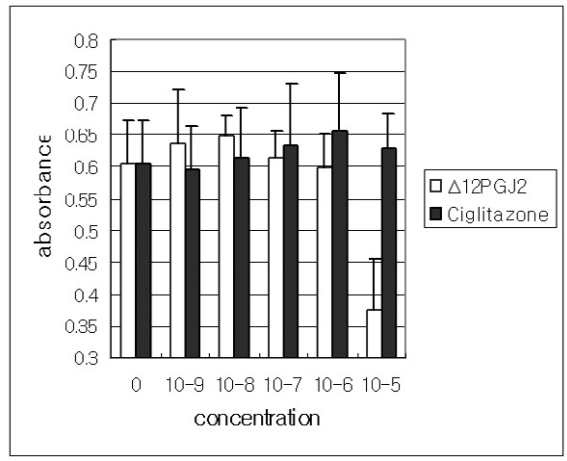
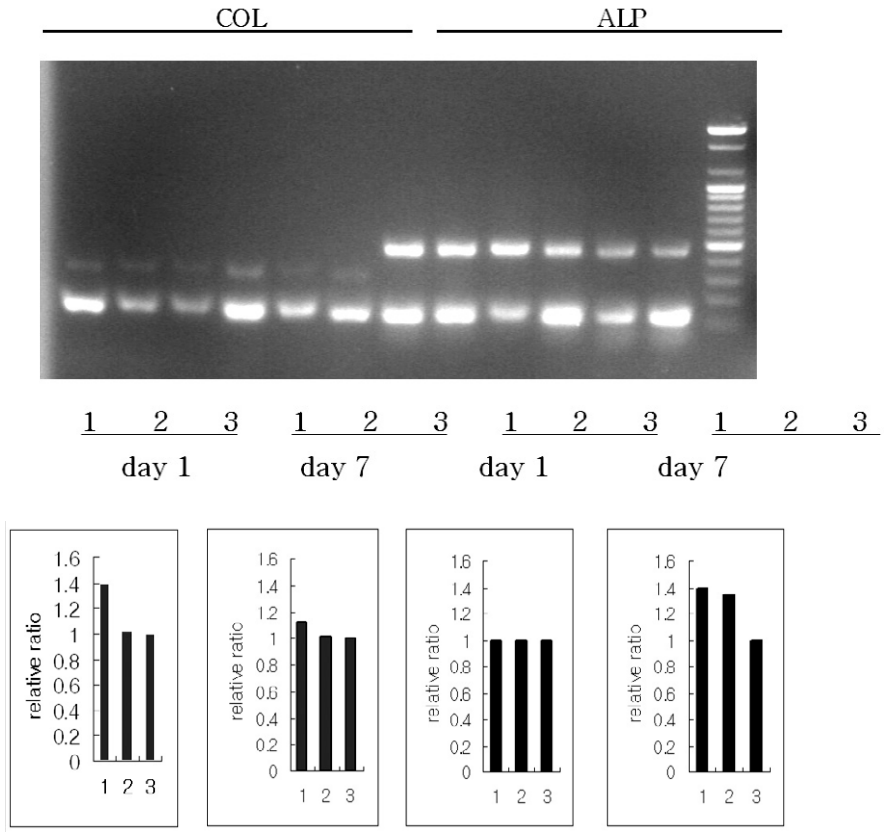


Figure 4. Effect of the concentration of $\Delta^{12}PGJ_2$ and ciglitazone on the ALPase activity of Saos-2 cells at day 2



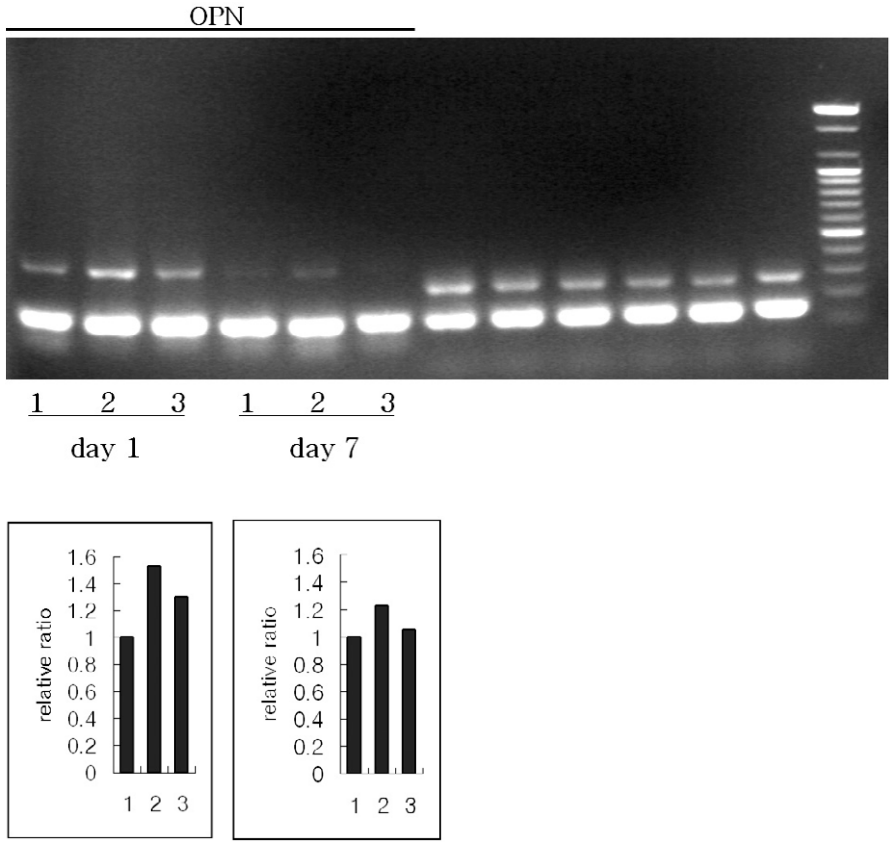
lane 1 : delta12 PGJ2 treated cells
lane 2 : Ciglitazone treated cells
lane 3 : Control cells

Figure 5. Effect of the mRNA expression of COL1 and ALPase

important cellular processes. Much of the prostaglandin literature on bone has focused on major PGs and the role of cell surface receptors in mediating the pleiotropic effects of these compounds. However, given their circulating concentrations, lower molecular weights, and lipophilicity, it seems plausible that a subset of prostaglandins could activate nuclear receptors directly or indirectly after diffusion into target cells.

In this study, we showed that PPAR $\gamma$is expressed in human osteoblastic cell line, Saos-2 cells at the mRNA levels. Jackson et al. also reported the expression of PPAR γ1, α, and δmRNA in mouse osteoblastic cell line, MC3T3-E1[58]. They showed these PPARs were functional in transfection study using a PPRE-luciferase promoter-reporter construct containing 110 bp of the acyl CoA oxidase promotor, harboring a binding site for PPARs. With this result, we next investigate the effect of $\Delta^{12}PGJ_2$, known as a natural PPAR γligand, on the proliferation and differentiation of human osteoblastic cell line, Saos-2 cell and compare the result with that of ciglitazone, a synthetic selective PPAR γagonist.

In present investigation, both of $\Delta^{12}PGJ_2$ and



lane 1 : Control cells
lane 2 : delta12 PGJ2 treated cells
lane 3 : Ciglitazone treated cells

Figure 6. Effect on the mRNA expression of OPN and OCN

ciglitazone directly stimulated the proliferation of human osteoblastic cells, but their maximum effective concentration was defferent. While $\Delta^{12}PGJ_2$ supported the proliferation of Saos-2 cells at low concentration, ciglitazone at relatively high concentration. And both of agents inhibited the proliferation of this osteoblastic cell line at 10^{-5} M. These findings also showed with mouse osteoblastic cell line, MC3T3-E1 in our laboratory (data not-shown). Consistant of this, Jackson et al. reported inhibitory effect of ciglitazone at 10^{-5} M concentration to mouse osteoblastic cell line[58]. Yu et al. also reported in their experiment with series of eicosanoids, that 10^{-4} M of PGD_2 caused death of the human osteosarcoma line, U2OS cells transfected with PPAR γ fusion protein[51]. Considering the fact that eicosanoids generally circulate at low levels (less than 1 nM) in the plasma, concentration below those at which they are normally able to elicit responses[61] and 10^{-5} M is non- physiologic, excessive concentration, it is thought that the cytotoxicity at this high concentration doesn't have significant meaning.

In this study, we have demonstrated that 10^{-8} M of

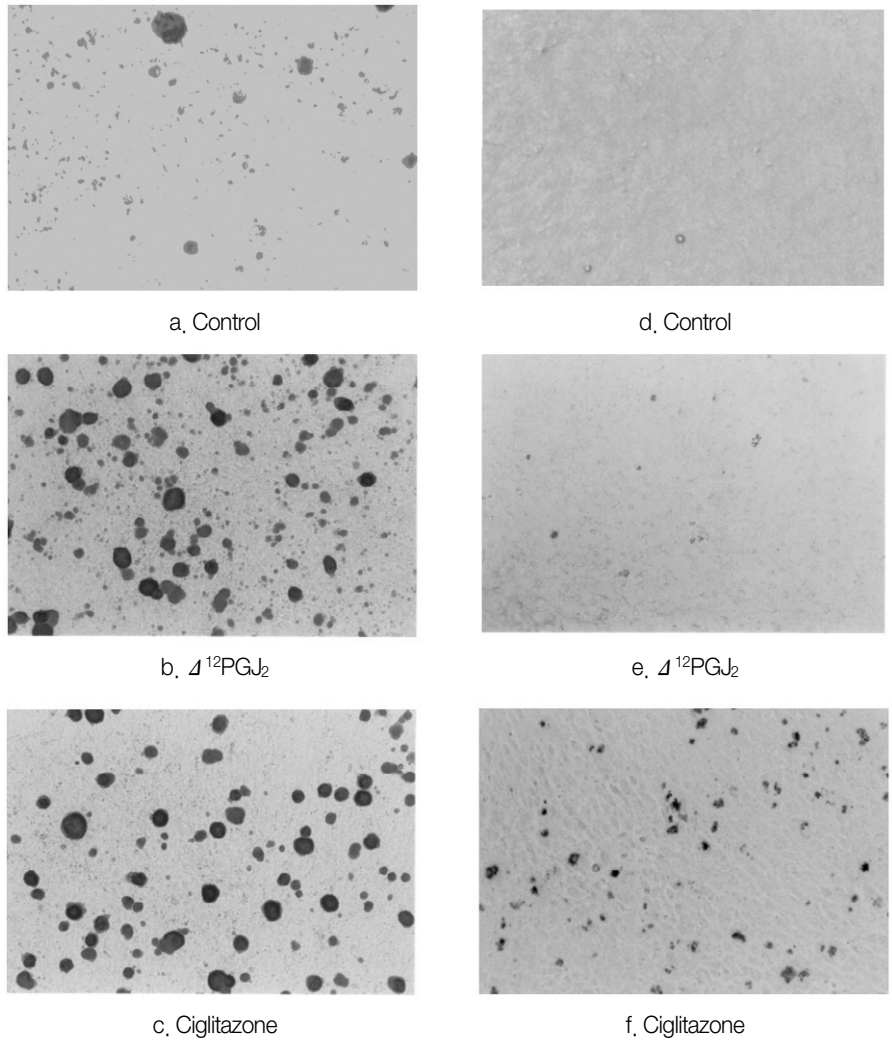


Figure 7. Effects on mineralization of MC3T3-E1 cells
(X100, a, b, c - Alizarin red S stain / d, e, f- von Kossa stain)

$\Delta^{12}PGJ_2$ and 10^{-6} M of ciglitazone stiumlated human osteoblastic differentiation using semi-quantitative RT PCR analysis. And we also showed that 10^{-6} M of $\Delta^{12}PGJ_2$ and 10^{-5} M of ciglitazone markedly increased formation of mineralized bone nodules by MC3T3-E1 cells. PPAR γ was originally known as a key player of adipocyte differentiation and Czernik *et al.* reported that PPAR γ ligand, 15-d PGJ_2 and rosiglitazone, stimulates adipogenesis and inhibits osteoblastogenesis in U-33/γ2 cells, mesenchymal progenitor of adipocytes and osteoblasts[57]. But, in their study, inhibition of osteoblastic differentiation required the induced expression of endogenous PPAR γ2, which was not detected in MC3T3-E1 cells. So, it is thought that PPAR γ ligands act differently to cells in different levels of mesenchymal lineage commitment. Our data are in agree with Koshihara et al. who reported in their human femur

explant cultural study, that PGD_2 stimulates ALPase activity and calcification unexpectedly, with potencies nearly equal to $1.25(OH)_2D_3$ at 10^{-8} M[21]. They suggested that active metabolite is $\Delta^{12}PGJ_2$, because PGD_2 is easily metabolite by plasma albumin to give $\Delta^{12}PGJ_2$ and PGD_2 added to a culture medium containing 10 % fetal bovine serum and incubated at 37 ℃ for 2 days is converted 100 % into $\Delta^{12}PGJ_2$. Tasaki et al. also reported that 10^{-5} M of $\Delta^{12}PGJ_2$ stimulate collagen synthesis by human osteoblasts from femur explant, though they didn't know the mechanism of action[22]. In our investigation, maximum effective concentraion of $\Delta^{12}PGJ_2$ on Saos-2 cell line was 10^{-8} M, lower than previous studies, indicating that this cell line is more sensitive to this bone inducing prostanoid.

Ciglitazone belongs to the thiazolidinedione (TZD) class of insulin-sensitizing drugs, and is clinically used as an oral antidiabetic agent for NIDDM patients. These drugs were originally developed without any knowledge of their molecular targets, but later known to act as direct agonists for PPARγ, and TNF-α, leptin, lipoprotein lipase, aP2 (fatty-acid binding protein) and GLUT4 are suggested as potential target genes for PPARγ relevant for the antidiabetic action of the TZD[54]. It is well known that periodontal tissue destruction is one of the serious complications of DM[62]. So, it is likely that favorable periodontal state of controlled diabetic patients is due partly to direct action of TZD on osteoblastic cells if pharmacological does of this drug is sufficient to stimulate PPARγ at these sites. This assumption awaits further research.

The underlying molecular mechanisms by which PPARγ affects proliferation and differentiation of osteoblastic cells are not fully understood. A putative PPAR binding site was identified in the promotor of the osteopontin gene[63] and PPAR induces expression of the glucose transporter, GLUT4 during adipocytic differentiation which was recently shown to be required for bone growth, perhaps related to increased energy requirements during matrix synthesis[64]. It is possible that the PPAR-independent pathway also exists because high affinity ligand, ciglitazone showed more modest effect than $\Delta^{12}PGJ_2$ in our investigation.

Many independent series of studies on $\Delta^{12}PGJ_2$ metabolites indicate that this agents mitigate the inflammatory process by inhibiting pro-inflammatory cytokines at the transcriptional level[32-38], and several observations suggests that $15dPGJ_2$ functions as a physiological negative feedback regulator of prostaglandin synthesis by repressing the COX-2 expression[65,66]. In view of the fact that proinflammatory cytokines play crucial roles in the connective tissue destruction seen in periodontitis[67], it would be beneficial to apply this agent for resolution of chronic inflammation of the periodontal tissue.

It is hasty to consider the clinical application of this bone inducing prostanoid in periodontal field, because the majority of studies are limited to in vitro study, and the physiological role of these compound in vivo needs further research. Nevertheless, it will be promising field of research considering the favorable biologic effect on osteoblastic cells and anti-inflammatory function of these compound.

VI. Conclusion

In this study, the expression of PPARγ was examined by RT-PCR analysis using specific primers on RNA. PPARγ mRNA was detected as a single band with the predicted size (474 bp) in human osteoblastic Saos-2 cell line. Next, the effects of $\Delta^{12}PGJ_2$ and ciglitazone on proliferation and differentiation of human and mouse osteoblastic cell line were examined. In MTT assay, both agents stimulated the proliferation of Saos-2 cells. When 10^{-8} M of

$\Delta^{12}PGJ_2$ and 10^{-6} M of ciglitazone are compared to control cells, they indicated higher proliferation rate in a time-dependent manner and their effect were observable after 1 day. When the effect on ALPase activity was investigated at day 2 in 10^{-5} - 10^{-9} M concentration range, the stimulation pattern was similar to that to proliferation, $\Delta^{12}PGJ_2$ stimulated the ALPase activity of Saos-2 cells significantly at 10^{-8} M and ciglitazone modestly at 10^{-6} M. To investigate the effect on the expression of the differentiation markers of osteoblast, we next performed semi-quantitative RT-PCR using specific primer sets. The relative mRNA levels of type I collagen and ALPase in the $\Delta^{12}PGJ_2$ treated cells were significantly higher that those in ciglitazone treated cells or control cells. The relative mRNA levels of osteopontin were also higher in $\Delta^{12}PGJ_2$ treated cells. MC3T3-E1 treated with 10^{-6} M of $\Delta^{12}PGJ_2$ and 10^{-5} M of ciglitazone showed marked mineralization whereas untreated cells showed only slight mineralization. These findings show favorable biologic effect of $\Delta^{12}PGJ_2$ on osteoblastic cells and its mechanism of action may involved PPAR γdependent pathway.

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-국문초록-

$\Delta^{12}PGJ_2$ 및 PPAR 감마 길항체가 조골세포의 증식 및 분화에 미치는 효과

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1. 목적

Prostaglandin은 치주질환과 관련된 국소적 골 대사에 중요한 역할을 한다. $\Delta^{12}PGJ_2$는 생체 내에서 혈장의 존재 하에 형성되는 천연 PGD_2 대사산물이며 peroxisome- proliferator에 의해 활성화되는 감마 수용체 (PPAR γ)에 대해 높은 친화성을 갖는 리간드로서 핵 수용체군에 속하는 전사조절인자이다. 이 연구의 목적은 골화 과정에서 $\Delta^{12}PGJ_2$의 역할을 규명하기 위해, 조골세포주의 증식과 분화에 미치는 영향과 그에 관련된 세포기전을 조사하는 데에 있다.

2. 방법

인간 골육종세포주인 Saos-2 (ATCC.HTB 85)와 쥐의 조골세포주 (MC3T3-E1)를 배양한 후 실험군에 농도가 각각 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} 몰인 $\Delta^{12}PGJ_2$와 ciglitazone (합성 PPAR 감마 길항체)를 첨가하였다. 조골세포에서 PPAR 감마의 발현을 관찰하기 위해 역전사효소-중합효소연쇄반응(RT-PCR)을 특정한 primer를 이용하여 시행하였다. 세포 증식은 1 일, 2 일, 3 일째에 MTT 분석법으로 측정하였고, 2 일째에 알칼리성 인산효소 (ALPase) 생산을 측정하였다. 위의 결과에서 얻은 적정한 농도에서 다양한 조골세포 분화의 표지자들-제 1 형 교원질, 알칼리성 인산효소, osteopontin 및 bone sialoprotein-에 대한 간이 정량적 역전사효소-중합효소연쇄반응 (semi-quantitative RT-PCR)을 실시하였으며 골결절 형성에 대한 효과를 알아보고자 석회화 분석도 시행하였다.

3. 결과

$\Delta^{12}PGJ_2$와 ciglitazone 모두 Saos-2 세포주의 증식을 촉진시켰다. 10^{-8} 몰의 $\Delta^{12}PGJ_2$와 10^{-6}몰의 ciglitazone을 첨가한 실험군을 대조군과 비교했을 때, 시간에 비례하여 세포 증식률이 증가되었다. 알칼리성 인산효소의 활성화 검사에서도 증식률에서와 유사한 결과를 보여주었다. 간이 정량적 RT-PCR에서는 $\Delta^{12}PGJ_2$로 처리한 군의 경우 제 1 형 교원질, 알칼리성 인산효소, osteopontin, 그리고 bone sialoprotein의 상대적 mRNA 수준이 유의하게 높았다. 석회화 분석에서는 MC3T3-E1 세포를 10^{-6} 몰의 $\Delta^{12}PGJ_2$로 처리한 군과 10^{-5} 몰의 ciglitazone으로 처리한 군에서 현저한 골결절 형성을 보였다. 이러한 결과들은 $\Delta^{12}PGJ_2$가 유용한 골 유도물질이 될 수 있으며 또한 그 작용기전이 PPAR 감마-의존형 경로와 연관되어 있음을 보여준다.

주요어 : $\Delta^{12}PGJ_2$, PPAR γ, ciglitazone, 조골세포, 역전사효소-중합효소연쇄반응