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치의학과

강 순 원

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유전자 발현에 미치는 효과

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ABSTRACT

Effect of ibuprofen on differentiation of osteoblastic cells

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Nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and indomethacin inhibit prostaglandin E_2 production, are commonly prescribed to reduce inflammation and pain, and slow bone loss in naturally occurring periodontal disease in beagle dogs and humans. In this study, we investigated the effects of ibuprofen on the proliferation, mRNA levels related to bone differentiation and pro-survival protein, b cell lymphoma-2 (bcl-2), of osteoblastic cells (MG63).

For the this study, a human osteogenic sarcoma cell line (MG63) was used for proliferation rate (MTT assay) and total RNA extraction. The mRNA levels of alkaline phosphatase (ALP), collagen type I (COL-1), osteopontin (OPN), osteocalcin (OC), and Bcl-2 were evaluated in cells cultured with various doses of ibuprofen (in doses of 0.03 mM, 0.06 mM, 0.08 mM, 1 mM, 3.5 mM, 7 mM, 10 mM) using reverse transcription polymerase chain reaction (RT-PCR) analysis.

The results were as follows;

1. At high concentration from 7 mM to 1 mM of ibuprofen, cell proliferation rate was decreased. But, at low concentration from 0.039 mM to 0.625 mM of ibuprofen, it was about 6~40% increased.

2. The effects of ibuprofen on COL-1 mRNA in MG63 cells showed a little decreased tendency at all concentrations of ibuprofen except 3.5 mM ibuprofen compared to control.
3. The effects of ibuprofen on ALP mRNA in MG63 cells showed the slightly increased tendency at all concentrations of ibuprofen compared to control.
4. The effects of ibuprofen on OC mRNA in MG63 cells revealed the slightly increased tendency at all concentrations of ibuprofen except 7.0 mM ibuprofen compared to control.
5. The effects of ibuprofen on OPN mRNA in MG63 cells revealed dose-dependently decreased tendency. Especially, the value of OPN mRNA in 7 mM was 4 times as high as control.
6. The effects of ibuprofen on bcl-2 mRNA in MG63 cells revealed the decreased tendency at all the concentrations of ibuprofen compared to control.

Taken altogether, the local regulatory factors (ALP, especially OPN, and OC) produced by ibuprofen-treated cells were greater than those of the control, in certain concentrations of ibuprofen. Also, anti-apoptosis related gene, such as Bcl-2 was down-regulated by Ibuprofen. Further studies will be needed in relation to periodontal regeneration and anti-apoptosis at various doses (from 0.625 mM to 7 mM) of ibuprofen.

I. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and indomethacin inhibit prostaglandin E_2 production, are commonly prescribed to reduce inflammation and pain. NSAIDs slow bone loss in naturally occurring periodontal disease in beagle dogs and humans. This effect occurs without changes in gingival inflammation and rebounds 6 months after cession of administration of the drug[1]. NSAIDS may have a potential adjunctive role in periodontal therapy[2]. In addition to, some NSAIDs inhibits proliferation and induces apoptosis of cancer cells at higher concentration[3], and ibuprofen as a NF-kappaB inhibitors may contribute to the reduction of invasiveness of pancreatic cancer[4]. However, use of NSAIDs increases the risk for gastrointestinal tract mucosal injury and other complications, especially bleeding, and the effects of platelet dysfunction[5].

In the present study, author evaluated the effect of ibuprofen on the expression of bone differentiation markers in osteoblastic cells (MG63). Ibuprofen belongs to the 2-aryl propionic-acid derivatives and consists of two enantiomers, of which S-ibuprofen is a potent COX-1 and COX-2 inhibitor whereas the R-enantiomer is two to three orders of magnitude less potent to inhibit COX[6].

The effect of ibuprofen on bone healing (formation/loss) is controversy. Obeid et al.[7] suggested that ibuprofen has an adverse effect on the healing of bone and cartilage in the temporomandibular joint of the rabbit. On the other hand, Yazdi et al.[8] reported that there appeared to be an enhancement of bone formation by ibuprofen. Offenbacher et al.[9] demonstrated that NSAIDs treatments significantly retarded the rate of bone loss.

However, the underlying molecular mechanism by which ibuprofen worked in differentiation of osteoblastic cells has been poorly understood. In this study, author investigated the effects of ibuprofen on the proliferation, mRNA levels related to differentiation and bcl-2, of osteoblastic cells (MG63).

Ⅱ. Materials and methods

1. Culture of human osteogenic sarcoma cell line (MG63)

A human osteogenic sarcoma cell line (MG63) was cultivated in Dulbecco's modified Eagle's Medium (DMEM, Gibco BRL, Rockville, MD) supplemented with and antibiotics (100 ㎍/㎖ penicillin, 100 ㎍/㎖ streptomycin, 50 ㎍/㎖ gentamicin, and 0.25 ㎍/㎖ fungizone) in free fetal bovine serum. Cultivation was performed at 37℃ in humidified atmosphere containing 5% CO_2. After 48 hours, the cells were plated $2X10^4$ cells/well in 60 ㎜ plates. When the cells were confluent, all cells were starved for 12 hour.

At the beginning of the culture, only antibiotics were added to DMEM. With 10 ~ 0.08 mM ibuprofen, after 24 hour of the culture, cells were washed with DMEM twice and scraped off and transferred to microtubes. They were used for proliferation assay and total RNA extraction. The mRNA levels of collagen type 1 (COL-1), osteopontine (OPN), alkaline phosphatase (ALP), osteocalcin (OC), and b cell lymphoma-2 (bcl-2) were compared treated cells with various dose ibuprofen.

2. Cell proliferation assay

To determine cell proliferation, the MG63 cells were plated at a density of $2X10^4$ cells per well in 96 well plates. After incubation for 24 hours, the culture medium was replaced by various doses of ibuprofen, in doses of 0.03 mM, 0.06 mM, 0.08 mM, 1 mM, 3.5 mM, 7 mM, and 10 mM. At 4hours before the end of incubation, the cells were washed twice with 10 mM phosphate-buffered saline (PBS, pH 7.2), and then incubated with 0.5

㎎/㎖ MTT, 2 ㎎/㎖ stock solution (Sigma, USA) for the last 4 hours. The medium was then decanted, the cells were incubated with 10% SDS and 0.01M HCl for 2 hours, and the absorbance was determined at 570 ㎚ using an enzyme linked immunosorbent assay reader (ELISA, BIO-TEK Instruments, USA).

3. RNA extraction and RT-PCR

Total RNA was extracted from cells by homogenizing with Trizol Reagent on 24 hour of culture. cDNA was synthesized by reverse transcription of 5 ㎍ samples of RNA in 20 ㎕ of master mix containing 200 U/㎕ superscript™ II (Invitrogen), 5 mM $MgCl_2$, first strand buffer, 1 mM dNTP, 1 U/㎕ RNase inhibitor™, and 2.5 mM oligodT in DEPC-treated distilled water. The master mix was incubated in a PCR at 42℃ for 50-min. and 96℃ for 10-min. Synthesized cDNAs were subjected to 30 cycles of amplification under the following conditions: 94℃ denaturing for 5-min., 65℃ annealing for 1-min. and 72℃ extension for 1-min (Table 1).

4. Statistical analysis

Numerical values are expressed as the mean ±SD, n=3 per group. In all studies, three similar experiments were performed for each group. Statistical differences among the experimental groups were evaluated by analysis of variance followed by Kruscal-Wallis test; *, p values < 0.05, **, p values < 0.005 versus control were considered statistically significant.

Table 1. Amplification primer sets used in polymerase chain reaction

Primer		Sequences 5′-3′	Product size	NCBI Accession No.
GAPDH	sense	5'-GGAGTCCACTGGCGTCTTCA-3'	182	NM_002046
	antisense	5'-AGCAGTTGGTGGTGCAGGAG-3'		
ALP	sense	5'-CGTGGTCACTGCGGACCATT-3'	219	NM_000478
	antisense	5'-GCAGACTGCGCCTGGTAGTT-3'		
COL-1	sense	5'-CTTCCTGCGCCTGATGTCCA-3'	192	NM_000088
	antisense	5'-CTCGTGCAGCCATCGACAGT-3'		
OPN	sense	5'-ACAGCCAGGACTCCATTGACTCGAACGACTCT-3'	198	NM_000582
	antisense	5'-CCACACTATCACCTCGGCCATCATATGTGTCT-3'		
OC	sense	5'-AGCGGTGCAGAGTCCAGCAA-3'	190	NM_199173
	antisense	5'-AGCCGATGTGGTCAGCCAAC-3'		
Bcl-2	sense	5′-CAATGGTGGGGAACTATAAA-3′	249	EU287875
	antisense	5′-ATTCCATCAATGTTTCAAGG-3′		

GAPDH; glyceraldehyde-3-phosphate dehydrogenase: ALP; alkaline phosphatase; COL-1; collagen type 1: OPN; osteopontin: OC; osteocalcin: Bcl-2; B cell lymphoma-2.

III. Results

1. Effect of ibuprofen on the proliferation of the MG63 cells

MTT assay for the proliferation of MG63 cells to ibuprofen was performed and showed a 6~40% increase in cells treated with 0.625 mM ~ 0.039 mM ibuprofen. At high concentration from 1 mM to 7 mM of ibuprofen, the value of MTT was decreased. But, at low concentration from 0.039 mM to 0.625 mM of ibuprofen, the value of MTT was about 6~40% increased (Fig. 1).

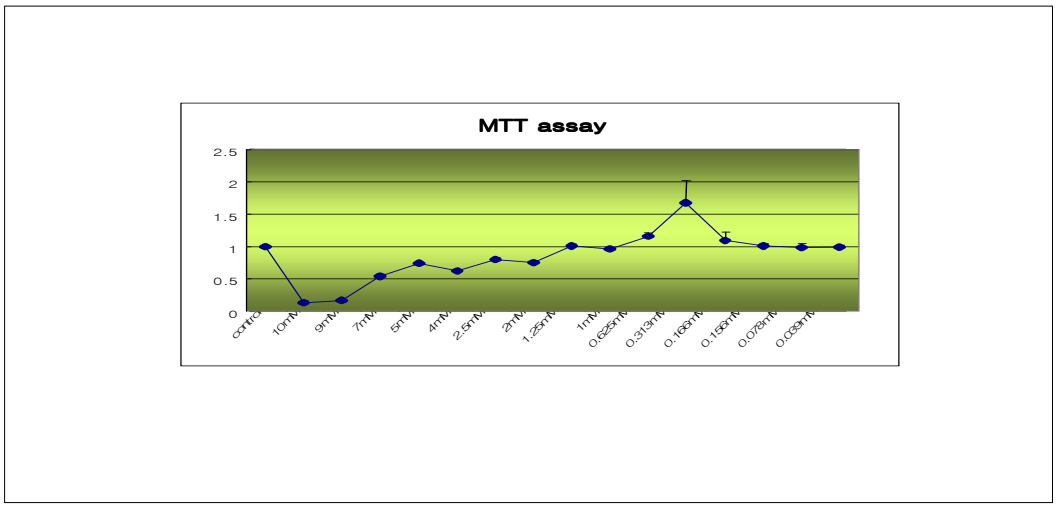

Fig. 1. MTT assay; the effect of ibuprofen on proliferation of MG63 cells.

2. Effect of ibuprofen on bone differentiation marker genes

1) Expression of COL-1 mRNA

The effects of ibuprofen on COL-1 mRNA in MG63 cells showed the decreased tendency at all concentrations of ibuprofen except 3.5 mM ibuprofen compared to control ($p>0.05$) (Fig. 2).

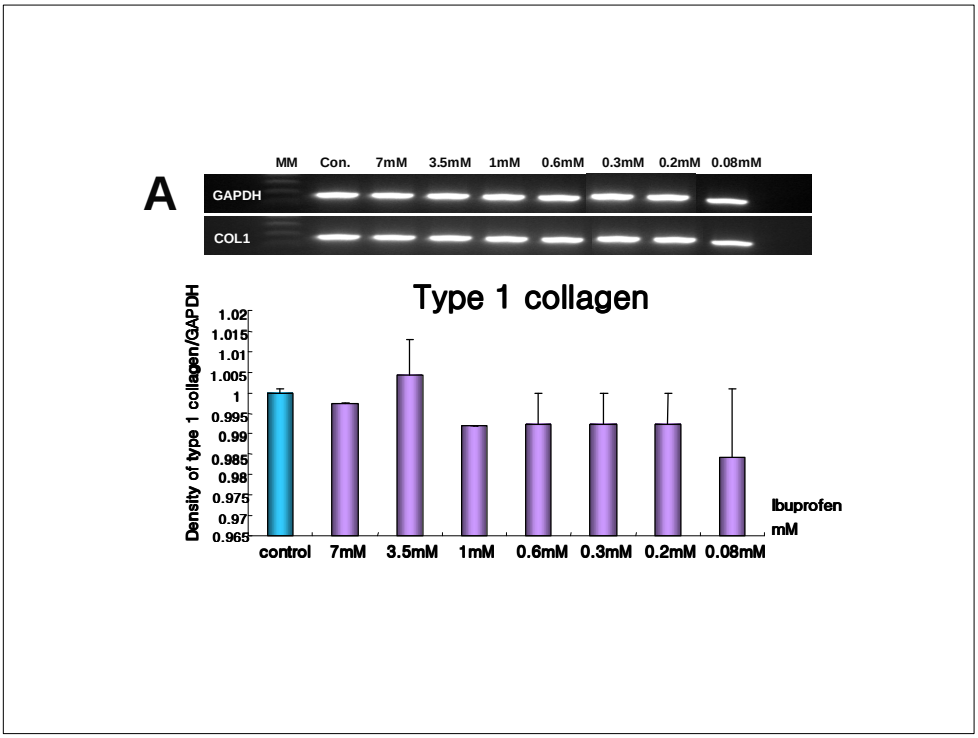

Fig. 2. Effect of ibuprofen on expression of COL-1 mRNA in MG63 cells.

2) Expression of ALP mRNA

The effects of ibuprofen on ALP mRNA in MG63 cells showed the slightly increased tendency at all concentrations of ibuprofen compared to control ($p>0.05$) (Fig. 3).

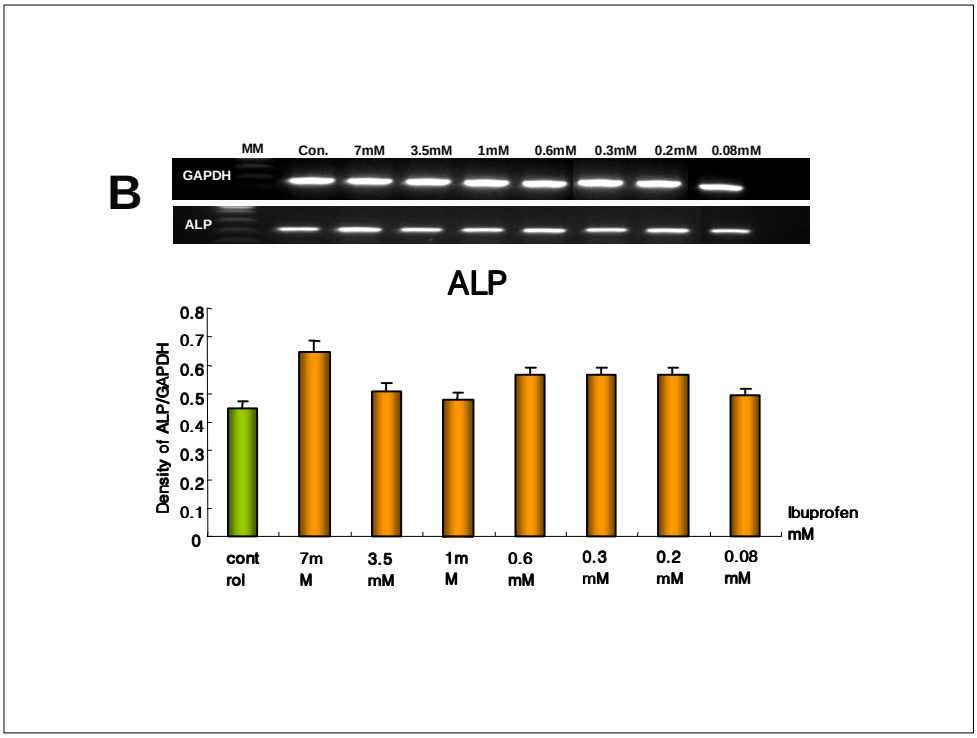

Fig 3 Effect of ibuprofen on expression of ALP mRNA in MG63 cells.

3) Expression of OC mRNA

The effects of ibuprofen on OC mRNA in MG63 cells revealed the slightly increased tendency at all concentrations of ibuprofen except 7.0 mM ibuprofen compared to control ($p>0.05$) (Fig. 4).

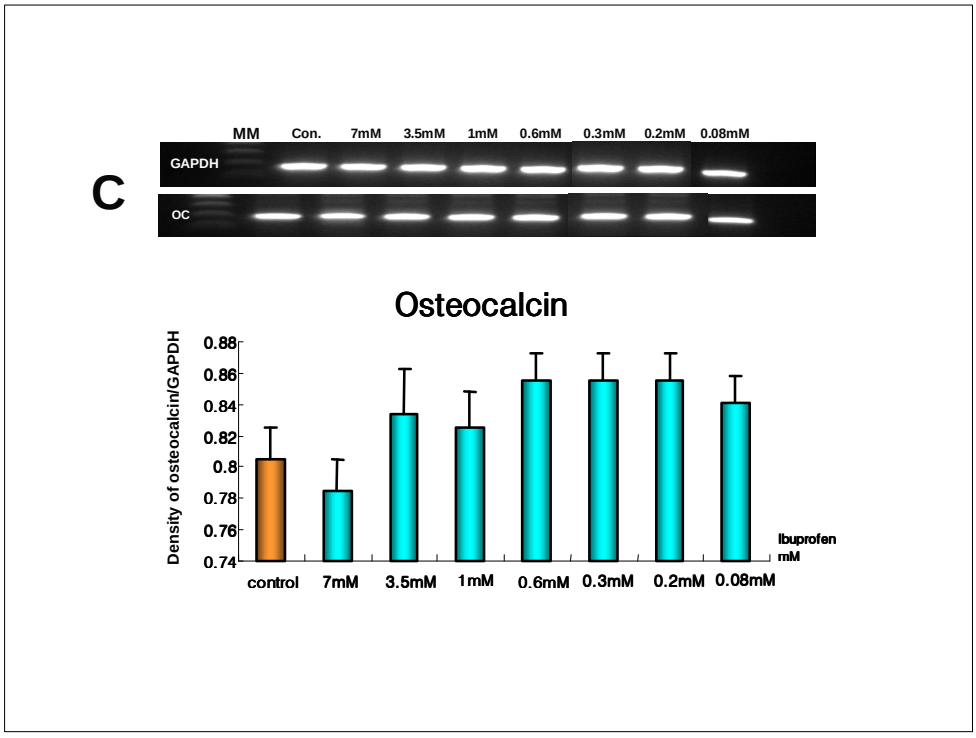

Fig 4 Effect of ibuprofen on expression of OC mRNA in MG63 cells.

4) Expression of OPN mRNA

The effects of ibuprofen on OPN mRNA in MG63 cells revealed dose-dependently decreased tendency. Especially, the value of OPN mRNA in 7mM was 4 times as high as control ($p>0.05$) (Fig. 5).

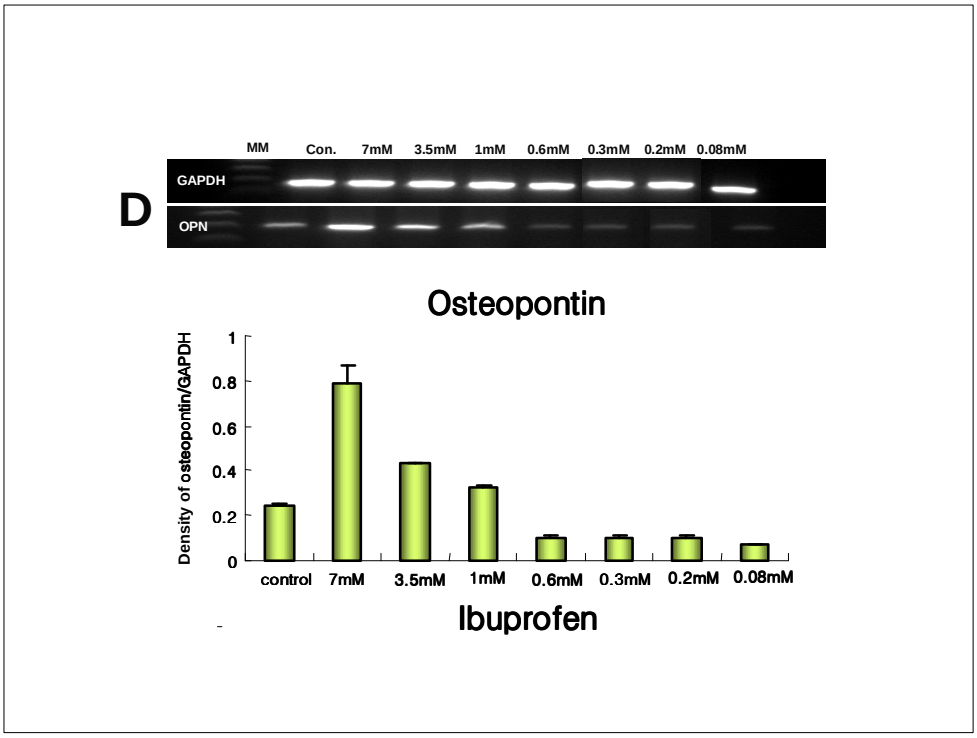

Fig 5 Effect of ibuprofen on expression of OPN mRNA in MG63 cells.

3. Expression of bcl-2 mRNA

The effects of ibuprofen on bcl-2 mRNA in MG63 cells revealed the decreased tendency at all the concentrations of ibuprofen compared to control ($p>0.05$)(Fig. 6).

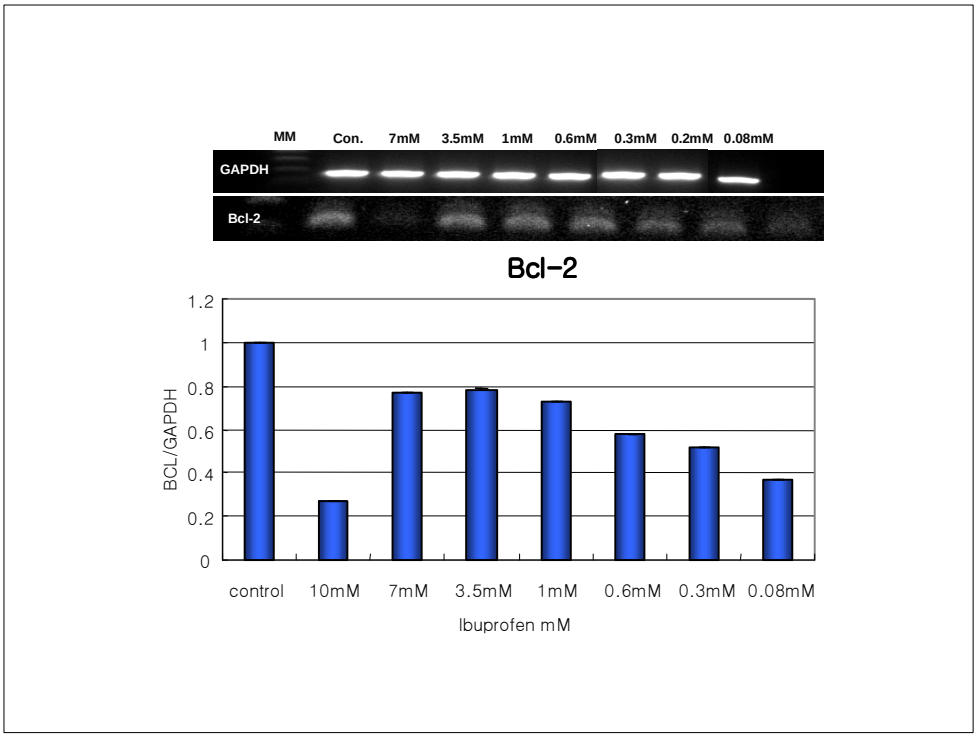

Fig 6 Effect of ibuprofen on expression of bcl-2 mRNA in MG63 cells.

IV. Discussion

Bone markers are aimed to assess bone cells activity. Some (OC, bone ALP, extension peptides of type I procollagen) are specific of bone formation; others (deoxypyridinoline and peptidebound forms) are specific of bone resorption[10].

In the present study, author studied the effect of ibuprofen on the proliferation and expression of bone differentiation markers as well as bcl-2 expression in MG63 cells. In semiquantitive RT-PCR analysis, mRNA expression was analyzed at 24 hours. The cells were cultured for 24-hr with ibuprofen (doses of 0.03 mM, 0.06 mM, 0.08 mM, 1 mM, 3.5 mM, 7 mM, and 10 mM).

For the studies, MG63 cells were used because established cell lines grow rapidly in general and, if appropriately subcultured, have unlimited lifespan[11], and provides phenotypically consistent and stable cell populations, large enough for biochemical analysis[12,13].

For the effect of ibuprofen on the proliferation of osteoblasts cultured, higher doses of ibuprofen (1.0 mM - 10 mM) exhibited negative effect of cell proliferation, while lower doses of ibuprofen (0.166 mM - 0.625 mM) do not. Abukawa et al.[14] demonstrate that high dose ibuprofen has a deleterious effect on porcine osteoblasts derived from bone marrow progenitor cells's proliferation and differentiation.

COL-I is widely distributed in the body, and synthesized by fibroblasts, odontoblasts, osteoblasts, and chondroblasts. It weakly interacted with glycosaminoglycans and its main function is resistance to tension[15]. In this study, the effects of ibuprofen on COL-1 mRNA in MG63 cells showed the decreased tendency at all concentrations of ibuprofen except 3.5 mM ibuprofen compared to control. This result indicated that ibuprofen inhibited COL-1 mRNA expression of MG63 cells, but author could not compare this results with other studies because there was no report with regard to ibuprofen and expression of

COL-1 mRNA in osteoblasts.

ALPs present in many tissues (e.g. liver, bile ducts, intestine, bone, kidney, placenta, and leukocytes), catalyze the release of orthophosphate from ester substrates at alkaline pH. The biologic function of ALP is unknown, except for an apparent role in the deposition of hydroxyapatite in osteoid to form bone[16]. In this study, the effects of ibuprofen on mRNA ALP in MG-63 cells showed the slightly increased tendency at all concentrations of ibuprofen compared to control. This result indicated that ibuprofen slightly up-regualted the mRNA ALP of MG63 cells. But, This result was in contrast to other report. Abukawa et al.[14] reported that differentiated osteoblasts cultured in 3 mM doses of ibuprofen produced significantly less ALP. The possible explanation of conflicting results was the type cells for studies.

OC, known as bone Gla protein, is a marker of bone formation. It is a vitamin K-, and vitamin D-dependent protein produced by osteoblasts and is most abundant and most widely studied of the non-collagenous proteins in bone.[17] The majority of OC secreted by the osteoblast is deposited in extracellular bone matrix; serum osteocaclin represents the fraction of total OC that has not adsorbed to hydroxyapatite[17]. In this study, the effects of ibuprofen on OC mRNA in MG63 cells revealed the slightly increased tendency at all concentrations of ibuprofen except 7.0 mM ibuprofen compared to control. This result indicated that ibuprofen could upregulate the OC mRNA of MG63 cells.

OPN is a secreted acidic phosphoglycoprotein containing an arginin- glycine-aspartate motif that interacts with integrin $\alpha_v\beta_3$ on the cell surface[18]. OPN is important for the function of fibroblasts, macrophages and lymphocytes during inflammation and wound healing[19], is a marker of osteogenic differentiation[20], and is a prominent component of the extracellular matrix of mineralized connective tissues that have been implicated in the formation and remodelling of bone. Chen et al.[21] reported that dexamethasone also stimulated a marked (>

5-fold) increase in OPN expression by osteoblasts and cells lining endosteal and periosteal bone surfaces. So, author didn't use the dexamethasone as a medium. But, in this present study, expression of OPN mRNA at higher dose ibuprofen was increased, too. This finding suggests that certain concentrations of ibuprofen stimulates especially OPN related bone formation by promoting osteoblastic differentiation and/or activation. Over-expression of OPN is a feature of haemopoietic malignancies, and as OPN as a bridge between bone and blood is reported[22], its role will be researched for bone defect healing in the future.

COX-2 synthesizes prostaglandin E_2 which stimulates bcl-2 and inhibits apoptosis[23]. Thurnher et al.[24] reported that the growth of the head and neck cancer cell lines was significant reduced and apoptosis was increased, possibly due to a reduction in bcl-2 expression after exposure to indomethacin (1 mM) and ibuprofen (1 mM) in the head and neck cancer cell lines tested. In the present study, the effects of ibuprofen on bcl-2 mRNA in MG63 cells revealed the decreased tendency at all the concentrations of ibuprofen compared to control, too. This finding suggests that certain concentrations of ibuprofen down-regulates bcl-2 mRNA in MG63 cells.

COX activation represents a major regulatory step in bone destruction and may thereby serve as an important site for pharmacological modulation[9]. As Yazi et al.[8] described previously, time course of the drug administration is critical, suggesting that early events in bone formation may be modulated by arachidonic acid metabolites.

Taken altogether, the local regulatory factors (ALP, especially OPN, and OC) produced by ibuprofen-treated cells were greater than those of the control, in certain concentrations of ibuprofen. Also, anti-apoptosis related gene, such as Bcl-2 was down-regulated by Ibuprofen. Further studies will be needed related to periodontal regeneration and anti-apoptosis at various doses (from 0.625 mM to 7 mM) of ibuprofen.

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ABSTRACT IN KOREAN

Ibuprofen이 골모세포의 분화표지자 및 bcl-2의 유전자 발현에 끼치는 효과

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비스테로이드성 소염제인 ibuprofen은 prostaglandin E_2의 생성을 억제하며, 염증과 통증을 감소시키며 골소실을 지연시킨다. 이 연구에는 ibuprofen이 골세포의 증식과 골분화표지자 (collagen type 1; COL-1: alkaline phosphatase; ALP: osteocalcin; OC: osteopontin; OPN) 그리고 bcl-2의 발현에 끼치는 효과를 평가하고자 시행되었다.

이 연구를 위해서, human osteogenic sarcoma cell line (MG63)에 다양한 농도의 ibuprofen (대조군:0 mM; 실험군: 0.03 mM, 0.06 mM, 0.08 mM, 1 mM, 3.5 mM, 7 mM, 10 mM)을 첨가한 후 배양하여 증식률과 RT-PCR법을 이용하여 COL-1, ALP, OC, OPN, bcl-2 의 mRNA 표현을 평가하였다. 그 결과는 다음과 같다.

1. 고농도의 Ibuprofen (1 mM에서 7 mM까지)은 세포의 증식을 억제하였으나, 저농도의 ibuprofen은 세포 증식이 약 6 - 40%정도 증가하였다.
2. Ibuprofen에 의한 COL-1 mRNA 발현은 대조군과 비교했을 때 모든 농도에서 감소하는 경향을 보였으나 3.5 mM에서는 증가되었다.
3. Ibuprofen에 의한 ALP mRNA 발현은 대조군과 비교했을 때 모든 농도에서 증가하는 경향을 보였다.
4. Ibuprofen에 의한 OC mRNA 발현은 대조군과 비교했을 때 모든 농도에서 증가하는 경향을 보였으나 7.0 mM에서는 감소되었다.
5. Ibuprofen에 의한 OPN mRNA 발현은 대조군과 비교했을 때 농도 의존적으로 감소되었다. 그러나, 특히 7 mM의 ibuprofen 농도에서는 대조군에 비해 4배 증가되어 나타났다.

6. Ibuprofen에 의한 bcl-2 mRNA 발현은 대조군과 비교했을 때 모든 농도에서 감소하는 경향을 보였다.

전체적으로 보면, ibuprofen과 함께 배양된 MG63세포에 의해 생성된 골분화유전자들은 대조군에 비해 증가되는 양상을 보였으며, bcl-2와 같은 antiapopotis 관련 유전자는 감소되어 나타났다. 따라서 향후에는 특정 농도의 ibuprofen을 치주인대섬유모세포와 배양하여 OPN과 bcl-2에 관련된 연구가 필요하리라 생각된다.

(별 지)

저작물 이용 허락서

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논문제목	한글 : Ibuprofen이 골모세포의 분화표지자 및 bcl-2의 유전자 발현에 끼치는 효과				
	영문 : Effect of ibuprofen on differentiation of osteoblastic cell				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) **반대**( )

2008년 2월 일

저작자: 강 순 원 (서명 또는 인)

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