원 저

Bee Venom induces apoptosis and inhibits COX-2 in human osteosarcoma cell line MG-63

Hwang Dae-yeon[*], Kim Ho-hyun[**], Kim Chang-ju[***] and Kim Ee-hwa[*]

Departments of Meridian & Acupoint[*], Physiology[**],
College of Oriental Medicine, Se-Myung University
Department of Physiology[***], College of Medicine, Kyung-Hee University

국문초록

봉독이 골육종세포주에서 세포사멸 및 COX-2 억제에 미치는 영향

황대연[*] · 김호현[**] · 김창주[***] · 김이화[*]

세명대학교 한의과대학 경혈학교실[*], 생리학교실[**]
경희대학교 의과대학 생리학교실[***]

목적 : 한의학에서 관절염이나 진통치료에 사용되어 왔던 봉독약침액이 인간 골육종 세포주인 MG-63 세포에서 항종양효과가 있는지를 연구하고자 한다. 특히 본 실험에서는 이러한 봉독의 종양발생 억제작용이 세포사멸과 관련이 있는지, 그리고 프로스타글란딘 합성 효소인 cyclooxygenase(COX)-2의 억제와 관련이 있는지를 연구하고자 한다.

방법 : 인간 골육종 세포주에서 세포사멸의 변화를 관찰하기 위해서 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay, 4,6-diamidino-2-phenylindole (DAPI), DNA fragmentation assay 및 reverse transcription-polymerase chain reaction(RT-PCR) 방법을 이용하였다.

결과 : 세포독성 검사에서 봉독은 MG-63 세포에서 농도-의존적으로 세포독성을 나타내었다. 이러한 봉독의 세포독성이 세포사멸로 인한 것인지를 여러 가지 형태로 검사한 결과 봉독에 의한 세포독성은 TUNEL 검사와 DAPI 염색시 세포사멸의 특징적인 소견들을 나타내었고, flow cytometric 분석에서도 세포사멸을 의미하는 세포주기의 변화들을 나타내었다. 봉독이 COX-2의 발현에 미치는 영향을 RT-PCR로 실험한 결과 봉독은 COX-2 mRNA의 발현을 선택적으로 억제하였다.

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· **교신저자** : 김이화, 충북 제천시 신월동 산 21-1 세명대학교 한의과대학 경혈학교실
Tel. 043-649-1348 E-mail : kimeh@semyung.ac.kr

결론 : 본 실험의 결과 봉독은 COX-2 mRNA의 발현을 억제함으로써 골육종 세포에서 세포사멸을 유발하고 그 결과 항종양효과를 나타내는 것으로 보여진다.

Key words : apoptosis, bee venom, COX-2, MG-63, osteosarcoma

Ⅰ. Introduction

In a traditional Oriental medicine, bee venom (BV) has been widely used for the treatment of inflammatory diseases such as rheumatoid arthritis[1] and relief of pain[2]. BV possesses anti-inflammatory effect[3], a general characteristic of non-steroidal anti-inflammatory drugs(NSAIDs). Two main components of BV are melittin and phospholipase A2(PLA2)[4]; of these, melittin, the major active ingredient of BV[5], has been reported to induce apoptosis[6] and possess anti-tumor effect[7]. In addition, Shin et al.[8] have shown that hybrid peptides derived from melittin exerts anti-tumor effect on small cell lung cancer cell lines.

Apoptosis plays crucial role in tumorigenesis and anti-tumor therapy[9]. A lot of drugs have been studied and put into practice for the treatment of human tumors[10),11]. Various potential therapeutic agents including NSAIDs which show inhibitory effect on tumorigenesis in the colon[12], breasts[13], and lungs[14] have been investigated[10),15),16]. Of particular interest is lung cancer, which is of great noxiousness and high mortality due to an ever-increasing population of smokers worldwide. Several studies have established that the agents inducing apoptosis in target organs suppress tumorigenesis[11),17].

Apoptosis, a process of central importance in the prevention of tumor development[18], is a genetically programmed cell death mechanism serving physiologic and homeostatic functions[19]. It has been implicated in the pathogenesis and pathophysiology of several human diseases such as cancer, autoimmune dysfunction, aquired immune deficiency syndrome (AIDS), and several neurodegenerative diseases[20),21].

Wardlaw et al.[22] reported that cyclooxygenase(COX)-2 was overexpressed in lung cancer cells and it was shown that COX inhibitors retard the growth of human cancer cells *in vitro*[23]. COX, an enzyme of the prostaglandins (PGs) synthesis pathway[24], has two isoforms : COX-1 and COX-2. COX-1 is constitutively expressed in normal physiologic functions. Inhibition of COX-1 is known to have serious adverse effects such as peptic ulcer formation and renal dysfunction. COX-2 is induced during inflammatory process[25]. Selective COX-2 inhibition has benn proposed as a way to avoid these toxic effects[26),27] during the maintaining

of the chemotherapeutic effect.

In the present study, the possibility of BV's application as an anti-tumor therapeutic agent in human osteocarcinoma cancer cells was investigated.

Ⅱ. Materials & Methods

2.1. Reagents

BV was purchased from You-Miel Bee Venom Ltd.(Kwangju, Korea). 3,3'-diamino-benzidine(DAB), 4,6-diamidino-2-phenylin-dole (DAPI), propidium iodide(PI) and paraformaldehyde(PFA) were obtained from Sigma Chemical Co.(St. Louis, MO, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay kit was purchased from Boehringer Mannheim(Mannheim, Germany), and the terminal deoxynuclotidyl transferase (TdT)-mediated dUTP nick end-labeling(TUNEL) assay kit was purchased from Intergen (Purchase, NY, USA). The DNA fragmentation assay kit was obtained from TaKaRa(Shiga, Japan).

2.2. Cell culture

The human osteocarcinoma cell line MG-63 was purchased from Korean Cell Line Bank (KCLB ; Seoul, Korea), and cultured by previous reported method[17]. Cells were cultured in RPMI 1640 media(Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum(Gibco BRL, Grand Island, NY, USA) at 37℃ in 5% CO_2, 95% O_2 in a humidified cell incubator. Cells were plated in culture dish(Corning Incorporated, Corning, NY, USA) at a density of 1×10^6 cells per dish, and the media was changed once every 2 days.

2.3. MTT cytotoxicity assay

Cell viability was determined by the MTT assay kit using as per the manufactures protocol. MG-63 cells were cultured in 96 well plates. Experimental groups are exposed to BV at final concentrations of 1㎍/ml and 10㎍/ml (diluted with saline) for 24 hrs, and saline of an equal volume was added to control group. Ten ml of the MTT labeling reagent was then added to each well, and the plates were incubated for 4 hrs. After the cells were incubated in 100 ml of the solubilization solution for 12 hrs, the absorbance was measured with a microtiter plate reader(Bio-Tek, Winooski, VT, USA) at a test wavelength of 595nm with a reference wavelength of 690nm. The optical density(O.D.) was calculated as the result of the subtraction of the absorbance at the reference wavelength from that of the test wavelength. Percent viability was calculated as(O.D. of drug-treated sample/control O.D.)×100.

2.4. TUNEL assay

For *in situ* detection of apoptotic cells, TUNEL assay was performed using ApoTag peroxidase *in situ* apoptosis detection kit. MG-63 cells were cultured on 4-chamber slides(Nalge Nunc International, Naperville, IL, USA) at a density of 2×10^4 cells/chamber. After 24 hrs

exposure to BV(10㎍/ml), cells were washed with phosphate-buffered saline(PBS) and fixed in 4% PFA for 10 min at 4℃. The fixed cells were incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase(TdT)-catalyzed reaction for 60 min at 37℃ in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. The DNA fragments were stained using DAB as the substrate for the peroxidase.

2.5. DAPI staining

In order to determine using morphologic markers whether apoptosis is induced by BV, DAPI staining was performed according to previously described protocol[28),29)]. In short, cells were cultured on 4-chamber slides. After treatment with BV(10㎍/ml) for 24 hrs, the cells were washed twice with PBS and fixed by incubation in 4% PFA for 30 min. Following a second washing in PBS, cells were incubated in 1㎍/ml DAPI solution for 30 min in the dark. The cells were then observed with a fluorescence microscope(Zeiss, Oberköchen, Germany).

2.6. Flow cytometric analysis

Flow cytometric analysis was performed as previously described[29)]. In brief, MG-63 cells were harvested after BV treatment for 24 hrs at a final concentration 10㎍/ml. Cells were then washed twice with PBS and fixed with 70% ethanol in PBS at -20℃. After washing twice with PBS, the cells were incubated with 100㎍/ml RNase(Sigma) and stained with 20㎍/ml PI. The stained cells were incubated for 30 min at 37℃ and were analyzed using FACScan (Becton Dickinson, San Jose, CA, USA).

2.7. DNA fragmentation

For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed using ApopLadder EX™ DNA fragmentation assay kit. In brief, cells were collected after BV treatment for 24 hrs at a final concentration of 10㎍/ml and washed in PBS. The cells were then lysed with 100㎕ of lysis buffer. The lysate was incubated with 10 ml of 10 % SDS solution containing 10㎕ of Enzyme A at 56℃ for 1 hr followed by treatment with 10㎕ of Enzyme B at 37℃ for 1 hr. This mixture was then centrifuged for 15 min after adding 70㎕ of precipitant and 500㎕ of ethanol. The DNA was extracted by washing in the resultant pellet in ethanol and resuspending it in TE(Tris-EDTA) buffer. DNA fragmentation was visualized by electrophoresis in a 2% agarose gel containing ethidium bromide.

2.8. RNA isolation and reverse transcription-polymerase chain reaction(RT-PCR)

Total RNA was extracted using RNAzol™B (TEL-TEST, Friendswood, TX, USA). Two ㎍ of RNA and 1㎕ of random hexamers(Promega, Madison, WI, USA) were mixed and heated at 65℃ for 5 min. One ㎕ of AMV reverse transcriptase(Promega, Madison, WI, USA), 5㎕ of

10 mM dNTP(Promega, Madison, WI, USA), 1 μℓ of RNasin(Promega, Madison, WI, USA), and 5μℓ of 10×AMV RT buffer(Promega, Madison, WI, USA) were then added and the final volume was brought up to 50μℓ with dimethyl pyrocarbonate(DEPC)-treated water, and the reaction mixture was then incubated at 42℃ for 1 hr.

COX-2 primer sequences as reported by Hla et al.[30] and Funk et al.[31] were used. For human COX-2, the primer sequences were 5-TTCAAATGAGATTGTGGGAAAATTGCT-3(a 27-mer sense oligonucleotide starting at position 573) and 5-AGATCATCTCTGCCTGAGTATCTT-3(a 24-mer antisense oligonucleotide starting at position 878). For cyclophilin, the internal control used in the study, the primer sequences were 5'-ACCCCACCGTGTTCTTCGAC-3'(a 20-mer sense oligonucleotide starting at position 52) and 5'-CATTTGCCATGGACAAGATG-3'(a 20-mer sense oligonucleotide starting at position 332). The expected sizes of the PCR products are 305 bp(for COX-2) and 299 bp(for *cyclophilin*).

PCR amplification was performed in a reaction volume of 40μℓ containing 1μℓ of each cDNA and 1μℓ of 10 ρM of each set of primers, 1μℓ of 10×buffer, 1μℓ of 2.5 mM $MgCl_2$, 1μℓ of 2.5 mM dNTP and 2 units Taq DNA polymerase(TaKaRa, Shiga, Japan). For COX-2, the PCR procedure was carried out under the following conditions : initial denaturation at 95℃ for 5 min, followed by 40 amplication cycles of denaturation at 95℃ for 30 sec, annealing at 58℃ for 30 sec, extension at 72 ℃ for 30 sec, and with an additional extension step at 72℃ for 5 min using a GeneAmp 9600 PCR system(Perkin Elmer, Norwalk, CT, USA). For *cyclophilin*, the conditions were similar, except that 25 amplification cycles were executed. The final amount of RT-PCR products for each of the mRNA were compared densitometrically using Molecular Analyst™ software version 1.4.1(Bio-Rad, Hercules, CA, USA).

2.9. Statistical analysis

Statistical analysis was performed using Student's t-test and the results were expressed as mean±S.E.M. Differences were considered significant for $p<0.05$.

Ⅲ. Results

3.1. MTT assay for cell viability

In order to find out the concentration at which the cytotoxic effect of BV on the MG-63 cell line become evident, cells were cultured with BV at final concentrations of 10μg/ml, 1μg/ml and 0.1μg/ml for 24 hrs, and MTT assays were carried out, with cells cultured in BV-free media as the control. The viabilities of cells incubated with BV at concentrations of 10 μg/ml, 1μg/ml and 0.1μg/ml were 73.89±4.41%, 97.56±3.93 % and 97.41±4.54% of the control value respectively. A trend of increasing cytotoxicity with increasing concentration was observed. Based on these results, the concentration of BV to be in subsequent experiments

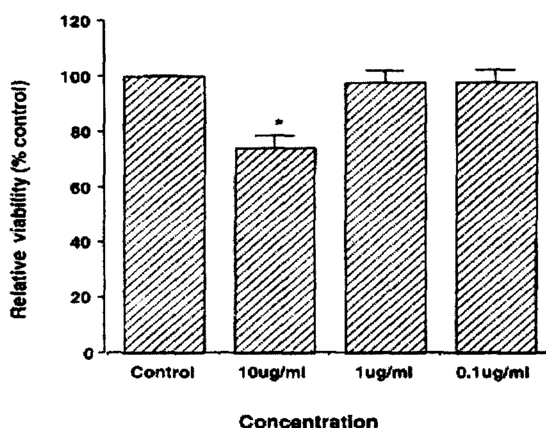

Fig.1. Cytotoxic effects of bee venom(BV).

Human osteosarcoma cell line MG63 cells were incubated with BV at various concentrations prior to the determination of cellular viability through MTT assay. Results are represented as mean±standard error. * represents $p<0.05$ compared to the control.

devised to elucidate the properties of BV-induced cytotoxicity as set at around 10㎍/ml.

3.2. Morphological changes induced by BV

To observe the effect of BV on cell morphology, cells were examined by phase-contrast microscopy. As shown in Figure 2, cells treated with 10㎍/ml BV for 24 hrs showed cell shrinkage, cytoplasmic condensation, and irregularity in shape. These morphological characteristics suggest that BV induces apoptotic cell death in MG-63 cells.

To further confirm the induction of apoptosis in MG-63 cells by BV, BV-treated cells were biochemically analyzed *via* TUNEL assay and DAPI staining. As shown in Figure 2, TUNEL-positive cells were stained dark brown under the light microscope, and TUNEL-positive cells in which the nuclei were condensed in culturesof MG-63 cells treated with BV at a concentration of 10㎍/ml for 24 hrs. In DAPI staining, cells were observed via fluorescence microscopy following treatment with DAPI, which specifically stains the nuclei. These re-

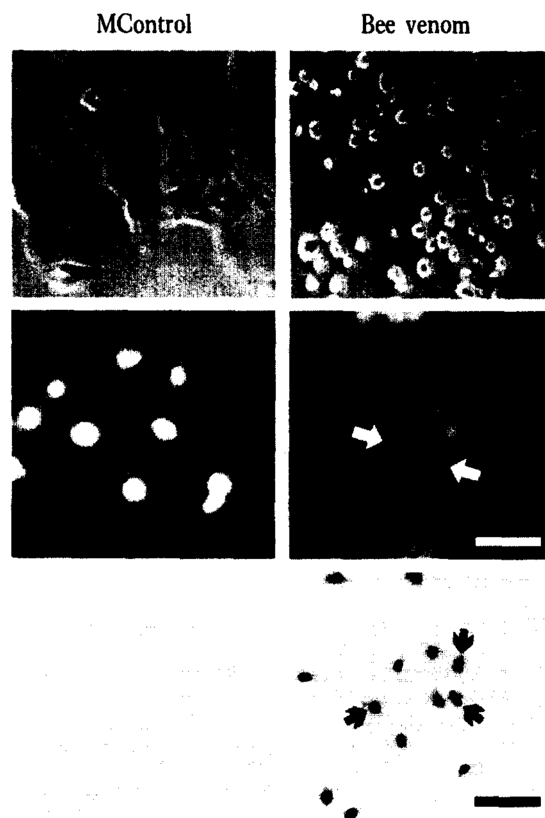

Fig. 2. Characterization of bee venom(BV)-induced cell death in MG63 cells.

Top ; Phase-contrast microscopy showed cell shrinkage, irregularity in shape, and cellular detachment in the BV-treated cultures; these morphological characteristics were not observed in the control(untreated) group. Middle : MG63 cells stained with DAPI. Bottom: TUNEL stain. The white and black arrows indicate condensed nuclei. Scale bar represents 100㎛.

sults have shown that the presence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon BV treatment.

3.3. Cell cycle distribution changes induced by BV

Through flow cytometric analysis, an increased number of cells in the sub-G1 phase(from 2.77% to 31.79% of total cells) and a decreased number of cells in the G1 phase(from 25.68% to 16.68% of total cells) were observed in the 10μg/ml BV-treated cells<Figure 3>.

3.4. DNA fragmentation characterization of apoptosis

In order to ascertain BV-induces cell death, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was analyzed. As seen in Figure 4, treatment with BV at a concentration of 10μg/ml for 24 hrs resulted in the formation of definite fragments which could be seen by electro-phoretic examination as a characteristic ladder pattern.

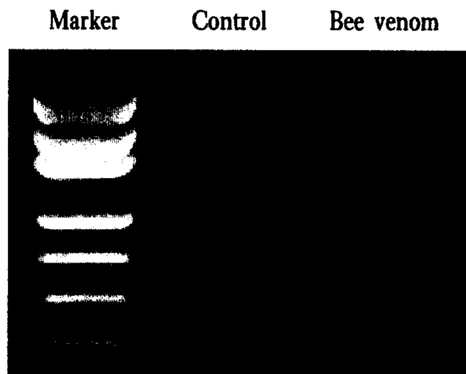

Fig. 4. Electrophoretic examination of genomic DNA of MG63 cells.

Genomic DNA was extracted and analyzed via electrophoresis on a 2% agarose gel containing ethidium bromide. Cells of 10μg/ml of bee venom(BV)-treated group exhibited the ladder pattern characteristic of apoptosis.

3.5. Effect of BV on the expression of COX-2 mRNA

RT-PCR analysis of the mRNA level of COX-2 was performed in order to provide an estimation of the relative level of expression of the COX-2 gene. Figure 5 shows the levels of

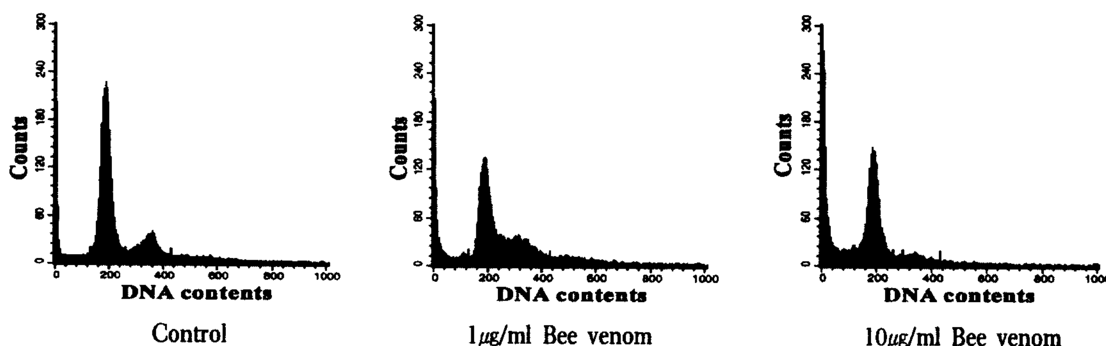

Fig. 3. Flow cytometric analysis of the effect of bee venom on cell cycle. Bee venpm(BV) treatment has shown to increase sub-G1 phase and decrease G1 phase of cell cycle.

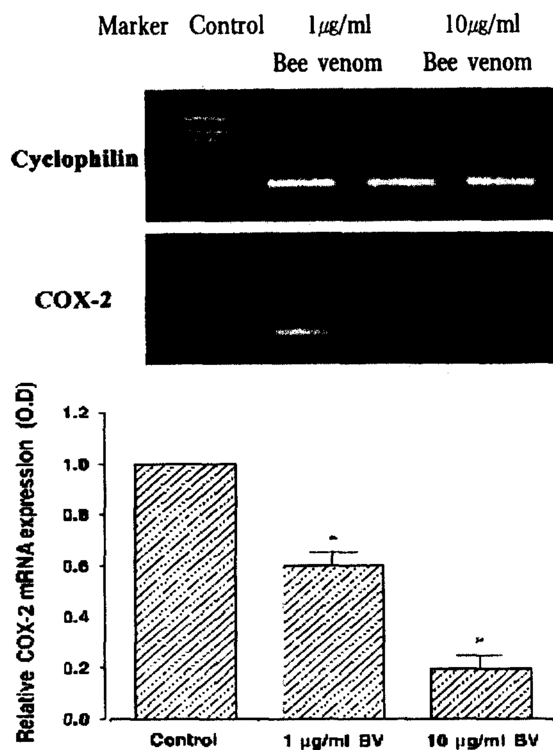

Fig. 5. Results of RT-PCR analysis of the m RNA levels of COX-2.

The level of expression of COX-2 in cells treated with bee venom(BV) was significantly lower than control group. As the internal control, *cyclophilin* mRNA was also reverse transcriptized and amplified.

mRNA expression of *cyclophilin* and COX-2 in the MG-63 cells. Following treatment with BV at a concentration of 1㎍/ml for 24 hrs, the level of expression of COX-2 was decreased to 60.93±3.42%, while at a BV concentration of 10㎍/ml, the respective figure was decreased to 18.12±4.53% of the control level. These results showed that BV selectively inhibits COX-2mRNA expression in a dose-dependent manner.

Ⅳ. Discussion

The purpose of the present study is to find out the possibility whether BV, through induction of apoptosis and inhibition of COX-2 gene expression, exerts a therapeutic action on human osteosarcoma. From the results, it was demonstrated that BV exerts two profound effects on the cells of the human lung carcinoma cell line MG-63. One is that BV induces apoptotic cell death ; the other is its selective inhibition on the COX-2 mRNA expression.

Analysis onalysis of DNA content using the PI, an increase in the fraction of cells in the early sub-G1 phase, which can be seen as a peak positioned close to the sub-G1 phase, was observed. The distribution of DNA content among the various fraction of the cell cycle observed in the lung carcinoma cells treated with BV seems to be indicative of the presence of apoptosis, and this result is similar to that reported by Hanif et al.[16] in a study involving colon cancer cells.

As further evidence for the presence of apoptosis, DNA fragmentation was clearly detected via agarose gel electrophoresis. It is well known that apoptosis is linked with the activation of endonucleases and that it results in the fragmentation of DNA into well defined fragments which can be seen by electrophoretic examination as a characteristic ladder pattern[16),32)]. Furthermore, DNA strand breaks occur during the process of apoptosis, and it is

known that the nicks in the DNA molecules can be detected *via* TUNEL assay[33]. In addition to the effects mentioned above, BV was also seen to produce notable changes in the morphology of the cells ; that these changes meet the stringent morphological criteria for apoptosis was confirmed by DAPI staining. Apoptotic bodies were characteristically presented in BV-treated cells stained with DAPI. It was also reported that cells undergoing apoptosis exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape and retraction of processes[34].

The definitive molecular mechanisms of the anti-tumor action of certain drugs are still unknown. However, it is well known that certain drugs, most notably NSAIDs, are potent inhibitors of the enzymatic activity of COX[35]. COX is a key enzyme in the PGs synthesis pathway ; COX-1 is a constitutive enzyme present in most cells, whereas COX-2 is inducible and is often up-regulated in several types of tumors[36]. Selective down- regulation of COX-2 is an important strategy for the development of anti-tumor agents[24]. In addition, Hanif et al.[16] reported that drugs with chemotherapeutic effect induce a shift in the distribution of cell population among the different phases of the cell cycle in colon cancer cells, and that it is mediated by the inhibition of PGs synthesis.

RT-PCR analysis of COX genes was performed in this study and the expression of COX-2 in MG-63 cells treated with BV(10㎍/ml) was decreased compared to that of the control <Figure 6>. It is possible that it will not incur the serious adverse effects, such as peptic ulcer formation and renal dysfunction, associated with COX-1 inhibition.

In the present study, it was shown that BV induces apoptosis and selectively inhibits on the COX-2 expression in MG-63 human lung carcinoma cells. Based on the results, it is possible that BV may exert anti-tumor effect on human osteosarcoma.

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