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Bee Venom Induces Apoptosis and Inhibits Expression of Cyclooxygenase-2 mRNA in Human Lung Cancer Cell Line NCI-H1299

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Abstract. To investigate whether bee venom (BV) induces apoptosis, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay, 4,6-diamidino-2-phenylindole staining, flow cytometric analysis, and DNA fragmentation assay were performed on NCI-H1299 lung cancer cells treated with BV. Through morphological and biochemical analyses, it was demonstrated that NCI-H1299 cells treated with BV exhibit several features of apoptosis. In addition, reverse transcription-polymerase chain reaction and prostaglandin E_2 (PGE_2) immunoassay were performed to verify whether BV possesses an inhibitory effect on the expression of *cyclooxygenase* (*COX*) and PGE_2 synthesis. Expression of COX-2 mRNA and synthesis of PGE_2 were inhibited by BV. These results suggest the possibility that BV may exert an anti-tumor effect on human lung cancer.

Keywords: bee venom, lung cancer, apoptosis, cyclooxygenase, prostaglandin E_2

Introduction

Bee venom (BV) has been used for the treatment of inflammatory diseases such as rheumatoid arthritis and relief of pain in Oriental medicine (1, 2). BV possesses anti-inflammatory effects (1, 2), a property of non-steroidal anti-inflammatory drugs (NSAIDs). The two main components of BV are melittin and phospholipase A_2 (PLA_2) (3). Of these, melittin, the major active ingredient of BV (4), has been reported to induce apoptosis (5) and to possess anti-tumor effects (6). Shin et al. (7) have shown that hybrid peptides derived from melittin exert anti-tumor effects on small cell lung cancer cell lines.

Evidence suggests that the regulation of apoptosis plays a crucial role in tumorigenesis and anti-tumor therapy (8). Various drugs have been studied and used critically that reduce the risk of development of a variety of tumors in humans (9, 10); representative agents include the NSAIDs (9, 11, 12), which were shown to have beneficial effects by inhibiting tumorigenesis in the colon (13), breasts (14), and lungs (15). Of particular concern is lung cancer, which has a high rate of morbidity due to an ever-increasing population of smokers worldwide. Several studies have established that the agents inducing apoptosis in target organs suppress tumorigenesis (10, 16).

Apoptosis, a process of central importance in the prevention of tumor development (17), is a programmed cell death mechanism serving homeostatic functions (18). It has been implicated in the pathogenesis and pathophysiology of several human diseases, such as cancer, autoimmune dysfunction, AIDS and neurodegenerative diseases (19). It has been shown in various studies that the process of apoptosis is regulated by the expression of several proteins. Two important groups of proteins involved in apoptotic cell death are members of the bcl-2 family (20) and a class of cysteine proteases

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known as caspases (21). The bcl-2 family is classified into two functionally distinct groups: anti-apoptotic proteins and pro-apoptotic proteins. Bcl-2, an anti-apoptotic protein, is known to regulate the apoptotic process and to protect against cell death, while bax, a pro-apoptotic protein, is expressed abundantly and selectively during apoptosis and promotes cell death (22). Caspases are known to mediate the crucial stage of the apoptotic process and is also expressed in pathological conditions. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of apoptosis, being responsible either partially or wholly for the proteolytic cleavage of many proteins (21).

In addition, Wardlaw et al. (23) found that cyclooxygenase (COX)-2 is overexpressed in lung cancer cells, and it has been demonstrated that COX inhibitors retard the growth of human lung cancer cells in vitro (24). COX is an enzyme responsible for understanding a crucial stage in the prostaglandin (PG) synthesis pathway (25); it has two isoforms, COX-1 and COX-2. While COX-1 is a constitutively expressed form required for normal physiologic functions, COX-2 is induced during inflammatory processes (26). Inhibition of COX-1 is known to have adverse effects such as peptic ulcer formation and renal dysfunction, and selective COX-2 inhibition has been proposed as a way to avoid these toxic effects (27, 28) while maintaining the chemotherapeutic effects. In the present study, the possibility of BV's application as an anti-tumor therapeutic agent was investigated by examining its induction of apoptosis and inhibition of COX, especially the COX-2 isoform, in human lung cancer cells.

Materials and Methods

Reagents

BV was purchased from You-Miel Bee Venom, Ltd. (Kwangju, Korea). Acetylsalicylic acid (ASA), 3,3'-diaminobenzidine (DAB), 4,6-diamidino-2-phenylindole (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 (Ohtsu).

Cell culture

The human lung carcinoma cell line NCI-H1299 was purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in RPMI 1640 media (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2, 95% O_2 in a humidified cell incubator, and the media was changed once every 2 days.

MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit using as per the manufacturer's protocol. NCI-H1299 cells were cultured in 96-well plates. Experimental groups were exposed to BV at final concentrations of 1 and 10 μg/ml (diluted with saline) for 6, 12, 24, and 48 h, and saline of an equal volume was added to the control group. ASA at a concentration of 100 μg/ml was also treated at same conditions. Ten microliters of the MTT labeling reagent was then added to each well, and the plates were incubated for 4 h. After the cells were incubated in 100 μl of the solubilization solution for 12 h, the absorbance was measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm and a reference wavelength of 690 nm. The optical density (O.D.) was calculated as absorbance at the reference wavelength minus that of the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample / control O.D.) × 100.

TUNEL assay

For in situ detection of apoptotic cells, TUNEL assay was performed using the ApoTag® peroxidase in situ apoptosis detection kit. NCI-H1299 cells were cultured on 4-chamber slides at a density of 2×10^4 cells/chamber. After a 24 h exposure to BV (10 μg/ml), cells were washed with phosphate-buffered saline (PBS) and fixed in 4% PFA for 10 min at 4°C. The fixed cells were incubated with digoxigenin-conjugated dUTP in a TdT-catalyzed reaction for 60 min at 37°C 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.

DAPI staining

In order to determine whether apoptosis is induced by BV, DAPI staining was performed. In short, cells were cultured on 4-chamber slides. After treatment with BV (10 μg/ml) for 24 h, 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 μg/ml DAPI solution for 30 min in the dark. The cells were then observed through a fluorescence micro-

scope (Zeiss, Oberköchen, Germany).

Flow cytometric analysis

After treatment with BV at a final concentration 10 μg/ml for 24 h, cells were collected and fixed with 75% ethanol in PBS at −20°C for 1 h. Afterwards, the cells were incubated with 100 μg/ml RNase and stained with 20 μg/ml propidium iodide in PBS. The stained cells were incubated for 30 min at 37°C and were analyzed using FACScan (Becton Dickinson, San Jose, CA, USA).

DNA fragmentation

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

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

Total RNA was extracted using RNAzol™B (TEL-TEST; Friendswood, TX, USA). Two micrograms of RNA and 1 μl of random hexamers (Promega, Madison, WI, USA) were mixed and heated at 65°C for 5 min. One microliter of AMV reverse transcriptase (Promega), 5 μl of 10 mM dNTP (Promega), 1 μl of RNasin, and 5 μl of 10 ×AMV RT buffer were then added, and the final volume was brought up to 50 μl with diethyl pyrocarbonate-treated water, and the reaction mixture was then incubated at 42°C for 1 h.

The primer sequences for *COX-1*, *COX-2*, *bax*, and *bcl-2* used in the study were as reported by Hla and Neilson (29), Funk et al. (30), Sawa et al. (31), Zhang et al. (32), and Wang et al. (33), respectively. For human *COX-1*, the primers sequences were 5'-TGCCCAGCTC CTGGCCCGCCGCTT-3' (a 24-mer sense oligonucleotide starting at position 516) and 5'-GTGCATCAACA CAGGCGCCTCTTC-3' (a 24-mer antisense oligonucleotide starting at position 819). For human *COX-2*, the primer sequences were 5'-TTCAAATGAGATTGTGG GAAAATTGCT-3' (a 27-mer sense oligonucleotide starting at position 573) and 5'-AGATCATCTCTGCCT GAGTATCTT-3' (a 24-mer antisense oligonucleotide starting at position 878). For human *bax*, the primer sequences were 5'-GTGCACCAAGGTGCCGGAAC-3' (a 20-mer sense oligonucleotide starting at position 375) and 5'-TCAGCCCATCTTCTTCCAGA-3' (a 20-mer anti-sense oligonucleotide starting at position 560). For human *bcl-2*, the primer sequences were 5'-CGACGA CTTCTCCCGCCGCTACCGC-3' (a 25-mer sense oligonucleotide starting at position 334) and 5'-AGATCA TCTCTGCCTGAGTATCTT-3' (a 25-mer anti-sense oligonucleotide starting at position 628). 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 antisense oligonucleotide starting at position 332). The expected sizes of the PCR products were 303 bp (for *COX-1*), 305 bp (for *COX-2*), 205 bp (for *bax*), 318 bp (for *bcl-2*), and 280 bp (for *cyclophilin*).

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

Caspase activity assay

Caspase activity was measured using the ApoAlert® caspase-3 assay kit according to the manufacturer's protocol. In brief, after treatment with BV at concentrations of 1 μg/ml and 10 μg/ml for 24 h, cells were lysed with 50 μl of chilled Cell Lysis Buffer. Then 50 μl of 2 × reaction buffer (containing dithioreitol) and 5 μl of the conjugated substrate at a concentration of 1 mM were added to each lysate. The mixture was incubated in a water bath at 37°C for 1 h, and the absorbance was measured with a microtiter plate reader at a test wavelength of 405 nm.

PGE_2 assay

Assessment of PGE_2 synthesis was performed using a commercially available PGE_2 competitive enzyme

immunoassay kit (Amersham Pharmacia Biotech, Inc, Piscataway, NJ, USA) according to the instructions provided by the manufacturer. To start, cells were lysed as directed, and cell lysates and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE_2 antibody and peroxidase-conjugated PGE_2 were then added to each of the wells, and the plate was incubated at room temperature for 1 h with shaking. The wells were then drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was added. The plate was incubated at room temperature with shaking, and the reaction was terminated after 30 min through the addition of H_2SO_4. The absorbance of the content of each of the wells at 450 nm was then measured.

Statistical analyses

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Scheffé's post-hoc test, and results were expressed as mean ± standard error mean (S.E.M.) for two independent experiments, each with a minimum of three cultures per group (n = 6). Differences were considered significant for $P<0.05$.

Results

MTT assay of cell viability

In order to find out the concentration at which the cytotoxic effects of BV on the NCI-H1299 cell line becomes evident, cells were cultured with BV at final concentrations of 1 μg/ml and 10 μg/ml and with ASA at a concentration of 10 μg/ml for 6, 12, 24, and 48 h; and MTT assays were carried out with cells cultured in BV-free media as the control. The viabilities of cells incubated with BV at a concentration of 1 μg/ml for 6, 12, 24, and 48 h were 84.96 ± 1.38%, 80.57 ± 2.14%, 75.53 ± 3.04%, and 52.55 ± 2.01% of the control value, respectively. The viabilities of cells incubated with BV at a concentration of 10 μg/ml for 6, 12, 24, and 48 h were 70.82 ± 2.01%, 50.57 ± 3.12%, 47.09 ± 2.52%, and 33.94 ± 1.28% of the control value, respectively. The viabilities of cells incubated with ASA at a concentration of 100 μg/ml for 6, 12, 24, and 48 h were 76.95 ± 1.59%, 55.32 ± 2.04%, 52.70 ± 3.12%, and 49.72 ± 1.92% of the control value, respectively. A trend of increasing cytotoxicity with increasing concentration and incubation time was observed (Fig. 1). Based on these results, the concentration of BV to be used in subsequent experiments devised to elucidate the properties of BV-induced cytotoxicity was set at around 10 μg/ml.

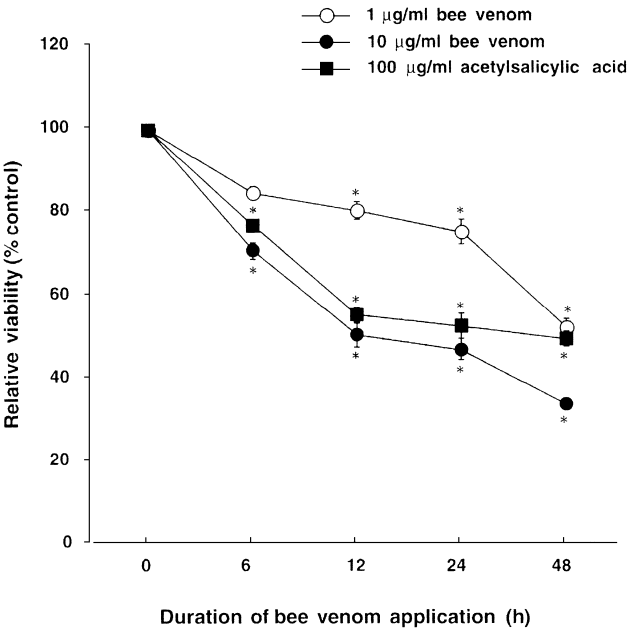

Fig. 1. Dose- and duration-dependent effect of bee venom on cell viability. Viability was determined via MTT assay. Results are presented as mean ± S.E.M. * represents $P<0.05$ compared to the control group.

Morphological changes induced by BV

To observe the effect of BV on cell morphology, cells were examined via phase-contrast microscopy. As shown in Fig. 2, cells treated with 10 μg/ml BV for 24 h exhibited cell shrinkage, cytoplasmic condensation, and irregularity in shape. These morphological characteristics suggest that BV induces apoptotic cell death in NCI-H1299 cells.

To further ascertain the induction of apoptosis in NCI-H1299 cells by BV, BV-treated cells were biochemically analyzed via TUNEL assay and DAPI staining assay. As shown in Fig. 3, cells in which the nuclei were condensed in cultures of NCI-H1299 cells treated with BV at a concentration of 10 μg/ml for 24 h were stained dark brown under the light microscope. In DAPI staining, cells were observed via fluorescence microscopy following treatment with DAPI, which specifically stains the nuclei. The assay revealed the occurrence of nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies upon BV treatment.

Cell cycle distribution changes induced by BV

Through flow cytometric analysis, it was determined that there was an increased number of cells in the sub-G1 phase (from 2.77% to 31.79% of total) and a decreased number of cells in the G1 phase (from 25.68% to 16.68% of total) in the 10 μg/ml BV-treated cells (Fig. 3).

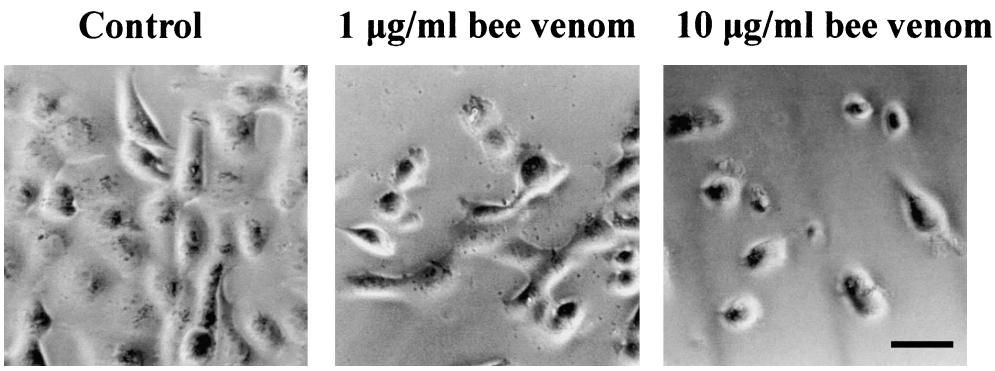

Fig. 2. Effect of bee venom (BV) on the morphology of NCI-H1299 cells. Photomicrographs from phase-contrast microscopy. Cell shrinkage, irregularity in cellular shape, and cellular detachment were seen in the BV-treated cultures, but not in the control. Scale bars represent 100 μm.

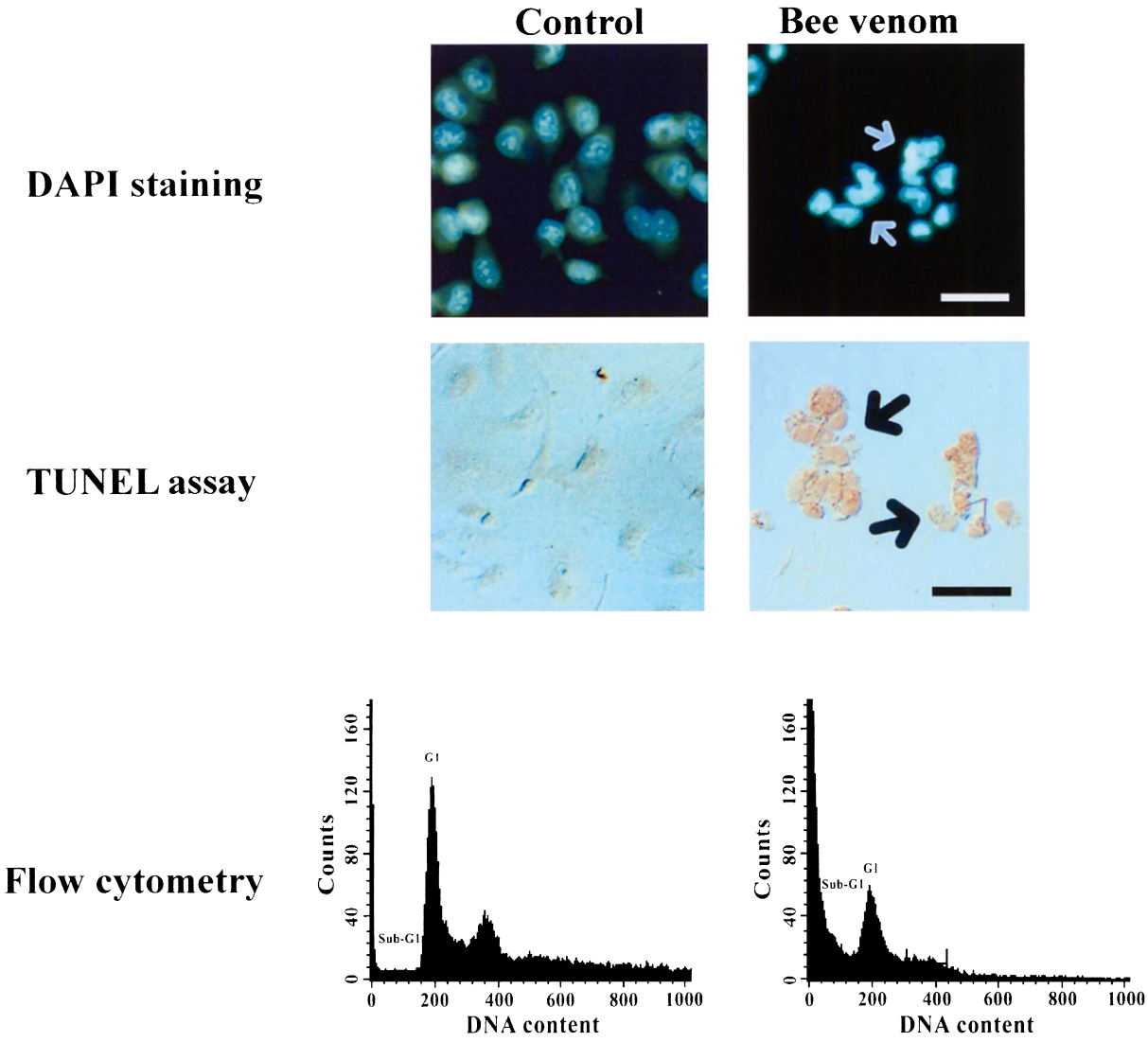

Fig. 3. Characterization of bee venom (BV)-induced cell death in NCI-H1299 cells. Cells were cultured without BV (control) and with 10 μg/ml BV for 24 h. Scale bars represent 100 μm. Top: Cells stained with DAPI. The white arrows indicate condensed nuclei. Middle: Cells stained using TUNEL assay. Black arrows indicate condensed and marginated chromatin. Bottom: Flow cytometric analysis. Note the decreased number of cells in the G1 phase and the increased number of cells in the sub-G1 phase after treatment with 10 μg/ml BV.

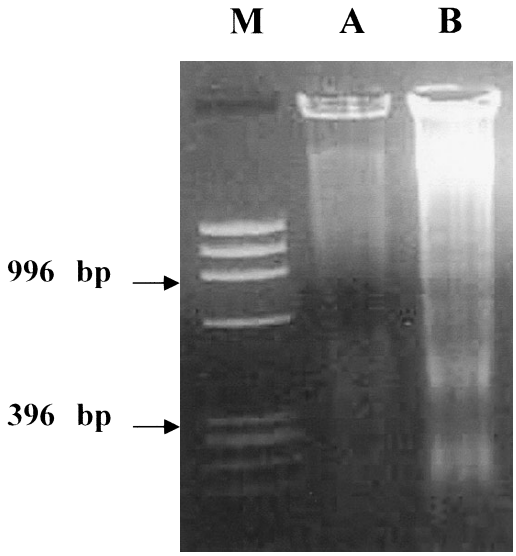

Fig. 4. Electrophoretic analysis of genomic DNA of NCI-H1299 cells treated with bee venom (BV). Cells were incubated for 24 h without BV (control) and with 10 μg/ml BV. The genomic DNA was extacted and analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide. NCI-H1299 cells treated with BV exhibited the ladder pattern characteristic of apoptosis. M, Marker; A, Control; B, BV-treated cells.

DNA fragmentation characterization of apoptosis

In order to ascertain the induction of cell death by BV, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was analyzed. As seen in Fig. 4, treatment with BV at a concentration of 10 μg/ml for 24 h resulted in the formation of definite fragments that could be seen via electrophoretic examination as a characteristic ladder pattern.

Effects of BV on expression of bax and bcl-2 mRNA

RT-PCR analysis of the mRNA levels of *bax* and *bcl-2* was performed in order to provide an estimate of the relative levels of expression of these genes. In the present study, bax and bcl-2 mRNA levels in the control cells were set at 1.00. The bax mRNA level was markedly increased following treatment for 24 h with BV at concentrations of 1 μg/ml and 10 μg/ml and ASA at a concentration of 100 μg/ml to 1.09 ± 0.02, 2.05 ± 0.02, and 1.93 ± 0.02, respectively (Fig. 5). The Bcl-2 mRNA level changed in a random fashion, to 1.01 ± 0.03 following treatment with BV for 24 h at a concentration of 1 μg/ml, to 0.59 ± 0.02 following treatment at 10 μg /ml, and to 0.70 ± 0.02 following treatment with ASA at a concentration of 100 μg/ml (Fig. 5).

Caspase-3 enzyme activity analysis

Caspase-3 enzyme activity was measured with a microtiter plate reader. From the enzyme activity assay, after 24 h of exposure to BV or ASA, the optical density was observed to increase from 0.61 ± 0.05 to 0.62 ± 0.03, 0.91 ± 0.04, and 0.87 ± 0.04 by treatment with BV at 1 μg/ml and 10 μg/ml and ASA at 100 μg/ml, respectively (Fig. 6).

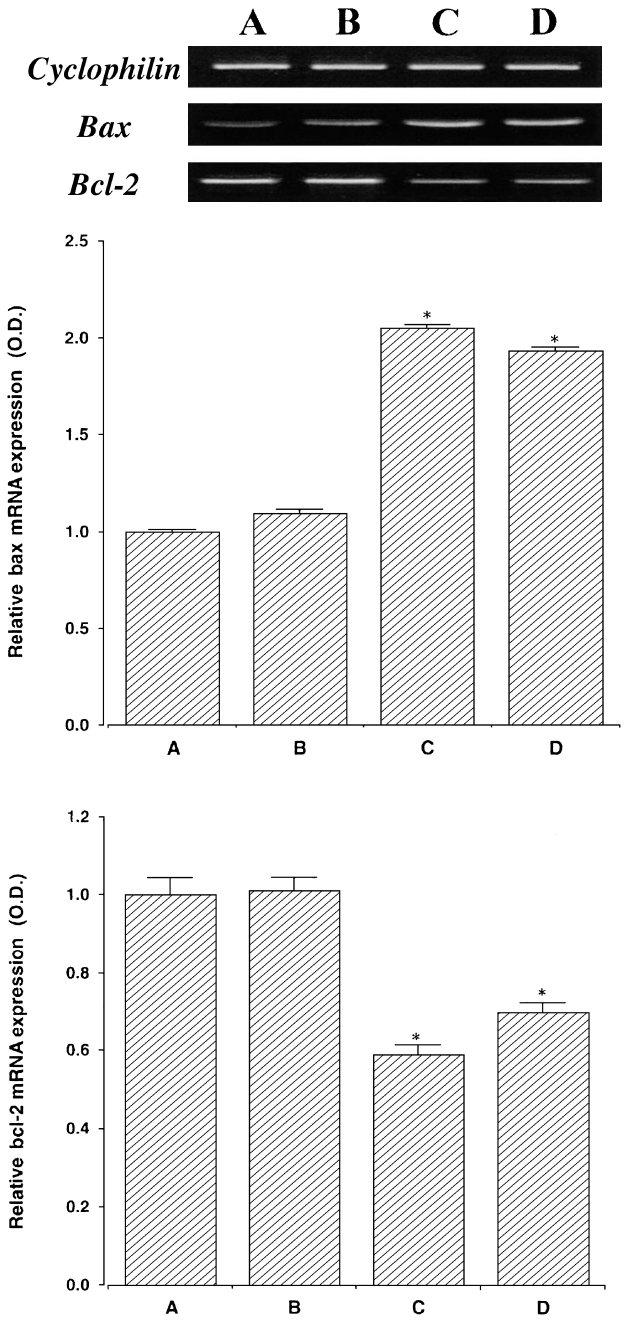

Fig. 5. Assessment of expression of bax and bcl-2 mRNAs in NCI-H1299 cells treated with bee venom (BV) by RT-PCR. RNA prepared from these cells was reverse-transcribed and amplified to examine the expression of *bax* and *bcl-2*. As the internal control, cyclophilin mRNA was also reverse-transcribed and amplified. * represents $P<0.05$ compared to the control group. A, Control; B, 1 μg/ml BV; C, 10 μg/ml BV; D, 100 μg/ml acetylsalicylic acid.

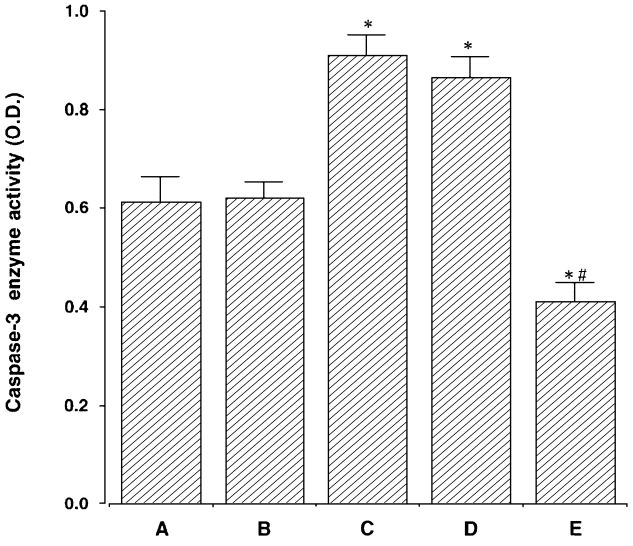

Fig. 6. Results of caspase-3 enzyme activity assay. The optical density was measured at 405 nm. * represents $P<0.05$ compared to the control group. # represents $P<0.05$ compared to the 10 μg/ml bee venom (BV)-treated group. A, Control; B, 1 μg/ml BV; C, 10 μg/ml BV; D, 100 μg/ml acetylsalicylic acid. E, 10 μg/ml BV with DEVD-fmk. DEVD-fmk is a caspase inhibitor.

Effects of BV on expression of COX-1 and COX-2 mRNA

RT-PCR analysis of the mRNA levels of *COX-1* and *COX-2* was performed in order to provide an estimation of the relative levels of expression of the two genes. Figure 7 shows the levels of expression of cyclophilin, COX-1 and COX-2 mRNAs in NCI-H1299 cells, and COX-1 and COX-2 mRNA levels in the control cells were set at 1.00. COX-1 mRNA level was slightly decreased, although the difference was of no statistical significance, to 1.01 ± 0.10 by treatment with BV for 24 h at a concentration of 1 μg/ml, to 0.95 ± 0.09 by 10 μg/ml BV, and to 0.89 ± 0.12 by treatment with ASA at a concentration of 100 μg/ml. COX-2 mRNA level was markedly decreased, to 0.98 ± 0.03 by treatment with BV for 24 h at a concentration of 1 μg/ml, to 0.67 ± 0.03 by treatment at 10 μg/ml BV, and to 0.65 ± 0.02 by treatment with ASA at a concentration of 100 μg/ml.

In addition, in order to study the effect of BV on COX-2 mRNA expression in cells of the NCI-H1299 cell line over time, cells were incubated with BV at final concentrations of 1 μg/ml and 10 μg/ml for 6, 12, 24, and 48 h. The COX-2 mRNA level was slightly decreased, to 0.98 ± 0.10, 0.96 ± 0.09, 0.93 ± 0.09, and 0.90 ± 0.94 following treatment with BV at a concentration of 1 μg/ml for 6, 12, 24, and 48 h, respectively; these decreases were of no statistical significance, however. On the other hand, when the concentration of BV was raised to 10 μg/ml, the COX-2 mRNA level was decreased to 0.95 ± 0.03, 0.88 ± 0.03, 0.57 ± 0.02, and 0.51 ± 0.02 after 6, 12, 24, and 48 h (Fig. 8).

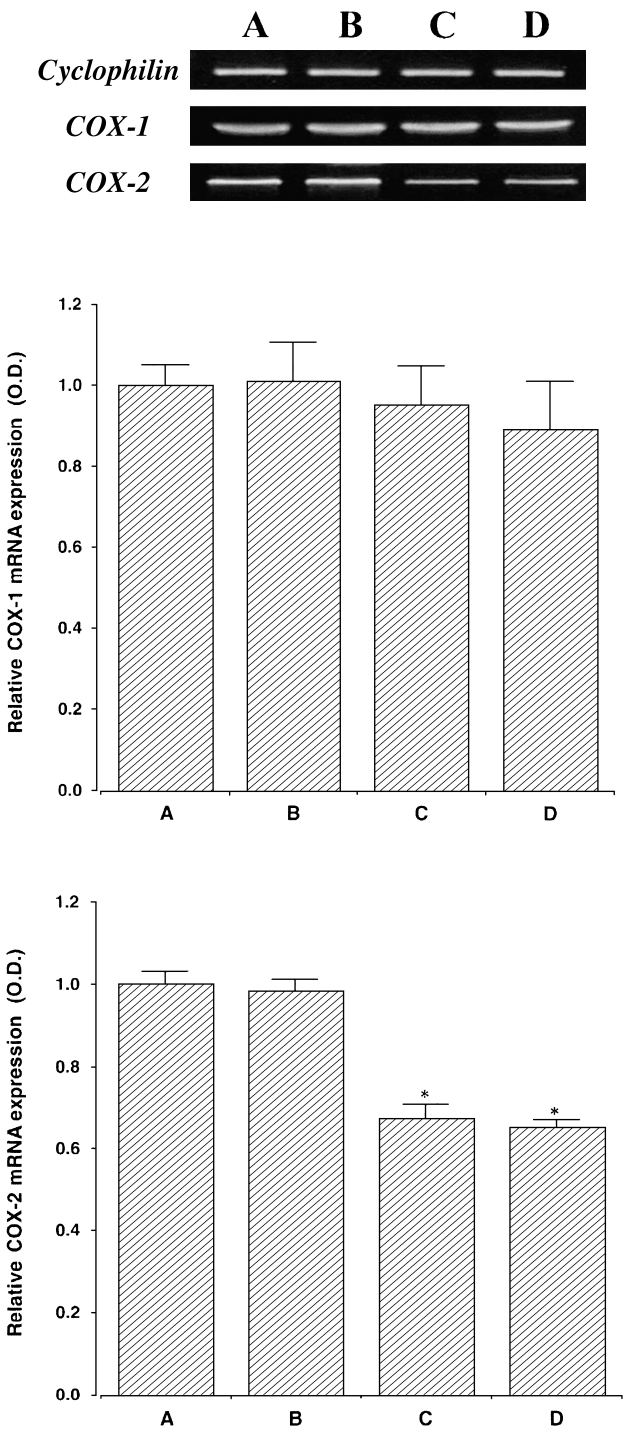

Fig. 7. Assessment of expression of COX-1 and COX-2 mRNAs in NCI-H1299 cells treated with bee venom (BV) by RT-PCR. A, Control; B, 1 μg/ml BV; C, 10 μg/ml BV; D, 100 μg/ml acetylsalicylic acid. RNA prepared from these cells was reverse-transcribed and amplified to examine the expression of *COX-1* and *COX-2*. As the internal control, cyclophilin mRNA was also reverse-transcribed and amplified. * represents $P<0.05$ compared to the control group.

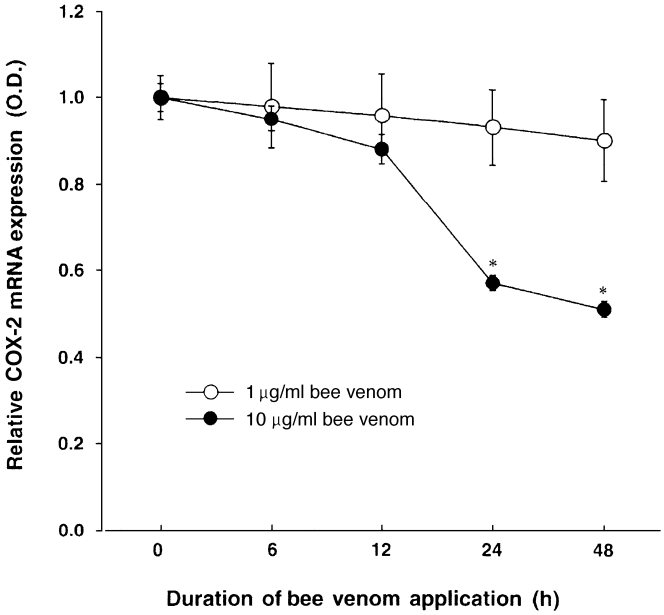

Fig. 8. Effect of bee venom (BV) on *COX-2* mRNA expression in NCI-H1299 cells over time. Administration of BV at a concentration of 10 μg/ml inhibits COX-2 mRNA expression in a time-dependent manner. * represents $P<0.05$ compared to the control group.

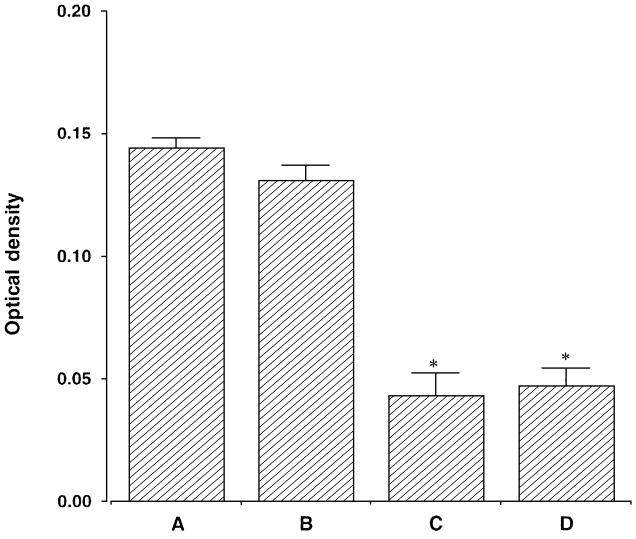

Fig. 9. Measurement of prostaglandin E_2 in NCI-H1299 cells treated with bee venom (BV) by immunoassay. A, Control; B, 1 μg/ml BV; C, 10 μg/ml BV; D, 100 μg/ml acetylsalicylic acid. * represents $P<0.05$ compared to the control group.

Effects of BV on PGE_2 synthesis

NCI-H1299 cells were treated with BV at concentrations of 1 μg/ml and 10 μg/ml and with ASA at a concentration of 100 μg/ml. From the PGE_2 immunoassay, after 24 h of exposure to BV and ASA, the rate of optical density was shown to increase from 0.144 ± 0.004 to 0.131 ± 0.006, 0.043 ± 0.009 and 0.047 ± 0.007 by treatment with BV at 1 μg/ml and 10 μg/ml and ASA at 100 μg/ml, respectively (Fig. 9).

Discussion

The purpose of the present study was to find out whether BV, through induction of apoptosis and inhibition of *COX*, possesses an inhibitive effect on human lung cancer cells. It was demonstrated that BV exerts two profound effects on cells of the human lung carcinoma cell line NCI-H1299. One is that BV induces apoptotic cell death; the other is its selective inhibition of COX-2 mRNA expression.

Analysis of cytotoxicity by MTT assay confirmed that BV is dose- and duration-dependent in its cytotoxic effects and that these effects are apparent at a concentration of 10 μg/ml. From flow cytometric analysis of DNA content using 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 cells in various stages 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. (12) in a study involving colon cancer cells.

In addition, 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 is seen upon electrophoretic examination as a characteristic ladder pattern (12, 32). DNA strand breaks are also known to occur during the process of apoptosis, and nicks in the DNA molecules can be detected via TUNEL assay (34); such strand breaks were shown to occur in the BV-treated lung cancer cells. 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 present in BV-treated cells stained with DAPI. It has also been reported that cells undergoing apoptosis exhibit cytoplasmic blebbing, nuclear shrinkage, chromatin condensation, irregularity in shape and retraction of processes (35).

Members of the bcl-2 family of proteins are characterized by their ability to form a complex combination of heterodimers with bax and homodimers with itself (36). When bax, the first pro-apoptotic homologue to be identified, is overexpressed in cells, apoptotic death in response to a death signal is accelerated; this finding

has resulted in its designation as a death agonist. When bcl-2 is overexpressed, it heterodimerizes with bax, and cell death is suppressed. Presumably, the ratio of bax to bcl-2 serves as a determinant of the susceptibility of cells to apoptosis (20). In the present study, BV treatment resulted in an increase in *bax* expression and a decrease in *bcl-2* expression.

Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway; caspase-3 in particular, when activated, has many cellular targets that, when severed and/or activated, produce the morphologic features of apoptosis (21). The present data indicates an up-regulation of caspase-3 activity in cells exposed to BV.

The definitive molecular mechanisms behind 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 (29). COX is a key enzyme in the synthesis of PGs. COX-1 is a constitutive enzyme present in most cells, whereas COX-2 is inducible and is often up-regulated in tumors (37), and selective down-regulation of COX-2 is an important strategy in the development of anti-tumor agents (25). Hanif et al. (12) reported that drugs with chemotherapeutic effects induce a shift in the distribution of cell population among the different phases of the cell cycle in colon cancer and that this appears to be mediated by the inhibition of the synthesis of PGs. RT-PCR analysis of *COX* genes was performed in this study; the expression of *COX-2* in NCI-H1299 cells treated with BV (10 μg/ml) was decreased compared to that of the control, and production of PGE_2 in cells treated with BV was also decreased compared to that of the control. On the other hand, unlike some NSAIDs, BV was not observed to inhibit *COX-1*, and 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 demonstrated that BV induces apoptosis and causes selective inhibition of *COX-2* in NCI-H1299 human lung carcinoma cells. It thus seems that BV may have an anti-tumor effect on human lung cancer.

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