Abstract. Our previous studies have shown that bee venom (BV) can induce apoptosis in human cervical cancer Ca Ski cells, but it can also affect human breast cancer cells, though its molecular mechanisms are not precisely known. In this study, the molecular mechanisms of apoptosis induced by BV in human breast cancer MCF7 cells were investigated. BV induced morphological changes (examined by phase-contrast microscopy) and inhibited the proliferation (examined by MTT assay) of MCF7 cells; both effects occurred in a dose- and time-dependent manner. Flow cytometric analysis demonstrated that BV induced the production of reactive oxygen species (ROS) and dysfunction of the mitochondrial membrane potential (Δψm), and led to cytochrome c release, an increase in the levels of caspase-9 and Poly (ADP-ribose) polymerase (PARP) and then apoptosis. It also showed that BV induced S-phase arrest in MCF7 cells which may occur through the promotion of p53, p21, p27 and the exhibition of Cdk2. Western blotting demonstrated that BV reduced Bcl-2 and increased Bax protein levels which may have caused the changes of Δψm. BV treatment led to ROS production up to but after treatment led to a decrease in the levels of ROS, which may be associated with the observations of BV affecting glutathion S-transferase (GST), Zn-superoxide dismutase (Zn-SOD), Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and catalase. The Comet assay also showed that BV induced DNA damage while DAPI staining also confirmed that BV induced apoptosis in examined MCF7 cells. Our results also showed that BV increased the levels of AIF and EndoG in MCF7 cells. In conclusion, our data demonstrated that BV induced apoptosis via a mitochondria-dependent pathway based on the changes of Δψm, AIF and EndoG release in MCF7 cells.

Bee venom (BV) has been used in oriental medicine for the treatment of chronic inflammatory diseases, particular in rheumatoid arthritis, and pain relief (1-3). It was also reported that BV has several biological activities such as inducing analgesic and anti-inflammatory effects (1, 4-6). BV was found to inhibit cyclooxygenase-2 expression in human lung cancer cells (7) and induce apoptosis in synovial fibroblasts of patients with rheumatoid arthritis through caspase-3 activation (8). It was also found that BV inhibited mammary carcinoma cell proliferation and tumor growth in vivo but it caused tumor rejection after the stimulation of the local cellular immune responses in lymph nodes (9, 10). Furthermore, BV inhibited the proliferation of vascular smooth muscle cells through induction of apoptosis via suppression of NF-κB and Akt activation, and down-regulation of Bcl-2 (11). BV induced apoptosis in human leukemia U937 cells through down-regulation of the ERK and Akt signaling pathway, with Bcl-2 and caspase-3 as the key regulators (12).

It is well documented that the best strategy for the function of an anticancer agent is to induce apoptosis of the target cancer cells. The reason is that: i) apoptosis plays a
critical role in the development and homeostasis of multicellular organisms; ii) apoptosis is a well-regulated and organized death process involved in physiological and pathological conditions (13). Apoptotic features include cellular morphological changes, membrane blebbing, chromatin condensation, oligonucleosomal DNA cleavage, translocation of phosphatidylserine of the plasma membrane from the inner to the outer leaflet, dysfunction
of mitochondria and activation of a family of caspases (14-16). Although there are many characters of apoptosis, the key hallmark for apoptosis is caspase activation and the dysfunction of mitochondria. Recent studies have shown that BV induced apoptosis in human cervical Ca Ski cancer cells through a mitochondrial pathway (17). However, there is no available information to address whether BV affects human breast cancer MCF7 cells. Therefore, in this study we investigated whether or not BV induced ROS production and the role of mitochondria in the induction of apoptosis brought about by BV in MCF7 cells.

Materials and Methods

Chemicals and reagents. DiOC₆, potassium phosphates and dimethyl sulfoxide (DMSO) were purchased from Merck Co. (Darmstadt, Germany). BV, 4,6-diamidino-2-phenylindole (DAPI), DCFH-DA, propidium iodide (PI), RNase, trypsin, fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

Human breast adenocarcinoma MCF cell line. MCF7 cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75 cm² tissue culture flasks containing RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% L-glutamine, and grown at 37°C under a humidified 5% CO₂ and 95% air at 1 Atm. Cells were cultured for several generations and the viability of each generation was verified (18).

BV-induced morphological changes and proliferation of human breast cancer MCF7 cells. Approximately 2x10⁵ cells/well MCF7 cells were plated in 12-well plates in RPMI-1640 + 10% FBS and were incubated with BV at 0, 2.5, 5, 7.5, 10 and 12.5 µg/ml for 24 h, or 10 µg/ml for 0, 6, 12, 24, 48 and 72 h, only adding DMSO (solvent) for the control regimen, and grown at 37°C in 5% CO₂ and 95% air. To determine morphological changes, phase-contrast microscopy was used as described elsewhere (18-22). To determine the inhibition of proliferation, the MTT assay was used (17).

BV-induced cell cycle arrest and apoptosis in human breast cancer MCF7 cells. Approximately 2x10⁵ cells/well MCF7 cells were plated in 12-well plates in RPMI-1640 + 10% FBS and were incubated with 10 µg/ml BV for 0, 6, 12, 24, 48 and 72 h, only adding DMSO (solvent) as the control, and grown at 37°C in 5% CO₂ and 95% air. Cells were harvested and fixed gently with the addition of 70% ethanol (in PBS) at 4°C overnight, then washed twice and re-suspended in PBS containing 40 µg/ml PI, 0.1 mg/ml RNase and 0.1% Triton X-100 in the dark. After 30 min at 37°C, the cells were analyzed with flow cytometry (Becton-Dickinson, San Jose, CA, USA) using an argon ion laser at 488 nm. The cell cycle distribution and proportion of the sub-G1 group (apoptosis) were determined and analyzed (22).

Comet assay of BV-induced apoptosis in human breast cancer MCF7 cells. MCF7 cells were plated in 12-well plates at a density of 2x10⁵ cells/well and grown for 24 h. The cells were incubated with BV at final concentrations of 0, 2.5, 5, 10 and 10.5 µg/ml, only adding DMSO (solvent) for the control regimen, and grown at 37°C in 5% CO₂ and 95% air, and were then isolated for the examination of DNA damage using the Comet assay as described elsewhere (18-22).

DAPI staining of BV-induced apoptosis in human breast cancer MCF7 cells. MCF7 cells were plated in 12-well plates at a density of 2x10⁵ cells/well and grown for 24 h. The cells were incubated with 10 µg/ml BV for 0, 6, 12, 24, 48 h, only adding DMSO (solvent) for the control regimen, and grown at 37°C in 5% CO₂ and 95% air. Cells were then stained by DAPI and photographed under fluorescence microscopy as described elsewhere (18-22).

DCFH-DA staining for examining the effects of BV on the production of reactive oxygen species from MCF7 cells. Approximately 5x10⁵ Ca Ski cells/ml in 12-well plates were treated with 10 µg/ml BV for 0, 1, 3, 6, 12, 24 and 48 h. The cells were harvested and washed twice, re-suspended in 500 µl of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and incubated with BV for different time periods. The cells were then visualized under a fluorescence microscope (22).
(10 μM) (DCFH-DA; Sigma) and incubated at 37°C for 30 min to detect the changes of ROS using flow cytometry, as described elsewhere (18-22).

The effect of BV on the mitochondrial membrane potential (Δψm) of MCF7 cells. Approximately 5x10^5 MCF7 cells/ml in 12-well plates were treated with 10 μg/ml BV for 0, 1, 3, 6, 12, 24 and 48 h. In order to detect the changes of Δψm from each sample of MCF7 cells, the cells were harvested and washed twice, resuspended in 500 μl of DIOc_6 (4 mol/l), incubated at 37°C for 30 min and analyzed by flow cytometry as described elsewhere (18-22).

**Figure 3.** BV induced cell cycle arrest and apoptosis of human breast cancer MCF7 cells. The MCF7 cells (2x10^5 cells/well; 12-well plates) were plated in RPMI-1640 + 10% FBS with 10 μg/ml BV treated for 0, 24 and 48 h. The cells were harvested and stained with PI, and then cell cycle and sub-G1 groups were examined by flow cytometry as described in Materials and Methods. Panel A: representative profiles of cell cycle and apoptosis; panel B: the distribution of cells in the cell cycle and proportion of the sub-G1 group. Data represent mean±S.D. of three experiments; *p<0.05, significantly different from the control.

Western blotting of cell cycle and apoptosis associated proteins from MCF7 cells after exposure to BV. Approximately 5x10^6 MCF7 cells/ml in 6-well plates were treated with 10 μg/ml BV for 0, 6, 12, 24 and 48 h. The cells were then harvested by centrifugation and were lysed in lysis buffer as described elsewhere (18-22). Total proteins were collected from each sample for staining by primary antibody for p53, p27, Cdk2, p21, p16, GST, SOD (Mn), SOD (Cu/Zn), catalase, Bax, Bcl-2, Bcl-xL, Apaf, pro-caspase-9, pro-caspase-3, AIF, EndoG, PARP, Fas/CD95, pro-caspase-8, t-Bid and β-actin from Santa Cruz, CA, USA, and were then stained by secondary antibody and measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described elsewhere (18-22).
Results

**BV induced morphological changes and inhibited proliferation of human breast cancer MCF7 cells.** Incubation of MCF7 cells with different concentrations of BV or 10 µg/ml BV treated for 0, 6, 12, 24, 48 and 72 h indicated that BV induced morphological changes (Figure 1) and inhibited proliferation (reduced the percentage of viable cells) (Figure 2). Both effects were dose- and time-dependent.

**BV induced cell cycle arrest and apoptosis in human breast cancer MCF7 cells.** After MCF7 cells were incubated with 10 µg/ml BV for 0, 24 and 48 h, they were isolated for examining the effects on cell cycle and apoptosis. The results showed that BV induced G0/G1 arrest and promoted the S-phase fraction and increased the proportion of cells in the sub-G1 group (apoptosis) (Figure 3). These results demonstrated that BV induced apoptosis in MCF7 cells.

**BV-induced apoptosis in human breast cancer MCF7 cells shown by DAPI staining and Comet assay.** After MCF7 cells were incubated with different concentrations of BV, it was demonstrated that BV induced apoptosis (Figure 4A) by nuclear condensation, and apoptosis induction effects were time-dependent. DNA damage examined by single cell electrophoresis (Comet assay) demonstrated that BV induced DNA damage (Figure 4B) and the effect was dose-dependent.

**DCFH-DA and DiOC₆ staining for the effects of BV on the production of ROS and the mitochondrial membrane potential (Δψm) of MCF7 cells.** The flow cytometric assay indicated that BV induced the production of ROS in the examined cells (Figure 5A). The production of ROS relative to the control increased up to 3 h treatment; after 3 h the ROS production decreased up to 48 h, finally becoming significantly lower than the control. The flow cytometric assay indicated that BV significantly reduced the level of Δψm in examined cells (Figure 5B); although remaining significantly lower than the control throughout the 48-h incubation, the loss of Δψm was somewhat restored in a time-dependent manner.

**Western blotting showed BV affected proteins associated with cell cycle arrest and apoptosis in MCF7 cells.** MCF7 cells were incubated with 10 µg/ml BV for 6, 12, 24 and 48 h. Then the cells were harvested for associated protein expressions, and were estimated using Western blotting. The results demonstrated that BV promoted the
expression of p53, p27, Bax, cytochrome c, EndoG, AIF, Fas/CD95 and t-Bid (Figure 6B-E). However, the expressions of Cdk2, p21, p16, GST, SOD (Zn), SOD (Cu/ZN), catalase, Bel-2, Bel-xL, pro-caspase-9, pro-caspase-3, PARP and pro-caspase-8 (Figure 6A, B, C, D and E) were inhibited. This may have led to the G0/G1 arrest and S-phase accumulation associated with apoptosis in the examined MCF7 cells. BV promoted Bax expression and inhibited Bel-2 and Bel-xL. This affected the ratio of Bax/Bel-2 that may have led to dysfunction of mitochondria followed by apoptosis.

**Discussion**

BV has been used for medicine for a long time in oriental populations, and it has been found to induce apoptosis in human cancer cell lines, such as human leukemia U937 cells (12), osteosarcoma MG-63 cells (23), breast cancer MCF7 cells (24), and lung cancer NCI-H1299 cells (25, 26). BV inhibited mammary carcinoma cell proliferation *in vitro* (7) and it also inhibited K1735M2 mouse melanoma cells *in vitro* and the growth of murine B16 melanomas *in vivo* (27). Furthermore, it was reported that BV inhibited proliferation and induced apoptosis by affecting Bax and Bel-2 in human breast cancer MCF7 cells (24) but the exact molecular mechanism and signal pathway is still unclear, particularly that involving the mitochondria.

The present study aimed at showing the role of mitochondria in BV-induced apoptosis in human breast cancer MCF7 cells. BV may induce apoptosis through Fas receptor based on the levels of Fas increasing in MCF7 cells after exposure to BV. Other investigators have already demonstrated that BV induced apoptosis through a decrease in Bel-2 expression and an increase in Bax, leading to caspase-3 activation then apoptosis (11, 12, 28). Our data also showed that BV not only induced apoptosis through mitochondria (reduction in the levels of Δψm and caspase-3 expression), but also promoted the levels of AIF and EndoG in the cytoplasm. The morphological changes and inhibition of proliferation in MCF7 cells after exposure to BV indicated that BV are in agreement with other reports on other cell lines (12, 17). We also used DAPI and flow cytometry assay which showed that BV induced apoptosis. When apoptosis occurs DNA strand breaks are present and it is known that the nicks in DNA molecules can be detected via the DAPI assay (29, 30). This result can also be confirmed by the Comet assay; in our case it indicated that BV induced DNA strand breaks in MCF7 cells. It is well-known that apoptosis is involved in the activation of endonucleases, whose activity results in the form of DNA fragmentation which can be seen upon electrophoretic examination (30). Our data also confirmed that of other investigators which demonstrated that BV affected Bax and Bcl-2, and promoted p53 and p21 (23). It is recognized that the ratio of Bax to Bcl-2 serves to determine the susceptibility of cells to apoptosis (31-32) because the ratio of Bax/Bcl-2 determines the level of Δψm. Interestingly, in this study BV changed the Δψm of MCF7. It was also demonstrated that BV affected Δψm, leading to apoptosis through caspase-3 dependent and independent (AIF and End G) pathways.

In conclusion, our results showed that BV affected ROS production, induced DNA damage through the Fas receptor, promoted caspase-8, affected the ratio of Bel-
Figure 6. Representative Western blot showing the changes in the levels of cell cycle- and apoptosis-associated proteins in human breast cancer MCF7 cells after exposure to BV. MCF7 cells (5x10^6/ml) were treated with 10 µg/ml BV for 6, 12, 24 and 48 h then harvested for associated protein expressions estimated using Western blotting as described in Materials and Methods. Panel A: GST, SOD (Mn), SOD (Cu/Zn) and catalase; Panel B: p53, p27, p21, p16 and Cdk2; Panel C: Bax, Bcl-2, Bcl-xL and t-Bid; Panel D: cytochrome c, Apaf-1, pro-caspase-9, AIF and EndoG; panel E: Fas/CD95, pro-caspase-8, pro-caspase-3 and PARP.
2/Bax, changed the levels of Δψm, caused cytochrome c release, and apoptosis was induced either by caspase-9 and -3 activation or through the release of EndoG and AIF from mitochondria (Figure 7).

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References


