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Melittin Inhibits Human Prostate Cancer Cell Growth through Induction of Apoptotic Cell Death

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ABSTRACT. It was previously found that melittin inhibited NF-κB activity by reacting with signal molecules of NF-κB which is critical contributor in cancer cell growth by induction of apoptotic cell death. We here investigated whether melittin inhibits cell growth of human prostate cancer cells through induction of apoptotic cell death, and the possible signal pathways. Melittin (0~1 μg/ml) inhibited prostate cancer cell growth in a dose dependent manner. Conversely related to the growth inhibitory effect, melittin increased the induction of apoptotic cell death in a dose dependent manner. Melittin also inhibited DNA binding activity of NF-κB, an anti-apoptotic transcriptional factor. Consistent with the induction of apoptotic cell death and inhibition of NF-κB, melittin increased the expression of pro-apoptotic proteins caspase-3, and Bax but down-regulated anti-apoptotic protein Bcl-2. These findings suggest that melittin could inhibit prostate cancer cell growth, and this effect may be related with the induction of apoptotic cell death via inactivation of NF-κB.

Keywords: Melittin, PC-3, Apoptotic cell death, NF-κB.

INTRODUCTION

Prostate cancer is the most common cancer as well as the second leading cause of cancer-related deaths in men of Western countries (Jemal *et al.*, 2003). One out of nine men over 65 years of age is frequently diagnosed with prostate cancer in the United States (Bosland *et al.*, 2002; Jemal *et al.*, 2003). Current therapy for prostate cancer is limited by the propensity of the disease to progress from androgen-dependent to an androgen-independent state. In the normal prostate, organ homeostasis is maintained by a dynamic balance between the rate of cell proliferation and the rate of apoptosis (Kyprianou and Issacs, 1988). Cytotoxic chemotherapies or radiotherapy also do not show any significant improvement in patient condition due to the high recurrence of apoptosis resistance hormone refractory prostate cancer, which is responsible for 28,000 deaths per year (Jemal *et al.*, 2003; Pilat *et al.*, 1998-99). At present, there is no effective therapy available for the treatment of androgen-independent stage of prostate cancer, which usually arises after hormonal deprivation/ablation therapy (Feldman and Feldman, 2001).

Distruption of the molecular mechanisms that regulate apoptosis and cell proliferation among the stroma and epithelial cell populations may underlie the abnormal growth of the gland that characterizes neoplastic development of the prostate (Bruckheimer and Kyprianou, 2000; Kyprianou *et al.*, 1996). Thus apoptosis induction provides a relevant endpoint for testing new drugs for therapeutic efficacy against prostate growth disorders, benign prostatic hyperplasis (BPH), and prostate cancer (Kyprianou *et al.*, 1996). Nuclear factor-κB (NF-κB) is an important element in regulating growth or apoptosis of tumors, including prostate cancers (Suh *et al.*, 2002). Thus, both NF-κB-mediated signals strongly affect prostate tumorigenesis through regulation of apoptosis. It is generally recognized that cancer cells have constituted high level of NF-κB to survival. Li *et al.*

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(2002) showed that NF-κB was up-activated in PC-3 cells. Thus, any compounds reducing NF-κB activity can be possible agents to inhibit cancer cell growth through induction of apoptotic cell death (Aggarwal, 2004; Suh *et al.*, 2002; Suh and Rabson, 2004).

Important molecular interfaces of specific toxin-receptor/ion channel complexes have been largely studied in drug discovery initiatives (Brown *et al.*, 1999; Samson *et al.*, 2002; Sher *et al.*, 2000; Van and Moyer, 1999). Melittin is a major component of bee venom (BV) which also contains a variety of different peptides including apamin, adolapin and mast cell degranulating (MCD) peptide (Price et al., 1983; Jentsch and Mucke, 1997). Increasing studies have demonstrated that melittin inhibits cancer cell growth, and induction of apoptotic cell death (Holle *et al.*, 2003; Chen *et al.*, 2004). However, systemic experiments demonstrating molecular mechanisms and effect of melittin on prostate cancer cells have not been reported. We recently also found that melittin and bee venom toxin inhibited NF-κB activation and target gene expression through the interaction with p50 in the NF-κB pathway, thereby inhibited inflammatory reaction in the *in vivo* and *in vitro* arthritis models (Park *et al.*, 2004). We also found that melittin inhibited smooth muscle cell proliferation via induction of apoptotic cell death through down activation of NF-κB (Son *et al.*, 2006). To investigate the effect of melittin on prostate cancer cell growth and to gain better insight into the action mechanisms, in this study, we conducted an in vitro analysis to evaluate the prostate cell response to melittin in order to determine the ability of this compound as a therapeutic agent to suppress prostate cell growth by affecting cell proliferation and apoptosis, and determine possible mechanisms related with inactivation of NF-κB and other cell survival signals in prostate cancer PC-3 cells.

METHODS

Cell culture

The PC-3 prostate cancer cell was obtained from ATCC (American Type Culture Collection, Rockville, MD). PC-3 cells were cultured in RPMI-1640 medium (Life Technologies Inc., Gaithersberg, MD) supplement with 10% fetal calf serum (FCS; Collaborative Biomedical Products, Bedford, MA) and antibiotics, penicillin/streptomycin (100 unit/ml, Bioproducts, Walkersville, MD) Cell cultures were then maintained at 37°C in a humidified atmosphere of 5% CO_2.

WST-1 assay

Cells were plated at a density of 1×10^5 cells per well in 96-well plates and then subconfluent cells were exposed to different doses (0~10 mg/ml) of melittin (Sigma Co., USA) for 12, 24, 48, 72 hr. After treatment, cell viability was measured by WST-1 assay (Dojin Laboratory, Kumamoto, Japan) according to the manufacturer's instructions. WST-1 solution was added to cells in 96-well plates, cells were incubated at 37.5°C for 1 hr, and the optical density of each well was read at 450 nm.

Evaluation of apoptotic cell death

Apoptotic cell death assay was performed using the 4,6-diamidino-2-phenylindole (DAPI) staining. PC-3 cells were cultured in the absence or presence of increasing concentrations of melittin, and apoptosis induction were evaluated after 24 hr. Apoptotic cells were determined by the morphological changes after DAPI staining under fluorescence microscopic observation (DAS microscope, 100 or 200×; Leica Microsystems, Inc., Deerfield, IL). For each determination, three separated 100-cell counts were scored. Apoptotic cell death was expressed as a percentage calculated from the number of cells with apoptotic nuclear morphology divided by the total number of cells counted. Total number of cells in a given area was determined by using DAPI nuclear staining.

Western blot analysis

Cells were homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM PMFS, 10 μl/ml aprotinin, 1% igapel 630 (Sigma-Aldrich, St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate], and centrifuged at 23,000 g for 1 hr. Equal amount of proteins (80 μg) were separated on a SDS/12%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were blocked for 2 hr at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% tween-20. The membrane was incubated for 5 hr at room temperature with specific antibodies: Bax, Bcl-2, caspase-3 (Santa Cruz, CA, USA Santa Cruz Biotechnology Inc.). The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the ECL western blotting detection system. The relative density of the protein bands was scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, California).

Preparation of nuclear extracts and electromobility shift assays

It was performed according to the manufacturer's recommendations (Promega, Madison, WI). Briefly, 1×10^6 cells/ml was washed twice with 1× PBS, followed by the addition of 1 ml of PBS, and the cells were scraped into a cold Eppendorf tube. Cells were spun down at 15,000 g for 1 min, and the resulting supernatant was removed. Solution A (50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 μg/ml phenylmethyl-sulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 0.5% Nonidet P-40) was added to the pellet in a 2 : 1 ratio (v/v) and allowed to incubate on ice for 10 min. Solution C (solution A + 10% glycerol and 400 mM KCl) was added to the pellet in a 2 : 1 ratio (v/v) and vortexed on ice for 20 min. The cells were centrifuged at 15,000 g for 7 min, and the resulting nuclear extract supernatant was collected in a chilled Eppendorf tube. Consensus oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP for 10 min at 37°C. Gel shift reactions were assembled and allowed to incubate at room temperature for 10 min followed by the addition of 1 μl (50,000~200,000 cpm) of ^{32}P-labeled oligonucleotide and another 20 min of incubation at room temperature. For supershift assays, nuclear extracts from cells treated with melittin (1 μg/ml) were incubated with specific antibodies against the p50, p65 and Rel-A NF-κB isoforms for 1 hr before EMSA. For competition assays, nuclear extracts from cells treated with melittin (0.1 mg/ml) were incubated with unlabelled NF-κB oligonuclaotide (50×, 100× and 200×) or labeled SP-1 (100×) and AP-1 (100×) for 30 min before EMSA. Subsequently 1 μl of gel loading buffer was added to each reaction and loaded onto a 6% non-denaturing gel and electrophoresed until the dye was three-fourths of the way down the gel. The gel was dried at 80°C for 1 hr and exposed to film overnight at 70°C. The relative density of the DNA-protein binding bands was scanned by densitometry using MyImage (SLB, Seoul, Korea), and quantified by Labworks 4.0 software (UVP Inc., Upland, California).

RESULTS

Inhibition of cell growth

Morphological alteration of the cells was demonstrated in Fig. 1. Once the cells were exposed to melittin, the cells were not growth cells, and died in a dose dependent manner. To evaluate an effect of melittin on the cell growth of human prostate cancer cells, we analyzed cell viability using WST-1 assay and direct cell counting. Melittin inhibited prostate cancer cell growth in a dose (0~10 mg/ml) and time-dependent manner (Fig. 2).

Induction of apoptotic cell death

To delineate whether the inhibition of cell growth by the melittin was due to increase of the apoptotic cell death, we evaluated change of the chromatin morphology of human prostate cancer cells using DAPI staining. Consistent with the loss of viability, apoptotic cell death determined after 24 hr treatment was increased in a dose dependent manner. The number of apoptotic cells from 10% in the control was increased up to about

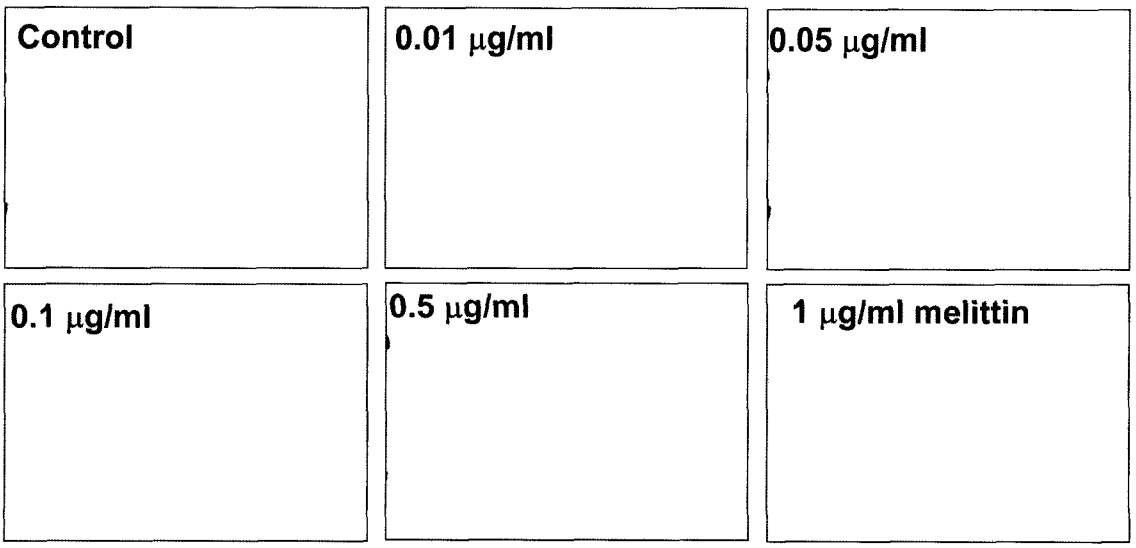

Fig. 1. Morphological changes of PC-3 cells by melittin. Morphological changes were observed under microscope (magnification, 200×). The figures are representative of three experiments, with triplicate of each experiment.

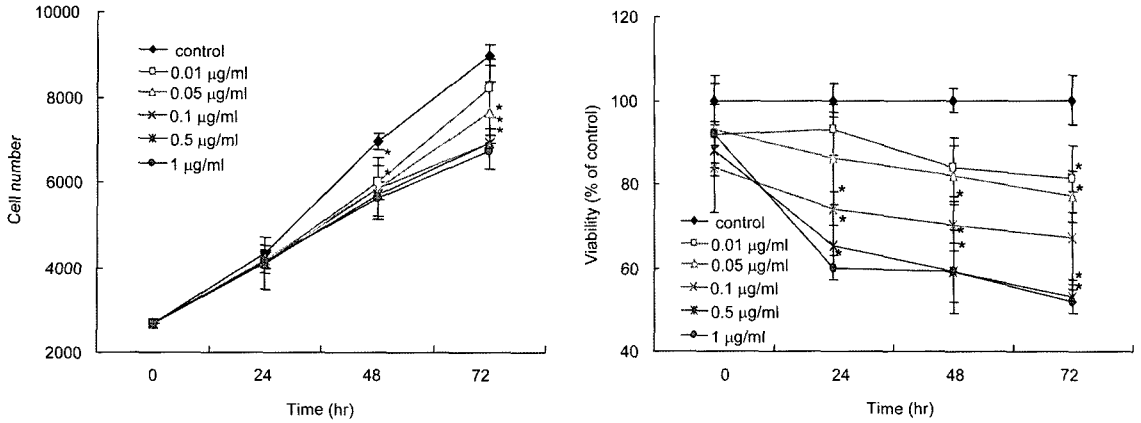

Fig. 2. Cell viability of PC-3 cells by melittin. Cell viability was determined by WST-1 assay as described under *METHODS*. Values are mean ± S.D. of three experiments, with triplicate of each experiment.

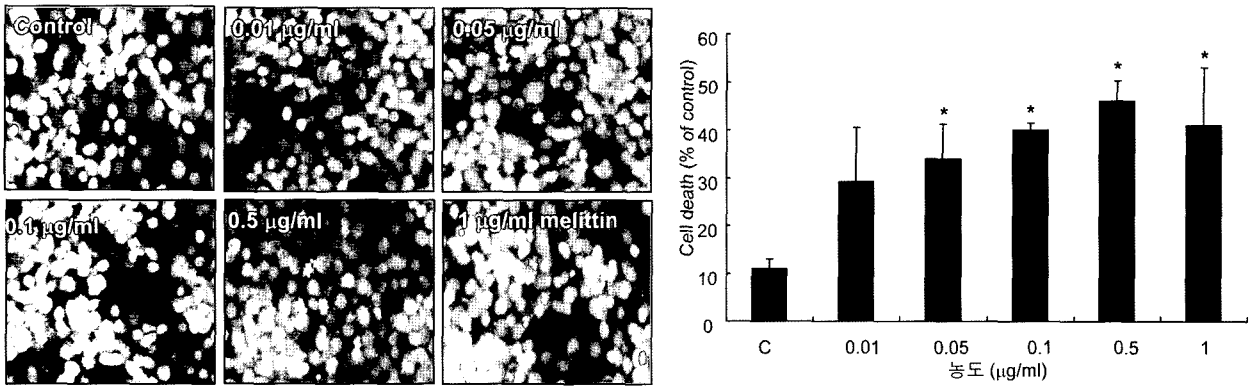

Fig. 3. Effects of melittin on induction of apoptosis of PC-3 cells. The apoptotic cells were examined by fluorescence microscopy after DAPI staining. Treatment of cobrotoxin for 24 hr caused apoptosis characterized by marked chromatin condensations, small membrane-bound bodies (apoptotic bodies), and cellular shrinkage. The cell indicated by the arrow is an example of a morphological characteristic of apoptosis (magnification, 250×). Apoptotic cells were estimated by direct counting of fragmented nuclei after DAPI staining. The values are means ± S.D. of three experiments, with triplicate of each experiment.

40~50% in the cells treated with melittin (0~10 mg/ml) in the PC-3 cells (Fig. 3).

Expression of apoptosis regulatory proteins

Execution of apoptosis occurs through activation of Bax and the caspase proteases and inactivation of Bcl-2. Caspases are a family of cysteine proteases that are expressed as inactive pro-enzymes in normal cells, and upon activation, they are capable of cleaving structural and functional proteins involved in key cellular processes (Wolf and Green, 1999). The increase of apoptotic action was confirmed by the ability of melittin to induce caspase-3 activation (increased expression of active form of csapse-3). Figure 4 reveals a western blot analysis of Bax, Bcl-2 and caspase-3 expression in PC-3 cells before and after treatment with a different dose of melittin. Expression of the Bax and active form of caspase 3 was increased, but the expression of Bcl-2 was decreased in a dose-dependent manner in the cells treated by melittin for 24 hr (Fig. 4).

Inhibition of NF-κB

It has been demonstrated that melittin negatively regulates nuclear transcription factor NF-κB by mean of protein-protein interaction (Park *et al.*, 2004). In addition, NF-κB is also known to inhibitory transcription factor of apoptotic cell death. To investigate the hypothesis whether melittin can inactivate NF-κB, and thereby prevent anti-apoptotic ability of NF-κB causing the cells go apoptotic cell death, we assessed NF-κB activity in the cells treated with different concentration of melittin for 1 hr. NF-κB was highly activated in these cells; however the activation of NF-κB was gradually decreased by the culture in the presence of melittin in

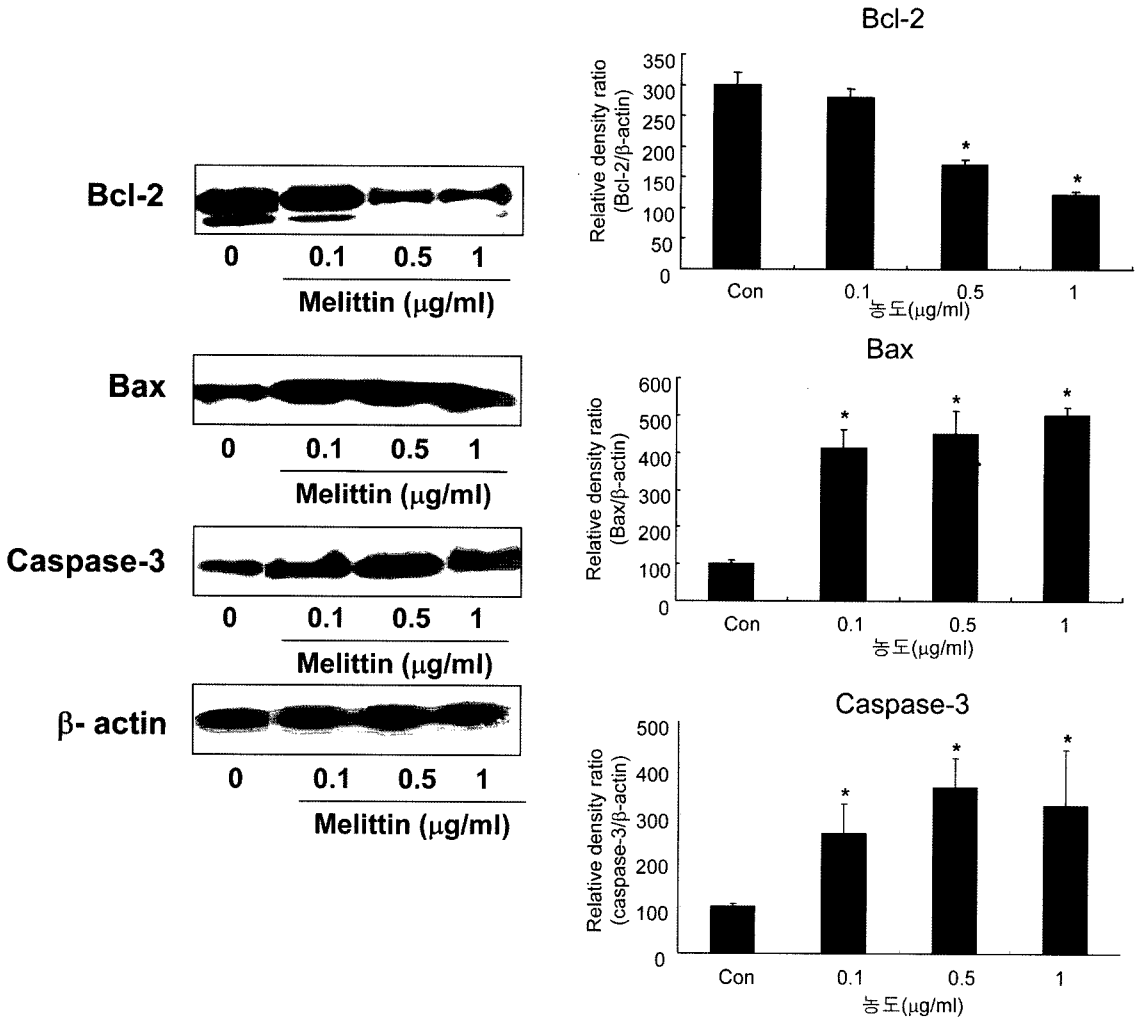

Fig. 4. Expression of apoptosis related molecules in PC-3 cells treated with melttin for 24 hr. Equal amounts of whole cell lysates 50 μg were subjected to electrophoresis and analyzed by Western blot for apoptosis regulatory molecules (caspase-3, Bax, and Bcl-2). Cell lysates were prepared, and levels of proteins were determined by using specific primary antibody followed by peroxidase-conjugated secondary antibody and visualization by enhanced chemiluminescence detection system. The protein bands were visualized with enhanced chemiluminescence, and relative density was analyzed by densitometry. Values are mean ± S.D. of three experiments, with triplicate of each experiment.

the cells (Fig. 5).

DISCUSSION

The central and novel finding in the present study is the identification of in vitro anticancer efficacy of melittin against advanced human prostate carcinoma PC-3 cells. Most of the present available cytotoxic anticancer drugs mediate their effect via induction of apoptosis in cancer cells (Lowe and Lin, 2000; Guseva *et al.*, 2004), and apoptosis is suggested as one of the major mechanisms for the targeted therapy of various cancers including prostate cancer (Gurumurthy *et al.*, 2001; Guseva *et al.*, 2004; Kantoff, 1995; Lowe and Lin, 2000). In case of advanced prostate cancer, cancer cells become resistant to apoptosis and do not respond to cytotoxic chemotherapeutic agents (Pilat *et al.*, 1998). The antiproliferative effects of melittin on cancer cell growth via apoptotic cell death have been reported in several cancer cells including hepatocarcinoma cells (Ling *et al.*, 2005), breast cancer cells (Leuschner *et al.*, 2003), and osteosarcoma cells (Chen *et al.*, 2004). Therefore, melittin showing induction of apoptotic death of prostate cancer cells could be useful in controlling this malignancy (Kantoff, 1995).

It is thought that melittin can cause cell death by cytolytic activity since like many other animal toxin protein, melittin is a membrane-interactive toxin (Phoenix *et*

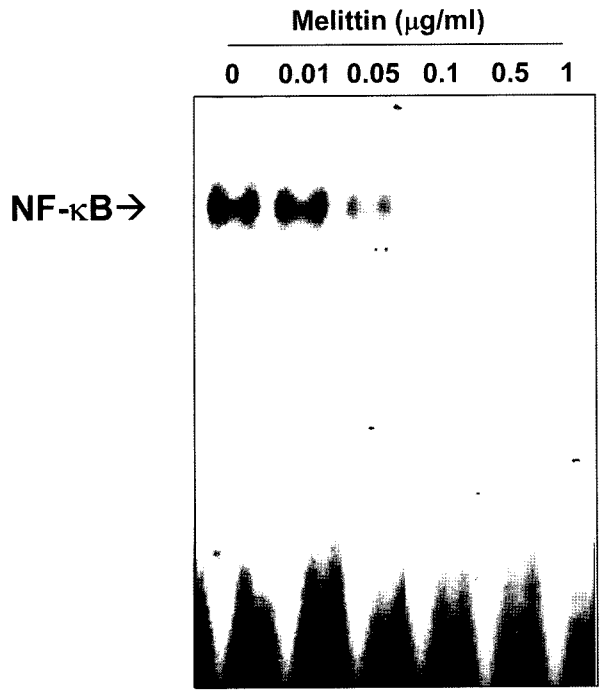

Fig. 5. Effect of melittin on the DNA binding activity of NF-κB in PC-3 cells. Nuclear extracts were prepared from PC-3 cells, which were incubated with 0.1~5 μg/ml melittin for 1 hr. Gel mobility shift assay was done as described under *Materials and Methods*. Similar pattern of DNA binding activity was seen from three different sets of experiments. NF-κB DNA binding activity in PC-3 cells. Values are mean ± S.D. of three experiments, with triplicate of each experiment.

al., 2001), and it mainly works on the cell membrane, thus kills the tumor cell directly. This action may be superior to that of bacterial and plant toxins. Breakdown of extracellular matrix may be another action mechanism of melittin. Consistent with this notion, melittin/avidin conjugate increased matrix metalloproteinase 2 (MMP2), an attractive target for cancer treatment since it breakdown the extracellular matrix, thereby cause cell death (Holle *et al.*, 2003). In this study, melittin (1~4 μg/ml) alone is extremely toxic to cells and induces immediate cell lysis, but becomes inactive when coupled with avidin in DU 145 prostate cancer cells and SK-OV-3 ovarian cancer cells. Our data showing that melittin cause cell growth inhibition via induction of apoptotic cell death rather than cytotoxic cell death may be related with the doses used (0.1~1 μg/ml). Therefore, due to its tumor targeting capabilities as well as its apoptotic cell death properties in vitro, the melittin displays the potential for use in cancer therapy. However, exact mechanism to cause apoptotic cell death is not clear.

Nuclear factor-κB (NF-κB) is an important element in regulating growth or apoptosis of tumors, including prostate cancers (Suh *et al.*, 2002). Thus, both NF-κB-mediated signals strongly affect prostate tumorigenesis through regulation of apoptosis. Li *et al.* (2002) showed that NF-κB was up-activated in PC-3 cells. In the present study, we also found that NF-κB was up-activated in untreated PC-3 cells, but melittin inhibited NF-κB activity. Down regulated NF-κB signals by melittin is consistent with cell growth inhibition. These data suggest that NF-κB signal may be significant contributor in melittin-induced PC-3 cell death. We previously found that melittin binds with NF-κB, IKKα and IKKβ resulting in down regulation of NF-κB activity in Raw 264.7 cells and astrocytes (Park *et al.*, 2004). We recently also found that melittin inhibited smooth muscle cell growth through induction of apoptotic cells via inhibition of NF-κB (Son *et al.*, 2006). Therefore, melittin may act as a very similar mechanism in PC-3 cells as found in Raw264.7 cells as well as smooth muscle cells. Consequently, the reduced activation of NF-κB proteins were associated with down-regulation of the constitutively over-expressed and NF-κB-dependent anti-apoptotic proteins Bcl-2, and/or upregulated apoptotic proteins such as Bax and caspase-3. In the present study, consistent with the increase of the induction of apoptotic cell death, the expression of apoptotic proteins; active caspase-3 and Bax was dose dependently increased, but Bcl-2 was decreased. Activation of Akt and/or MAP kinase (especially ERK) pathway may be also involved in the melittin-induced prostate cance r cell death since we found that melittin induced phosphorylation of Atk as well as ERK kinase. These data suggest that melittin induced apoptotic cell death of PC-3 cells, and the alteration of the expression of apoptosis regulatory proteins resulting in a shift the cells favoring apoptotic cell death.

In conclusion, our present findings showing the *in vitro* anticancer efficacy of melittin, with mechanistic rationale (apoptosis induction), against advanced human prostate cancer cells, warrant its further *in vivo* efficacy. The positive outcomes of such an *in vivo* study could form a basis for the development of melittin as a novel agent for human prostate cancer prevention and/or intervention.

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