Original Article

Melittin Inhibits DU-145 Cell Proliferation Through Induction of Apoptosis

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국문초록

멜라틴이 세포자멸사 유발에 의해 DU-145 세포증식에 미치는 영향

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목적 : 본 연구는 봉약침의 주요성분인 멜리틴이 전립선 암세포주인 DU-145 세포성장에 어떤 영향을 미치는지를 알아보기 위하여 시행하였다.

방법 : 멜리틴이 DU-145의 성장에 미치는 영향을 알아보기 위한 cell viability 측정으로는 WST-1 assay를, 세포자멸사의 관찰에는 DAPI(4,6-diamidino-2-phenylindole)와 TUNEL staining assay를 시행하였으며, 세포자멸사 조절단백질(calpain, Bax, caspase-3, -9, cleaved caspase-3, cleavaged PARP, cleaved caspase-9, Bcl-2, XIAP, cIAP2, Akt, p-Akt, MMP-2, MMP-13)의 관찰을 위하여 western blot analysis를 시행하였다.

결과 : 1. DU-145 세포에서 멜리틴을 처리한 후 세포자멸사가 유도되어 세포성장이 억제되었다.
2. 세포자멸사 관련 단백질 중 분리된 caspase-3, caspase-9은 유의한 증가를, Bcl-2, p-Akt, XIAP, cXIAP는 유의한 감소를 나타내었다.

결론 : 이상의 결과는 멜리틴이 인간 전립선암세포주인 DU-145의 세포자멸사를 유발함으로써 증식억제 효과가 있음을 나타낸 것으로, 전립선암의 예방과 치료에 대한 효과적인 치료제 개발에 도움이 될 것으로 기대된다.

검색어 : 멜리틴, 전립선암, DU-145, 세포자멸사, 세포자멸사 조절단백질

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Ⅰ. Introduction

Prostate cancer is the most commonly diagnosed male cancer in most western countries[1] and is currently the second leading cause of cancer death among men in the United States[2].

Early prostate cancers are eminently treatable due to their androgen dependency, however most of them eventually become resistant to androgen deprivation therapy at advanced stage[3,4], leading to the development of androgen-independent prostate cancer[5].

The androgen-refractory prostate cancer grows faster than the androgen-sensitive cancer, and the androgen-refractory prostate cancer has been considered as chemoresistant[6,7].

Despite related studies[6-9], the molecular mechanisms responsible for activated proliferation and chemoresistance of androgen-independent prostate cancer have not yet been clear.

DU-145 cells have been well characterized and commonly used as a androgen-refractory prostate cancer cell line, which are resistant to most of anticancer drugs, because the proliferation rate in DU-145 cells is much higher than in androgen-sensitive prostate cancer cells such as LNCaP cells[10].

Melittin is a major component of Bee Venom[11]. Increasing studies have demonstrated that melittin inhibits cancer growth, and induction of apoptotic cell death[12]. Park et al[13] reported that melittin inhibited human prostate cancer cell growth through induction of apoptotic cell death via down regulation of NF-κB and alteration of expression of apoptosis regulatory proteins.

In the present study, to reconfirm the effect of melittin on prostate cancer cell growth and to gain better insight into the action mechanism of tumorigenesis and chemoresistance, I therefore conducted the study determining whether melittin exerts inhibitory effect on human prostate cancer DU-145 cell proliferation via induction of apoptotic cell death.

Ⅱ. Materials and method

1. Materials

Melittin(MT) was purchased from Sigma Chemical Co(St Louis, MO, USA). All of the secondary antibodies such as Akt, phosphorylated Akt, Bax, Bcl-2, PARP, caspase-3, -9, cleaved caspase-3, -9, cIAP2, XIAP, MMP-2, -13, used in Western blot analysis were purchased from Santa Cruz Biotechnology(Santa Cruz, CA). All other reagents were purchased from Sigma unless otherwise stated.

2. Cell culture

The DU-145 human prostate cancer cell was obtained from ATCC(American Type Culture Collection, Rockville, MD). Prostate 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 (100unit/ml, Bioproducts, Walkersville, MD) Cell cultures were then maintained at 37℃ in a humidified atmosphere of 5% CO2

3. Cell viability

WST-1 assay

Cells were plated at a density of 1× 105 cells per well in 96-well plate and then subconfluent cells were exposed to different doses of melittin(0.5-2.5㎍) for 12, 24, 48, 72hr. 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℃ for 1hr, and the optical density of each well was read at 450nm.

4. Apoptosis evaluation

Apoptosis assays were performed using the 4,6-

diamidino-2-phenylindole(DAPI) staining. DU-145 cells were cultured in the absence or presence of increasing concentrations of melittin, and apoptosis induction were evaluated after 24hr. Apoptotic cells were determined by the morphological changes after DAPI staining under fluorescence microscopic observation(DAS microscope, 100 or 200x; Leica Microsystems, Inc, Deerfield, IL). For each determination, three separate 100-cell counts were scored. Apoptosis was expressed as a percentage calculated from the number of cells with apoptotic nuclear morphology divided by the total number of cells counted.

Apoptosis was also evaluated by TUNEL staining assay. In short, cells were cultured on 8-chamber slides. After treatment with melittin (0.5-2.5㎍) for 24hr, the cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 1 h at room temperature. TUNEL assays were performed by using the in situ Cell Death Detection Kit(Roche Diagonostics GmbH, Mannheim, Germany) according to manufacturer's instructions. Total number of cells in a given area was determined by using DAPI nuclear staining. The apoptotic index was determined as the number of TUNEL-positive stained cells divided by the total cell number counted 100.

5. Western blot analysis

Cells were homogenized with lysis buffer [50mM Tris pH 8.0, 150mM NaCl, 0.02% sodium azide, 0.2% SDS, 1mM PMFS, 10μl/ml aprotinin, 1% igapel 630(Sigma-Aldrich, St Louis, MO, USA), 10mM NaF, 0.5mM EDTA, 0.1mM EGTA and 0.5% sodium deoxycholate], and centrifuged at 23,000g for 1hr. Equal amount of proteins(80μg) were separated on a SDS/12%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2hr at room temperature with 5%(w/v) non-fat dried milk in Tris-buffered saline[10mM Tris(pH 8.0) and 150mM NaCl] solution containing 0.05% tween-20. The membrane was incubated for 5hr at room temperature with specific antibodies Akt, phosphorylated Akt, Bax, Bcl-2, PARP, caspase-3, -9, cleaved caspase-3, -9, cIAP2, XIAP, MMP-2, -13. 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, USA).

6. Statistical analysis

Data were analyzed using one-way analysis of variance followed by Tuckey test as a post-hoc test. Differences were considered significant at $p < 0.05$.

Ⅲ. Results

1. Inhibition of DU-145 cell growth

Morphological alteration of the cells was demonstrated in Fig. 1. Once the cells were exposed to MT(0.5-2.5㎍), the cells were not grown, and died in a dose dependent manner. To evaluate an effect of MT on the cell growth of DU-145 cells, I

Table 1. DU-145 Cell Growth after Treatment of MT

Group		Time			
		0hr	24hr	48hr	72hr
MT (μg/ml)	0	100.0	100.0±3.5	100.0±4.6	100.0±4.4
	0.5	100.0	103.7±9.9	114.2±4.5	96.1±5.3
	1	100.0	84.6±0.7	66.5±3.2*	60.5±3.8*
	2.5	100.0	24.0±7.9*	17.4±9.3*	16.6±4.6*

Values are the mean and SEM of 3 independent experiments performed in triplicate.
* : represents $p<0.05$, significant difference compared with control.

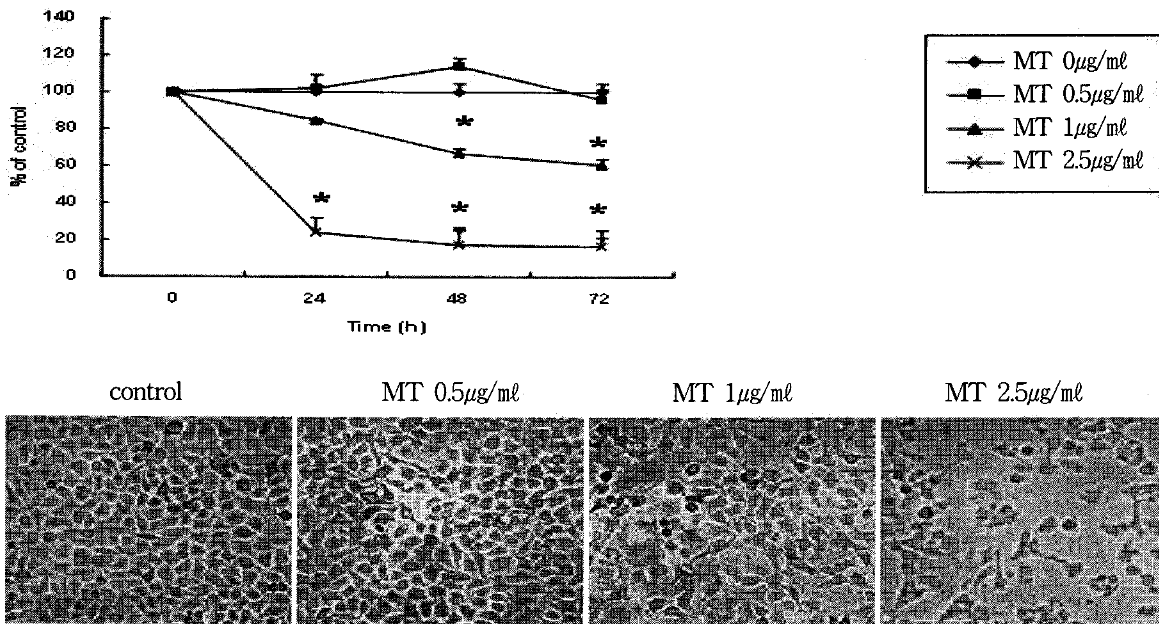

Fig. 1. Morphological changes of DU-145 cells by MT

Morphological changes were observed under microscope(magnification, × 200). Treatment of MT for 24hr caused apoptosis characterized by marked chromatin condensations, small membrane-bound bodies(apoptotic bodies), and cellular shrinkage. The figures are representative of three experiments, with triplicate of each experiment.

* : represents $p<0.05$, significant difference compared with control.

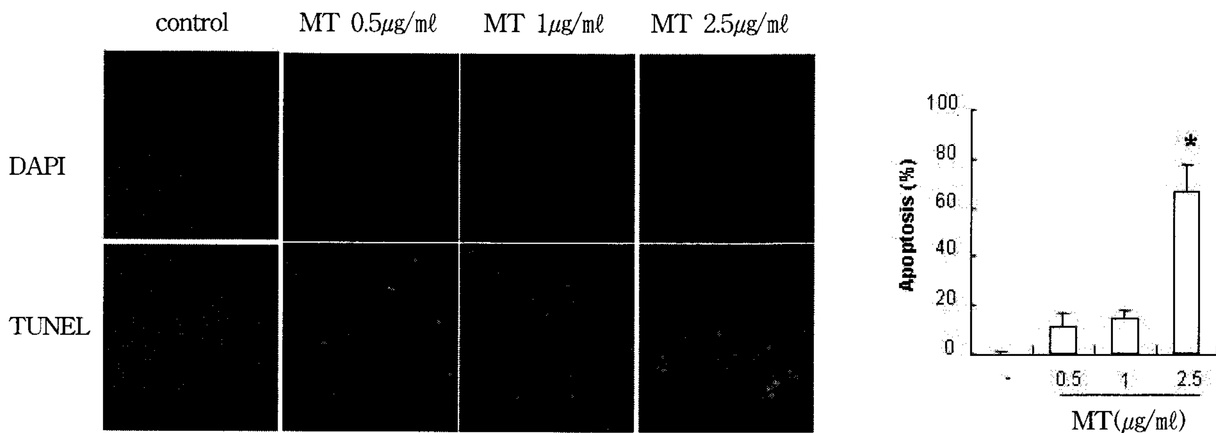

Fig. 2. Effect of MT on induction of apoptosis of DU-145 cells

The apoptotic cells were examined by fluorescence microscopy after DAPI staining, which were estimated by direct counting of fragmented nuclei.

* : represents $p<0.05$, significant difference compared with control.

analyzed cell viability using WST-1 assay and direct cell counting. MT inhibited prostate cancer cell growth in a dose dependent manner.

The percentage of control significantly decreased by 1μg/mℓ of MT at 48 and 72hr was 66.5±3.2 and 60.5±3.8%, which by 2.5μg/mℓ of MT at 24, 48 and 72hr was 24.0±7.9, 17.4±9.3 and 16.6±4.6%(Table 1, Fig. 1).

2. Induction of apoptosis

To delineate whether the inhibition of cell growth by the MT was due to increase of the induction of apoptosis, I evaluated change of the chromatin morphology of human prostate cancer cells using DAPI staining. Consistent with the loss of viability, apoptosis determined after 24hr treat-

ment was increased in a dose dependent manner. The percentages of the control significantly increased by MT 2.5μg/㎖ was 65.8±12.0%(Fig. 2). I also evaluated DU-145 cell apoptosis by TUNEL assay. As shown in Fig. 2, TUNEL-positive cells(stained green) were dose-dependently increased in MT treated DU-145 cells, and the nuclei(stained blue) were found to be condensed(Fig. 2).

3. Expression of apoptosis regulatory Proteins

To gain insight into mechanisms controlling apoptosis in DU-145 cells, we looked at the effect of MT on antiapoptotic and proapoptotic proteins. The increase of apoptotic action was confirmed by the ability of MT to induce calpain, Bax, caspase-3, -9, cleaved caspase-3, cleavaged PARP, cleaved caspase-9 up-regulation and Bcl-2, XIAP, cIAP2,

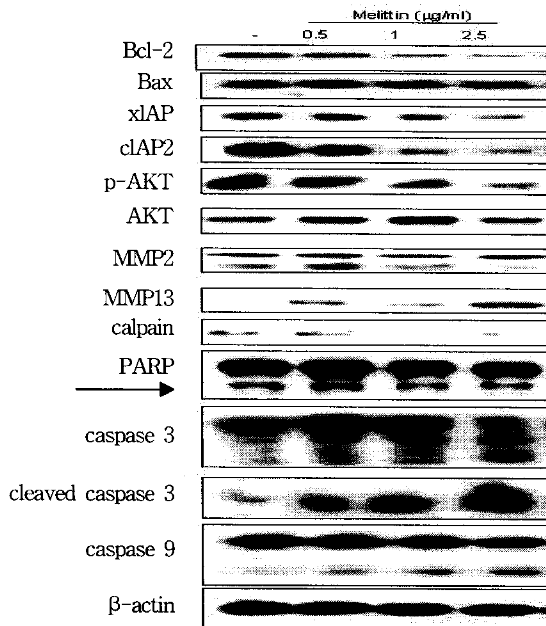

Fig. 3. Effect of MT or BV on expression of apoptosis related proteins in DU-145 cells

Equal amounts(50μg) of whole cell lysates were subjected to electrophoresis and analyzed by apoptosis regulatory molecules(Bax, Bcl-2, caspase-3, -9, cleaved caspase-3, -9, PARP, calpain, XIAP, cIAP2, Akt, p-Akt, MMP-2 and MMP-13). The relative density was analyzed by densitometry. Similar patterns of protein expression were obtained from three experiments. Values are mean and SEM of two experiments, with triplicate of each experiment.

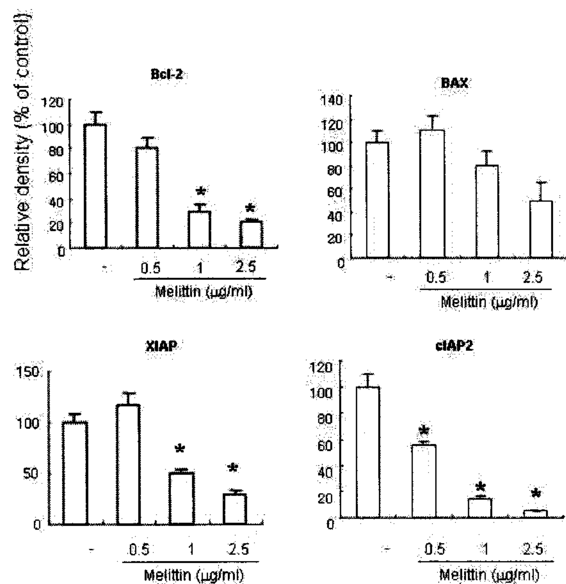

Fig. 4. Effect of MT on expression of Bcl-2, Bax, XIAP and cIAP2 in DU-145 cells

* : represents p<0.05, significant difference compared with control respectively.

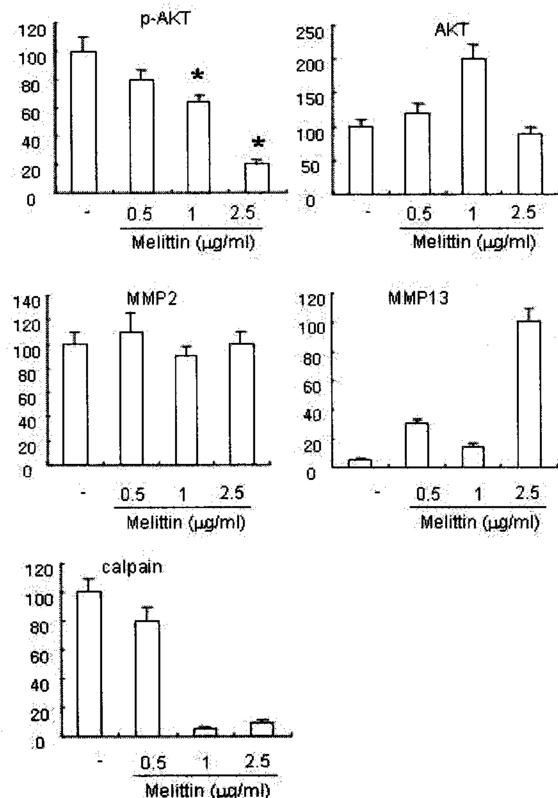

Fig. 5. Effect of MT on expression of p-AKT, AKT, MMP-2, MMP-13 and calpain in DU-145 cells

* : represents p<0.05, significant difference compared with control and Melittin respectively.

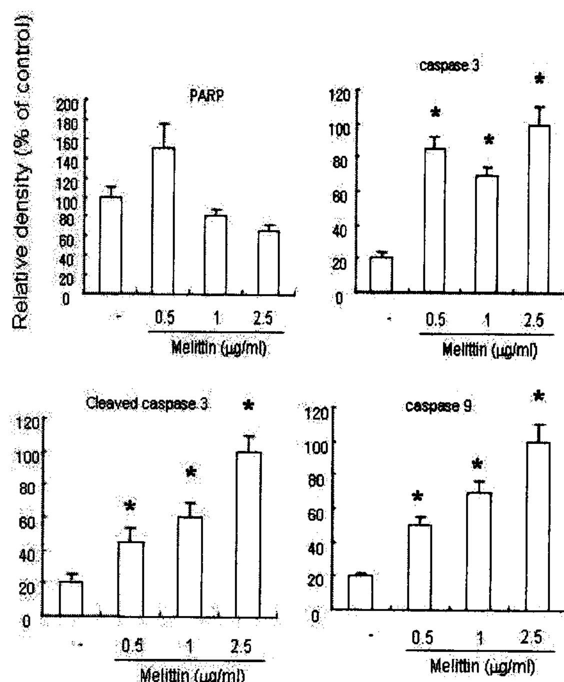

Fig. 6. Effect of MT on expression of PARP, caspase 3, cleaved caspase 3 and caspase 9 in DU-145 cells

* : represents $p<0.05$, significant difference compared with control respectively.

Akt, p-Akt, MMP-2, MMP-13 down regulation. Fig. 3 reveals a western blot analysis of calpain, Bax, cleaved caspase-3, cleavaged PARP, cleaved caspase-9, Bcl-2, XIAP, cIAP2, Akt, p-Akt, MMP-2 and MMP-13 expressions in DU-145 cells treated with a different dose of MT. Compared with control, Expression of Pro-apoptotic proteins such as cleaved caspase-3, and cleaved caspase-9 in the DU-145 cells treated by 0.5, 1 and 2.5μg of MT was increased in a dose dependent manner, whereas anti-apoptosis related proteins including Bcl-2, XIAP, cIAP2, p-Akt were decreased by 1 and 2.5μg of MT dose dependently.(Fig. 3-6)

Cleavaged caspase-3 significantly increased by 0.5, 1 and 2.5μg of MT was 85.0±8.0, 70.0±4.0 or 100.0±10.0%, and cleaved caspase-9 significantly increased by 0.5, 1 and 2.5μg of MT was 50.0±5.0, 70.0±7.0 and 100.0±10.0%. Bcl-2 significantly decreased by 1 and 2.5μg of MT was 30.0±5.0 and 20.0±3.0%. XIAP significantly decreased by 1 and 2.5μg of MT was 50.0±4.5 and 30.0±3.5%, and cIAP2 significantly decreased by 0.5, 1 and 2.5μg of MT was 55.0±3.1, 15.0±1.8 and 5.0±0.6%. p-Akt significantly decreased by 1 and 2.5μg of MT was 65.0±4.0 and 20.0±3.0%(Fig. 4-6).

Ⅳ. Discussion

The central and novel finding in the present study is the identification of anticancer efficacy of MT against advanced human prostate carcinoma DU-145 cells. Most of the present available cytotoxic anticancer drugs mediate their effect via induction of apoptosis in cancer cells[14,15], and induction of apoptosis is suggested as one of the major mechanisms for the targeted therapy of various cancers including prostate cancer[14-17].

In case of advanced prostate cancer, cancer cells become resistant to apoptosis and do not respond to cytotoxic chemotherapeutic agents[16]. Therefore, the agents that induce apoptotic cell death of prostate cancer cells could be useful in controlling this malignancy[17]. Consistent with this approach, my datashowing an induction of apoptotic cell death in prostate cancer control suggest that extremely low concentration(below 2.5μg/ml of MT) of natural toxin could be useful as a anti-cancer agent.

Apoptosis is modulated by antiapoptotic and pro-apoptotic effecters, which involve a large number of proteins. Bcl-2 protein functions as a suppressor of apoptosis[18]. Bax is a proapoptotic protein and its predominance over Bcl-2 promotes apoptosis. Translocation of Bax protein activated by calpain causes change in mitochondrial transmembrane potential that plays an important role in induction of apoptosis. Permeabilization of this membrane allows matrix condensation and release of cytochrome c to the intermembrane space by decrease in MMP and activation of subsequent downstream caspases such as caspase-3, -9. Activated caspase-3 cleaves intracellular proteins such as PARP, which are vital to cell survival and growth, and PARP cleavage has been used as an important marker of apoptosis.

Contrast, Akt phosphorylation confers cell survival signals through upregulation of antiapoptotic protein Bcl-2 and downregulation of pro-apoptotic factors, such as caspase-9. The human inhibitor of apoptosis (IAP) family includes cellular IAP1 (cIAP1), cIAP2, X-linked IAP (XIAP), neuronal apoptosis inhibitor protein, and survivin. cIAP2 is inducible by a variety of NF-κB-inducing stimuli in multiple cell lines, whereas induction of cIAP1 and XIAP appears to be cell type- and stimulus-dependent.

Apoptosis plays a crucial role in eliminating the mutated hyperproliferating cells from the system. Thus, induction of apoptosis in tumor cells may be considered as a protective mechanism against development and progression of cancer. Apoptosis triggered by various stimuli, is characterized by a series of distinct biochemical and morphological changes[19], including increase in ROS level[20], activation of caspases, cell shrinkage, chromatin condensation and nucleosomal degradation[21]. One of the most significant events in apoptosis is mitochondrial dysfunction. Loss of mitochondrial transmembrane potential (MTP) elicits the release of cytochrome c from mitochondria to cytosol[22]. After release, it promotes caspase-9 and -3 subsequently activating downstream caspase-3[23]. Activated caspase-3 cleaves intracellular protein PARP that is an important marker of apoptosis[24]. It has been evident that the proapoptotic protein Bax plays an essential role for the onset of MTP changes and induces cytochrome c release which is inhibited by the antiapoptotic protein Bcl-2[21]. Studies have also shown that Bax/Bcl-2 ratio increases during apoptosis[24]. Bax is a p53 target and is known to be transactivated in a number of systems during p53-mediated apoptosis[25]. p53 is known to mediate cell cycle arrest[26] and also acts as a transcription factor, that binds to a p53-specific DNA consensus sequence in responsive genes such as p21[27]. Upregulation of p21 functions as the inhibitor of cell cycle kinetics[28]. Previous reports showed that Akt phosphorylation confers cell survival signals through upregulation of antiapoptotic protein Bcl-2 and downregulation of pro-apoptotic factors, such as caspase-9[29,30]. The human inhibitor of apoptosis(IAP) family including cellular IAP1 (cIAP1), cIAP2, X-linked IAP (XIAP), neuronal apoptosis inhibitor protein, and survivin also down regulates apoptosis as an antiapoptotic protein[31,32]. Matrix metalloproteinases including a gelatinase MMP-2 and a collagenase MMP-13 contribute to the local growth and spread of malignant lesions in a number of ways, which facilitate local and metastatic spread by destroying the ECM, promote tumor angiogenesis, and have a variety of other actions, including activation and deactivation of growth factors and other active molecules. These actions, and the observation that MMPs are upregulated in many tumors, have made them an attractive target for tumor drug development[33,34].

All the aboves indicate that the balance between the pro and anti- apoptotic proteins, dysfunction of mitochondria and activation of caspases are the important factors deciding apoptotic cell death of tumor cells.

Several apoptosis related proteins such as Bcl-2, and/or up-regulated apoptotic genes: Bax, caspase-3 and -9, and p53 are regulated by NF-κB. Bcl-2 protein is prototypic cell death regulator whose function is modulated by complex homo- and heterodimerizations with their proapoptotic homologues such as Bax[35,36]. Bcl-2 delays or prevents apoptotic cell death to virtually all of the chemotherapeutic agents, suggesting that it may be crucial for the antitumor activity[36-38]. Deregulated overexpression of Bcl-2 has been described in numerous malignant tissues including prostate cancer[35,36]. In addition, antisense Bcl-2 oligonucleotide constructs or down-regulation of Bcl-2 can be utilized to sensitize apoptosis-resistant prostate cancer cells to chemotherapeutic agents[37]. This altered expression of Bcl-2 family members triggered the activation of initiator caspase-9 and -8 followed by activation of effector caspase-3. Activation of caspase-3 activity has been known to be significant in the cancer cell death, and compounds inhibiting caspase-3 activity have been suggested as powerful agents for cancer therapy. For these reasons,

Bcl-2[36,38,39] and caspase-3[40-42] have been considered as a major new strategic target for the development of gene knockdown therapies, and activation of caspase-3 may be significant in the prostate epithelial cancer cell death[42-44].

According to previous Lee et al[45]'s Report, Bax, cleavaged PARP and cleaved caspase-9 was significantly activated, and Bcl-2, XIAP, cIAP2, p-Akt and MMP-13 was significantly down regulated by MT or BV in androgen sensitive LNCaP cells. Similarly the present study showed that in androgen refractory DU-145 cells treated by 0.5 to 2.5μg of MT, Compared with control, cleaved caspase-3 and cleaved caspase-9 of Pro-apoptotic proteins were significantly activated ,and Bcl-2, XIAP, cIAP2 and p-Akt of anti-apoptosis related proteins significantly down regulated by MT. These data suggest that melittin exerts inhibitory effect on androgen refractory DU-145 cell proliferation via induction of apoptotic cell death, and it could be used as a novel agent for human prostate cancer prevention and/or intervention.

V. Acknowledgement

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VI. References

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