대한침구학회지 제23권 제2호(2006년 4월)
The Journal of Korean Acupuncture & Moxibustion Society

Original Article

The Effect of Bee Venom and Melittin on FBS-induced Vascular Smooth Muscle Cells Proliferation

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Abstract

In the present study, We have investigated the bee venom (BV) and melittin (a major component of BV)-mediated anti-proliferative effects, and defined its mechanisms of action in cultured rat aortic vascular smooth muscle cells (VSMCs). BV and melittin (0.4~0.8 μg/ml) effectively inhibited 5% FBS-induced VSMCs proliferations. The regulation of apoptosis has attracted much attention as a possible means of eliminating excessively proliferating VSMCs. In the present study, the treatment of BV and melittin strongly induced apoptosis of VSMCs. These results suggest that the anti-proliferative effects of BV and melittin in VSMCs should be related with induction of apoptosis. Further study about Influence of BV and melittin upon apoptosis mechanism is therefor thought to be necessary to confirm the above results.

Key words : Bee venom, Melittin, VSMCs, Proliferation, Apoptosis

Ⅰ. Introduction

The increased potential for growth of vascular smooth muscle cells (VSMCs) is a key abnormality in the development of atherosclerosis lesions and post angioplasty restenosis[1]. Thus, inhibition of VSMCs proliferation represents a potentially important therapeutic strategy for the treatment of disease such as atherosclerosis and restenosis[2]. It is well known that in response to a variety of stimuli, including many growth factors such as platelet derived growth factor (PDGF) or Fetal Bovine Serum (FBS), VSMCs can initiate

· **Acceptance** : 2006. 1. 30. · **Adjustment** : 2006. 3. 21. · **Adoption** : 2006. 3. 21.
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highly conserved signaling events, which lead to either cell migration or proliferation[2]. However, the proliferative potential can be regulated by induction VSMCs apoptosis[3].

Apoptosis (programmed cell death), which plays a critical role in both the normal development and pathology of a wide variety of tissues, is characterized by cytoplasmic shrinkage, nuclear condensation and DNA fragmentation[4]. In recent years, apoptosis has been implicated in atherosclerosis, and numerous recent investigations on the development and morphology of atherosclerotic lesions have shown apoptosis to be an important factor in atherogenesis[5-8]. It has become more evident that the balance between changes in regulation of cell growth and cell death is an important determinant of vascular integrity and lesion formation[9]. Although the regulation of apoptosis in the vessel wall is complex and likely to consist of multiple interacting pathways within atherosclerotic plaques, the regulation of apoptosis has attracted much attention as a possible means of eliminating excessively proliferating VSMCs[10,11]. After vessel injury, diverse signaling mechanisms become activated in VSMCs, leading to neointimal hyperplasia. Therefore, it is suggested that VSMCs apoptosis is beneficial in that it offers protection to the walls of arteries against proliferative restenosis induced by arterial injury including arterial balloon angioplasty or stent implantation[8,12-16].

Bee venom (BV) is known to be a very complex mixture of active peptides, including melittin (a major component of BV), phospholipase A_2, apamin, adolapin, and mast cell degranulating peptide[17]. Many studies on the biological and pharmacological activities of BV have been carried out. The anti-inflammatory and anti-rheumatoid arthritis effect[17], relief of pain[18], and immune modulatory activity[19] of BV have been described. BV and melittin also have been reported to induce apoptosis in several cancer cells and rheumatoid arthritis synovial fibroblasts *in vitro* and *in vivo*[20-23]. It was also reported that target inactivation of nuclear factor-kappa B (NF-κB) by directly binding to the p50 subunit is an important mechanism of the anti-arthritic effect of BV[24], and that NF-κB is an important regulator of gene expression in cell proliferation and apoptosis[25], which is considered as a potential therapeutic target in atherosclerosis and restenosis[9,26-28].

In this study, We therefore evaluated the effect of BV and melittin on FBS-induced proliferation and apoptosis in cultured rat aortic VSMCs.

Ⅱ. Materials and Methods

1. Chemicals

Dried BV was purchased from You-Miel Bee Venom Ltd. (Hwasoon, Jeonnam, Korea). The composition of the BV was as follow: 45~50% melittin, 2.5~3% apamin, 2~3% MCD peptide, 12% PLA_2, 1% lyso-PLA, 1~1.5% histidine, 4~5% 6pp lipids, 0.5% secarpin, 0.1% tertiapin, 0.1% procamine, 1.5~2% hyaluronidase, 2~3% amine, 4~5% carbohydrate, and 19~27% other, including protease inhibitor, glucosidase, invertase, acid phosphomonoesterase, dopamine, norepinephirne, and unknown amino acids, with >99.5% purity. Melittin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). FBS was obtained from Upstate Biotechnology (Lake Placid, NY, USA). ERK1/2, phospho-ERK1/2, Akt, phospho-Akt and active-caspase-3 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). IκBα, phospho-IκBα, NF-κB p50, p53, Bcl-2, Bax and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [^{3}H]thymidine was from Amersham Pharmacia Biotech (Buckinghamshire, UK). The cell culture materials were obtained from Gibco-BRL (Rockville, MD, USA), and other chemical reagents were from Sigma Chemical Co.

2. Cell culture

Rat aortic VSMCs were isolated by enzymatic dispersion as previously described[29]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 IU/ml penicillin, 100 g/ml streptomycin, 8 mM HEPES, 2 mM L-glutamine at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2 incubator. The purity of VSMCs culture was confirmed by immunocytochemical localization of smooth muscle actin.

3. VSMCs proliferation assays

The rat aortic VSMCs proliferation was measured using by cell counting and DNA synthesis assay as previously described[29]. For cell counting, cells were seeded in 12-well culture plates at 1×10^5 cells/ml, and cultured in DMEM with 10% FBS at 37 ℃ for 24 h. The cells were then cultured with serum-free medium containing BV and melittin (0.4~0.8 μg/ml each) or vehicle (DMSO). Twenty four hours later, the cells were stimulated with 5% FBS, and then trypsinized with trypsin-EDTA and counted using a hemocytometer under microscopy.

DNA synthesis was assayed by measurement of the [^{3}H]thymidine incorporation into cell DNA. Cells were seeded in 24-well culture plates under the same conditions. The medium was then replaced by serum-free medium containing BV and melittin, or vehicle. 24 hours later, cultures were then exposed to 5% FBS for 20 h before 2 μCi/ml of [^{3}H]thymidine was added to the medium. Four hours later, labelling reactions were terminated by aspirating the medium and subjecting the cultures to sequential washes on ice with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v). Acid-insoluble [^{3}H]thymidine was extracted into 300 μl of 0.5 M NaOH per well, and this solution was mixed with 3 ml scintillation cocktail (Ultimagold, Packard Bioscience Co., Meriden, CT, USA), and quantified using a liquid scintillation counter (model LS3801, Beckman, Düsseldorf, Germany).

4. Trypan blue

Cultures were incubated with 0.1% Trypan blue in PBS 5 min at 22 ℃, rinsed with PBS, and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate. Trypan blue-positive and -negative cells were counted by an unbiased observer. More than 300 cells were evaluated in each experimental group (three cultures per group).

5. Apoptosis assay

In order to determine whether apoptosis is induced by BV and melittin, TUNEL and DAPI staining were performed. In short, cells were cultured on 8-chamber slides. After treatments with BV and melittin (0.4~0.8 μg/ml each) for 24 h, 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. For the DAPI staining, slides were incubated 30 min at room temperature in the dark with mounting medium for fluorescence with DAPI (Vector Laboratories Inc., Burlingame, CA, USA). The cells were then observed through a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany). 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.

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 $P<0.05$ and $P<0.01$.

Ⅲ. Results

1. BV and Melittin inhibit proliferation of VSMCs

We first sought to determine whether BV and melittin can inhibit the proliferation of rat aortic VSMCs. Inhibitory effects of BV and melittin on the proliferation of VSMCs were examined by direct cell counting and DNA synthesis assay. The cell number was significantly increased by treatment with 5% FBS for 24 h, and decreased significantly in a concentration-dependent manner by 24 h pretreatment with BV or melittin. The percentages of the control cell number significantly decreased by BV 0.6 and 0.8 μg/ml were 85.0 ± 3.6 and 75.3 ± 4.2% on FBS-stimulated cells (Fig. 1). The percentages of the control cell number significantly decreased by melittin 0.6 and 0.8 μg/ml were 57.0 ± 4.0 and 45.8 ± 3.9% on FBS-stimulated cells (Fig. 2).

Effects of BV or melittin on DNA synthesis in rat aortic VSMCs were tested using [^{3}H]thymidine incorporation. As shown in Figure 3, 4, BV and melittin concentration-dependently inhibited [^{3}H]thymidine incorporation induced by FBS. The percentages of the control significantly decreased by BV 0.8 μg/ml were 45.8 ± 3.9% on FBS- stimulated cells (Fig. 3). The percentages of the control significantly decreased by melittin 0.4, 0.6 and 0.8 μg/ml were 58.1 ± 7.2, 24.8 ± 6.1 and 13.4 ± 3.5% on FBS-stimulated cells (Fig. 4). The inhibitory effects were also dependent on concentration and corresponded with the inhibition of cell number. Taken together, these results indicate that BV and melittin significantly inhibit rat aortic VSMCs proliferation, and melittin especially exhibited the strongest inhibition against FBS-induced VSMCs proliferation.

2. BV and Melittin induce apoptosis of VSMCs

Given the potent inhibition on cell proliferation by BV and melittin, We evaluated VSMCs apoptosis by cell morphologic analysis and TUNEL assay. Cytotoxicity effect of BV in VSMCs was first measured by Trypan blue exclusion assay to confirm that the inhibitory effect of BV and melittin mentioned above should not be the resulted from toxic effect (cell death by BV and melittin) (Fig. 5, 6).

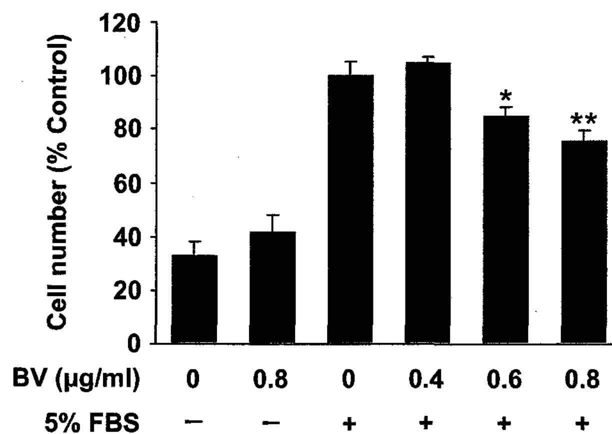

Fig. 1. The Effect of BV on Cell Number in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml BV for 24 h, and then stimulated with 5% FBS.

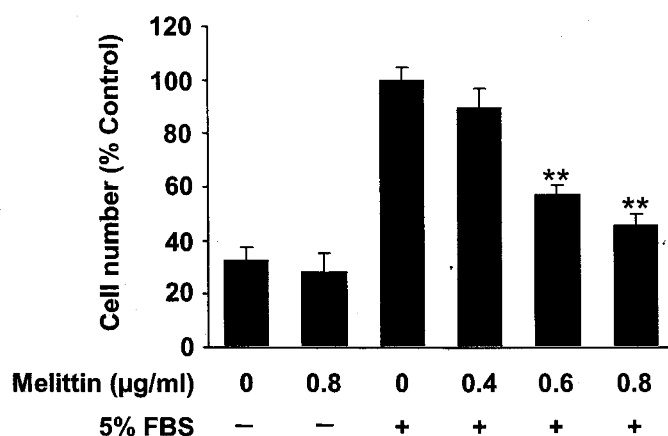

Fig. 2. The Effect of Melittin on Cell Number in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml Melittin for 24 h, and then stimulated with 5% FBS.

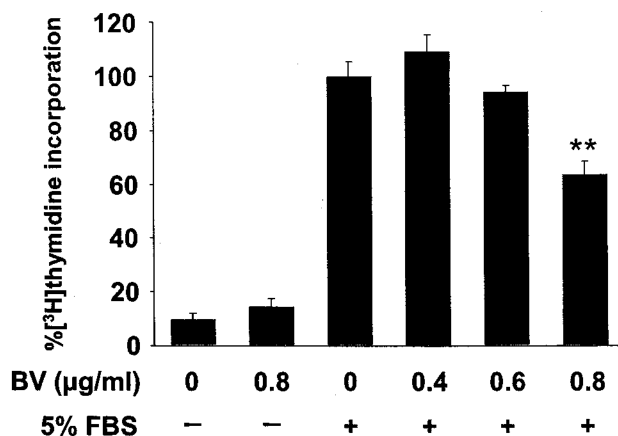

Fig. 3. The Effect of BV on DNA Synthesis in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml BV for 24 h, and then stimulated with 5% FBS.

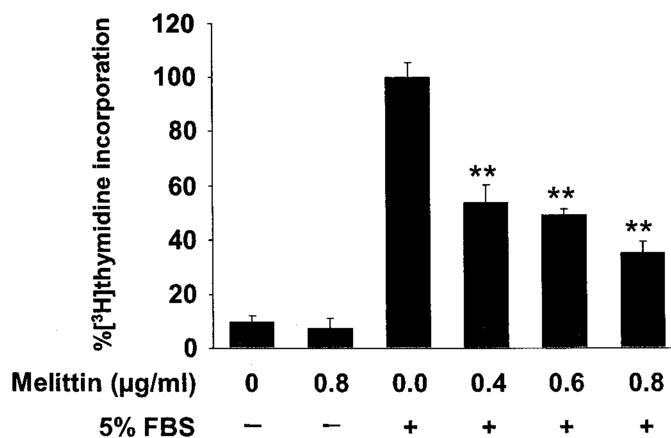

Fig. 4. The Effect of Melittin on DNA Synthesis in VSMCs
Cells were pre-treated with 0.4~0.8 μg/ml Melittin for 24 h, and then stimulated with 5% FBS.

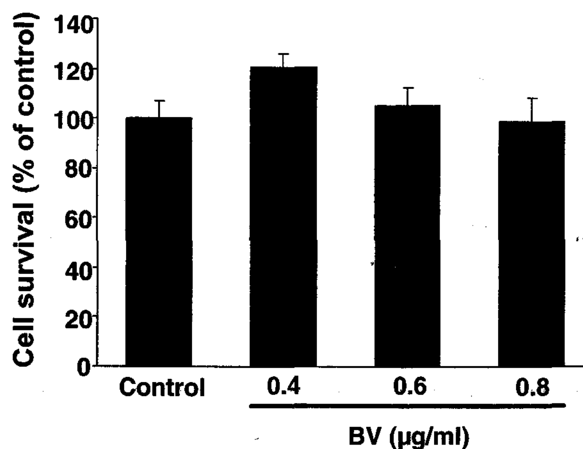

Fig. 5. The Cytotoxicity Effect of BV in VSMCs
VSMCs were pre-cultured in serum-free medium at the different (0.4, 0.6 and 0.8 μg/ml) concentrations of BV. After 24 h, cells were transferred to hemocytometer. Trypan blue exclusion assay was performed as described in materials and methods. Data are expressed as mean ± S.E.M. from three different sets of experiments.

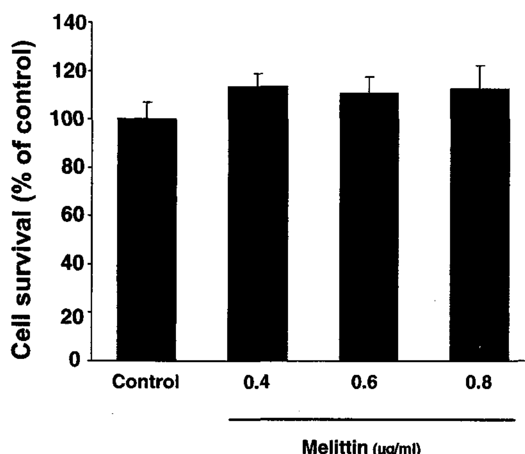

Fig. 6. The Cytotoxicity Effect of Melittin in VSMCs
VSMCs were pre-cultured in serum-free medium at the different (0.4, 0.6 and 0.8 μg/ml) concentrations of Melittin. After 24 h, cells were transferred to hemocytometer. Trypan blue exclusion assay was performed as described in materials and methods. Data are expressed as mean ± S.E.M. from three different sets of experiments.

To observe the effect of BV and melittin on cell morphology, cells were examined via phase-contrast microscopy (ECLIPSE TE-300, Nikon Instech Co., Kawasaki, Kanagawa, Japan). In the morphologic analysis, BV and melittin were seen to cause characteristic changes of VSMCs. Under the phase-contrast microscopy, BV-and melittin-treated VSMCs for 24 h presented with cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (Fig. 7, 8). These morphological characteristics suggest that BV and melittin induce apoptotic cell death in VSMCs.

To further ascertain the induction of apoptosis by BV and melittin in VSMCs, We evaluated the TUNEL assay with DAPI staining. Apoptotic bodies, the presence of which is stringent morphological

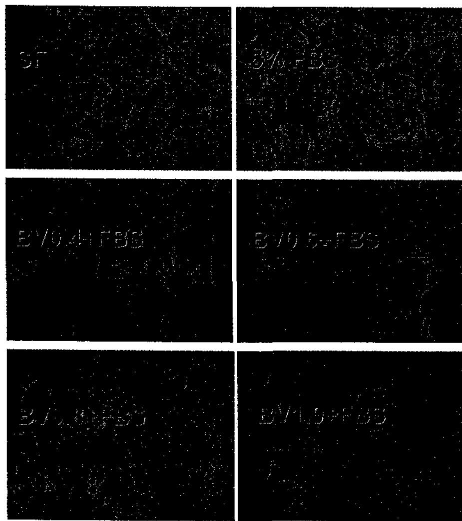

Fig. 7. The Effect of BV on Cell morphological Changes in VSMCs

Treatment of BV for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (phase-contrast microscopy magnification, ×200).

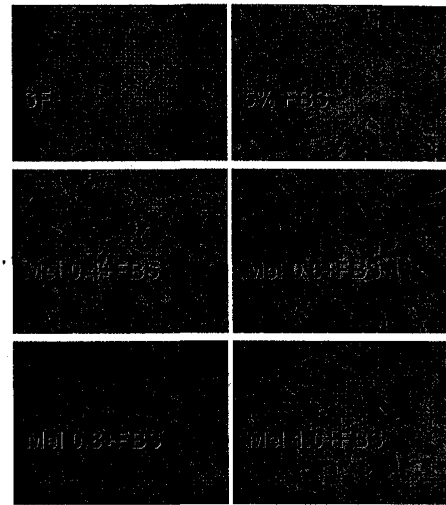

Fig. 8. The Effect of Melittin on Cell morphological Changes in VSMCs

Treatment of Melittin for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (phase-contrast microscopy magnification, ×200).

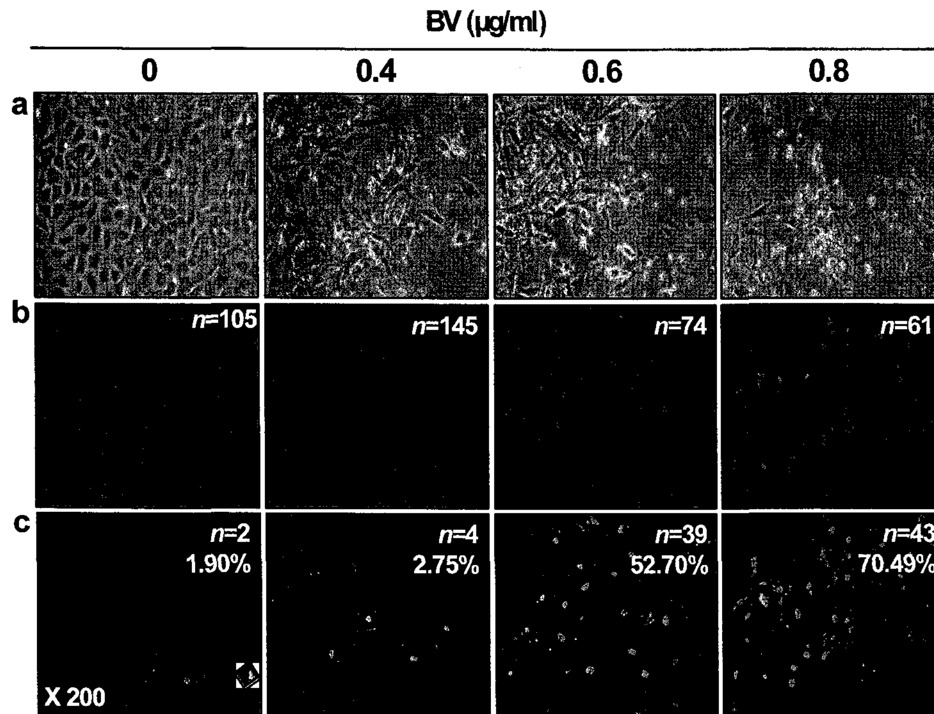

Fig. 9. The Effect of BV on Apoptosis Induction of VSMCs

The apoptotic cells were examined by morphologic analysis (a), DAPI staining (b) and TUNEL assay (c). Treatment of BV for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape(phase-contrast microscopy magnification, ×200). 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 (fluorescent microscopy magnification, ×100). The percentage of TUNEL-positive cells calculated (low panel).

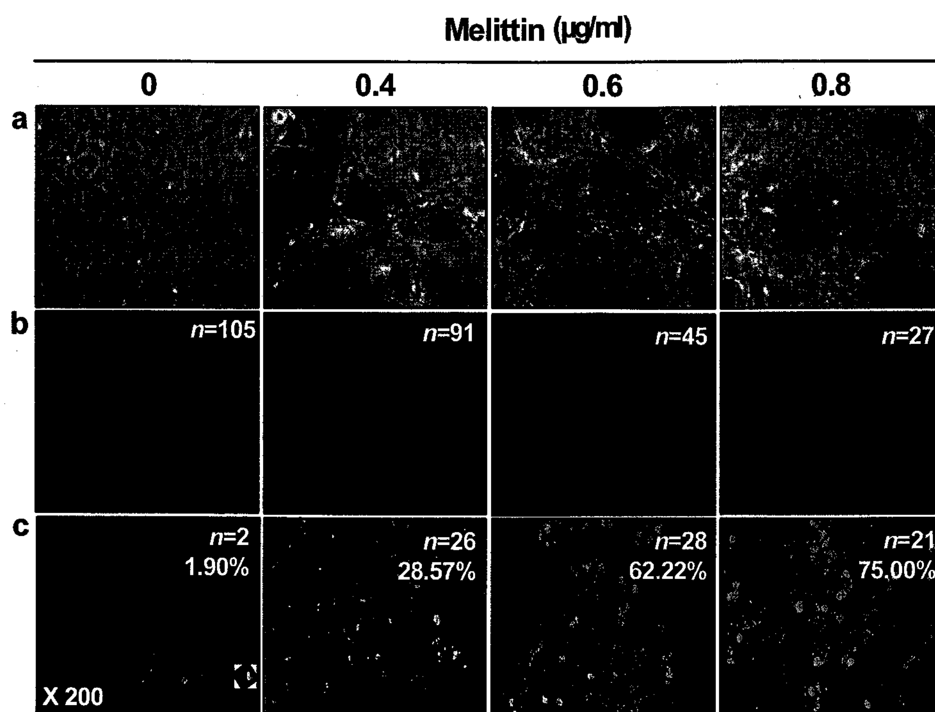

Fig. 10. The Effect of Melittin on Apoptosis Induction of VSMCs

The apoptotic cells were examined by morphologic analysis (a), DAPI staining (b) and TUNEL assay (c). Treatment of Melittin for 24 h caused apoptosis characterized by cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape(phase-contrast microscopy magnification, ×200). 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 (fluorescent microscopy magnification, ×100). The percentage of TUNEL-positive cells calculated (low panel).

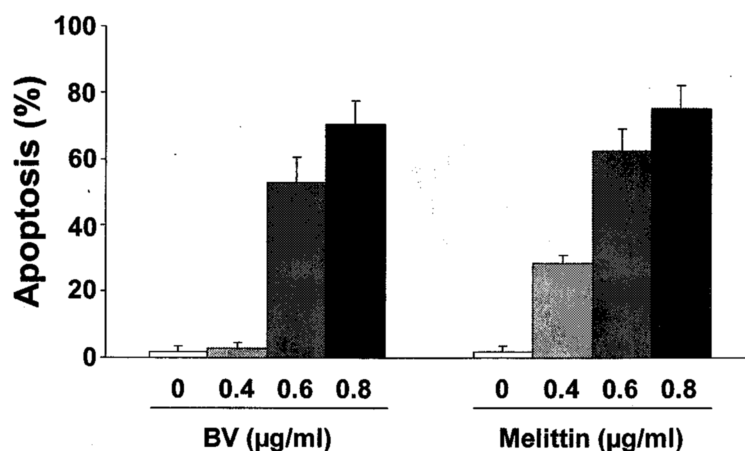

Fig. 11. The Effect of BV and Melittin on Apoptosis Induction of VSMCs

criteria for apoptosis, were observed in BV- and melittin-treated VSMCs stained with DAPI (Fig. 9, 10). In deed, TUNEL-positive cells (stained green) were increased in BV and melittin-treated VSMCs (Fig. 11).

In the plot of DNA content analyzed by flow

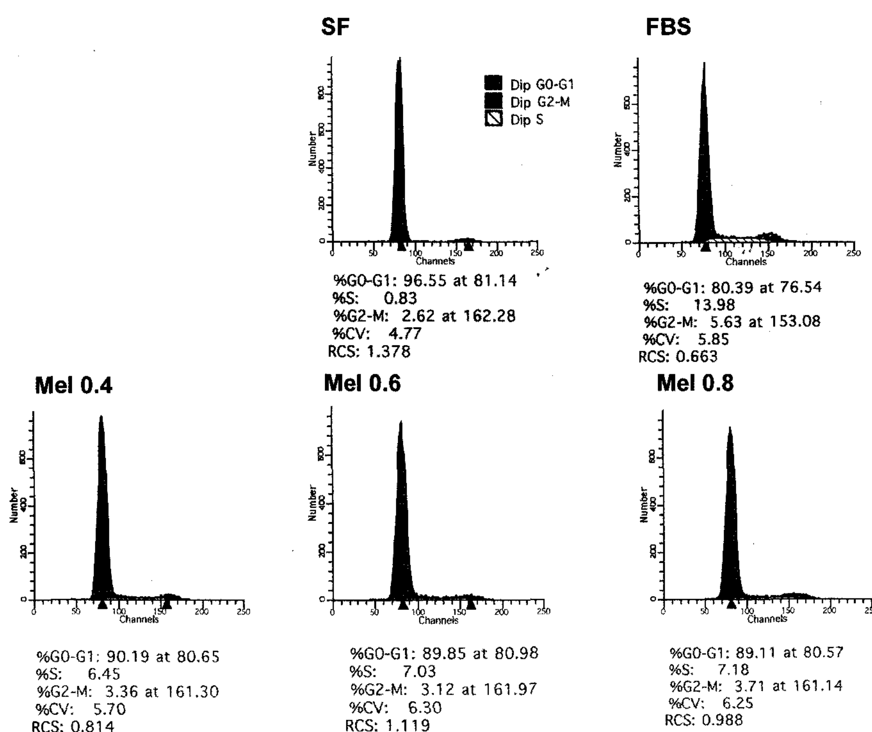

Fig. 12. The Effect of Melittin on Cell Cycle Progression
VSMCs were pre-cultured in the presence and absence of 0.4~0.8 μg/ml Melittin in serum-free medium for 24 h., and then induced by 5% FBS for 24 h. Individual nuclear DNA content is as reflected by fluorescence intensity of incorporated propidium iodide. Each item is derived from a representative experiment, where data from at least 10,000 events were obtained. The distribution of cell population in each cell cycle was shown as based on FACS analysis.

cytometry also showed that melittin increased the proportion of the cells in the sub-G_1 phase (apoptotic cells). The percentage of the cells presented in the sub-G_1 phase was 80.39% (control), 90.19% (0.4 μg/ml), 89.85% (0.6 μg/ml), and 89.11% (0.8 μg/ml) in VSMCs, respectively (Fig. 12).

Ⅳ. Discussion

The purposes of the present study were to find out whether BV and melittin possess an inhibitory effect on rat aortic VSMCs proliferation. It demonstrated the anti-proliferative effect of BV and melittin in VSMCs.

The abnormal growth of VSMCs is prominent features of vascular disease, including atherosclerosis, post-angioplasty restenosis[1]. Neointimal thickening is mainly due to VSMCs, which proliferate and migrate from the media. Excessive proliferative potential can be regulated by apoptosis[15]. In the present study, We showed that BV and melittin significantly inhibited FBS-induced proliferations of VSMCs (Fig. 1−4) and that the inhibitory effect of BV and melittin mentioned above should not be the resulted from toxic effect (cell death by BV and melittin) (Fig. 5, 6).

In the morphologic analysis, BV and melittin were seen to cause characteristic changes of VSMCs such as cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (Fig. 7, 8). These morphological characteristics suggest that BV and melittin induce apoptotic cell death in VSMCs.

Apoptotic bodies were observed in BV- and

melittin-treated VSMCs stained with DAPI (Fig. 9, 10) and TUNEL-positive cells (stained green) were found increased in BV and melittin-treated VSMCs (Fig. 11) and the proportion of the cells in the sub-G_1 phase were shown increased, which make firm the induction of apoptosis by BV and melittin in VSMCs.

Since my results also demonstrated that BV- and melittin-induced apoptosis of VSMCs, We therefore believe that the inhibitory effect of BV and melittin on cell proliferation of VSMCs results from pro-apoptotic properties and that further study about Influence of BV and melittin upon apoptosis mechanism is thought to be necessary to ascertain the above results.

V. References

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