Food Sci. Biotechnol. Vol. 17, No. 5, pp. 947 ~ 952 (2008)

Food Science and Biotechnology

Momordica charantia Protects against Cytokine-induced Apoptosis in Pancreatic β-Cells

Kyong Kim and Hye Young Kim*

Food Function Research Center, Korea Food Research Institute, Seongnam, Gyeonggi 463-746, Korea

Abstract The unripe fruit of *Momordica charantia* (MC) has been shown to possess antidiabetic activity. However, the mechanism of its antidiabetic action has not been fully understood. In this study, the effects of the aqueous ethanolic extract of MC (AEE-MC) were evaluated on the apoptosis in pancreatic β-cells treated with a combination of the cytokines, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. In MIN6N8 cells, the inhibitory effect of AEE-MC was significantly observed at 2 to 50 μg/mL: a 26.2 to 55.6% decrease of cytoplasmic DNA fragments quantified by an immunoassay. The molecular mechanisms, by which AEE-MC inhibited β-cell apoptosis, appeared to involve the inhibition on the expression of p21, Bax, and Bad, the up-regulation of Bcl-2 and Bcl-X_L, and the inhibition on the cleavage of caspase-9, -7, and -3 and poly (ADP-ribose) polymerase. This study suggests that MC may inhibit cytokine-induced apoptosis in β-cells and, thus, may contribute via this action to the antidiabetic influence in diabetes.

Keywords: *Momordica charantia*, cytokine, apoptosis, MIN6N8 cell, diabetes, Bcl-2 family

Introduction

Momordica charantia Linn. (MC; balsam apple, balsam pear, bitter gourd, bitter melon, or karela) of the family Cucurbitaceae is a plant native to the semi-tropical climate of Asia, whose fruits are used as a vegetable (1). In Korea, MC is allowed to be used as a source for foods by Korea Food & Drug Administration. The unripe fruits of MC have been used to treat diabetes (2-5). Studies in animal models of type 1 (1,6-9) and type 2 diabetes mellitus (10-12) support the claim that MC possesses antidiabetic function. However, the exact mechanism responsible for its antidiabetic action has yet fully been understood.

Apoptosis, programmed cell death, is probably the main form of β-cell death in both type 1 and type 2 diabetes. In type 1 diabetes, β-cell destruction is believed to be mediated by inflammatory cytokines such as interleukin (IL)-1β, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α produced by auto-aggressive T lymphocytes (13). A variety of cytokines play a role in the pathogenesis of type 2 diabetes. The discovery that there is local induction of IL-1β production within islets in response to chronic glucose implies that IL-1β plays a role in inducing β-cell apoptosis in type 2 diabetes, as well as in type 1 diabetes. In obesity-linked diabetes, certain adipocyte-derived cytokines are elevated in the circulation, including TNF-α and IL-6. Some of these cytokines can induce β-cell apoptosis through induction of signaling pathways that activate the transcription factor NFkB (14).

Apoptosis is the end result of a cascade of interactions and activities of intracellular death signaling molecules. Mammals have two main networks of apoptosis signaling molecules, which are largely distinct. One is initiated by death receptors at the cell surface and is not controlled by Bcl-2 family members; the other is initiated by pro-apoptotic BH3-only members of the Bcl-2 family (Bax, Bad, and Bak), and is inhibited by anti-apoptotic Bcl-2 homologues (Bcl-2 and Bcl-X_L)(15). The Bcl-2 family possesses both antiapoptotic and proapoptotic members. In general, the antiapoptotic members display sequence conservation throughout all four Bcl-2 homology domains (BH1-4). Proapoptotic Bcl-2 members can be subdivided into more fully conserved, 'multidomain' members possessing homology in BH1-3 domains and 'BH3 domain-only' members that display sequence homology only within this amphipathic α-helical segment which serves as the critical death domain (16). The ratio between the antiapoptotic and the multidomain proapoptotic Bcl-2 members helps determine the susceptibility of cells to a death signal (17). Evolving evidence indicates the multidomain members demonstrate active and inactive conformations.

When death receptors bind their ligand, the receptors cluster, and signaling complexes are assembled, including first-level pro-caspases (such as caspase-9). Multiple apoptotic signals release cytochrome c from the mitochondrial intermembrane space to activate Apaf-1, coupling this organelle to caspase activation. Bcl-2 family members are major regulators of mitochondrial integrity and mitochondria-initiated caspase activation. The initiator caspase-9 is activated upon binding with a CED-4 homolog, Apaf-1 (18), in complex with cytochrome c, which subsequently activates the effector caspase-3 (19). These pro-caspases are cleaved to generate active caspase forms, which activate second-level caspases (such as caspases-3 and 7), which in turn cleave PARP to effect apoptosis (15).

It was reported that the juice of MC reduced the streptozotocin-induced apoptosis in a rat insulinoma cells (RINm5F)(20). In order to elucidate the mechanisms by which MC exerts antidiabetic action in diabetes, it was tested that the hypothesis that MC could protect β-cells against apoptosis induced by cytokines in a mouse

*Corresponding author: Tel: +82-31-780-9267; Fax: +82-31-709-9876
E-mail: khyey@kfri.re.kr
Received November 13, 2007; Revised March 3, 2008;
Accepted March 3, 2008

insulinoma cells (MIN6N8). To explain the anti-apoptotic mechanism of MC, it was evaluated that the effects of MC on the induction of p21, Bax, Bad, Bcl-2, and Bcl-X_L, and the cleaved activation of caspase-9, -7 and -3, and PARP in cytokine-treated β-cells.

Materials and Methods

***Momordica charantia* (MC)** Unripe fruits of MC were purchased in fresh forms from a local farm (Jeongup, Jeonbuk, Korea) in the year 2005. Seeds were removed and flesh was freeze-dried. After 72 hr of freeze-drying, the yield was about 67.3 g/kg fresh fruit. The freeze-dried sample was stored in the Laboratory of Food Function Research Center, Korea Food Research Institute. The MC sample was kept in airtight containers at −70°C until use.

Preparation of MC extract The MC sample was ground to pass an 80-mesh sieve and extracted under reflux with water or water:ethanol (50:50, v/v) at a material-to-solvent ratio of 20 mL/g for 2 hr, followed by filtration with filter papers (Toyo Advantec No. 2 and 4; Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and then, the process including the addition of solvent, extraction under reflux, and filtration was repeated 3 times. The combined filtrate was concentrated with a rotary vacuum evaporator at 40°C until the volume was reduced to less than 20% of the original volume of the filtrate. And the extraction solvent was completely removed by freeze-drying so that solid residue was obtained. The yield was 46.0% for aqueous extract (AE) and 51.4% for aqueous ethanolic extract (AEE) by weight of the original MC powder. The residue was dissolved in distilled water or dimethyl sulfoxide (DMSO) and used for subsequent bioassays. The final concentration of DMSO in cell incubation medium never exceeded 0.1%, which we determined did not affect apoptosis in preliminary experiments (unpublished observations).

Cell lines and reagents As a model of pancreatic β-cells, SV40 T-transformed insulinoma cells derived from nonobese diabetic (NOD) mice were used (MIN6N8). The cells were kindly provided by Prof. Myung-Shik Lee (Sungkyunkwan University, School of Medicine, Seoul, Korea) under the permission of Prof. Junichi Miuagaki (Osaka University, Osaka, Japan) (21). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY, USA). Reagents were obtained from the following sources: high glucose DMEM, FBS, trypsin-ethylenediamine tetracetic acid (EDTA) 100 IU/mL, penicillin, and 100 μg/mL streptomycin from Gibco. Sodium carbonate, β-mercaptoethanol from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), recombinant mouse, *Escherichia coli* derived IL-1β, IFN-γ, and TNF-α from R&D systems (Minneapolis, MN, USA). The antibodies against polyclonal cleaved caspase-3, -7, -9, and cleaved poly (ADP-ribose) polymerase (PARP) were purchased Cell Signaling Technology (Berverly, MA, USA). The antibodies against PARP, p21, Bax, Bad, BcL-2, Bcl-X_L, and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary antibodies were obtained from Vector Laboratories (Burlingame, CA, USA); horseradish peroxidase-conjugated anti goat or anti rabbit, and exposed to an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech., Piscataway, NJ, USA), and electrophoresis equipment from Bio-Rad Laboratories (Richimond, CA, USA).

Exposure to proinflammatory cytokines Cytokine-mediated apoptosis was induced by incubation with a cytokine mixture of 1, 5, and 10 ng/mL each of recombinant mouse IL-1β, IFN-γ, and TNF-α (R&D Systems) for 48 hr. The choice of cytokine concentrations was based on the results. Test reagents (MC extracts) were added simultaneously with cytokines.

Cell survival assay Cell viability was determined by the reduction of yellow 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) into a purple formazane product by mitochondrial dehydrogenase of metabolically active cells. Briefly, cells were seeded at a density of 2×10^6 cells/well into 96-well plate. After overnight growth, cells were treated with cytokines and a series of concentrations of MC extracts for 48 hr. At the end of treatment, 30 μL of MTT was added, and cells were incubated for a further 4 hr. Cell viability was obtained by scanning with an enzyme-linked immuno-sorbent assay (ELISA) reader with a 570 nm filter (22).

Measurement of cell death For quantitative determination of apoptotic cell death, cytoplasmic histone-associated DNA fragments were measured with the Cell Death Detection ELISA Plus kit from Roche (Mannheim, Germany) according to the manufacture's instructions. This assay is based on a quantitative sandwich enzyme immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes but not free histone or DNA that may generate during nonapoptotic cell death (23) in the cytoplasmic fraction of cell lysates. At the end of the culture period, cells were washed with phosphate-buffered saline (PBS), lysed according to the manufacturer's protocol, centrifuged (200×g, 10 min), placed in a streptavidin-coated microtiter plate, and incubated with a mixture of antihistone (biotin-labeled) and anti-DNA (conjugated with peroxidase) antibodies. After removal of the unbound antibodies by a washing step, the amount of nucleosomes was quantified photometrically by the peroxidase retained in the immunocomplex. Glucagon-like peptide-1 (GLP-1; Sigma-Aldrich) was used as a positive control.

Preparation of cell lysates and immunoblotting Cells were collected by centrifugation at 800×g for 3 min, washed twice with ice-cold PBS and centrifuged at 800×g for 3 min. Cells were lysed by suspending the pellet in lysis buffer (cellLytic MT Mammalian Tissue lysis/extraction reagent, Sigma-Aldrich) for 30 min at 4°C. The lysates were centrifuged at 13,000×g at 4°C for 20 min, and the protein contents in the supernatants were measured using an assay kit for protein determination (Quick Start Bradford Dye Reagent; Bio-Rad). The supernatant containing 50 mg of protein was separated on 10% sodium dodecyl sulfate

(SDS)-polyacrylamide gels at 20 mA (100 V) and blotted onto immunoblot PVDF membranes (Bio-Rad) in Tris-borate-EDTA buffer. Membranes were incubated for 4 hr at room temperature with primary antibody (diluted 1:200-1:1,000). In all cases, the excess of primary antibody was removed by 3 washes with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20 (TBST), membranes were then incubated with anti-goat or anti-rabbit IgG conjugated with peroxidase (1 mg/mL) for 60 min, and immuno-reactive bands were detected by enhanced chemiluminescence. Immunoblots were analyzed with a GS-800 calibrated densitometer (Bio-Rad).

Statistical analysis Experiments were performed in triplicate and replicated 3 times. All values were expressed as means and standard errors of means (SEM). One-way ANOVA followed by Duncan's multiple range test was used for statistical analysis (SAS Software, SAS Inc., Cary, NC, USA).

Results and Discussion

Effects of cytokines on cell viability, apoptosis, and pro-apoptotic proteins in MIN6N8 cells It was established that an experimental *in vitro* condition where cytotoxicity and apoptosis was induced in MIN6N8 cells with cytokines in the previous work (24). Interleukin (IL)-1β, IFN-γ, or TNF-α alone did not cause significant cytotoxicity and apoptosis. However, the combined treatment of IL-1β, IFN-γ, and TNF-α caused significant cytotoxicity and apoptosis (24). After 48 hr of the cytokine treatment, cell viability was determined by MTT assay. As shown in Fig. 1A, the combined treatment of IL-1β, IFN-γ, and TNF-α did not affect cell viability at 1 ng/mL, but significantly decreased the percentage of viable cells at 5 and at 10 ng/mL compared to the cytokine-untreated group. After 48 hr of the cytokine treatment, the nucleosomal release was measured as an early biochemical feature and quantitative marker of apoptosis (25). Incubating MIN6N8 cells with the cytokine combination of IL-1β/IFN-γ/TNF-α resulted in a significant stimulation of DNA fragmentation, i.e., compared to 2.07 folds at 1 ng/mL, 2.45 folds at 5 ng/mL, and 2.87 folds at 10 ng/mL (Fig. 1A).

The cleaved activation of PARP and caspases were examined 48 hr after the treatment with the combinations of cytokines. Treatment with cytokines caused a proteolytic cleavage of PARP, dose-dependently for 48 hr with accumulation of the 89 kDa species, confirming that cytokine-activated caspase-3 was functionally active (Fig. 1B). We evaluated whether the processing and activation of caspase-3, which is considered to play a central role in many types of stimuli-induced apoptosis. Caspase-3, one of effector caspases, was cleaved yielding a 17-kDa fragment 48 hr after the cytokine treatment. Caspase-7, another effector caspase, was also cleaved 48 hr after the cytokine treatment. Our results showed the cleavege of caspase-3 and -7 after the treatment of TNF-α/IFN-γ /IL-1β in MIN6N8 cells.

The expressions of the cleavage of PARP and caspases were mostly maximal at the combination with 10 ng/mL each of cytokine. The effects on cell viability and apoptosis, and the expressions of the cleaved PARP and caspases

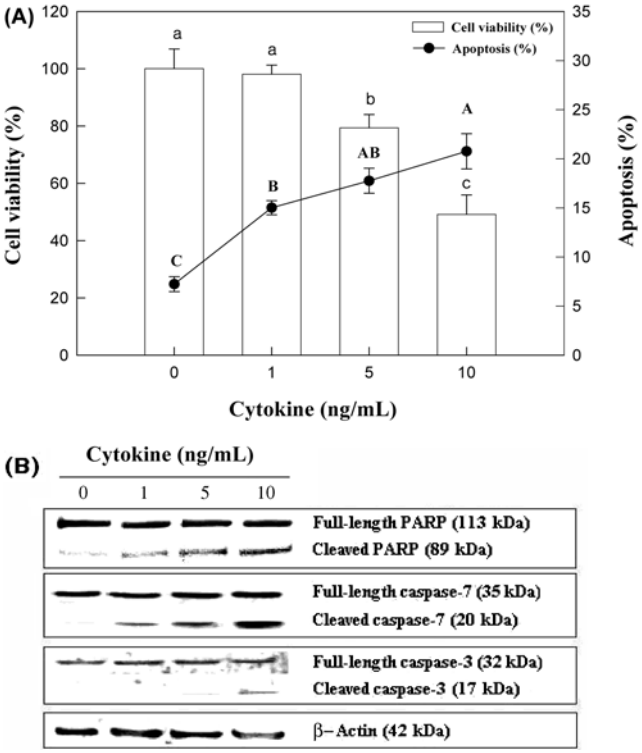

Fig. 1. (A) Effects of cytokines on cell viability and apoptosis in MIN6N8 cells. The cells were cultured with the combined treatment of IL-1β, IFN-γ, and TNF-α at different concentrations as indicated for 48 hr. Value are means±SEM, n=9. Means with different letters differ significantly among groups (a, b, and c for cell viability; A, B, and C for apoptosis) at $p<0.05$ using Duncan's multiple range test. **(B) Effects of cytokines on the cleaved activation of PARP, and caspase-9, -7, and -3 in MIN6N8 cells.** All data are representative of 3 independent experiments in triplicates. Similar results were obtained for each of the 3 experiments.

were maximal at 10 ng/mL each of the cytokine. Therefore, it was evaluated that the effects of MC extracts on cytotoxicity, apoptosis, and signaling pathways induced by 10 ng/mL each of the cytokine.

Effect of MC extracts on cell viability, cytokine-induced cytotoxicity, and apoptosis in MIN6N8 cells To examine whether or not the extracts of MC are toxic to MIN6N8 cells, the cytotoxicity of the aqueous extract of MC (AE-MC) and the aqueous ethanolic extract of MC (AEE-MC) on the cells was observed. The extracts were applied in increasing concentrations up to 1,000 mg/mL to the cells for 48 hr. It was found that AE-MC and AEE-MC in the concentration range 0.01-1,000 μg/mL had no cytotoxic effect on the cells, when the cytotoxicity was evaluated by MTT assay (data not shown). Based on this result, AE-MC and AEE-MC were used in concentration up to 50 μg/mL. The viability of the cells treated with AE-MC extract (2-10 μg/mL) did not show protection against the cytotoxicity induced by cytokines. The cells treated with AE-MC extract (50 μg/mL) showed significant protection against the cytotoxicity induced by cytokines. The cells treated with AEE-MC (2-50 μg/mL) dose-dependently showed significant protection against the cytotoxicity induced by

Table 1. Effects of MC extracts on cell viability and apoptosis in MIN6N8 cells treated with cytokines

Extract	Concentration	Cell viability (%)	Apoptosis (fold of cytokine-untreated control)
Cytokine-untreated group	-	100.05±6.75[1)]	1.00±0.11
Cytokine-treated control	-	49.16±6.78d	3.47±0.70a
Aqueous extract of MC (AE-MC)	2 μg/mL	53.28±3.36d	3.31±0.12a
	10 μg/mL	55.49±7.56d	3.04±0.38b
	50 μg/mL	61.25±3.47c	2.43±0.27b
Aqueous ethanolic extract of MC (AEE-MC)	2 μg/mL	60.02±4.32c	2.56±0.06b
	10 μg/mL	70.86±5.85b	2.05±0.14bc
	50 μg/mL	81.71±5.27a	1.54±0.13c
Glucagon-like peptide-1 (GLP-1)	1 nM	60.16±2.10c	1.74±0.11c
	10 nM	69.87±5.17b	1.38±0.11d
	100 nM	82.13±3.82a	1.35±0.11d

[1)]Values are means±SEM, n=9. Values within columns among cytokine-treated groups having the same letters are not significantly different at $p<0.05$ using Duncan's multiple range test.

Table 2. Quantitative analysis on effects of AEE-MC on the expressions of pro-apoptotic and anti-apoptotic proteins in response to 48 hr exposure of cytokines (10 ng/mL) in MIN6N8 cells

	Cytokine-untreated control	Cytokine-treated control	AEE-MC (μg/mL)		
			2	10	50
Pro-apoptotic molecules					
Cleaved PARP	2.10±0.41[1)]	10.72±1.27a	6.15±0.33b	4.15±0.17c	3.47±0.26d
Cleaved caspase-3	1.98±0.11	15.44±1.92a	12.68±1.41b	8.14±0.73c	7.59±0.34c
Cleaved caspase-7	2.11±0.87	24.68±2.82a	18.45±1.78b	13.94±2.16c	10.77±2.15d
Cleaved caspase-9	2.39±0.20	13.08±1.66a	11.15±2.52ab	7.72±0.34b	3.34±0.99c
Cytochrome C	8.29±2.27	37.49±5.68a	24.72±2.30b	14.82±1.83c	8.37±0.97d
P21	9.55±0.80	15.19±4.32a	14.16±0.10a	10.84±0.07b	6.70±0.07c
Bax	4.90±0.26	12.85±1.16a	8.59±0.81b	7.25±0.77bc	5.24±0.40c
Bad	1.25±0.89	18.2±1.77a	10.79±1.35b	8.79±0.44c	6.42±0.97d
Anti-apoptotic molecules					
BcL-2	13.29±0.38	5.49±0.93b	5.12±0.49b	10.41±0.82ab	12.62±0.19a
Bcl-X_L	12.62±0.89	4.86±0.77c	6.16±0.97b	11.08±0.44a	12.28±0.35a
(Bax+Bad)/(BcL-2+BcL-xL) ratio	0.24±0.02	3.00±0.28a	1.72±0.15b	0.75±0.05c	0.04±0.001d

[1)]Expressed as relative density of the signaling molecules to β-actin in arbitrary units. Values are means±SEM, n=9. Values within rows among cytokine-treated groups having the same letters are not significantly different at $p<0.05$ using Duncan's multiple range test.

the cytokines. The glucagon-like peptide (GLP)-1 was used as a positive control. The incubation with GLP-1 for 16 hr inhibited H_2O_2-induced apoptosis of insulin-secreting MIN6 cells (26). The result suggests that MC protected against cytokine-mediated cytotoxicity. The inhibitory effect of AEE-MC was higher than that AE-MC of in the cells (Table 1).

AE-MC and AEE-MC showed no significant influence on nucleosomal release in cytokine-untreated cells (not shown in data). Cytokine-mediated nucleosomal release was significantly inhibited in the presense of AE-MC at the concentrations of 50 μg/mL by 30%. Cytokine-mediated nucleosomal release was significantly inhibited in the presense of AEE-MC at the concentrations of 2, 10, and 50 μg/mL by 26.2, 40.9, and 55.6%. This result suggested that MC protected against cytokine-mediated apoptosis. The inhibitory effect of AEE-MC was higher than that of AE-MC in the cells (Table 1).

Effects of AEE-MC on cytokine-mediated activation of PARP and caspases The effect of AEE-MC on the expression of PARP cleavage was examined by Western blot analysis. As shown in Fig. 2, the cleavage of the 113 kDa PARP to its active 89 kDa was inhibited by AEE-MC dose-dependently. The levels of the active 89 kDa fragment of PARP with AEE-MC at 2, 10, and 50 μg/mL were significantly and dose-dependently reduced by 42.6, 61.3, and 67.6% of the control level, respectively, in the cytokine-treated MIN6N8 cells (Table 2).

The cleavage of the 49 kDa caspase-9 to its active 37 kDa was inhibited by AEE-MC dose-dependently (Fig. 2). The levels of the active 37 kDa fragment of caspse-9 with AEE-MC at 2, 10, and 50 μg/mL were significantly and dose-dependently reduced by 14.8, 41.0, and 74.5% of the control level, respectively, in the cytokine-treated MIN6N8 cells (Table 2).

The cleavage of the 35 kDa caspase-7 to its active 20

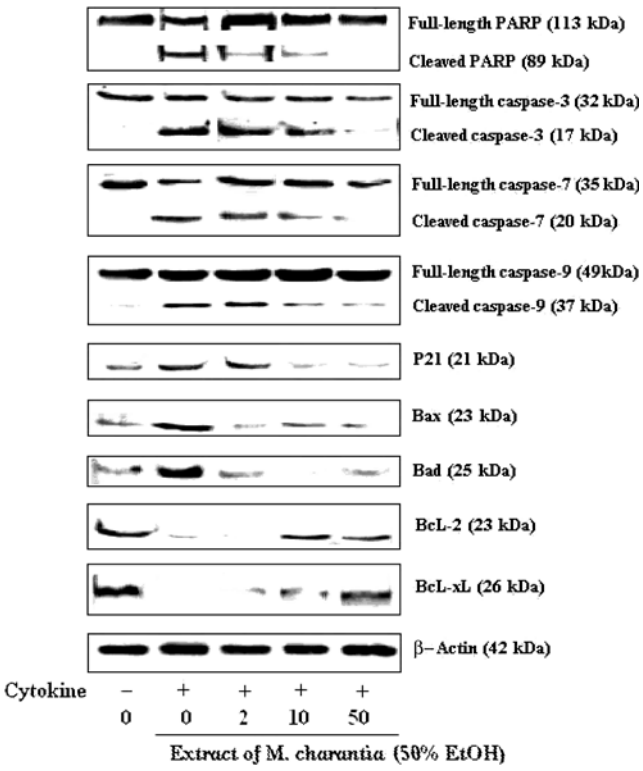

Fig. 2. Effects of aqueous ethanolic extract from MC (AEE-MC) on the expression of pro-apoptotic and anti-apoptotic proteins in MIN6N8 cells treated with cytokines for 48 hr. Typical example of Western blot analysis was shown.

kDa was inhibited by AEE-MC dose-dependently (Fig. 2). The levels of the active 20 kDa fragment of caspase-7 with AEE-MC at 2, 10, and 50 μg/mL were significantly and dose-dependently reduced by 25.2, 43.5, and 56.4% of the control level, respectively, in the cytokine-treated MIN6N8 cells (Table 2).

The cleavage of the 32 kDa caspase-3 to its active 17 kDa was inhibited by AEE-MC dose-dependently (Fig. 2). The levels of the active 17 kDa fragment of caspase-3 with AEE-MC at 2, 10, and 50 μg/mL were significantly reduced by 17.9, 47.3, and 50.8% of the control level, respectively, in the cytokine-treated MIN6N8 cells (Table 2).

Effects of AEE-MC on the expressions of Bcl-2 family and p21 in cytokine-treated MIN6N8 cells Since Bax, Bad, Bcl-2, and Bcl-X_L play a crucial role in apoptosis, we studied the dose-dependent effects of AEE-MC on the protein levels of Bax, Bad, Bcl-2, and Bcl-X_L in MIN6N8 cells. The Western blot analysis exhibited a significant decrease in the protein expression of Bax at the concentrations of 2, 10, and 50 μg/mL, by 33.2, 43.6, and 59.2% of the cytokine-treated control, respectively. The Western blot analysis exhibited a significant decrease in the protein expression of Bad at the concentrations of 2, 10, and 50 μg/mL, 40.7, 51.7, and 64.7% of the cytokine-treated control, respectively. In contrast, the protein expression of Bcl-2 was significantly increased at the concentrations of 10 and 50 μg/mL, by 89.6 and 130% of the cytokine-treated control, respectively. The protein expression of Bcl-X_L was significantly increased at the concentrations of 2, 10, and 50 μg/mL, by 27.0, 128, and 153% of the cytokine-treated control, respectively. A dose-dependent decrease in the ratio of Bax and Bad to Bcl-2 and Bcl-X_L was significantly observed after the AEE-MC treatment in cytokine-treated cells, indicating the inhibition of apoptotic process (Table 2).

The expression of p21, a well-known potent pro-apoptotic protein involved in the mitochondrial apoptotic pathway, was significantly increased for 48 hr cytokine treatment (Table 2) (27). The AEE-MC at the concentrations of 10 and 50 μg/mL significantly and dose-dependently caused the decrease of p21 expression, by 28.6 and 55.9% of the cytokine-treated control, respectively.

We demonstrated that MC extract protected pancreatic β-cells from cytokine-induced apoptosis in a dose-dependent manner. This is the first time the anti-apoptotic effect of MC extract has been demonstrated in cytokine-mediated apoptosis in MIN6N8 cells. The inhibitory effect of AEE-MC was greater than that AE-MC on the cytokine-induced apoptosis of MIN6N8 cells.

The p21 is a critical initiator of the intrinsic apoptosis pathway in response to stress from oncoproteins, DNA damage, hypoxia, and survival factor deprivation (28). The p21 protein can induce apoptosis by transcriptional activation of pro-apoptotic Bcl-2 family members and repression of anti-apoptotic Bcl-2 proteins and inhibitors of pro-apoptotic proteins (29). In addition, p21 can also regulate apoptosis by activating mitochondrial genes to enhance reactive oxygen species generation (30). Our results showed that AEE-MC decreased the level of p21 in cytokine-treated cells (Fig. 2 and Table 2), suggesting that the p21 pathway was regulated by AEE-MC. From the observation in this study that AEE-MC decreased the expression of Bax and Bad, and increased the expression of Bcl-2 and Bcl-X_L in cytokine-treated cells (Fig. 2 and Table 2), it is suggested that AEE-MC inhibited apoptosis through p21 by regulating Bcl-2 family.

We observed that AEE-MC inhibited the cleaved activation of caspase-9 in the cytokine-treated cells (Fig. 2 and Table 2). Disruption of mitochondrial membrane potential results from the opening of permeability transition pores, causing a local disruption of the outer mitochondrial membrane, and leads to the release of soluble intermembrane proteins, including cytochrome c, and the cytochrome c release contributes to the activation of caspase-9 (first-level caspase; activator). It might be estimated that AEE-MC leads to the release of cytochrome c.

It has been reported that caspases play an essential role in β-cell death. The caspase family is a class of cysteine proteases that is involved in a cascade of protective cleavages leading to the eventual fragmentation of DNA in mammalian cells. Of particular interest is caspase-3, the most widely studied member of the caspase family and the most important executioner of apoptosis, since it is responsible for the direct proteolytic cleavage of various cellular target proteins. Thus, caspase-3 is often used as a marker for detection of apoptosis. Activation of caspase-3 is an essential step in the execution of apoptosis and its inhibition blocks apoptotic cell death (15). As demonstrated in our experiment, cytokine-induced apoptotic signaling leading to the activation of caspase-3, yet cotreatment of MIN6N8 cells with AEE-MC apparently inhibited the

cleavage and activation of caspase-3. This result indicates that AEE-MC exerts antiapoptotic activity partly by preventing the apoptotic signaling that leads to the activation of caspase-3.

In conclusion, our results demonstrated that AEE-MC protected cytokine-induced apoptosis in a dose-dependent manner in MIN6N8 cells. AEE-MC decreased the expression of p21, Bax, and Bad, and the cleaved activation of caspase-9, -3, and -7 and PARP, and increased the expression of Bcl-2 and Bcl-X_L in the cytokine-treated cells. Therefore, we speculate that the inhibition of apoptosis observed in this study may provide a mechanism for the antidiabetic function of MC, and that the down-regulation of p21, Bax, Bad, cleaved caspases, and cleaved PARP, and the up-regulation of Bcl-2 and Bcl-X_L may provide a mechanism for the anti-apoptotic activity of MC.

Acknowledgments

This work was supported by the Korea Research Foundation granted by the Korean Government (MOEHRD) (KRF-2006-531-F00017), and by the Korea Food Research Institute (the grant number E080802).

References

1. Sarkar S, Pranava M, Marita R. Demonstration of the hypoglycemic action of *Momordica charantia* in a validated animal model of diabetes. Pharmacol. Res. 33: 1-4 (1996)
2. Krawinkel MB, Keding GB. Bitter gourd (*Momordica charantia*): A dietary approach to hyperglycemia. Nutr. Rev. 64: 331-337 (2006)
3. Grover JK, Yadav SP. Pharmacological actions and potential uses of *Momordica charantia*: A review. J. Ethnopharmacol. 93: 123-132 (2004)
4. Basch E, Gabardi S, Ulbricht C. Bitter melon (*Momordica charantia*): A review of efficacy and safety. Am. J. Health Syst. Pharmacol. 60: 356-359 (2003)
5. Raman A, Lau C. Anti-diabetic properties and phytochemistry of *Momordica charantia* L. (Cucurbitaceae). Phytomedicine 2: 349-362 (1996)
6. Shetty AK, Kumar S, Sambaiah K, Salimath PV. Effect of bitter gourd (*Momordica charantia*) on glycaemic status in streptozotocin-induced diabetic rats. Plant. Food Hum. Nutr. 60: 109-112 (2005)
7. Virdi J, Sivakami S, Shahani S, Suthar AC, Banavalikar MM, Biyani MK. Antihyperglycemic effects of three extracts from *Momordica charantia*. J. Ethnopharmacol. 88: 107-111 (2003)
8. Day C, Cartwright T, Provost J, Bailey CJ. Hypoglycemic effect of *Momordica charantia* extracts. Planta Med. 56: 426-429 (1990)
9. Akhtar MS, Athar MA, Yaqub M. Effect of on blood glucose level of normal and alloxan-diabetic rabbits. Planta Med. 42: 205-212 (1981)
10. Miura T, Itoh Y, Iwamoto N, Kato M, Ishida T. Suppressive activity of the fruit of *Momordica charantia* with exercise on blood glucose in type 2 diabetic mice. Biol. Pharm. Bull. 2: 248-250 (2004)
11. Miura T, Itoh C, Iwamoto N, Kato M, Kawai M, Park SR, Suzuki I. Hypoglycemic activity of the fruit of the *Momordica charantia* in type 2 diabetic mice. J. Nutr. Sci. Vitaminol. 47: 340-344 (2001)
12. Vikrant V, Grover JK, Tandon N, Rathi SS, Gupta N. Treatment with extracts of *Momordica charantia* and *Eugenia jambolana* prevents hyperglycemia and hyperinsulinemia in fructose fed rats. J. Ethnopharmacol. 76: 139-143 (2001)
13. Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes. Diabetes 54: S97-S107 (2005)
14. Rhodes CJ. Type 2 diabetes - a matter of β-cell life and death? Science 307: 380-384 (2005)
15. Mathis D, Vence L, Benoist C. β-Cell death during progression to diabetes. Nature 414: 792-798 (2001)
16. Wang K, Yin X-M, Cao DT, Milliman CL, Korsmeyer SJ. BID: A novel BH3 domain-only death agonist. Gene Dev. 10: 2859-2869 (1996)
17. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, bax that accelerates programmed cell death. Cell 74: 609-619 (1993)
18. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90: 405-413 (1997)
19. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91: 479-489 (1997)
20. Sitasawad SL, Shewade Y, Bhonde R. Role of bittergourd fruit juice in stz-induced diabetic state *in vivo* and *in vitro*. J. Ethnopharmacol. 73: 71-79 (2000)
21. Yagi N, Yokono K, Amano K, Nagata M, Tsukamoto K, Hasegawa Y, Yoneda R, Okamoto N, Moriyama H, Miki M, Tominaga Y, Miyazaki JI, Yagita H, Okumura K, Mizoguchi A, Miki A, Ide C, Maeda S, Kasuga M. Expression of intercellular adhesion molecule 1 on pancreatic β-cell destruction by cytotoxic T-cells in murine autoimmune diabetes. Diabetes 44: 744-752 (1995)
22. Di Matteo MA, Loweth AC, Thomas S, Mabley JG, Morgan NG, Thorpe JR, Green IC. Superoxide, nitric oxide, peroxynitrite, and cytokine combinations all cause functional impairment and morphological changes in rat islets of langerhans and insulin-secreting cell lines but dictate cell death by different mechanism. Apoptosis 2: 164-177 (1997)
23. Kumar R, Mandal M, Lipton A, Harvey H, Thompson CB. Overexpression of HER2 modulates Bcl-2, Bcl-X_L, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. Clin. Cancer Res. 2: 1215-1219 (1996)
24. Kim HY, Kim K. Protective effect of ginseng on cytokine-induced apoptosis in pancreatic β-cells. J. Agr. Food Chem. 55: 2816-2823 (2007)
25. Allen RT, Hunter WJ III, Agrawal DW. Morphological and biochemical characterization and analysis of apoptosis. J. Pharmacol. Toxicol. 37: 215-228 (1997)
26. Hui H, Nourparvar A, Zhao X, Perfetti R. Glucagon-like peptide-1 inhibits apoptosis of insulin-secreting cells via a cyclic 5'-adenosine monophosphate-dependent protein kinase A- and a phosphatidyl-inositol kinase-dependent pathway. Endocrinology 144: 1444-1455 (2003)
27. Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvessen GS, Dixit VM. Yama/CPP32β, a mammalian homology of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81: 801-809 (1995)
28. Sansome C, Zaika A, Marchenko ND, Moll UT. Hypoxia death stimulus induces translocation of p53 protein to mitochondria. FEBS Lett. 488: 110-115 (2001)
29. Donath MY, Gross DJ, Cerasi E, Kaiser N. Hyperglycemia-induced β-cell apoptosis in pancreatic islets of *Psammomys obesus* during development of diabetes. Diabetes 48: 738-744 (1999)
30. Li P-F, Dietz R, Harsdorf R. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J. 18: 6027-6036 (1999)