

The Apoptotic Effect of the Methanol Extract of *Polygonum cuspidatum* through Up-Regulation Death Receptor 5 and CHOP in HSC-2 Human Oral Cancer Cells

Hyun-Ju Yu¹, Ji-Ae Shin¹, Eun-Sun Choi¹, Jae-Gyu Jeon², Nam-Pyo Cho¹, Sung-Dae Cho^{1*}

¹Department of Oral Pathology, School of Dentistry, Institute of Oral Bioscience, Chonbuk National University, Jeonju, Korea;

²Department of Preventive Dentistry, School of Dentistry, Institute of Oral Bioscience, Chonbuk National University, Jeonju, Korea.

Email: *efiwdsc@chonbuk.ac.kr

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ABSTRACT

Polygonum cuspidatum is used as a traditional medicinal herb for the therapy of various diseases including several types of cancers. In the present study, we focused on addressing the anti-cancer activity and molecular mechanism of methanol extract of *Polygonum cuspidatum* (MEPC) in HSC-2 human oral cancer cells. The effect of MEPC on oral cancer cells was estimated by 3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay, 4'-6-diamidino-2-phenylindole (DAPI) staining and Western blot analysis. MEPC inhibited the cell viability and induced apoptosis through the induction of death receptor 5 (DR5). MEPC also increased the expression of C/EBP homologous protein/growth arrest and the DNA damage-inducible gene 153 (CHOP), a transcription factor induced by ER stress. Thus, we concluded that the induction of CHOP leading to DR5 up-regulation is required for the anti-cancer activity of MEPC in HSC-2 cells and MEPC may be a promising drug candidate for oral cancer.

Keywords: *Polygonum cuspidatum*; Endoplasmic Reticulum Stress; C/EBP Homologous Protein/Growth Arrest and the DNA Damage-Inducible Gene 153 (CHOP); Death Receptor 5 (DR5); Apoptosis; Human Oral Cancer Cell

1. Introduction

Oral cancer is one of the leading causes of cancer-related deaths in developed countries [1], and it is estimated that 36,540 new cases diagnosed in the United States in last year (2010) [2]. Several factors are associated with increased risk of oral cancer, and these include smoking and alcohol usage [3-5]. Because oral cancer is frequently not detected in early stages, oral cancer patients have a relatively low five-year survival rate [2]. Therefore, the clinical trials such as early detection and therapeutic strategies for oral cancer are still required.

The function of the endoplasmic reticulum (ER) is essential for cellular activities and survival in eukaryotic cells. The ER is the primary organelle for protein synthesis, initial posttranslational modification, proper protein folding and assembling of secreted, membrane-bound and some organelle-targeted proteins [6,7]. The accumulation of misfolded and unfolded protein in ER lumen results in the condition referred to as ER stress, and then leads to ER stress response known as unfolded protein response (UPR). To resolve this problem, cells evolved various

protective strategies such as transcriptional induction of chaperone genes for protein folding, translational attenuation of protein synthesis and protein degradation by proteasome [8,9]. However, if misfolded and unfolded protein aggregation is persistent and cannot be resolved, this response also results in apoptosis. Earlier studies showed that ER stress-induced apoptosis up-regulates the transcription factor C/EBP homologous protein/growth arrest and the DNA damage-inducible gene 153 (CHOP/GADD153) [10,11]. In addition, induction of DR5 through activation of CHOP plays an important role in ER-stress induced apoptosis [12-18].

The naturally occurring products derived from plant sources are used as traditional medicines due to their potential chemotherapeutic activity. *Polygonum cuspidatum*, a traditional medicinal herb, has been used for the therapy of various diseases including inflammation, hepatitis and diarrhea [19-23]. In many previous studies, *Polygonum cuspidatum* was found that it contains several constituents such as resveratrol, emodin, polydatin, physicon and rhein [24-26]. The extract of *Polygonum cuspidatum* including these constituents have been reported to possess an important role for anti-inflammatory

*Corresponding author.

and anti-cancer activities [21,27-30]. In particular, our group reported that emodin in *Polygonum cuspidatum* has anti-proliferative and pro-apoptotic effects in several oral cancer cells and Sp1 and survivin played important role for *P. cuspidatum*-induced apoptosis [31]. However, the apoptotic effect of *Polygonum cuspidatum* in oral cancer cells related to ER stress is not well studied yet.

In the present study, we investigated the growth-inhibitory effect of MEPC in oral cancer and the molecular target for MEPC-induced apoptosis. The results showed that MEPC induced apoptosis and it modulated the expressions of DR5 and CHOP in HSC-2 human oral cancer cells.

2. Materials and Methods

2.1. Reagents and Antibodies

The methanol extracts of *Polygonum cuspidatum* (MEPC) was kindly given by Dr. Jeon in Chonbuk National University (Jeonju, Korea). Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), 100× antibiotic solution, Trypsin, and D-PBS was obtained from WelGENE Inc. (Dae-gu, Korea). The Poly (ADP-ribose) polymerase (PARP) antibody was purchased from BD Pharmingen™ (San Jose, CA, USA). Antibodies recognizing cleaved caspase-3 (Asp175) and DR5 were purchased from Cell Signaling Technology (Denver, MA, USA). The antibodies for CHOP (Cat No. sc-793; R-20) and actin (Cat No. sc-1615; C-11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DR4 antibody was obtained from R&D Systems Inc. (Minneapolis, MN, USA). Cell Titer 96® Aqueous One Solution Cell Proliferation assay kit for 3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay was purchased from Promega (Madison, WI, USA). DAPI (4'-6-Diamidino-2-phenylindole) was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.2. Cell Culture and Chemical Treatment

HSC-2 cells were obtained from Prof. Shindo in Hokkaido University (Hokkaido, Japan). HSC-2 cells were cultured in DMEM supplemented with 10% FBS and 100U/ml each of penicillin and streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. An equal number of cells were seeded and allowed to attach. When the cells reached 50% confluence, the cells were treated with DMSO or MEPC (25, 50 and 100 g/ml). MEPC was dissolved in 0.1% DMSO (vehicle control)

2.3. MTS Assay

The effects of MEPC on cell viability were estimated

using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit. The cells were seeded in a 96-well plate and then incubated for 24 hr. After MEPC treatment for 24 and 48 hr, a MTS solution was added to each well and the plates were incubated at 37°C in humidified 5% CO₂ atmosphere for 2 hr. The absorbance at 490 nm was recorded using ELISA plate reader (BIO-TEK Instruments, Inc., Madison, WI, USA). The data were expressed as the percentage of cell viability compared to the vehicle control.

2.4. DAPI Staining

Apoptotic cell death was determined morphologically using a fluorescent nuclear dye, DAPI staining. HSC-2 cells were incubated with DMSO or MEPC (25, 50 and 100 g/ml) for 48 hr, HSC-2 cells were harvested by the trypsinization and fixed in 70% ethanol overnight at 4 C. The cells were re-suspended in PBS, deposited on poly-L-lysine coated slides, stained with a DAPI solution, deposited onto the slides and finally viewed to detect apoptotic characteristics with a fluorescent microscope.

2.5. Western Blot Analysis

Whole-cell lysates were extracted and quantified with DC Protein Assay kit (BIO-RAD, Hercules. CA, USA). Equal amount of protein from each sample was mixed with 5X loading buffer and heated at 95°C for 5 min. Equal amount of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane (BIO-RAD, Hercules. CA, USA). The membranes was blocked with 5% skim milk in TBST buffer at RT for 2 hr, washed with TBST and maintained overnight at 4°C with primary antibody. Washing was done with TBST and incubation with horseradish peroxidase (HRP)-conjugated secondary antibody was done at RT for 2 hr. After washing with TBST, the antibody-bound proteins were detected using an ECL Western Blotting Luminol Reagent (Santa Cruz, CA, USA).

2.6. Statistical Analysis

The data were assessed for statistical significance using a Student's t-test. A value of $p < 0.05$ compared with the solvent control was considered statistically significant.

3. Results

3.1. MEPC Inhibits the Cell Viability and Induces Apoptosis in HSC-2 Human Oral Cancer Cells

The effect of MEPC on the viability of HSC-2 cells was investigated using the MTS assay. The HSC-2 cells were

treated with various doses of MEPC (25, 50 and 100 $\mu\text{g/ml}$) for 24 and 48 hr. The result shown in **Figure 1(a)** indicated that the viability of HSC-2 cells significantly inhibited upon treatment with MEPC in dose-dependent manner. To evaluate why MEPC inhibits the cell viability, we next investigated the apoptotic activity of MEPC by Western blot analysis. As shown in **Figure 1(b)**, MEPC-treated cells showed decreased total PARP and activated caspase-3 than control cells. We also examined the apoptotic activity of MEPC by DAPI staining. As shown in **Figure 2**, nuclear staining with DAPI revealed that nuclear condensation and fragmentation exhibited in the cells treated with higher doses of MEPC for 48 hr. These results show that MEPC has anti-proliferative and apoptotic effect in HSC-2 cells.

3.2. MEPC Increases DR5 Expression, but Not DR4

To further investigate that whether MEPC-induced apoptosis is involved in extrinsic pathway by death receptors, the protein levels of death receptor 4 and 5 were detected. As shown in **Figure 3(a)**, MEPC increased the expression of DR5 in dose-dependent manner compared to vehicle control, but did not affect DR4 expression. Based on these results, we performed experiments in triplicate. As

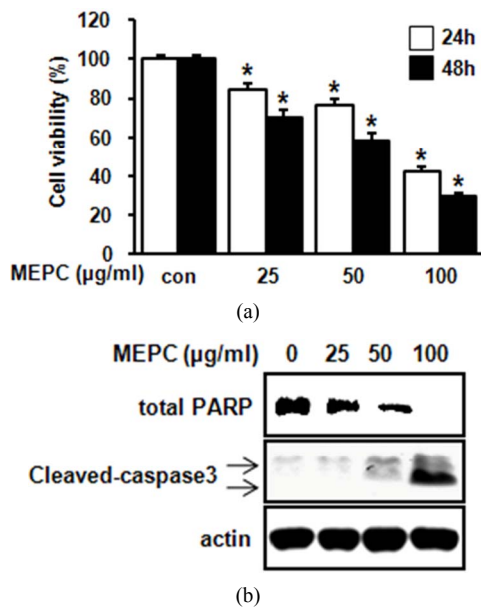


Figure 1. HSC-2 cell were treated with DMSO or various doses of methanol extract of *Polygonum cuspidatum* (MEPC). (a) Cell viability was determined using the MTS assay in HSC-2 cells for 24 and 48 hr. The bars are the means \pm SD of triplicate experiments. *, $p < 0.05$ compared to the vehicle control group; (b) The effect of MEPC on the total PARP and cleaved caspase-3 in HSC-2 cells was determined by Western blot analysis. Actin was used to normalize the protein loading from each treatment.

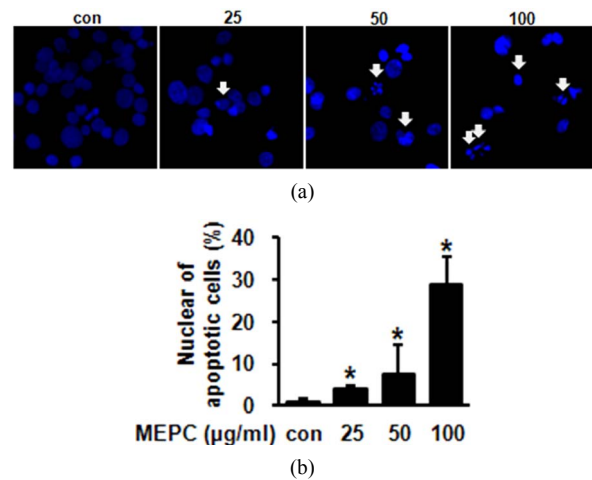


Figure 2. (a) HSC-2 cells were treated with DMSO or various doses of MEPC for 48 hr. Cells were harvested and the apoptotic effect of MEPC in HSC-2 cells was determined by DAPI staining. DNA fragmentation and nuclear condensation were detected by fluorescence microscopy (Magnification, $\times 400$); (b) The apoptotic cells were counted and the data shown in the graphs expressed the means \pm SD of triplicate experiments. *, $p < 0.05$ compared to the vehicle control group.

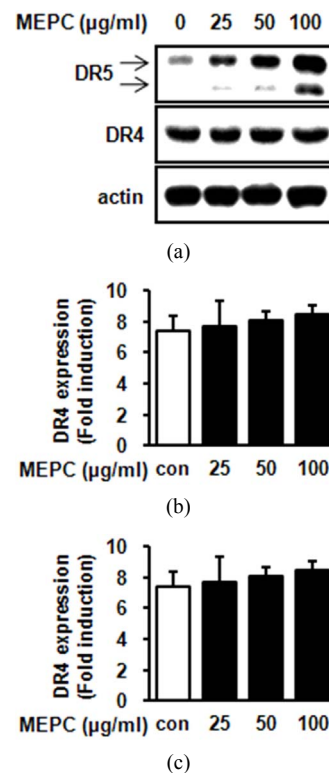


Figure 3. HSC-2 cells were treated with DMSO or 25, 50 and 100 $\mu\text{g/ml}$ of MEPC for 48 hr. (a) The protein levels of DR5 and DR4 were analyzed by Western blot analysis; (b) and (c) The protein levels of DR5 and DR4 were confirmed in triplicate experiments, and then the graphs expressed the means \pm SD, *, $p < 0.05$ compared to the vehicle control group.

shown in **Figures 3(b)** and **(c)**, HSC-2 cells treated with 100 $\mu\text{g/ml}$ of MEPC increased DR5 expression up to 12 folds compared to vehicle control whereas MEPC did not cause significant change in DR4 expression. These results showed that the extrinsic pathway by DR5 contributed to MEPC-induced apoptosis in HSC-2 cells.

3.3. MEPC Up-Regulates CHOP Expression through Endoplasmic Reticulum Stress

In previous studies, it was reported that the up-regulation of CHOP by ER stress was required for DR5 expression [12,13]. Thus, we examined whether MEPC affected the expression of CHOP, transcription factor related to ER stress. The result shown in **Figure 4(a)** indicated that MEPC-treated cells increased CHOP expression compared to vehicle control. Based on these results, we performed experiments in triplicate. As shown in **Figure 4(b)**, cells treated with 100 $\mu\text{g/ml}$ of MEPC increased CHOP expression up to 6 folds. Therefore, these results suggest that the up-regulation of CHOP was involved in MEPC-induced apoptosis.

4. Discussion

The naturally occurring products derived from plant are abundant source of bioactive constituents that have played an important role in pharmacology. Based on traditional therapeutic applications, several natural products have been demonstrated their beneficial effects in various cancer cells. *Selaginella tamariscina*, an oriental medicinal plant, induced p53 expression and G1 arrest, and suggests that this might contribute to cytotoxic effects by causing apoptotic DNA fragmentation in human leukemia

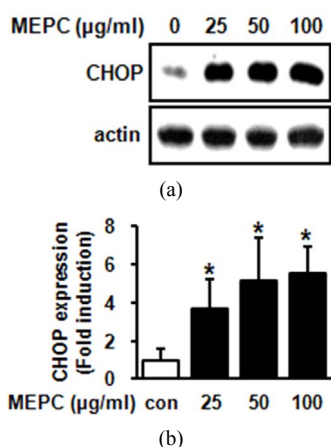


Figure 4. (a) The protein level of CHOP was analyzed by Western blot analysis in HSC-2 cells treated with various doses of MEPC for 48 hr; (b) The protein level of CHOP was confirmed in triplicate experiments, and then the graphs expressed the means \pm SD. *, $p < 0.05$ compared to the vehicle control group.

cells and human ovarian cancer cells [32]. Other study also reported that the extract of *Alpinia oxyphylla* inhibited the cell proliferation of skin cancer cells by inducing apoptosis [33]. In addition, the extract of *Solanum muricatum* suppressed tumor growth both *in vivo* and *in vitro* by inducing apoptosis [34]. These studies showed that the products derived from naturally occurring plants are useful for the prevention of cancer. In particular, chemopreventive agents such as naturally occurring product consist of a diverse group of compounds with different mechanisms of action, but, their ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention [35]. Therefore, these studies that explain their ability to induce apoptosis and mechanism of action are valuable for their clinical application in prevention and therapy of cancer.

In the present study, we examined the effect of MEPC on the growth inhibition of HSC-2 human oral cancer cells and what kind of key molecular target is involved in MEPC-induced apoptosis. We found that MEPC clearly decreased the number of viable cells in a dose-dependent manner and induced apoptosis as evidenced by total PARP decrease, and activation of caspase-3 in HSC-2 oral cancer cells (**Figure 1**). We also found that MEPC increases the nuclear condensation and fragmentation (**Figure 2**). These results suggest that MEPC induces apoptosis to inhibit the growth of oral cancer cells. Because death receptors (DR4 and DR5) are essential for extrinsic pathway of apoptosis, targeting death receptor-mediated apoptosis is effective strategy for prevention of cancer. Previous studies reported that Honokiol, a natural product purified from *magnolia*, is effective sensitizer of death receptor-mediated apoptosis in lung cancer cells [36]. Dihydroflavonol derived from *Blumea balsamifera*, are also known to induce apoptosis through DR5 up-regulation in leukemia cells [37]. Thus, we investigated whether MEPC can cause the expression of death receptors in oral cancer cells. The data showed that MEPC clearly increased DR5 expression, but did not affect DR4 expression (**Figure 3**). These results suggest that MEPC stimulates the extrinsic pathway by DR5.

ER stress is an important pathway that responds to ER dysfunction such as accumulation of misfolded and unfolded protein. The expression of genes associated with ER stress, including CHOP, GRP78 and ATF4, is increased by ER stress responses [6,8,9]. In particular, CHOP, an important marker for ER stress, is up-regulated in ER stress-induced apoptosis [16,38]. Interestingly, earlier studies suggested that ER-stress induced apoptosis through up-regulation of CHOP is associated with induction of the DR5 expression [12,13,16]. Our recent study in several oral cancer cells also show that C-DIMs activate the ER stress pathway leading to the

up-regulation of DR5 and cleaved Bid and caspase 8 [39]. In this study, MEPC potently stimulated the expression of CHOP (**Figure 4**), suggesting that up-regulation of CHOP contributes to MEPC-induced apoptosis.

5. Conclusion

In conclusion, this study showed that MEPC decreased cell viability and induced apoptosis through the induction of DR5 in HSC-2 oral cancer cells. Moreover, MEPC induced CHOP expression, a typical marker for ER stress. Taken together, we suggest that MEPC-induced apoptosis through the induction of DR5 might be mediated by ER stress leading to the up-regulation of CHOP in oral cancer cells, and MEPC may have a clinical potential for effective treatment of oral cancer although we need to investigate the anti-cancer efficacy and toxicity of MEPC in the *in-vivo* animal model.

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