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Apoptotic Activity of Methanol Extract of Tongcao in HEp-2 Human Cervical Cancer Cells

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ABSTRACT - Although tongcao has been reported to have anti-oxidative, anti-inflammatory and antipyretic effects, there is no report of the chemopreventive effect of tongcao in cancer cells. In the present study, we investigated the anti-proliferative activity of methanol extract of tongcao (MET) and its molecular target in HEp-2 human cervical cancer cells using MTS assay, western blot analysis, and DAPI staining. MET significantly decreases cell viability and induces apoptotic cell death. It affects Bid protein to be truncated resulting in the release of cytochrome c from mitochondria to cytosol whereas it did not affect other Bcl-2 family members. Thus, we clearly suggest that tongcao can be a potential naturally occurring plants having chemopreventive activity in cervical cancer.

Key words: Tongcao, Chemoprevention, Apoptosis, Cervical cancer

Introduction

Cervical cancer is the second leading cause of women cancer deaths in the world with an approximate morbidity of 500,000 female per year[1,2]. Due to inadequate or non-existent screening, disease is usually detected at the late stage. Thus, the development of treatment options for this cancer is highly necessary.

Human papollomaviruses (HPVs) are the primary cause of cervical cancer and the two early HPV genes, E6 and E7, play crucial role in tumor formation. Especially, E6 and E7 are deeply related with apoptotic cell death by modulating p53, IGFBP3 and TNF-alpha[3-5], so apoptotic cell death might be a good strategy for the prevention of cervical cancer.

Tongcao is an evergreen shrub in the family Araliaceae, the sole species in the genus Tetrapanax. It is endemic to Taiwan, but widely cultivated in East Asia and sometimes in other tropical regions as well[6]. In spite of wide cultivation, there are a few studies from only China available for its pharmacological actions. These studies reported that Tongcao has anti-inflammatory, antipyretic, diuretic and antioxidative effects[7-10]. However, there is no report of its action related to the cancer prevention. In this study, we extracted Tongcao using methanol (MET) and we determine whether MET affects the proliferation of HEp-2 human cervical cancer cells and evaluate unique molecular targets related with chemopreventive action of this extract.

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Materials and Methods

Reagents

The antibodies for Poly (ADP-ribose) Polymerase (PARP) (Asp175), caspase 3, Bid, Bad, Bak, Bax, Bim, Bcl-XL and Mcl-1 were obtained from Cell Signaling Technology (Danvers, MA, USA). Actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Methanol extract of tongcao (MET) was kindly provided by Prof. Ki-Han Kwon (Kwangju University, Republic of Korea). 4'-6-Diamidino -2-phenylindole (DAPI) was acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Cell Culture and Chemical Treatment

The HEp-2 human cervical cancer cells were cultured in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 μg/*mL streptomycin* (WelGENE Inc., Daegu, Korea) in a humid atmosphere of 5% CO_2. Equal numbers of cells were seeded and allowed to attach overnight. The cells were treated with MET (50, 100 and 150 μg/ml) diluted in DMEM with 5% FBS for 72 hr.

MTS assay

The effects of MET on cell viability were estimated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cells were seeded in 96-well plates and incubated with various doses of MET. After the treatment with MET for 72 hr, 30 μl of MTS solution was added to each well and the cells were incubated for 2 hr at 37°C. MTS solution was analyzed using a microplate reader (BioTeck Instruments Inc., Winooski, VT, USA) at 490 nm and 690 nm (background).

Western blot analysis

Whole-cell lysates were extracted with lysis buffer and quantified with *DC* Protein Assay (Bio-RAD). Equal amount of protein from each treatment group were separated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel and the separated proteins were transferred to Immun-Blot™ PVDF membrane (Bio-RAD). The membrane was blocked with 5% skim milk in TBS-T for 2 hr at RT and maintained overnight at 4°C with the primary antibody, followed by maintained with horseradish peroxidase-conjugated secondary antibodies. The antibody-bound proteins were detected using an ECL Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.).

Detection of morphological apoptosis

Cell death was measured by DAPI staining (Sigma Chemical Co, MO). After an experimental treatment with 50, 100 and 150 μg/ml of MET for 72 hr, HEp-2 cells were harvested by trypsinization and fixed in 4% paraformaldehyde at RT for 20 min. The cells were resuspended in PBS, deposited them on poly-L-lysin-coated slides, stained with a DAPI solution (2 μg/ml) and viewed under a fluorescence microscope.

Preparation of cytosolic and mitochondrial fractions

Cytosolic and mitochondrial fractions were isolated by digitonin or triton X-100 permeabilization. Briefly, cells were washed with ice-cold PBS, and cell pellets were resuspendend for 1 min on room temperature in plasma membrane extraction buffer containing 0.05% digitonin. Following a centrifugation step at 15,000 g at 4°C for 5 min, the supernatant was separated from the pellet consisting of cellular debris. The supernatant containing cytosolic proteins was collected, and the mitochondrial pellet was harvested by centrifugation at 15,000 g at 4°C for 5 min. The pellet consisting of mitochondrial proteins was resuspended by plasma membrane extraction buffer containing 0.5% triton X-100. The supernatant containing mitochondrial proteins was collected from last centrifugation.

Statistical Analysis

Statistical significance was assessed using a Student's t-test. A value of $p < 0.05$ compared to the solvent control was considered statistically significant.

Results and Discussion

The growth-inhibitory and apoptotic effect of MET in HEp-2 cells

A large number of natural compounds have shown the potential of chemopreventive effects in a variety of cancers[11,12]. In the present study, MET was tested *in vitro* for its potential growth inhibitory effect on HEp-2 cell lines using MTS assay which widely used to quantify cell viability. The results showed that MET significantly exhibited a cytotoxic effect on HEp-2 cells in a dose-dependent manner (Fig. 1A). At higher dose (150 μg/ml) for 72 hr, the cell viability was lower than 50%. To examine whether MET induced apoptosis, Western blot analysis using PARP and caspase 3 antibodies was performed. The results showed that the levels of cleaved PARP and caspase 3 were clearly detected in 100 or 150 μg/ml of MET (Fig. 1B). Also, the cells treated with MET were stained with DAPI to confirm the level of apoptosis and the results showed that the condensed chromatin and fragmented nuclear morphologies were observed in HEp-3 cells (Fig. 1C). It suggested that the growth inhibition of HEp-2 cells by MET is due to apoptotic cell death.

Anti-inflammatory compounds of plant origin such as quercetin and genistein can have chemopreventive effects in human cervical cancer cells[13,14]. Previously, tongcao has

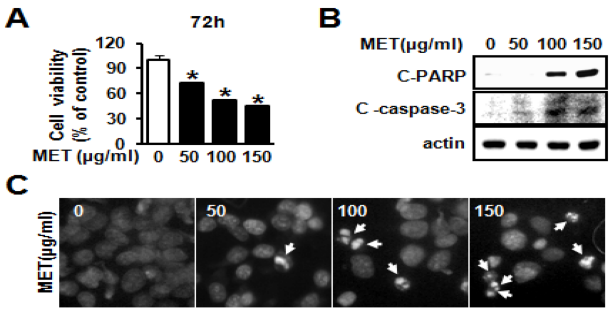

Fig. 1. (A) The effect of MET on the viability of HEp-2 cells using MTS assay. HEp-2 cells treated with 50, 100 and 150 μg/ml of MET for 72 hr. Cell viability was determined using MTS assay. The graph was representative of three independent experiments and bar is mean ± SD. *$p < 0.05$ compared to DMSO-treated group; (B) The effect of MET on the cleavage of PARP in HEp-2 cells. The protein levels of cleaved PARP and caspase 3 were determined by Western blot analysis. Actin was used to normalize the protein loading from each treatment; (C) The effect of MET on the condensation and fragmentation of nucleus in HEp-2 cells. Fluorescence microscopy images of DAPI-stained HEp-2 cells showing the appearance of apoptotic morphology.

pharmacological actions including anti-inflammatory activity and it means that tongcao can have anti-proliferative and apoptotic activity to show chemopreventive actions[7]. However, there is no such a study that investigates the anti-cancer activity of tongcao and its molecular target using cancer cells. In this study, our data revealed that MET has a growth-inhibitory effect on HEp-2 human cervical cancer cells and MET induced apoptosis in HEp-2 cells indicating that MET has a potential as chemopreventive agent.

MET truncates Bid resulting in the release of cytochrome c from mitochondria and cytosol

Targeting the B-cell lymphoma/leukemia 2 (Bcl-2) family promises to offer improved predictive biomarkers for oncologists and improved therapies for patients with cancer[15]. In order to investigate the critical role of the Bcl-2 family members in the MET-induced apoptosis, we examined the effects of MET on the expression of Bcl-2 family members. MET did not alter the expression of Bcl-2 family members in cervical cancer cells after being treated with 50, 100 and 150 μg/ml for 72 hr (Fig. 2C), but it cleaved Bid protein implying that Bid might contribute to MET-induced apoptosis (Fig. 2A). Previously, other study implied that proapoptotic Bcl-2 members including Bid might release cytochrome c from mitochondria to cytosol[16]. Thus, we determined whether MET affects this phenomenon and the result showed that MET clearly induced the expression level of cytosolic cytochrome c (Fig. 2B). It suggests that the cleavage of Bid by MET induces apoptosis in HEp-2 cells causing the release of cytochrome c.

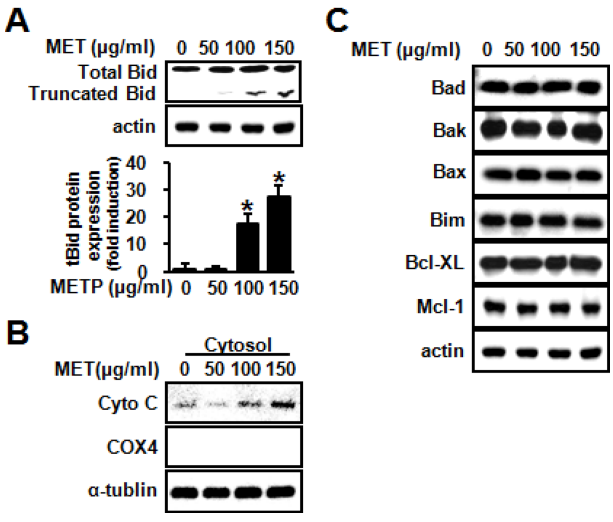

Fig. 2. (A) The effect of MET on the regulation of Bid protein. HEp-2 cells treated with 50, 100 and 150 μg/ml of MET for 72 hr. The protein levels were analyzed by Western blot analysis and the graph was representative of three independent experiments and bar is mean ± SD. $*p < 0.05$ compared to DMSO-treated group; (B) Cytosolic fractions were prepared and analyzed by Western blots to detect cytochrome c; (C) Other Bcl-2 family proteins (Bad, Bak, Bax, Bim, Bcl-xL and Mcl-1) were analyzed by Western blot analysis.

In summary, the results presented in this manuscript showed for the first time that MET inhibits cell growth and induces apoptotic cell death by truncating Bid protein in HEp-2 human cervical cancer cells. Although we need much deeper molecular mechanistic study for MET-induced apoptosis in the *in vitro* cell culture model, all of these results indicate that MET may be a potential chemopreventive agent against cervical cancer.

요 약

비록 천연유래물질 중의 하나인 통초가 항산화작용, 항염증작용 및 해열작용과 같은 효능이 있다고 알려져있지만, 종양에서의 암예방효능에 대한 연구는 보고된 바가 없다. 본 연구에서는 사람 자궁경부암세포주인 HEP-2세포에서 통초메탄올추출물의 증식억제 효능 및 관련 분자표적을 확인하고자 하였다. 통초메탄올추출물은 세포증식을 유의성있게 억제하고, 세포사멸을 유도하는 것으로 나타났다. 또한, Bid의 발현에 영향을 주어 truncation을 유도하고, 이로 인해 cytochrome c가 마이토콘드리아에서 세포질로 이동하도록 유도하였지만, Bid 이외의 다른 Bcl-2 family 에는 영향을 주지 못했다. 따라서, 이러한 결과를 종합해 볼 때, 통초메탄올추출물은 자궁경부암에서 암예방효능을 가진 잠재성있는 천연추출물이 될 수 있을 것으로 사료된다.

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