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Induction of apoptosis in human cervical carcinoma HeLa cells with active components of *Menispermum dauricum*

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ABSTRACT. *Menispermum dauricum* DC possesses a wide range of pharmacological effects. In this study, the mechanism of apoptosis induced by active components of *M. dauricum* was investigated in the human cervical carcinoma HeLa cell line. HeLa cells were treated with different *M. dauricum* concentrations over different time periods. The proliferation-inhibitory rate and cytotoxic effect of HeLa cells were measured by using the methyl thiazolyl tetrazolium (MTT) assay, and the apoptotic rate was detected by flow cytometry. Expressions of caspase-9, caspase-8, caspase-3, Bcl-2, and Fas proteins, in the apoptotic pathway, and the expression of nuclear factor-kappa B (NF-κB) were detected by SP immunocytochemistry. The MTT assay showed that active components of *M. dauricum* could significantly inhibit the growth of HeLa cells in a dose- and time-dependent manner ($P < 0.01$). The Sub-G_1 peak was found by flow cytometry, and the maximal apoptosis rate was 24.93%. Immunocytochemistry showed that after treatment with *M. dauricum*, the expressions of caspase-8, caspase-9, caspase-3, Fas protein, and NF-κB all increased, and the expression of

the Bcl-2 protein decreased, with significant differences relative to the control group ($P < 0.01$). Apoptosis in HeLa cells could be induced by active components of *M. dauricum* through the NF-κB signal transduction pathway and the caspase pathway, which was related to the downregulation of Bcl-2 expression and the upregulation of Fas expression.

Key words: *Menispermum dauricum*; HeLa cells; Apoptosis; Mechanism

INTRODUCTION

Apoptosis, also called programmed cell death, is a type of cell extinction regulated in an orderly manner by a series of signal cascades in certain situations, and is an important physical process involved in regulating growth, development, and immune responses (Rein et al., 2002; Fan et al., 2005; Valdespino and Valdespino, 2006). Induction of apoptosis in tumor cells with drugs or phytochemicals might be an important therapeutic approach for cancer. Recently, much attention has been paid to the anticancer activity of inducing cell apoptosis.

Menispermum dauricum DC is an herbal plant species that possesses a wide range of pharmacological effects, including antidotic, anti-inflammatory, anti-mutational, and anti-thrombotic effects (Yeh et al., 2003; Liu et al., 2006). In recent years, *M. dauricum* has been examined for its anticancer activity. It has been reported to have cytotoxic effects on some tumor cell lines by arresting the cell cycle and inducing cell apoptosis (Shi et al., 2003). However, the effect of *M. dauricum* on human cervical carcinoma cells remains unclear. Therefore, the purpose of the present study was to investigate the apoptotic pathway along with its molecular mechanism of action in human cervical carcinoma HeLa cells.

MATERIAL AND METHODS

Cell culture

HeLa cells (provided by Jilin University, Changchun, China) were cultured in RPMI-1640 medium (Gibco Co., USA) containing 10% fetal bovine serum (Hyclone Co., USA), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a 5% CO_2 humidified atmosphere. The cells were seeded on culture plates or dishes at appropriate concentrations.

Methyl thiazolyl tetrazolium (MTT) assay

Cancer cells were dispensed at 200 µL volumes on 96-well plates at a density of 10 x 10^4 cells/mL at 37°C in a 5% CO_2 humidified atmosphere, and allowed to adhere for 12 h. The cells were then treated with different *M. dauricum* concentrations, 10, 20, 40, and 80 µg/mL, for 24, 48, and 72 h. Twenty microliters 5 mg/mL MTT (Sigma Co., USA) was added to each well, followed by incubation for 4 h at 37°C. Finally, 150 µL dimethyl sulfoxide was added to each well, after which optical absorbance was read at 492 nm on a microplate reader. An empty well was used as a blank. The results were assessed as the percent cell viability compared to the vehicle-treated control cells, which were arbitrarily assigned 100% viability. Assays were carried out in triplicate.

Determination of apoptosis rate

Flow cytometric analysis was used to measure the cellular DNA content. Briefly, 10 x 10^4 cancer cells/mL were seeded in culture flasks and allowed to adhere overnight. The cells were treated with 40 μg/mL *M. dauricum* for 48 h and were then harvested by treatment with trypsin (Sigma), washed with ice-cold phosphate-buffered saline (PBS), isolated by centrifugation at 1200 rpm for 5 min, resuspended in 1 mL PBS, and finally stained with propidium iodide solution (50 mg/mL propidium iodide, 100 mg/mL RNase, and 0.1% Triton X-100 in PBS). The stained cells were analyzed for DNA histograms and cell cycle phase distribution by flow cytometry. The apoptotic rate was determined, and all experiments were performed in triplicate.

Immunocytochemical staining

Immunocytochemical staining was conducted according to manufacturer instructions, with slight modifications. After washing with PBS, slices were incubated in 0.3% H_2O_2 solution at room temperature for 20 min, and were then incubated with monoclonal antibodies for Bcl-2, Fas, caspase-9, caspase-8, caspase-3, or nuclear factor kappa B (NF-κB) (Neomarkers Inc., USA) at 4°C overnight. After washing with PBS, the secondary antibody was added, and cells were incubated at 37°C for 1 h. After washing with PBS, the ABC compound was added and incubated at room temperature for 10 min. DAB was used as the developer. After 10 min, the brown color signifying the presence of antigens bound to antibodies was detected by light microscopy. Controls were treated in the same manner as the experimental group except for the incubation of primary antibody. The positive rate was calculated as follows: positive rate = (number of positive cells/total number) x 100%.

Statistical analysis

Data are reported as means ± SD. Differences between groups were analyzed using the Student *t*-test. Data analysis was performed using the SPSS13.0 software, and $P < 0.05$ was considered a statistically significant difference.

RESULTS

MTT assay

The MTT assay showed that HeLa cell proliferation was significantly inhibited after incubation with 40 μg/mL *M. dauricum* for 24, 48, and 72 h in a dose- and time-dependent manner. The proliferation inhibitory rate of cells ranged from 22.67 to 86.59% after 48 h treatment with *M. dauricum*, and was close to 50% at a 40 μg/mL *M. dauricum* concentration (Figure 1).

Determination of apoptosis rate

Treatment of HeLa cells with 40 μg/mL *M. dauricum* for 24 and 48 h resulted in a clear increase in the percentage of apoptotic cells. The maximal apoptosis rate was 24.93%,

which was much higher than that of the control (2.45%). The flow cytometry analysis showed an obvious Sub-G_1 peak.

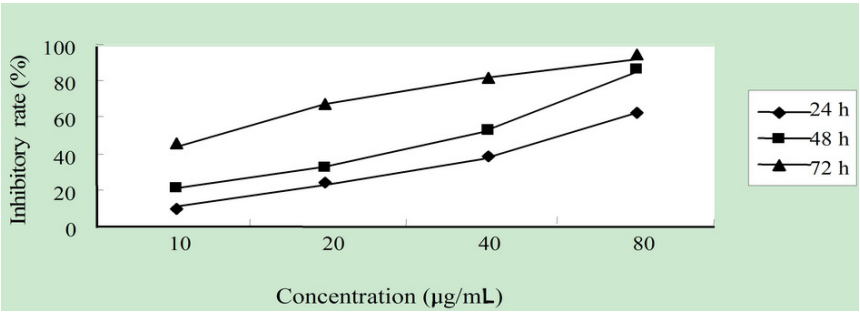

Figure 1. Dose- and time-dependent cytotoxicity of *Menispermum dauricum* in HeLa cells. Data are reported as means ± SD (N = 3).

Expression of related proteins in the apoptotic pathway

Immunocytochemical staining showed positive staining of Bcl-2 and Fas proteins in the cytoplasm and positive staining of caspase-8, caspase-9, and caspase-3, mainly in the cytoplasm or in both the cytoplasm and nucleus. After treatment with active components of *M. dauricum*, the expressions of caspase-8, caspase-9, caspase-3, and Fas proteins increased, and that of the Bcl-2 protein decreased, with significant differences from those of the control group ($P < 0.01$) (Figure 2).

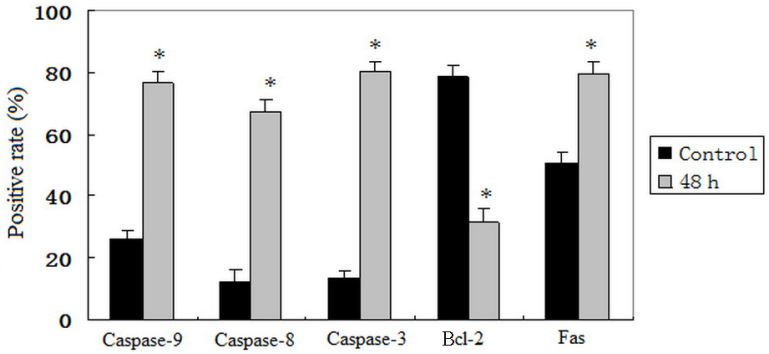

Figure 2. Detection of expression levels of apoptosis-related proteins by immunocytochemical staining. Data are reported as means ± SD (N = 5). *P < 0.01.

Expression of NF-κB p65

Positive staining of NF-κB in control cells was relatively low and was mainly located in the cytoplasm. However, treatment of cells with 40 μg/mL *M. dauricum* for 48 h resulted in a significant increase in NF-κB expression, and the positive staining was mainly located in the nucleus (Figures 3 and 4).

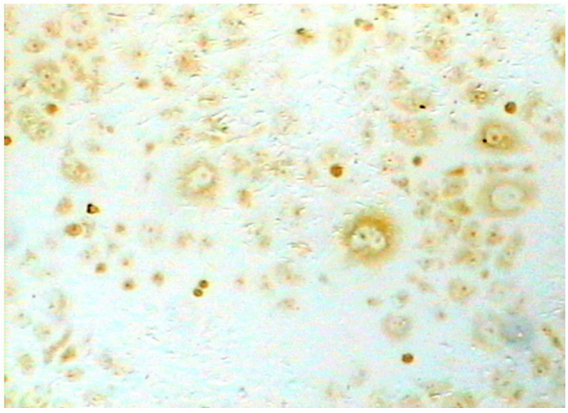

Figure 3. Expression of NF-κB in control cells (400X).

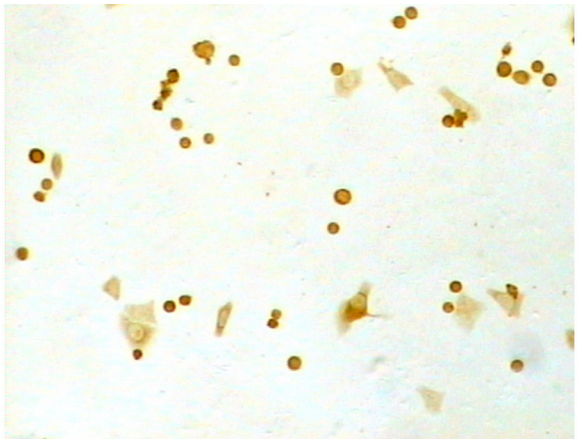

Figure 4. Expression of NF-κB after treatment of HeLa cells with 40 μg/mL *Menispermum dauricum* for 48 h (400X).

DISCUSSION

In this study, the viability of treated HeLa cells was measured by the MTT assay. When these cells were exposed to *M. dauricum* over various periods and concentrations, their viability was significantly inhibited in a dose- and time-dependent manner. This indicated that *M. dauricum* could effectively inhibit the proliferation of HeLa cells.

Flow cytometric analysis was used to measure the cellular DNA content and form the histogram (Duchrow et al., 1995; Nakatani et al., 2005; Niu and Wei, 2006). The Sub-G_1 peak was considered to indicate apoptosis. The percentage of apoptotic cells was determined by measuring the altitude of the Sub-G_1 peak (Elstein and Zucker, 1994; Moroni et al., 2004; Smith et al., 2006). The Sub-G_1 peak was found after treatment of HeLa cells with 40 μg/mL *M. dauricum*. This phenomenon demonstrated the presence of apoptotic cells. The treated cells resulted

in a clear increase in the percentage of apoptotic cells compared with control cells ($P < 0.01$).

Apoptosis involves two main pathways: the extrinsic pathway, which is initiated by binding of ligands to specific death receptors, and the intrinsic pathway, which is initiated at the mitochondria (Gupta, 2003). Triggering the extrinsic apoptosis pathway leads to the formation of the death-inducing signaling complex, which contains the Fas-associated death domain adaptor protein and pro-caspase-8 and -10. Activated caspase-8 cleaves and activates the effector caspase-3, and activated caspase-3 cleaves targeted "death proteins", such as poly-ADP-ribose polymerase (PARP), leading to apoptosis (Li et al., 2001; Jiang and Wang, 2004). Intrinsic death stimuli directly or indirectly activate the mitochondrial pathway by inducing the release of cytochrome C from the mitochondria, and form the apoptosome complex with Apaf-1 and pro-caspase-9 in the cytoplasm. Caspase-9 is activated at the apoptosome, and in turn activates caspase-3 and cleaves PARP. This death pathway is largely controlled by the pro-apoptotic (e.g., Bax, Bad, Bid, and Bak) and anti-apoptotic (e.g., Bcl-2 and Bcl-xL) Bcl-2 family proteins.

We detected the expression levels of apoptosis-related proteins by immunocytochemical staining. The results showed that the expression of caspase-8, caspase-9, caspase-3, and Fas proteins in the treated group dramatically increased compared to those in the control group, whereas the expression of the Bcl-2 protein was significantly decreased in comparison to the untreated group. Therefore, induction of apoptosis in HeLa cells with active components of *M. dauricum* seems to have occurred by the downregulation of the Bcl-2 protein and the upregulation of the Fas protein, which then activated the caspase pathway.

NF-κB is a ubiquitous transcription factor that is activated by a variety of cytokines and mitogens. It is now clear that in addition to its widely recognized role as a key regulator of immune and inflammatory responses, NF-κB has emerged as a decisive factor in the cell's response to apoptotic challenge (Yeh et al., 2003). NF-κB family members have been shown to be expressed in all cells, including NF-κB1-(p105/p50), NF-κB2 (p100/p52), REL-A (p65), cREL, and RELB, which associate to form various heterodimeric and homodimeric combinations (Van Waes, 2007). Classic NF-κB is a heterodimer of p50 (NF-κB-1) and p65 (REL-A) (Greten and Karin, 2004; Liu et al., 2006). NF-κB is retained in an inactive form in the cytoplasm through its association with one of the IκB inhibitory proteins. After cellular stimulation, the phosphorylation, ubiquination, and subsequent proteolysis of IκBα in proteosomes enable NF-κB to translocate into the nucleus. Therefore, the expression of NF-κB in nuclei indicates that NF-κB is activated (Deregowski et al., 2002; Kucharczak et al., 2003).

Recently, abundant evidence has implicated cellular NF-κB transcription factors in the control of apoptosis in many systems. A number of studies have implicated NF-κB in apoptosis-resistant tumor cells. NF-κB is most commonly involved in suppressing apoptosis by transactivating the expression of anti-apoptotic genes, whereas it can promote programmed cell death in response to certain death-inducing signals and in certain cell types. In diverse cell types, the NF-κB signaling pathway has been shown to have a critical role in regulating the apoptotic program. Whether NF-κB promotes or inhibits apoptosis appears to depend on the specific cell type, the inducing stimulus, and the experimental conditions, among other factors.

There are a number of instances in which NF-κB can promote apoptosis. For instance, IκB kinase activation was shown to be involved in the regulation of paclitaxel-induced apoptosis in human tumor cell lines (Huang and Fan, 2002; Meteoglu et al., 2008). In addition, some studies showed that NF-κB was involved in inducing apoptosis by upregulating the

expression of apoptotic genes and suppressing the expression of anti-apoptotic genes. For instance, the tumor necrosis factor-related apoptosis-inducing ligand could induce apoptosis of MCF-7 and HEK-293 cells by activating death receptor 5 via NF-κB. Treatment of U20S and MEF cells with daunorubicin, Adriamycin, or UV-C radiation resulted in the activation of NF-κB, the downregulation of Bcl-xL, xIAP, and A20 expressions, and the induction of apoptosis. The death domain of P100 could recruit the death receptor, activate the caspase pathway, and induce apoptosis of the cells.

In this study, we detected the expression of NF-κB after treatment of HeLa cells with 40 μg/mL *M. dauricum* using an NF-κB (p65) immunohistochemistry detection kit. The results showed low-positive staining of NF-κB in the control cells that was mainly located in the cytoplasm. However, positive staining of treated cells was mainly located in the nucleus, and the expression of NF-κB increased significantly. These results showed that *M. dauricum* could induce apoptosis of HeLa cells. Simultaneously, NF-κB was activated and nuclear transfer occurred. This result, along with the expression patterns of related proteins in the apoptotic pathway, suggested that the NF-κB signal transduction pathway might play a promoting role in the apoptosis of HeLa cells induced by *M. dauricum*. The mechanism underlying the apoptosis-inducing activity of *M. dauricum* involves inducing the degradation of IκB to activate NF-κB. Activated NF-κB can then upregulate the Fas protein, downregulate the Bcl-2 protein, and activate the caspase pathway. Apoptosis initiation and execution would then be completed by the collective activity of the Fas-dependent pathway and the mitochondria pathway.

As mentioned above, *M. dauricum* can inhibit proliferation and induce apoptosis in HeLa cells. The results reported in the present study suggest that *M. dauricum* contributes to HeLa cell apoptosis to some extent, which involves more than one single mechanism. Particularly relevant in this regard is the role of *M. dauricum* in the regulation of key molecules of the NF-κB signaling pathway. Our study provides evidence that could encourage the further study of *M. dauricum* as a promising therapeutic agent in the clinical treatment of human cervical carcinoma.

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