RESEARCH ARTICLE

Radix Tetrastigma Hemsleyani Flavone Induces Apoptosis in Human Lung Carcinoma A549 Cells by Modulating the MAPK Pathway

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Abstract

   Radix Tetrastigma Hemsleyani Flavone (RTHF) is widely used as a traditional herb for its detoxification and anti-inflammation activity. Recently, several studies have shown that RTHF can inhibit growth and induce apoptosis in human cancer cell lines. However, the mechanisms are not completely understood yet. In this study we investigated the potential effects of RTHF on growth and apoptosis in human lung adenocarcinoma A549 cells as well as its mechanisms. A549 cells were treated with RTHF at various concentrations for different times. *In vitro* the MTT assay showed that RTHF had obvious anti-proliferation effects on A549 cells in a dose- and time-dependent manner. Cell morphological changes observed by inverted microscope and Hoechst33258 methods were compared with apoptotic changes observed by fluorescence microscope. Cell apoptosis inspected by flow cytometry showed significant increase in the treatment group over the control group ($P<0.01$). Expression of apoptosis related Bax/Bcl-2, caspases and MAPK pathway proteins were detected by Western blotting. The results showed that RTHF up-regulated the Bax/Bcl-2 ratio and cle-caspase3/9, cle-PARP expression in a dose-dependent manner. Expression of p-p38 increased, p-ERK decreased significantly and that of p-JNK was little changed in the RTHF group when compared with the control group. These results suggest that RTHF might exert anti-growth and apoptosis activity against lung cancer A549 cells through activation of caspases and Bcl-2 family proteins and the MAPK pathway, therefore presenting as a promising therapeutic agent for the treatment of lung cancer.

Keywords: *Radix Tetrastigma Hemsleyani Flavone* - human lung carcinoma cell - apoptosis - MAPKs pathway

Introduction

   Lung cancer is the leading cause of cancer deaths throughout the world. Approximately 80-85% of all lung cancers are classified as non-small cell lung cancer (NSCLC), Current therapies include active surveillance, surgery, radiation therapy, chemotherapy and immunotherapy. Chemotherapy still plays an important role in this setting, however, these drugs are highly toxic with a low survival profile (De et al., 2006). Therefore, it is imperative to search for chemotherapeutic agents with high effectiveness and low adverse reactions. In the past few decades, traditional Chinese herbal medicine has begun to attain great popularity for disease prevention as well as being used as complementary medicines for a variety of diseases worldwide. Many scientists have focused on the potential roles of extracts of traditional herbs as alternative and complementary medications for cancer treatment. Herb extracts are often administered together with conventional cancer therapies to increase survival rates and enhance the quality of patient life (Li et al., 2009; Messina et al., 2011; Liu et al., 2012).

   Radix Tetrastigma Hemsleyani (RTH) is a traditional Chinese herb endowing with detoxification, anti-inflammation, antivirus and anticancer bioactivities. Flavone is the major composition extracted from the Chinese herb RTH, which has now been shown that *Radix Tetrastigma Hemsleyani Flavone* (RTHF) induces growth inhibition and apoptosis in various cancer cells including the human liver carcinoma cell line HepG2, gastric carcinoma cell line SGC-7901 and leukemic cell line K562 (Ding et al., 2005; Feng et al., 2006; Xu et al., 2010). It also can inhibit tumor growth (Ni et al., 2008; Li et al., 2012). However, the mechanism is not well described. This study investigated how RTHF affects cell growth and apoptosis of A549 human NSCLC cells. The anticancer mechanism of RTHF was also elucidated by analyzing apoptosis in A549 cells through activation of caspases and Bcl-2 family proteins and MAPKs pathways.

Materials and Methods

Materials and reagents
   Radix Tetrastigma Hemsleyani was purchased from

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Zhejiang Academy of traditional Chinese Medicine. Flavone extract from Radix Tetrastigma Hemsleyani (RTHF) was obtained from the Key Laboratory of Research and Development of new drugs of Traditional Chinese Medicine in Zhejiang Province. Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from HyClone (USA). Hoechst staining kit purchased from Beyotime Institute of Biotechnology (Shanghai, China). Annexin V-FITC Apoptosis Detection Kit was purchased from Keygen Institute of Biotechnology (Nanjing, China). The antibodies to pro-caspase3/9, cle-caspase3/9, PARP, p-p38, p-ERK, p-JNK, Bax and Bcl-2 were purchased from Cell Signaling, USA.

Cell line and culture

Human lung cancer A549 cells obtained from Zhejiang Province Academy of medical science, China and cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 100 IU/ml penicillin, 100 mg/ml streptomycin, incubated in 5% CO_2 incubator at 37°C. Cells were passaged every 2-3 days and used at about 80-90% of the confluence.

MTT assay

The cell proliferation was measured by MTT method. A549 cells were seeded in 96-well plates at a density of 5×10^3 cells/well, treated with various concentrations of RTHF (0, 0.5, 1, 5 and 10 mg/mL). After incubation for 24 h, 48 h or 72 h, discard medium and replaced with fresh medium (180 μl/well). 20 μl of MTT solution (5 mg/ml) added to each well and the plates were incubated for additional 4 h at 37°C. Then, discard the medium and 150 μl of DMSO were added to each well. The absorbance was read at 570 nm as test wavelengths using a microplate reader (Thermo Scientific, USA). Cell viability was calculated as percentage of viable cells in total population.

Cell morphological changes

Cells were treated with RTHF (0, 1, 5 and 10 mg/mL) for 48 h, then morphological changes of the cells were observed under a inverted microscope (Olympus IX71, Japan), recorded with a CCD camera (Olympus DP72, Japan).

Nuclear staining with Hoechst 33258

A549 cells were seeded in 6-well plates, and incubated with various concentrations of RTHF (0, 1, 5 and 10 mg/mL)for 48 h. Cells were fixed and stained with DNA fluorochrome Hoechst 33258 for 20 min. After washed with PBS, the morphological features of apoptosis (including cellular nucleus shrinkage, chromatin condensation, intense fluorescence and nuclear fragmentation) were monitored by fluorescence microscopy (Olympus IX71, Japan), recorded with a CCD camera (Olympus DP72, Japan).

Flow cytometry analysis of apoptosis

A549 cells were cultured in 6-well plates, incubated overnight, and then exposed to different concentrations of RTHF (0, 1, 5 and 10 mg/mL)for 48 h. cells were harvested by trypsin and washed with PBS. The cells
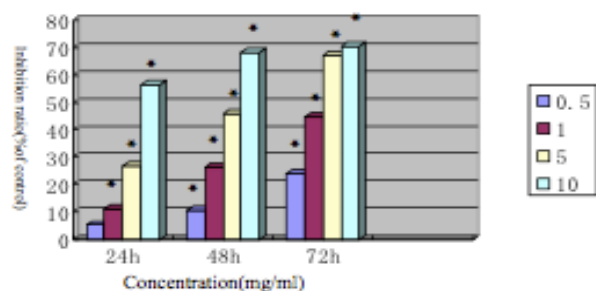

Figure 1. Proliferative Inhibition Effect of RTHF on A549 cells. Cell survival rates were measured using MTT assay. The cells were treated with various concentrations of RTHF for 24 h,48 h and 72 h. All data represent the mean values ±SD of at least three independent experiments.*$p<0.01$ vs. control group

were resuspended in binding buffer and then stained sequentially with Annexin V-FITC and PI as manufacture's instructions. The apoptotic cells were analyzed using a flow cytometry (BD FACSCalibur).

Western blot analysis

After treatments, A549 cells were harvested, and homogenized in 200 μl RIPA lysis buffer. then were extracted and the protein concentration was determined by Lowry method. Protein lysates (40 μg) from each sample were subjected to SDS-PAGE on 10% acrylamide gel and the separated proteins transferred to a PVDF membrane. After transfer, the membranes were blocked with 5% non-fat dry milk in TBS for 1 h at room temperature, then incubated with primary antibodies overnight at 4°C followed by secondary horseradish peroxidase-labeled antibody (1:2000). The bound antibodies were visualized using the ECL blotting detection system.

Statistical analysis

The data were expressed as means ± SD and were analyzed by one-way analysis of variance (ANOVA) by using SPSS software (version 13.0). $P<0.05$ was considered statistically significant.

Results

Effect of RTHF on A549 cell proliferation

A549 cell viabilities were determined using MTT assay at 570 nm. As shown in Figure 1, After incubate with RTHF (0, 0.5, 1, 5 and 10 mg/ml) for 48 h, the inhibition rates of cells were increased from 10.46 ± 0.75% to 68.36 ± 1.13%. After 24 h, 48 h and 72 h of treatment, the inhibition rates were elevated from 5.14 ± 0.68% to 23.88 ± 2.04%, 10.93 ± 1.33% to 44.96 ± 1.91%, 26.64 ± 2.13% to 67.31 ± 1.40% and 56.50 ± 2.24% to 70.79 ± 1.43%, respectively. The 50% inhibit concentration (IC50) was 6.5mg/ml at 48 h. The results showed that RTHF dramatically decreased the proliferation of A549 cells in a dose- and time-dependent manner ($p<0.01$).

RTHF induced cell morphological changes of A549 cells

A549 cell morphological changes were observed under a inverted microscope. Cells were treated with various concentrations of RTHF (0, 1, 5 and 10 mg/mL) for 48 h. As compared to control group, treated group showed a
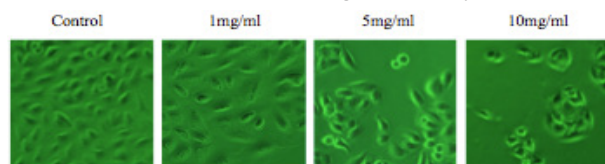
Figure 2. Effects of RTHF on Morphological Characteristics of A549 Cells
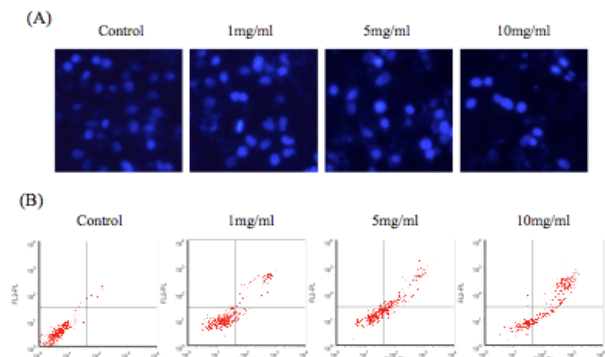

Figure 3. Apoptosis Induced by RTHF in A549 Cells. Cells were treated with various concentrations of RTHF for 48 h. (A) Cells stained with Hoechst 33258 were imaged with a fluorescence microscope. (B) Apoptotic cells were analyzed by flow cytometry with Annexin V-FITC and PI staining

significant decrease in adherent cells which accompanied an increase in floating cells in culture medium. This study also revealed that the RTHF treated A549 cells acquired a round and shrunken shape significantly in contrast to normal polyclonal structure of normal cells (Figure 2).

RTHF induced apoptosis of A549 cells

Apoptotic cell is characterized by major features like DNA fragmentation and loss of plasma membrane integrity. Morphologic changes in the nucleus representing cellular apoptosis were assessed by staining with the membrane-permeable DNA binding dye Hoechst 33258. As showed in Figure 3(A), A549 cells were treated with different concentrations of RTHF (0, 1, 5 and 10 mg/mL) for 48 h, normal cells exhibited regular and round shaped nuclei with a pallid blue, whereas apoptotic cells were characterized by the condensation and the fragmentation of nuclei with bright fluorescence. To further investigate RTHF-induced apoptotic effect, Flow cytometry analysis was used to quantify the apoptotic A549 cells were double stained with Annexin V-FITC and PI after treatment with different concentrations of RTHF (0, 1, 5 and 10 mg/mL) for 48 h. The proportion of apoptotic cells was increased from $3.45 \pm 2.61\%$ to $66.80 \pm 0.98\%$ (Figure 3B). The results revealed that RTHF induced apoptosis in A549 cells in a dose-dependent manner ($p<0.01$).

Effect of RTHF on expressions of Bcl-2/Bax and caspase family proteins

To further examine the possible mechanism of RTHF-induced apoptosis, we tested the protein expressions of Bcl-2, Bax, pro-caspase3/9, cle-caspase3/9, PARP. Western blotting analysis revealed that the Bax expression was obviously increased, whereas the Bcl-2 expression was decreased. In addition, there was significantly decrease in the pro-caspase3/9, pro-PARP expression and
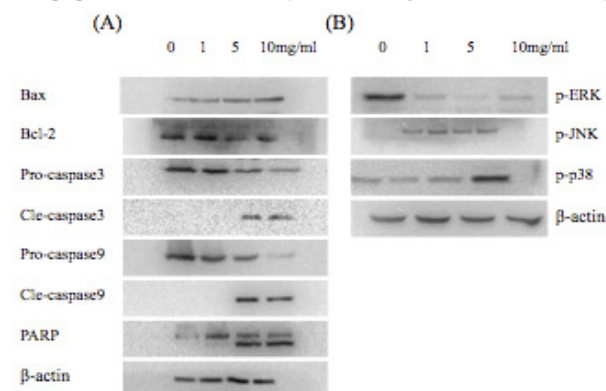

Figure 4. Protein Expression Levels in A549 Cells. The protein expression was measured by western blotting. (A) The protein expression of Bax, Bcl-2, pro-caspase3/9, cle-caspase3/9 and PARP. (B) The protein expression of p-ERK, p-JNK and p-p38

increase in the cle-caspase3/9, cle-PARP expression as compared to control (Figure 4A). These results indicated that RTHF up-regulation the Bax/Bcl-2 ratio and cle-caspase3/9, cle- PARP expression in a dose-dependent manner.

Effects of RTHF on MAPKs pathway

In order to better elucidate the molecular basis of RTHF induced apoptosis, we tested the expression of p-p38, p-ERK, p-JNK. The results revealed that RTHF increased the protein levels of p-p38, and decreased p-ERK via a dose-dependent manner, meanwhile little change of p-JNK (Figure 4B).

Discussion

Herbal medicines have been proven to be a major source of novel agents with various pharmaceutical activities (Mishra et al., 2011; De et al., 2012; Mondal et al., 2012). Natural products have provided a rich source of drugs for many diseases, including cancer. Radix Tetrastigma Hemsleyani (RTH) is a traditional Chinese herbal medicine have long been used in China in medical practice widely. Flavone is the major composition extracted from RTH (RTHF) have the functions of improving immune response, anti-inflammation and anticancer. In this study, we used RTHF to interfere in the growth of lung carcinoma A549 cell lines *in vitro*. The results showed that RTHF inhibited the proliferation of A549 cells in a dose- and time-dependent manner, meanwhile, the apoptotic rates of RTHF group showed statistically significance compared to control group.

Apoptosis is an important regulator in developmental processes, maintenance of homeostasis and elimination of the damaged cells. Generally, apoptosis pathways includes signals through the death receptor (extrinsic) or the mitochondria (intrinsic) pathway (Denault et al., 2004), The intrinsic pathway of apoptosis is regulated by the Bcl-2 family proteins. A balance between pro-apoptotic (BAX, BID, BAK or BAD) and anti-apoptotic (Bcl-XL, Bcl-2, BCLW or MCL1) proteins of the Bcl-2 family controls the mitochondrial apoptosis pathway (Zinkel et al., 2006). Many anticancer agents induced apoptosis by targeting the

proteins of Bcl-2 family and the ratio of Bax/Bcl-2 played a critical role in apoptosis of cells (Gupta et al., 2002; Emi et al., 2005). The caspase family is an executioner of apoptosis, in which caspase-3 is the key point (Heimlich et al., 2004). Mitochondrial proteins, such as cytochrome c, which bind with Apaf-1 and procaspase-9 in a dATP-dependent manner to form the apoptosome (Mignotte et al., 1998). The apoptosome can induce activation of caspase-9, thereby initiating apoptotic caspase cascades (Hengartner., 2000; Sun et al., 2004). Sequential activation of caspase-9 and caspase-3 in the intrinsic pathway results in PARP cleavage, leading to apoptosis (Shi, 2002). Thus caspases have been shown to be activated during apoptosis in many cells and play critical roles in both initiation and execution of apoptosis (Liu et al., 2009). In this study, we examined the effects of RTHF on expression of Bax, Bcl-2 and caspases proteins using Western blot analysis, and found that RTHF increased Bax, cle-caspase-3/-9 and cle-PARP expression and decreased Bcl-2, pro-caspase-3/-9 and pro-PARP expression, leading to up-regulate the ratio of Bax/Bcl-2 and inducing caspase-dependent apoptosis. This might be one of the molecular mechanisms in which RTHF induces apoptosis in A549 cells.

MAP kinase cascades consist of three core protein kinases such as ERK1/2, p38, and JNK pathways (Pearson et al., 2001; Raman et al., 2007). They are activated by phosphorylation induced by various mitogens and cell stressors, including osmotic stress, heat shock and proinflammatory cytokines. MAPK signaling pathways can induce cell proliferation, differentiation and apoptosis (Johnson et al., 2002). The expression of ERK is known as a prominent feature in many human cancers including nonsmall cell lung cancer (Lee et al., 2002). ERK can antagonize apoptosis by up-regulating pro-apoptotic Bcl-2 proteins such as Bax (Kim et al., 2005). The p38 and JNK MAPK pathways are activated by a variety of cell stressors, including ultraviolet light (UV), radiation, cytotoxic drugs, and cytokines such as tumor necrosis factor alpha and interleukin, and related to the occurrence, development and apoptosis of tumor cells.

Thus, to further understand the molecular mechanism of RTHF, the potential involvement of MAPK pathway in RTHF induced apoptosis was investigated by western blotting. This study showed that ERK and p38 phosphorylation in A549 cells was found enhanced in response to RTHF treatment, which plays an important role in apoptotic signaling through regulating the activities of Bcl-2 family proteins and mediating caspase activation (Tournier et al., 2000).

In conclusion, this study demonstrates that RTHF significantly inhibited proliferation and induced apoptosis in human lung cancer A549 cells, which is associated with activating of MAPKs pathways, then increased Bax/Bcl-2 ratio and caspases proteins expression. Therefore, RTHF may as a promisingly effective chemopreventive agent against lung adenocarcinoma.

Acknowledgements

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