

Effects of maslinic acid on the proliferation and apoptosis of A549 lung cancer cells

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Received December 3, 2014; Accepted October 27, 2015

DOI: 10.3892/mmr.2015.4552

Abstract. Maslinic acid (MA) is a pentacyclic triterpene acid that is present in numerous dietary plants. Although certain studies have demonstrated that MA has anti-cancer properties in different cell types, the effect of MA on lung cancer cell proliferation and apoptosis and the potential underlying mechanisms remain to be elucidated. In the present study, A549 lung cancer cells were treated with different doses of MA and it was found that MA significantly inhibited A549 cell growth in a dose-dependent manner. In addition, Annexin V/propidium iodide flow cytometric analysis demonstrated that MA induced apoptosis of A549 cells. The present study also confirmed that MA induced apoptosis by observing morphological alterations. In addition, the effect of MA treatment on the levels of apoptosis-associated proteins was examined. The results demonstrated that MA treatment suppressed the expression of caspase-3, -8 and -9, and increased the expression of cleaved caspase-3, -8 and -9 in a dose-dependent manner. The level of inhibitors of apoptosis (IAPs) and Smac, which are possible upstream factors of caspase proteins, were also examined. It was found that MA treatment increased the protein expression of Smac and decreased the protein levels of c-IAP1, c-IAP2, X-linked inhibitor of apoptosis protein (XIAP) and Survivin in a dose-dependent manner. These results suggested that MA inhibited proliferation and induced apoptosis of A549 cells through regulation of caspase cleavage as well as Smac, c-IAP1, c-IAP2, XIAP and Survivin.

Introduction

Lung cancer is the leading cause of cancer-associated mortality in the world (1) and non-small cell lung cancer (NSCLC) composes the majority of all lung carcinomas (2). Despite previous advances in diagnosis and treatment, the 5-year

survival rate has remained <15%, with only a 5-10% survival rate for advanced NSCLC (3,4). Therefore, identifying new therapeutic targets and agents to improve the prognosis of NSCLC is urgently required.

There has been a growing interest in the use of natural compounds as a new source of anti-tumor agent owing to their wide range of biological activities, low toxicity and weak side effects. Studies investigating the anti-tumor mechanisms of traditional Chinese herbal medicine, which are predominantly extracted from natural plants and animals, provide a theoretical basis and new strategies for the treatment of cancer. Currently, several anti-tumor components isolated from Chinese herbal medicines, including cephalotaxine, paclitaxel, podophyllotoxin, 10-hydroxycamptothecin and vinblastine, are used in clinical practice. Increasing attention has been paid to uncovering the anti-tumor potential and mechanisms of Chinese herbal medicine. Chinese herbs have been found to inhibit cell proliferation, angiogenesis and tumor metastasis, induce cell apoptosis and differentiation and regulate tumor-associated signaling pathways and the immune system (5-7), thus exhibiting anti-tumor potential *in vitro* and *in vivo*.

Maslinic acid (MA), a pentacyclic triterpene acid, is widely present in dietary plants and is particularly abundant in olive fruit skins. This compound has attracted significant interest due to its pharmacological safety and its various biological activities, including its anti-inflammatory, anti-bacterial, anti-viral and anti-oxidative properties (8-10). It has previously been reported that MA exerts anti-tumor effects on HT29 colon cancer cells, DU145 human prostate cancer cells and a mouse melanoma cell line, which were at least partially associated with apoptotic induction (11-13).

However, the effects of MA on various types of lung cancer remain to be elucidated. Thus, in the present study, the effects of MA on the proliferation and apoptosis of A549 lung cancer cells and the possible underlying mechanisms were examined.

Materials and methods

Reagents. MA was purchased from Shanghai Pure One Biotechnology Co., Ltd. (Shanghai, China). The extract used was a white powder comprising 98% MA and 2% oleanolic acid. This extract was stable when stored at 4°C. It was dissolved prior to its use at 10 mg/ml in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). A stock solution was frozen and stored at -20°C.

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Key words: maslinic acid, apoptosis, caspase, inhibitors of apoptosis, Smac

Cell culture. The A549 lung cancer cell line was obtained from the Department of Cell Biology (China Medical University, Shenyang, China) and cultured in RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal calf serum (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37°C in 5% CO₂. The medium was changed daily and the cells were digested using 0.25% trypsin (Biological Industries, Beit HaEmek, Israel). Cells were treated with different doses of MA (0, 9, 12, 15, 18 and 21 µg/ml) in their logarithmic growth phase.

MTT assay. A549 cells (1x10⁵/well) were plated in 96-well plates and cultured overnight. Subsequently, cells were incubated with different concentrations of MA (0, 9, 12, 15, 18 and 21 µg/ml) for 24 h, respectively. The corresponding culture medium was used as an empty control. Briefly, 20 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China) solution was added to each well and incubated for 4 h at 37°C. The supernatant was then removed from each well and DMSO (150 µl) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a microplate reader (ELx808; BioTek Instruments, Winooski, VT, USA). Each experiment was performed in triplicate. The following formula was used to calculate the inhibition ratio: Inhibition ratio (%) = (1 - M/C) x 100%, where M is the absorbance of MA-treated cells and C is the absorbance of control cells.

Flow cytometry. A549 cells (5x10⁵/well) were plated in 6-well plates and cultured overnight. Subsequently, cells were treated with different concentrations of MA (0, 9, 12, 15, 18 and 21 µg/ml) for 24 h and were harvested by 0.25% trypsin. The corresponding culture medium was used as the empty control. For Annexin V/propidium iodide (PI) apoptosis analysis, the cells were resuspended in 500 µl of binding buffer and adjusted to 1x10⁶/ml. Staining solution containing 5 µl Annexin V/fluorescein isothiocyanate and 5 µl PI (Nanjing KeyGen Biotech. Co., Ltd.) was added to the cells and then incubated at 2-8°C for 15 min in the dark. Following this, the cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). CellQuest version 5.1 software (BD Biosciences, San Jose, CA, USA) was used to analyze the data. Each experiment was performed in triplicate.

Immunofluorescence. A549 cells (5x10⁵/well) were seeded on slides in 6-well plates and cultured overnight. Subsequently, the cells were treated with 18 µg/ml MA at 37°C and 5% CO₂ for 24 h. Corresponding culture medium was used as the empty control. Cells were washed twice with cold phosphate-buffered saline (PBS), fixed with methanol and glacial acetic acid (3:1) for 15 min, stained with Hoechst 33342 (5 mg/l) at 37°C for 15 min (Sigma-Aldrich), and mounted with 1% glycerol. Morphological alterations were observed using fluorescence microscopy (BX53; Olympus, Tokyo, Japan). Each experiment was performed in triplicate.

Western blot analysis. Cells were seeded in culture flasks, allowed to attach overnight and incubated with 12 or 18 µg/ml MA for 24 h. An equal quantity of RPMI-1640 was added

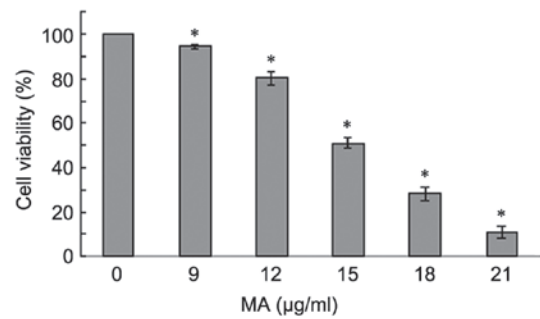


Figure 1. MA treatment inhibits the proliferation of A549 lung cancer cells. A549 cells were treated with different doses of MA (0, 9, 12, 15, 18 and 21 µg/ml) for 24 h. MTT assay demonstrated that the cell growth rate significantly decreased as the treatment dose increased. Columns show the mean value of three duplicates; bars represent standard deviation. *P<0.05, compared with the 0 µg/ml MA group. MA, maslinic acid.

as a control. Following that, cells were harvested (cell number >5x10⁶/ml) and washed twice with cold PBS. Western blot analysis was then performed. Briefly, the cell pellets were resuspended in lysis buffer (Nanjing KeyGen Biotech. Co., Ltd.) at 4°C for 1 h. Following centrifugation at 12,000 x g for 20 min, the supernatant was collected and stored at -80°C. The protein was quantified using a bicinchoninic acid quantification kit (Beyotime Institute of Biotechnology, Haimen, China). A total of 50 µg of protein was separated by 10% SDS-PAGE (Beijing Solarbio Science & Technology Co., Ltd.) and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with the following antibodies: Mouse monoclonal anti-caspase-3 (1:200; cat. no. sc-7272), mouse monoclonal anti-caspase-8 (1:500; cat. no. sc-81656), mouse monoclonal anti-caspase-9 (1:1,000; cat. no. sc-73548), rabbit polyclonal anti-cleaved caspase-3 (1:500; cat. no. sc-22171), rabbit polyclonal anti-cleaved caspase-8 (1:500; cat. no. sc-7890), goat polyclonal anti-cleaved caspase-9 (1:500; cat. no. sc-22182), mouse monoclonal anti-X-linked inhibitor of apoptosis protein (XIAP; 1:500; cat. no. sc-55552), mouse monoclonal anti-c-IAP1 (1:500; cat. no. sc-271419), rabbit polyclonal anti-c-IAP2 (1:500; cat. no. sc-7944), rabbit polyclonal anti-Survivin (1:500; cat. no. sc-10811), rabbit polyclonal anti-Smac (1:500; cat. no. sc-22766) and rabbit polyclonal anti-GAPDH (1:2,000; cat. no. sc-25778), all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Following incubation with peroxidase-conjugated anti-goat IgG (1:3,000; cat. no. SC-2020; Santa Cruz Biotechnology, Inc.) and peroxidase-conjugated anti-rabbit IgG (1:3,000; cat. no. SC-2004; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h, proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA) and detected using BioImaging Systems (UVP Inc., Upland, CA, USA).

Statistical analysis. All data were analyzed with SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). All values are expressed as the mean ± standard deviation. One-way analysis of variance and Fisher's least significant difference test was used to compare the differences between individual groups. P<0.05 was considered to indicate a statistically significant difference.

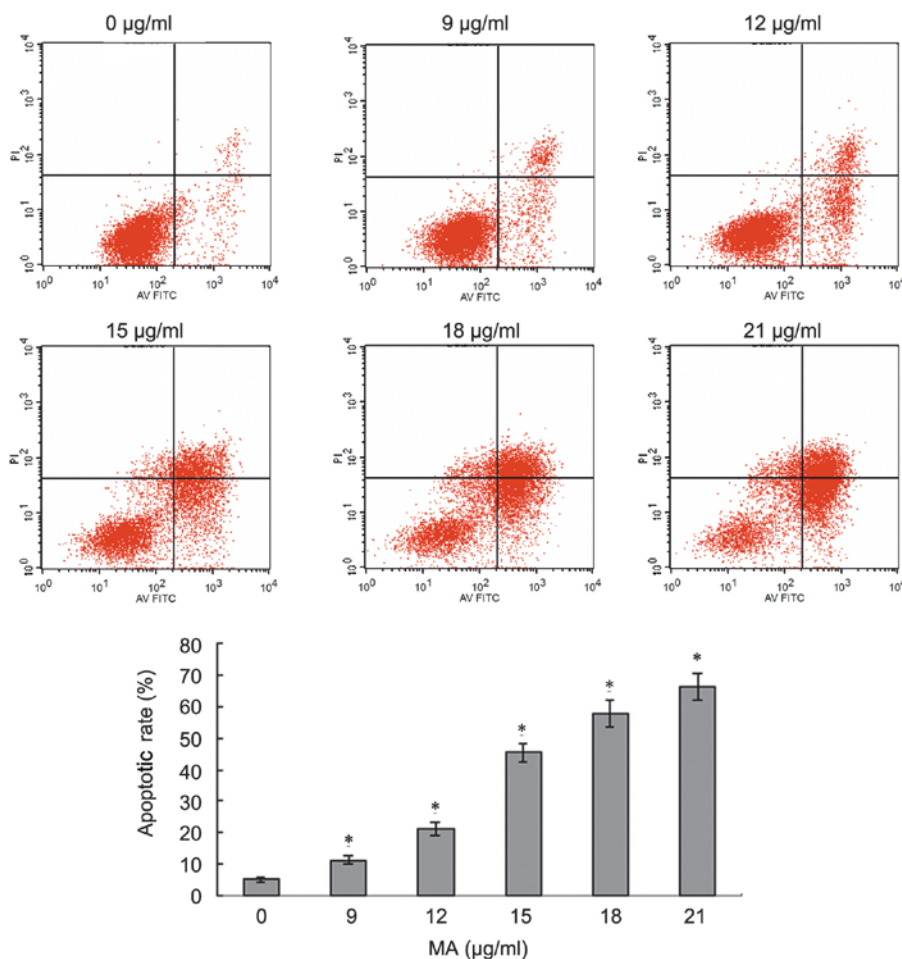


Figure 2. MA treatment induces apoptosis of A549 lung cancer cells. A549 cells were treated with different doses of MA (0, 9, 12, 15, 18 and 21 µg/ml) for 24 h. Annexin V/propidium iodide flow cytometric analysis demonstrated that the apoptotic rates induced by MA were significantly higher compared with that in untreated cells of the control group. When the dose of MA increased, more apoptotic cells were observed. Columns show the mean value of three duplicates; bars represent standard deviation. * $P < 0.05$, compared with the 0 µg/ml MA group. MA, maslinic acid.

Results

MA treatment inhibits the proliferation of A549 lung cancer cells. To examine the effect of MA on the proliferation of A549 cells, an MTT assay was performed in cells treated with different doses of MA (0, 9, 12, 15, 18 and 21 µg/ml) for 24 h. As the treatment dose increased, cell growth rate significantly decreased ($P < 0.05$; Fig. 1), suggesting that MA treatment suppressed A549 cell proliferation in a dose-dependent manner.

MA treatment induces apoptosis of A549 lung cancer cells. MA was reported to have anti-tumor effects on HT29 colon cancer cells, DU145 human prostate cancer cells and B16F0 mouse melanoma cell line, due to its role in apoptosis induction (12-14). Therefore, the effect of MA on the apoptosis of A549 cells was examined. A549 cells were incubated with different doses of MA (0, 9, 12, 15, 18 and 21 µg/ml) for 24 h and then Annexin V/PI flow cytometric analysis was performed to investigate the effect of MA on the apoptosis of NSCLC cells. As shown in Fig. 2, early apoptosis and late apoptosis markedly increased as the concentration of MA increased. The percentages of apoptotic cells were 5.73, 11.31, 21.06, 44.72, 57.71 and 66.10% following treatment with 0, 9, 12, 15, 18 and 21 µg/ml MA, respectively. The apoptotic rates induced by different

doses of MA were significantly higher compared with that in untreated cells of the control group ($P < 0.05$). When the dose of MA increased, the number of apoptotic cells increased. The results indicated that MA induced apoptosis of A549 cells in a dose-dependent manner.

MA treatment induces apoptotic morphological alterations in A549 lung cancer cells. Morphological alterations of cells is another important index of apoptotic detection. A549 cells were treated with 18 µg/ml MA for 24 h, stained with Hoechst 33342 and were then observed under fluorescence microscopy. It was found that MA treatment caused marked morphological alterations, including chromatin condensation, karyopyknosis and nuclear fragmentation, which are characteristic features of apoptotic cells (Fig. 3). In accordance with flow cytometric analysis, when the dose of MA increased, more apparent morphological alterations and more apoptotic cells were observed.

MA treatment regulates the expression of apoptosis-associated proteins. To further examine the mechanism of MA-induced apoptosis, the effects of MA on the protein expression of caspase-3, -8 and -9 and cleaved caspase-3, -8 and -9, which are important apoptosis-associated proteins,

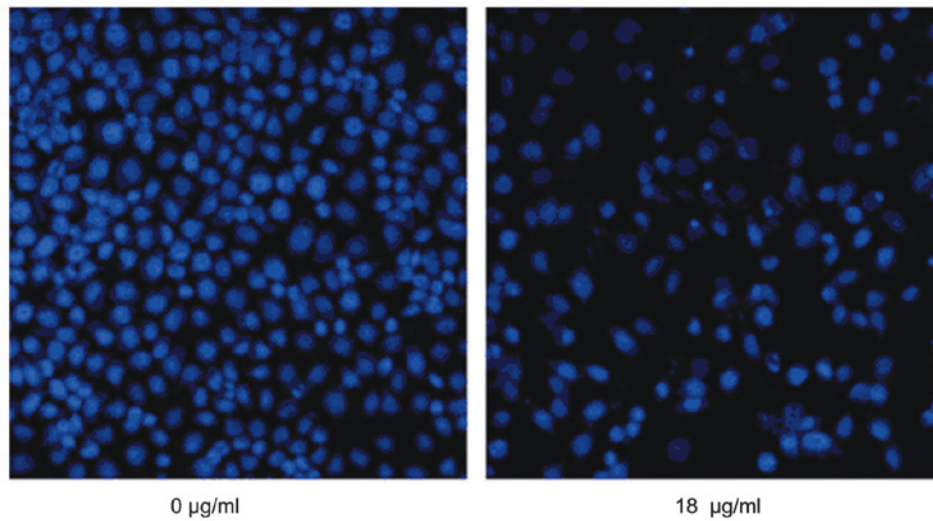


Figure 3. MA treatment induces apoptotic morphological alterations in A549 lung cancer cells. A549 cells were treated with 0 or 18 $\mu\text{g/ml}$ MA for 24 h and stained with Hoechst 33342. Using fluorescence microscopy, it was found that MA treatment caused marked morphological alterations with apoptotic features. MA, maslinic acid.

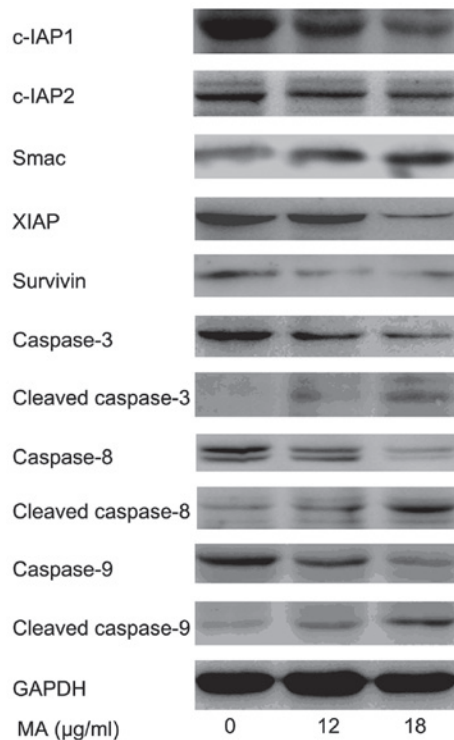


Figure 4. MA treatment regulates the expression of apoptosis-associated proteins. A549 cells were treated with different doses of MA (0, 12 and 18 $\mu\text{g/ml}$) for 24 h. MA treatment increased the protein levels of Smac, cleaved caspase-3, -8 and -9, and decreased the protein levels of c-IAP1, c-IAP2, XIAP, Survivin and caspase-3, -8 and -9 in a dose-dependent manner. MA, maslinic acid; XIAP, X-linked inhibitor of apoptosis protein; IAP, inhibitor of apoptosis.

were examined. A549 cells were treated with either 12 or 18 $\mu\text{g/ml}$ MA for 24 h and then the levels of caspase family proteins were analyzed by western blot analysis. As shown in Fig. 4, MA treatment suppressed the expression of caspase-3, -8 and -9, but promoted the expression of cleaved caspase-3, -8 and -9. In addition, as the doses increased, caspase-3, -8

and -9 decreased and cleaved caspase-3, -8 and -9 increased, suggesting that MA regulated the cleavage of caspase-3, -8 and -9 in a dose-dependent manner.

Smac and inhibitors of apoptosis (IAPs) family proteins also have a critical role in the regulation of apoptosis by inhibiting caspase family proteins. Thus, the expression of Smac and IAP family proteins, including c-IAP1, c-IAP2, XIAP and Survivin was investigated in cells treated with different concentrations of MA (0, 12 and 18 $\mu\text{g/ml}$). MA treatment increased the protein level of Smac and decreased the protein levels of c-IAP1, c-IAP2, XIAP and Survivin in a dose-dependent manner (Fig. 4).

Discussion

Apoptosis is an active form of cellular suicide encoded by an endogenous program that can be triggered by either internal or external cues. It is well established that resistance to apoptosis is a hallmark of cancer (15) and suppression of apoptosis is closely associated with the progression of various types of cancer, including NSCLC.

Caspases are a family of cysteine-containing proteolytic enzymes that have a central role in the execution-phase of cell apoptosis. Currently, 14 mammalian caspases have been found, which exist as inactive proenzymes distributed in different cellular compartments. The caspases consist of two sub-groups, initiator caspases, including caspase-2, -8, -9 and -10, and executioner caspases, including caspase-3, -6 and -7, which form a caspase-cascade system that has a central role in the induction, transduction and amplification of intracellular apoptotic signals (16). Caspase-3 is a major caspase, which amplifies signals from intrinsic and extrinsic pathways (17). Caspase-8 is important in the death receptor-mediated extrinsic pathway. Caspase-9 is regarded as the canonical caspase in the intrinsic mitochondrial pathway that is regulated primarily by Bcl-2 family and Bcl-2 homologous domain-3 only proteins (18). Caspase-3, -8 and -9 are synthesized as inactive pro-enzymes that are activated by proteolytic cleavage in cells undergoing apoptosis.

In addition, IAP family proteins are important in the regulation of apoptosis by inhibiting caspases. This protein family includes XIAP, cellular IAP1/2 and Survivin. IAPs are often found to be overexpressed in several types of human cancer and contribute to chemoresistance (19,20). XIAP can inhibit apoptosis by binding and inactivating caspases, including initiator caspase-9 and the effector caspase-3. XIAP is an important member of the mammalian IAP protein family, as it is the only member capable of inhibiting active caspases (21,22). cIAP-1 and cIAP-2 are predominantly involved in the regulation of the extrinsic pathway of apoptosis, through the inhibition of caspase-8 activation (23,24). Survivin has been demonstrated to inhibit caspase-dependent apoptosis through co-operation with XIAP and interference with caspase-3/9 (25,26). By contrast, Smac is a mitochondrial apoptogenic molecule that is released from the mitochondria in response to apoptotic stress. Smac is known to antagonize the function of IAPs (27-29).

Dysregulation of cell proliferation and apoptosis has been verified to be closely associated with tumor progression, and a number of anticancer drugs have been designed to induce apoptosis of cancer cells by targeting cellular processes, including cell growth, metabolism and proliferation (30). There has been a growing interest in the use of traditional Chinese medicines as a new source of anti-tumor agents owing to their wide range of biological activities, low toxicity and side effects. It is reported that certain terpenoids originating from Chinese medicine, particularly certain triterpenoids, have potential anti-tumor activities, which are often associated with apoptosis induction (31). MA is a pentacyclic triterpene acid, which is present in several dietary plants. This compound has been demonstrated to have abundant biological activities, including anti-inflammatory, anti-bacterial, anti-viral, anti-oxidative, anti-proliferative, anti-angiogenic properties as well as the ability to induce apoptosis (32). Previous studies have demonstrated that MA has anti-cancer capacity in different cell types, including liver, breast, pancreatic and prostate cancer (11,12,33-35). Specifically in colon malignancy, MA induced apoptosis in HT29 human colon cancer cells through the mitochondrial apoptotic pathway (35).

In the present study, the A549 lung cancer cell line was treated with different doses of MA and it was found that MA significantly inhibited A549 cell growth in a dose-dependent manner. In addition, AnnexinV/PI flow cytometric analysis was performed to investigate the effect of MA on the apoptosis of A549 cells. The results demonstrated that MA induced A549 apoptosis in a dose-dependent manner. Similarly, it was confirmed that MA induced apoptosis by observing the morphological alterations of cells, which exhibited typical apoptotic morphological characteristics. In addition, the effects of MA treatment on the protein expression of caspase-3, -8 and -9 and cleaved caspase-3, -8 and -9, which are important apoptosis-associated proteins, were examined. As shown in the results, MA treatment suppressed the expression of caspase-3, -8 and -9, but promoted the expression of cleaved caspase-3, -8 and -9. As the dose increased, caspase-3, -8 and -9 decreased and cleaved caspase-3, -8 and -9 increased, suggesting that MA regulated caspase cleavage in a dose-dependent manner.

IAP family proteins are considered to regulate apoptosis by inhibiting caspases, while Smac is known to antagonize the function of IAPs. In order to examine the possible mechanism

by which MA regulated the activity of caspase-3, -8 and -9, the levels of IAPs and Smac, which are possible upstream regulators of caspases, were examined. The present study demonstrated that MA treatment increased the protein level of Smac and decreased the protein levels of c-IAP1, c-IAP2, XIAP and Survivin in a dose-dependent manner.

Taken together, the results indicated that MA treatment inhibited proliferation and induced apoptosis of A549 lung cancer cells. MA promoted apoptosis by regulating the cleavage of caspase-3, -8 and -9 in a dose-dependent manner. Furthermore, the results suggested that MA increases the expression of Smac and decreases the expression of c-IAP1, c-IAP2, XIAP and Survivin, which leads to caspase cleavage. MA treatment inhibited the proliferation of A549 lung cancer cells in a dose-dependent manner by inducing cell apoptosis. MA induced apoptosis via regulating the cleavage of caspase-3, -8 and -9, by increasing Smac expression and decreasing c-IAP1, c-IAP2, XIAP and Survivin expression.

Acknowledgements

This study was supported by the Liaoning Province Science and Technology Plan project (grant no. 2013225021) and the Outstanding Scientific Fund of Shengjing Hospital (grant no. 201205).

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