Abstract. An extract from a traditional Chinese herb, *Marsdenia tenacissima* (trade name, Xiao-Ai-Ping) has been approved for use on the Chinese market as a cancer chemotherapeutic agent for decades. Previous studies have demonstrated the cytostatic and pro-apoptotic effects of *M. tenacissima* extract (MTE) in multiple cancer cells. However, the contributions of MTE to the proliferation and apoptosis of hepatoma carcinoma cells and the underlying mechanisms remain unclear. In the present study, Bel-7402 cells were incubated with increasing concentrations of MTE ranging from 0-320 µl/ml to explore the effects and potential mechanisms of MTE on the proliferation and apoptosis of Bel-7402 cells. 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium, inner salt and propidium iodide (PI)‑stained flow cytometry assays demonstrated that MTE significantly suppressed the proliferation of Bel-7402 cells in a dose-dependent manner by arresting the cell cycle at S phase (P<0.05). Annexin V‑fluorescein isothiocyanate PI‑stained flow cytometry confirmed the significantly pro-apoptotic effect of MTE at both 160 and 240 µl/ml (P<0.001). Reverse transcription-quantitative polymerase chain reaction and western blot analysis demonstrated that MTE (both 160 and 240 µl/ml) induced a significant downregulation of B-cell lymphoma (Bcl)-2 (P<0.01), upregulation of Bcl-2-associated X protein (P<0.01) and activation of caspase‑3 (P<0.05). Furthermore, a significant downregulation of murine double minute-2 (MDM2) (P<0.001) and activation of p53 (P<0.001) in Bel-7402 cells following treatment with 160 or 240 µl/ml MTE was observed, accompanied by the inhibition of the nuclear factor (NF)-κB pathway (P<0.001). These results suggested that MTE inhibited growth and exhibited pro-apoptotic effects in Bel-7402 cells, which was mediated by downregulation of the MDM2‑induced p53‑dependent mitochondrial apoptosis pathway and blocking the NF-κB pathway. Overall, these data serve as preliminary identification of the significant roles of MTE in hepatic carcinoma cells, and suggest that MTE may be a promising candidate for hepatocellular carcinoma therapy.

Introduction

Hepatocellular carcinoma (HCC) is the most frequent form of primary liver malignancy and the third leading cause of cancer-related mortality worldwide, with at least 700,000 deaths annually (1-3). Unlike the majority of solid tumors, HCC always develops in patients with underlying liver disease, such as alcohol abuse and viral hepatitis B or C (4,5). At present,
the main clinical strategy for HCC is partial hepatic resection combined with chemotherapy; however, this is limited by the high recurrence, with a rate of 75-100%, which leads to a poor 5-year survival rate of 40-50% (6). The high recurrence rate is largely attributed to the unlimited growth and anti-apoptotic abilities of hepatoma carcinoma cells (7). As a result, novel approaches to control recurrence and promote apoptosis of hepatoma carcinoma cells may be effective in reducing the mortality of HCC.

*Marsdenia tenacissima* caulis is a traditional herbal medicine comprised of the dried stems of *M. tenacissima* (Roxb.) Wight et Arn, which is an Asclepiadaceae plant widely distributed in the Guizhou and Yunnan Provinces of China (8). *M. tenacissima* caulis has been demonstrated to be clinically effective against asthma, trachitis, tonsillitis, pharyngitis, cystitis, pneumonia and rheumatism, with few side effects (8). *M. tenacissima* extract (MTE) injection has been used for the treatment of cancer in China for decades due to the bioactive constituents of polyoxypregnanol glycosides (9,10). Both *in vivo* and *in vitro* studies have reported that MTE enhances the sensitivity of various tumors to gefitinib, paclitaxel and doxorubicin, and also inhibited gefitinib metabolism by interfering with hepatic cytochrome P450 (CYP) 3A4 and CYP2D6 enzymes (10-15). Furthermore, *M. tenacissima* or MTE alone have been demonstrated to repress the proliferation and promote apoptosis of human esophageal carcinoma cells, hematologic neoplasm cell line cells and Burkitt's lymphoma cells (16-19). A study by Lin et al (8) reported that MTE reduced the migration of A549 lung cancer cells via regulation of the C-C chemokine (CC) receptor type 5-CC ligand 5 axis, Rho C and phosphorylated focal adhesion kinase (8). A study by Huang et al (20) demonstrated that the anti-angiogenic effect of MTE is achieved by downregulation of vascular endothelial growth factor (VEGF)-A in human hepatoma cells (HeP2), as well as VEGF-A and VEGF receptor-2 in human umbilical vein endothelial cells (20). As for HCC, a study by Jiang et al (21) identified that the *M. tenacissima* polysaccharide was able to inhibit tumor growth in H22 tumor-bearing mice in a dose-dependent manner (21). However, limited attention has been directed towards the underlying mechanism by which MTE suppresses proliferation and promotes apoptosis in hepatoma carcinoma cells.

The results of the present study demonstrated that MTE inhibited growth and induced mitochondrial pathway apoptosis of hepatoma carcinoma cells by triggering the murine double minute-2 (MDM2)-mediated p53 pathway and reducing the nuclear factor (NF)-κB pathway simultaneously.

**Materials and methods**

**Cell culture.** The Bel-7402 cell line was kindly provided by the Science Experimental Center of Liaoning Medical University (Jinzhou, China). Cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Beyotime Institute of Biotechnology, Haimen, China) and 100 U/ml streptomycin/penicillin at 37°C in an atmosphere containing 5% CO₂.

When 80-90% confluence was reached, Bel-7402 cells were digested by 0.25% trypsin (Beyotime Institute of Biotechnology) as previously described (22) for subsequent experiments.

**Drug treatment.** MTE (trade name, Xiao-Ai-Ping; 1 g crude/ml) was obtained from Nanjing Sanhome Pharmaceutical Co., Ltd. (Nanjing, China). The voucher specimen was 200907-T009-05, and was identified by Professor De-Kang Wu from Nanjing University of Chinese Medicine (Nanjing, China). The stem of *M. tenacissima* was collected from Yunnan, China and MTE was prepared as previously described (14). Briefly, 1 kg of *M. tenacissima* stem powder was extracted with water three times, filtered and concentrated. Following this, concentrates were resuspended with 85% (v/v) ethanol and centrifuged three times at 4°C. The suspension was evaporated to give a final volume of ~200 ml. The purchased MTE was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Bel-7402 cells were incubated at 37°C with various concentrations of MTE (0, 40, 80, 160, 240 and 320 µl/ml; equivalent of 0, 40, 80, 160, 240 and 320 mg/ml crude drug) for 24 h for MTS experiments. Control cells were exposed to 8,000 µg/ml 5-fluorouracil (5-FU; Sigma-Aldrich; Merck KGaA) at 37°C for 24 h as a positive control. For reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, cells were treated with various concentrations of MTE (0, 160 and 240 µl/ml) at 37°C for 24 h. For flow cytometric analysis, cells were incubated at 37°C with 160 and 240 µl/ml MTE for 24 and 48 h, respectively.

**Flow cytometric analysis of the cell cycle.** Bel-7402 cells in each group were trypsinized and fixed with 70% ethanol at 4°C for 12 h. Subsequent to washing with phosphate-buffered saline (pH 7.4), cell cycle analysis was carried out with a Cell Cycle Detection kit (C1052, Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The kit contained binding buffer, propidium iodide (PI) staining buffer (20x) and RNase A (50x). In brief, cells were stained in 500 µl binding buffer containing 25 µl PI staining buffer with 10 µl RNase A for 30 min at 37°C in the dark. The cell cycle was analyzed immediately using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). The percentage of cells across the cell cycle was obtained using CellQuest 3.3 software (BD Biosciences).

**Flow cytometric analysis of cell apoptosis.** According to the protocol of the Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (Nanjing KeyGen Biotech...
Co., Ltd., Nanjing, China) which contains binding buffer, annexin V-FITC and PI staining buffer, the collected Bel-7402 cells were resuspended in 500 µl binding buffer and sequentially mixed with 5 µl Annexin V-FITC and 5 µl PI. The mixture was incubated for 15 min at room temperature in the dark. Cell apoptosis was calculated with flow cytometry (FACS Calibur; BD Biosciences) and CellQuest 3.3 software.

**RT-qPCR.** Total RNA from each group was extracted with RNAPrep Pure Cell/Bacteria kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions, and 50 ng RNA was reverse transcribed into cDNA. qPCR was performed using a thermocycler (MiniOpticon Real-Time PCR System; Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 2X SYBR-Green PCR Mastermix (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), which contains buffer, dNTP, HotStart Taq DNA polymerase, SYBR-Green I dye and MgCl₂, according to the manufacturer's instructions. The cycling profile was as follows: Initial denaturation at 95°C for 5 min, 30 cycles consisting of 95°C for 20 sec, 60°C for 20 sec and 72°C for 30 sec. Primer sequences were as follows: B-cells lymphoma-2-associated X (Bax), forward 5'-CCCAGGGAGGTTCTTTCGCAG-3' and reverse 5'-CCAGCCCATGATGGTCTCGAT-3'; B-cell lymphoma (Bcl)-2, forward 5'-ATGACTTCTCTGTCGCTACT-3' and reverse 5'-CCCATCCTGGAAAGGTTCCGA-3'; caspase-3, forward 5'-CATGGGCTCTGTCGCTACT-3' and reverse 5'-TACCCGAGTAAGACTGTGC-3'; and β-actin, forward 5'-TGAACCGGAAGCTCACTGTGG-3' and reverse 5'-TCCACACCTCTTGTGCTGGA-3'. Relative mRNA expression levels were calculated using the 2^ΔΔCq method (23) from three independent experiments and β-actin was used as an internal control.

**Western blotting.** Total proteins were extracted by lysing Bel-7402 cells with radiomunoprecipitation assay lysate containing 1% phenylmethanesulfonyl fluoride (both Beyotime Institute of Biotechnology). For NF-xB p65 detection, nuclear and cytoplasmic proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The concentrations of proteins were subsequently quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Following this, 40 µg protein was loaded and separated by 10% SDS-PAGE and subsequently electro-transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at 37°C and probed with specific primary antibodies against Bax (AB026), Bcl-2 (AB112), cleaved-caspase-3 (AC033), phosphorylated (p)-p53 (AP068) (all 1:1,000; Beyotime Institute of Biotechnology), NF-xB p65 (A00284; 1:400; Boster Systems, Inc., Pleasanton, CA, USA) and MDM2 (ab137413; 1:1,000; Abcam, Cambridge, UK) at 4°C overnight. Subsequently, the membranes were incubated at 37°C with their corresponding secondary antibodies (A0208 and A0216; 1:5,000; Beyotime Institute of Biotechnology) for 45 min. Target bands were visualized using enhanced chemiluminescence solution (Qihai Biotec, Shanghai, China) and measured using Gel-ProAnalyzer software v4.0 (Media Cybernetics, Inc., Rockville, MD, USA). GAPDH and histone H3 were employed as internal controls. The experiment was repeated three times.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism v. 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). All data were presented as the mean ± standard deviation. One-way analysis of variance followed by the Bonferroni post hoc test was used to compare differences between groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**MTE suppresses the proliferation of Bel-7402 cells by arresting the cell cycle at S phase.** To explore the effect of MTE on the proliferation of Bel-7402 cells, cells were exposed to increasing concentrations of MTE (0, 40, 80, 160, 240 and 320 µl/ml) for 24 h, and the inhibitory histograms were plotted according to MTS results. The growth inhibitory rate of Bel-7402 cells was significantly increased in a dose-dependent manner with MTE treatment compared with negative control cells, particularly at dosages of 160, 240 and 320 µl/ml (Fig. 1A; P<0.001). The inhibitory rate of Bel-7402 cells at 240 and 320 µl/ml MTE was significantly higher than that in the 5-FU-treated positive control cells (Fig. 1A; P<0.001). The growth inhibitory effect was only slightly higher at 320 than at 240 µl/ml MTE treatment; therefore 160 and 240 µl/ml MTE were used for subsequent experiments. Flow cytometry analysis revealed the number of Bel-7402 cells arrested in the S phase was significantly higher in the MTE-treated cells compared with negative control cells, resulting in a significant decrease of cells in the G1/G0 phase at both 24 and 48 h of incubation (Fig. 1B and C; P<0.05). These results suggested a cytostatic effect of MTE via arresting the cell cycle at S phase in Bel-7402 cells.

**MTE enhances the apoptosis of Bel-7402 cells.** An Annexin V-FITC/PI staining-based flow cytometry assay was employed to evaluate the contribution of MTE to Bel-7402 cell apoptosis. The total apoptosis rate of Bel-7402 cells was demonstrated to be significantly increased with 240 but not 160 µl/ml MTE treatment at 24 h compared with negative control cells (Fig. 2A and B; P<0.01). The total apoptosis rate was increased significantly (3.36- and 5.38-fold, respectively) in Bel-7402 cells treated with 160 and 240 µl/ml MTE for 48 h compared with the respective negative controls (Fig. 2C; P<0.001). These results suggested that MTE has pro-apoptotic effects on Bel-7402 cells.

**MTE triggers the mitochondrial apoptosis pathway in Bel-7402 cells.** To address the status of the mitochondrial apoptosis pathway in Bel-7402 cells with MTE treatment, RT-qPCR and western blotting were performed to detect the expression levels of Bax, Bcl-2 and caspase-3 (Fig. 3). The results demonstrated that both mRNA and protein expression levels of Bax in Bel-7402 cells were significantly increased with 160 and 240 µl/ml MTE administration compared with untreated control cells (Fig. 3A and C; P<0.001). Furthermore, the expression of Bcl-2 at both mRNA and protein levels was
significantly decreased (Fig. 3A and C; P<0.001) compared with untreated controls. Bel-7402 cells treated with 160 and 240 µl/ml MTE demonstrated a significant increase in the expression of caspase-3 mRNA after treatment with 160 and 240 µl/ml MTE (P<0.01 and P<0.001, respectively; Fig. 3B) compared with untreated controls. Additionally, the level of cleaved caspase-3 in Bel-7402 cells was significantly increased compared with the negative control by 1.6-fold with 160 µl/ml MTE treatment (P<0.05) and 2.6-fold with 240 µl/ml MTE treatment (P<0.001; Fig. 3D). These results suggested that MTE induces the apoptosis of Bel-7402 cells by triggering the mitochondrial apoptosis pathway.

MTE downregulates MDM2 to activate the p53-dependent mitochondrial pathway and inhibit the NF-κB pathway. Western blotting was used to assess the expression levels of MDM2, p53 and p65. The protein expression levels of MDM2 in cells treated with 160 and 240 µl/ml MTE were significantly decreased by 1.52-fold and 2.78-fold, respectively, compared with control cells (Fig. 4A; P<0.001). The levels of p-p53 were significantly elevated by treatment with 160 and 240 µl/ml (Fig. 4B; P<0.001). Furthermore, cytoplasmic p65 was significantly elevated in MTE-treated cells compared with the control (Fig. 4C; P<0.01), and p65 levels in the nucleus were significantly downregulated compared with the control (Fig. 4C; P<0.001). These results suggested that the cytostatic and pro-apoptotic effects of MTE were induced by downregulating the MDM2-mediated activation of the p53-dependent mitochondrial pathway and inhibition of the NF-κB pathway.

Discussion

Previous studies have demonstrated that MTE is an effective treatment for various types of malignant cancer (10-21). However, the anti-cancer effects of MTE and the underlying mechanisms are not fully understood in HCC. The results of
the present study indicated that MTE is able to suppress the proliferation and promote the apoptosis of hepatoma carcinoma cells via activating the p53-dependent mitochondrial pathway and inhibiting the NF-κB pathway.

Clinical studies have demonstrated that administration of MTE represses hematologic neoplasm growth in tumor models of nude mice (17), and _M. tenacissima_ polysaccharide dose-dependently inhibited the growth of hepatoma H22 tumor-bearing mice (21). In vitro studies have reported that MTE induces G0/G1 cell cycle arrest in human esophageal carcinoma cells and hematologic neoplasm cells (16). In the present study, it was demonstrated that MTE suppressed the proliferation of Bel-7402 cells. Notably, a significant decline in the number of cells at the G1/G0 phase and a notable increase at the S phase was observed, indicating that the growth inhibition of MTE was due to S phase arrest in Bel-7402 cells.

To the best of our knowledge, the effects of MTE on apoptosis in hepatoma carcinoma cells have not previously been reported. The results of the present study demonstrated that MTE significantly enhanced the apoptosis of Bel-7402 cells at doses of 160 and 240 µl/ml. Among the molecules involved in the mitochondrial apoptosis pathway, Bax is a pro-apoptotic protein sequestered in the cytosol and on the outer membrane of mitochondria (MOM) and endoplasmic reticulum (ER) (24,25). Bcl-2 is an anti-apoptotic protein located predominantly in the cytosol but also on the MOM and ER in different proportions (24,25). Caspase-3 is a major effector of the intrinsic apoptotic pathway (26). A study by Li et al (19) reported that MTE components, Tenacigenoside A and 11α-O-benzoyl-12β-O-acetyltenacigenin B, increased tumor cell apoptosis in mice with lymphoma via downregulating Bcl-2 and Bcl-xl and upregulating BH3 interacting-domain death agonist. A study by Ye et al (17) revealed that MTE promoted the apoptosis of human hematologic neoplasm cells by downregulating Bcl-2 and upregulating Bax and caspase-3. Accordingly, a notable upregulation of Bax, downregulation of Bcl-2 and activation of caspase-3 in Bel-7402 cells was observed in the present study following MTE treatment. These results suggested that MTE downregulates Bcl-2, enabling the oligomerized Bax to re-localize to the MOM, which subsequently formats mitochondrial pores to increase the permeability of the mitochondrial membrane, ultimately releasing cytochrome c (cyt c) and activating the mitochondrial apoptotic pathway. Thus, the
activation of caspase-3 in Bel-7402 cells may eventually result in cell death.

MDM2 is an E3 ubiquitin ligase that is overexpressed in various malignancies and is capable of suppressing cell cycle arrest or apoptosis and promoting cell survival and growth (27). p53 is a tumor suppressor, and activated p53 directly binds to Bcl-x1 and Bcl-2 to induce cyt c release, ultimately resulting in quiescence, senescence or death of cancer cells (28,29). Previously, detailed studies have identified that MDM2 negatively regulates p53 via ubiquitin-mediated degradation of p53 (30). Once MDM2 and p53 are activated by acetylation and phosphorylation on specific residues, the MDM2-p53 complex is dissociated, resulting in the stabilization of p53 and, consequently, transcriptional upregulation of p53 leading to cell-type specific cell death (31). In the present study, it was demonstrated that MTE was able to significantly decrease the expression of MDM2, thereby activating the p53-induced mitochondrial apoptotic pathway.

It is a well-established fact that the majority of malignancies are associated with long-term activation of NF-κB (32,33). MDM2 has been identified as a co-transcription factor for NF-κB as it may directly induce p65 transcription by interacting with specificity protein 1 binding sites at the p65 gene promoter (34). p53 competes with NF-κB for limited transcription co-factors (35) and, in the present study, increased cytoplasmic p65 and decreased nuclear p65 in MTE-treated Bel-7402 cells were observed, indicating that downregulation of MDM2 inactivates the NF-κB pathway. These findings suggested that the growth inhibition and pro-apoptotic effects of MTE in Bel-7402 cells are mediated by activating the p53-induced mitochondrial apoptotic pathway and inactivating the NF-κB pathway.

In conclusion, the results of the present study suggested that MTE suppresses proliferation and promotes apoptosis in Bel-7402 cells via downregulating MDM2 to activate the p53-mediated mitochondrial apoptotic pathway and...
inactivate the NF-κB pathway. These findings highlight the significant influence of MTE on the survival of Bel-7402 cells and preliminarily suggest the relative underlying molecular mechanisms involved. The present study indicates that MTE may be a promising candidate for HCC therapy.

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