Research Paper

Matrine Treatment Triggers Apoptosis in Colon Cancer Cells

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Chang and Liu: Matrine Triggers Apoptosis in Colon Cancer Cells

The aim of the present study was to investigate the role of matrine, a natural alkaloid from *Sophora flavescens* with antiproliferative and proapoptotic activities, in human colon cancer using *in vitro* studies. Caco-2 cell line was treated with increasing doses (2.0 and 32 mg/ml) of matrine for 24 h. The MTT assay was used to measure abnormal cell multiplication, while flow cytometry was applied to determine the degree of apoptosis and cell growth. Western blot analysis was also used to detect the expression of various proteins. The results indicated that matrine blocked Caco-2 cell division in a dose-dependent and time-dependent manner. Matrine triggered apoptosis and blocked mitosis by altering the protein expression levels of B-cell lymphoma-2, B-cell lymphoma-2-associated X protein, cytochrome c, caspase-9 and caspase-3. In conclusion, matrine is found to interfere with the proliferation of colon cancer cells *in vitro* by triggering apoptosis through enhancing the expression of X protein, caspase-3 and caspase-9, as well as suppressing B-cell lymphoma-2 expression. Therefore, matrine is a potential natural compound that could be used in colon cancer treatment..

Key words: Matrine, colon cancer Caco-2 cells, antiproliferative

Colon cancer is one of the leading malignancies worldwide, with a high incidence and mortality rate in China[1,2]. Studies have suggested an increase in colon cancer incidence in adults aged <50 y[3]. Surgery remains the important curative therapy for colon cancer[4]; however, the outcome is not always favourable. Although 70 % of all colorectal cancers are resectable, 50 % of all newly diagnosed patients ultimately develop metastasis. Numerous patients also require adjuvant chemotherapy[5,6], however, this is associated with side effects. Natural products are biologically active with an acceptable safety profile in animal models and thus show promise as therapeutic alternatives for treating colon cancer and hepatoma[7,8].

Sophora flavescens Ait belongs to the Leguminosae family and is grown in South East Asia and Europe for its root, which is dried and extensively used in traditional Chinese medicine to ameliorate infectious and metabolic diseases[9-13]. Matrine, an alkaloid with low toxicity, is purified from the dry root of *Sophora flavescens* Ait. Matrine, $C_{15}H_{24}N_2O$ with a molecular weight of 248.36 is shown in fig. 1. Its antitumor activity is not known to be associated with obvious toxicity or side-effects[14-16]. Recent studies[17-19] suggested that treatment with matrine interfered with the expansion and/or induction of apoptosis in cervix, stomach, liver, lung and breast cancers and blood malignancies. It also enhanced the differentiation of leukaemia K-562 cells[20].

Furthermore, matrine blocked the proliferation of HeLa cells, inhibited the metastasis of human malignant melanoma A375 cells and interfered with the gastric tumor progression in mice[21-25]. However, the effects of matrine on human colon cancer and the underlying mechanisms of its action are largely unknown. Therefore, the present study sought to investigate

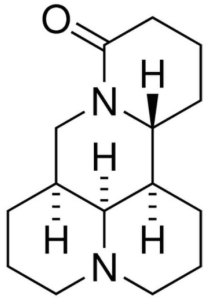

Fig. 1: Chemical structure of matrine ($C_{15}H_{24}N_2O$)

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the role of matrine as an anticancer agent in colon carcinogenesis *in vitro*.

MATERIALS AND METHODS

Matrine was obtained from Sigma-Aldrich (USA). The purity of the compound was >98 %, as confirmed using high-performance liquid chromatography. Matrine stock solution (200 mg/ml) was stored at -20° and was freshly diluted using medium prior to use. Fetal bovine serum was purchased from Zhejiang Tianhang Biological Technology Co., Ltd. (China). RPMI 1640 medium was obtained from Keygen Biotechnology Co., Ltd. (China). Sodium dodecyl sulfate (SDS), MTT, L-glutamine and the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit were purchased from Beijing Biosea Biotechnology Co., Ltd. (China). Antibodies specific for B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), cytochrome c and β-actin were obtained from R&D Systems Inc, (USA). Anticaspase-3 and anticaspase-9 were purchased from Wuhan Boster Bio-engineering Co., Ltd. (China).

In vitro evaluations:

The human colon cancer Caco-2 cell line, obtained from the Department of Oncology, Zhongnan Hospital of Wuhan University (Wuhan, China), was incubated in RPMI 1640 medium supplemented with 10 % fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., USA) under conditions of 5 % CO_2 at 37°. Matrine was dissolved dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) and serially diluted in RPMI 1640 immediately prior to experiments.

MTT assay:

The cytotoxic activity of matrine was assessed using the MTT assay with different concentrations of the plant extract. The cells were added into 96-well microtitre plates at a density of 5×10^3-10^4 cells per well, depending on the cell line. Wells at different concentrations were investigated in triplicate. After incubation at 37° for 24 h, the medium was substituted with different concentrations of matrine and incubated for an additional 24, 36 and 48 h. The initial concentration of matrine was 1.0 mg/ml in DMSO, which was serially diluted to prepare 5 final concentrations ranging between 2 and 32 mg/ml. cells were exposed for an additional 4 h to a humidified atmosphere at 37°, followed by removal of the medium containing MTT. Subsequent to further treatment with DMSO, the absorbance of samples was read at 570 nm. The cell viability was determined in comparison with the negative control containing the solvent without matrine. The percent inhibition of proliferation was determined based on the measured optical density (OD), using the following formula, 1 - (average OD of wells containing matrine/average OD of control wells) ×100. All procedures were performed in triplicate.

Mitosis:

Mitotic analysis was conducted to assess rate of cell death rate as described by Wells[26]. Approximately 1×10^5 cells exposed to various concentrations of matrine were mixed with phosphate buffered saline (PBS) at 4° and centrifuged at 12 000 g for 5 min. The samples were then resuspended in 70 % ethanol and incubated overnight at 4°. The supernatant was discarded and the cells were collected and resuspended in 500 µl PI solution (PBS containing 0.2 % Triton X-100, PI at 50 µg/ml and RNase A at 100 µg/ml) at room temperature. After 30 min, the status of mitosis was determined using flow cytometry (Sysmex Partec GmbH, Görlitz, Germany) employing Cell Quest software.

Flow cytometry:

An Annexin V/PI assay was performed using the appropriate kit, according to the manufacturer's instructions. Briefly, cells were plated into 6-well plates and incubated for 24 h with matrine (4, 8 and 16 mg/ml). The cells were collected, treated with cold PBS at 4° and centrifuged at 12 000 g. Subsequently, cells were resuspended in 100 µl binding buffer containing 2.5 µl FITC-conjugated Annexin V and 1 µl 100 µg/ml PI at room temperature.

Cytochrome c release:

Western blot analysis was performed to determine the level of cytochrome c released into the cytosol. Briefly, the cells were harvested after the ice-cold PBS treatment. The mitochondria and cytosol were separated by sonicating the cells in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 175 mM sucrose and 12.5 mM disodium edetate (EDTA). Subsequently, the cell extract was centrifuged at 1000 g for 10 min to pellet the nuclei. The supernatant obtained was then centrifuged at 18 000 g for 30 min to yield the cytosolic fraction from the supernatant and the mitochondrial pellet as previously described. The protein content was measured in the 2 fractions using Bradford's method. Equal amounts of protein were separated by 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro transferred to a polyvinylidene difluoride membrane. The membrane

was then incubated in 5 % non-fat milk in Tris-buffered saline-Tween 20 [TBST; containing 50 mM Tris, 150 mM NaCl (pH 7.6) with 0.1 % Tween 20] for 2 h, followed by overnight incubation with the primary antibody separately. The membranes were extensively washed with TBST prior to incubation for 2 h with the secondary antibody. After repeated washing with TBST, the immune complexes were detected using enhanced chemiluminescence.

Western blot analysis:

Western blot analysis was performed to determine the protein levels of caspase-3, caspase-9, Bcl-2 and Bax, as previously described[27]. Briefly, Caco-2 cells were exposed to different levels of matrine (4, 8 and 16 mg/ml) for 24 h, followed by lysis and denaturation. A bicinchoninic acid assay system (Beyotime Institute of Biotechnology) with BSA as a standard was used to estimate the total protein content of the cell extracts. Equal quantities (80 µg protein per lane) of total proteins were resolved by SDS-PAGE (12 % gels) under reducing conditions. After electrophoretically transferring the proteins to nitrocellulose membranes, a 5 % skimmed milk was used to block the membranes. Anticaspases-3, anticaspase-9, antiBcl-2, antiBax and antiβ-actin primary antibodies were added to the membranes and incubated at 4° overnight. A goat antirabbit/antimouse secondary antibody conjugated with horseradish peroxidase (1:5000; Abcam, USA) was subsequently added. Immunoblotting of the membranes with β-actin antibodies after separating previous primary antibodies was conducted. The immunoreactive bands were analyzed using densitometry (Molecular Dynamics, USA).

Statistical analysis:

All values are presented as the mean±standard deviation. Data were analyzed with the Student's t-test, using SPSS version 13.0 program (SPSS, Inc., USA). A P-value of <0.05 was considered to indicate statistically significant differences in the results.

RESULTS AND DISCUSSION

The MTT assay was used to assess the antiproliferative activity of matrine on Caco-2 cells. Cells were treated with increasing doses of matrine (2-32 mg/ml) for 24, 36 and 48 h. The results indicated that matrine significantly interfered with the proliferation of Caco-2 cells, with an increasing effect observed upon treatment with increasing matrine doses and longer treatment duration ($P<0.05$ fig. 2).

To investigate the role of matrine on mitotic progression, cells were cultured at specific doses of matrine (0, 4, 8 and 16 mg/ml), followed by flow cytometric analysis. As shown in fig. 3, increasing doses of matrine significantly increased the cell population in the G_0/G_1 stage and reduced the population in the G_2/M stage. This result suggested that matrine arrested Caco-2 cells at G_0/G_1 phase, resulting in depletion of cells at S and G_2-M stages.

Changes in the cell membrane during early apoptosis result in the extracellular translocation of

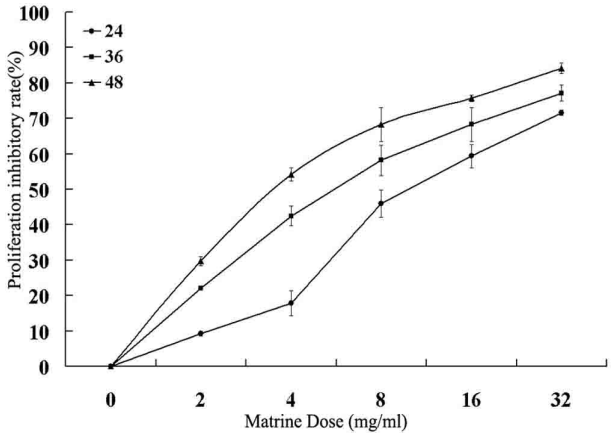

Fig. 2: Proliferation inhibitory effect of matrine

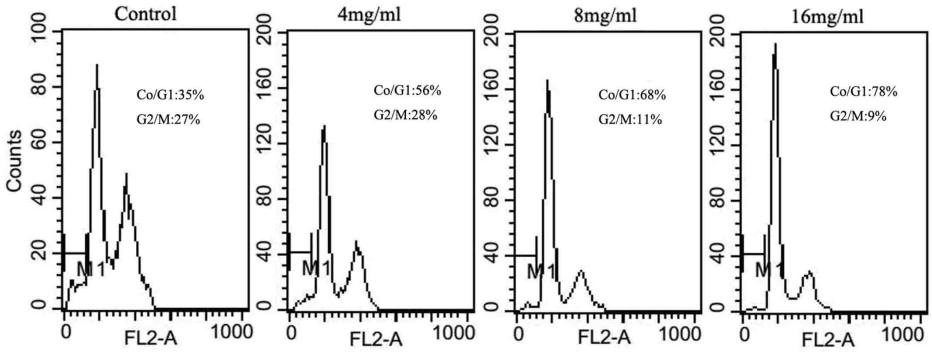

Fig. 3: Cell cycle analysis

Representative DNA fluorescence histograms of PI stained cells. Caco-2 cells were untreated or treated with 4, 8, 6 mg/ml matrine, with proportions of 35, 56, 68 and 78 % in G_0/G_1, respectively, as assessed by flow cytometry.

phosphatidylserine. The binding of Annexin V to phosphatidylserine demonstrates the feasibility of using FITC-conjugated Annexin V to identify apoptotic cells[28,29]. Thus, Annexin-V-FITC/PI dual staining was used to detect apoptosis of Caco-2 cells in the present study. Histochemical analysis using PI was used to separate the initial stages of apoptosis from necrosis. The proportion of cells at different stages, including viable, dead or apoptotic phases, was tested in 10 000 events collected with a FACScan flow cytometer. The results are illustrated in fig. 4. The living cells were identified in the bottom left quadrant (absence of Annexin V-FITC and PI staining). Cells in the early stage apoptosis were found in the bottom right quadrant (positive for Annexin V-FITC). Delayed apoptosis was characterized by the presence of membrane and nuclear blebbing and cells at this stage were found in the top right quadrant (double staining for Annexin V-FITC and PI). As shown in fig. 4, an increase in early and late stage apoptosis was observed with increasing daily dosage of matrine (4, 8 and 16 mg/ml). Furthermore, the proportion of delayed apoptotic cells was higher than that of early apoptotic cells.

Apoptotic stimuli triggered the mitochondrial release of cytochrome c into the cytosol. Along with apoptotic protease activating factor-1, cytochrome c induces apoptosome formation and activates caspase-9 and other downstream caspases in a cascade effect. It is evident that matrine is causing apoptosis by initiating the initiator capases-3, progressing the cell death by stimulating caspases-9 continuing the apoptotic activity. Western blot analysis in the current study revealed an increased cytochrome c content in the cytoplasm, depending on the dose of matrine administered, and a concomitant decrease in mitochondrial cytochrome c fraction (fig. 5). The role of matrine on Caco-2 cell proliferation was also investigated using the Western blot analysis to determine the levels of apoptosis-associated proteins. The results indicated that at doses ranging between 4 to 16 mg/ml, matrine abrogated Bcl-2 expression and enhanced Bax levels, resulting in a reduced Bcl-2/Bax ratio (fig. 5). Previous studies[30,31] have demonstrated that Bcl-2 and its potent inhibitor Bax served a critical role in regulating the cellular expansion and apoptosis. Upregulated Bcl-2 increases cell survival by interfering with apoptosis, whereas elevated expression of Bax accelerates cell death. Therefore, the antiproliferative activity of matrine in Caco-2 cells might be mediated by triggering apoptosis and mitotic arrest and by decreasing the Bcl-2/Bax ratio.

Caspases are cysteine proteases that mediate the lethal phase of apoptosis. Matrine was found to enhance the generation of activated caspases-9 and -3 from their precursors, procaspases-9 and -3, respectively (fig. 6). The levels of proteolytically activated caspase-9 plateaued at a matrine dose of 16 mg/ml, whereas the 17 kDa active caspase-3 was identified even after 24 h. Advances in neoadjuvant chemotherapy over the past decade resulted in enhanced survival prospects for patients with colon cancer[32,33]. However, chemoresistance is a major challenge, warranting development of innovative therapeutic approaches targeting the malignant behaviour of colon cancer cells for improved prognosis. Matrine is a naturally occurring compound extracted from plants of the family Leguminosae. Plant extracts, particularly matrine infusions, are renowned for efficacy in several diseases and health conditions. Matrine is a safe and effective Chinese medicinal preparation, indicated for inflammatory conditions even in children. Ophthalmological and histological analyses have confirmed the safety of low-dose matrine, when administered to the eyes of rabbits[34-37].

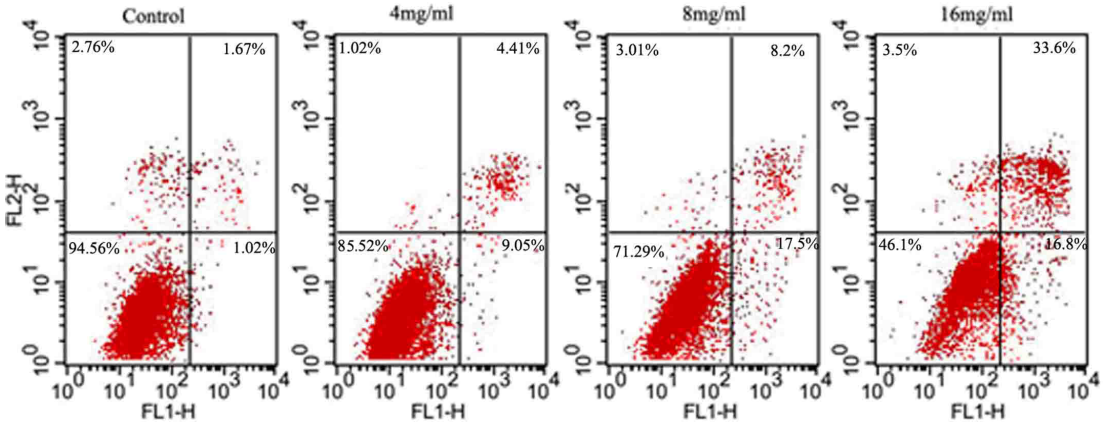

Fig. 4: Apoptosis of Caco-2 cells induced by matrine
Caco-2 cells were treated with or without matrine (4, 8 and 16 mg/ml) for 24 h followed by annexin V/PI staining and flow cytometric analysis

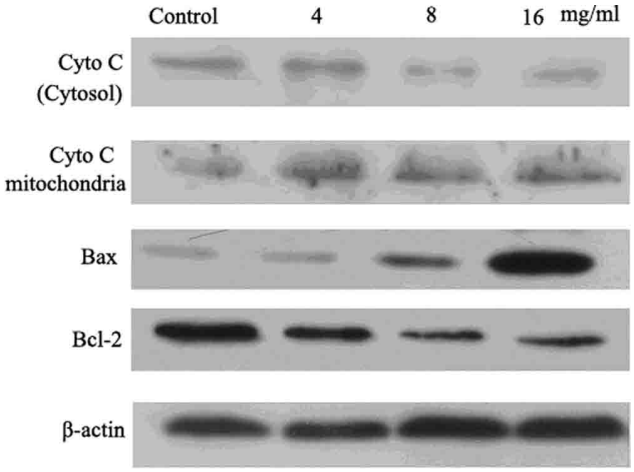

Fig. 5: Effect of matrine on the expression of cytochrome C, Bax and Bcl-2 proteins in Caco-2 cells

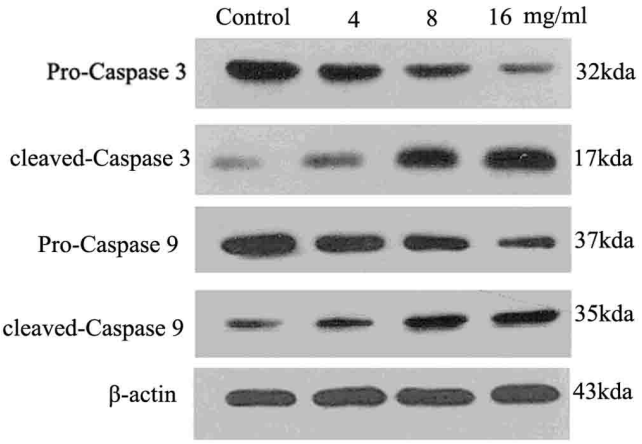

Fig. 6: The expression of caspase-3 and -9 in matrine-treated Caco-2 cells

In the present study, the role of matrine treatment in colon cancer was investigated. The results highlighted the pathophysiology of cancer chemoprevention and chemotherapy. In addition, the anticancer effects of matrine were established *in vitro* on a colon cancer cell line. As shown by the MTT assay, the growth of the Caco-2 cells was blocked in a dose-dependent manner, upon exposure to 2-32 mg/ml matrine. Flow cytometry analysis revealed growth arrest of Caco-2 cells at the G_0/G_1 phase followed by depletion of cells in the S and G_2-M stages.

Apoptosis mediates not only normal physiology, but also pathophysiology of several diseases and health conditions. Imbalance in apoptosis and proliferation results in tumor development and progression[38-40]. Combination anticancer therapies block tumor cell expansion and induce apoptosis. In the current study, the proapoptotic properties of matrine were initially investigated to determine the mechanisms underlying its anticancer effects. Matrine treatment increased the proportion of cells in apoptosis *in vitro* when compared with the baseline value ($P<0.05$). The Bcl-2 group of proteins, including antiapoptotic Bcl-2 and proapoptotic Bax among other members, regulate cellular growth and expansion, pluripotency and apoptosis[39-43]. Bcl-2 prevents the secretion of cytochrome c without disrupting the outer mitochondrial membrane. By contrast, Bax promotes the cytochrome c release[44-46]. In the present study, matrine treatment downregulated Bcl-2 and upregulated Bax, thereby increasing the permeability of the mitochondrial membrane. Immunoblotting revealed that matrine interfered with the proliferation of colon cancer cells *in vitro* by modulating the expression levels of Bax and Bcl-2.

Caspases mediate the lethal phase of apoptosis. The extrinsic and intrinsic signalling pathways are induced by caspases-8 and -9, respectively. Procaspase-8 is cleaved by mitochondrial protein clusters. Furthermore, caspase-3 is implicated in apoptosis as a key enzyme[47,48] and is known to induce apoptosis via cleavage of DNA repair molecules, degradation of antiapoptotic proteins, proteolytic modification of extracellular matrix, expression of specific skeletal polypeptides and other molecules. In the present study, matrine treatment was found to induce the mitochondrial release of cytocrome c and eventually increased caspase-3 activity. Caspase-3 is expressed as a 35-kDa inactive precursor (procaspase-3), which is activated to a 17-kDa enzyme[49,50]. Apoptotic Caco-2 cells exhibited increased levels of activated caspase-3 following exposure to matrine at a dose range of 4-16 mg/ml.

In conclusion, the results of the present study revealed that matrine prevented the progression of human colon cancer cells *in vitro*. Matrine induced apoptosis in colon cancer cells by lowering the Bcl-2/Bax ratio, and increased the activation of caspases-3 and -9. Therefore, matrine may be useful in human colon cancer treatment or adjuvant therapy.

Conflict of interest:

The authors report no conflicts of interest in this work.

Acknowledgements

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