Matrine induces apoptosis of human multiple myeloma cells via activation of the mitochondrial pathway

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Abstract
Multiple myeloma (MM) is a hematological malignancy characterized by the uncontrolled proliferation of clonal plasma cells in bone marrow in the elderly. Although there have been tremendous advances in the treatment of MM, it remains an incurable disease. Matrine, a main alkaloid of the traditional Chinese herb Sophora flavescens Ait, has been shown to inhibit cellular proliferation and induce apoptosis of various cancer cells. The aim of this study was to investigate the possibility of matrine as a novel therapeutic agent for patients with MM. We investigated the effects of matrine for its anti-myeloma activity in vitro, and further examined the mechanisms of apoptosis induced by matrine. Matrine inhibited the proliferation of human myeloma cell lines as well as freshly isolated myeloma cells from patients in a dose- and time-dependent manner. Matrine showed a potent induction of apoptosis of myeloma cells. Mitochondrial membrane potential (ΔΨm) was lost and cytochrome c (cyt c) was released from mitochondria to cytosol in myeloma cells treated by matrine for 24 h in a dose-dependent manner. The ratio of Bcl-2/Bax protein decreased, and the percentage of activated caspase-3 increased in myeloma cells treated by matrine for 48 h, but this matrine-induced activity of caspase-3 was completely canceled by the addition of Z-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone (Z-DEVD-FMK), a caspase-3 inhibitor. The addition of Z-DEVD-FMK partially blocked the apoptotic effect of matrine on myeloma cells. These data indicated that matrine could exert antiproliferative effects on myeloma cells and induce apoptosis of myeloma cells in vitro. The induction of apoptosis appeared to proceed via the mitochondrial pathway, including down-regulation of Bcl-2/Bax ratio, loss of ΔΨm, release of cyt c from mitochondria to cytosol, and activation of caspase-3. These findings support the view that matrine may be a useful candidate as a chemotherapeutic agent against MM.

Keywords: Myeloma, pharmacotherapeutics, cell cycle and apoptosis changes

Introduction
Multiple myeloma (MM) is an incurable plasma-cell malignancy that accounts for slightly more than 10% of all hematologic cancers [1]. Front-line combination chemotherapy offers complete response rates of 13–33% in patients with MM [2,3]. High-dose therapy plus autologous stem cell transplant has achieved higher complete response rates than conventional chemotherapy, but in the long run most patients relapse [4]. Therefore, a more effective therapy for MM is urgently required.

Many components of herbs, including curcumin, baicalein, and cantharidin, have been identified as effective in the treatment of human carcinoma [5–7]. Matrine, a monomer of traditional Chinese medicine that comes from Leguminosae plants such as Sophora flavescens Ait, is a tetracyclic quinolizidine with the molecular formula C15H24N2O. Matrine has been shown to possess pharmacological activities including anti-inflammatory, anti-viral, and anti-arrhythmic activities, leading to wide clinical use in China for the clinical treatment of chronic liver disease and heart failure. Recently, several studies demonstrated that matrine had potent anti-tumor activity against various human cancer cell lines such as the melanoma cell line A375 [8], lung cancer cell line A549 [9], and gastric cancer cell line MKN45 [10].
Summarizing these studies, we found that induction of apoptosis was one of the main mechanisms of the anti-tumor activity of matrine. Anticancer agents have been shown to induce apoptosis by overcoming the cytoprotective effect of the Bcl-2-like proteins, losing mitochondrial membrane potential ($\Delta$ψm), and consequently releasing cytochrome c (cyt c) from the mitochondria to the cytosol and activating caspase cascades [11]. Caspase-3 is the major effector caspase involved in apoptotic pathways [12]. In the present study, we investigated the effect of matrine on myeloma cell proliferation and the mechanism of suppression.

Materials and methods

Cell lines and reagents

The human multiple myeloma cell lines RPMI8226 and U266 (obtained from the Cancer Institute & Hospital, Chinese Academy of Medical Sciences, Beijing, China) were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in a humidified 5% CO$_2$ atmosphere at 37°C. Matrine, purchased from the Xi’an Tianyuan Biological Agent Plant (Xi’an, Shanxi, China), was first dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) and then serially diluted in RPMI1640 immediately prior to experiments.

Isolation and culture of bone marrow mononuclear cells from patients with myeloma

Bone marrow (BM) aspirates were obtained from five patients with newly diagnosed MM. Approval for the study was obtained from the First Affiliated Hospital of Wenzhou Medical College institutional review board. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki protocol. Bone marrow mononuclear cells (BMMNCs) from BM aspirates were separated by Ficoll–Hypaque centrifugation. BMMNCs were seeded at density of 0.5–1.0 x 10$^6$ cells/mL and cultured in RPMI1640 medium supplemented with 10% FBS. The cells were stained with phycoerythrin (PE)-labeled anti-CD138 (BD Biosciences, San Jose, CA, USA) and then serially diluted in RPMI1640 immediately prior to experiments.

Cell survival assay

Cell survival was determined by assaying viable cell numbers using Cell Counting Kit-8 (CCK-8) dye according to the manufacturer’s protocol (Dojindo Laboratories, Kumamoto, Japan). Myeloma cells (RPMI8226, U266, and BMMNCs) were plated in 96-well microtiter plates and treated by different doses (0.25, 0.5, 1.0, 1.5, 2.0, 3.0 g/L) of matrine for 24 h, 48 h, and 72 h, and cell viability determined at the indicated times. The absorbance (A) of the solution was read by a microplate reader (ELX800; Bio-Rad, Foster City, CA, USA), using a test wavelength of 450 nm. Cell viability rate (%) was calculated from: $A_{450, \text{matrine}}/A_{450, \text{control}} \times 100\%$. Half maximal inhibitory concentration (IC$_{50}$) was calculated by SPSS13.0 software using linear regression analysis of percent survival versus log drug concentration.

Apoptosis detection

Myeloma cells were plated in 12-well plates at 2.0 x 10$^5$ cells/well in 2 mL of medium. Various concentrations (0.25, 0.5, 1.0, 1.5, 2.0, 3.0 g/L) of matrine were added for 48 h, and a time course for a certain concentration was carried out at 24 h, 48 h, and 72 h. Apoptosis was detected using a fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit II (BD Pharmingen™, San Diego, CA, USA) according to the manufacturer’s instructions. Data acquisition and analysis were performed using FCM with CellQuest software.

$\Delta$ψm analysis

Rhodamine 123 is a popular green fluorescent mitochondrial dye that stains mitochondria in living cells in a membrane potential-dependent fashion. The loss of $\Delta$ψm induces apoptotic events. Cells were plated in 12-well plates and treated with matrine. After 24 h of treatment, cells were collected and washed twice with cold phosphate buffered saline (PBS), resuspended in 1 mL of fresh culture medium containing 5 $\mu$M Rhodamine 123 (Sigma), and incubated at 37°C for 30 min in the dark. Then, the cells were washed twice and analyzed with FCM.

Cyt c assay

After matrine application, the mitochondrial-free cytosolic fraction and solubilized mitochondrial fraction were extracted from myeloma cells according to our previous study [13], and were then subjected to quantitation by employing a bicinchoninic acid protein concentration assay kit (Beyotime, Jiangsu, China). Cyt c protein was measured with a commercially available cyt c enzyme-linked immunosorbent assay (ELISA) kit (Calbiochem, San Diego, CA, USA) according to the manufacturer’s instructions. The absorbance was measured at 450 nm using a microplate reader.
**Bcl-2 and Bax protein assays**

The expressions of Bcl-2 and Bax protein were analyzed with FCM after matrine treatment. According to the manufacturer’s protocol for the Cytofix/ Cytoperm™ Fixation and Permeabilization kit (BD Pharmingen™), myeloma cells were stained with Bcl-2 FITC (Caltag Laboratory, Burlingame, CA, USA), Bax FITC, Bax PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and their isotype control, then analyzed with FCM.

**Activated caspase-3 analysis**

Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17 and 12 kDa subunits which is derived from the 32 kDa proenzyme. The CPP32 antibody has been reported to specifically recognize the active form of caspase-3 in human and mouse cells [14]. After matrine treatment, cells were collected and treated by the Cytofix/ Cytoperm™ Fixation and Permeabilization kit according to the manufacturer’s protocol, and then labeled with caspase-3 PE (BD Pharmingen™). Rabbit immunoglobulin G (IgG)-PE was used as isotype control. The cells were analyzed with FCM. Z-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone (Z-DEVD-FMK; Sigma) was dissolved in DMSO at a stock concentration of 10 mM, and stored at −20°C and subsequently diluted with serum-free RPMI1640 medium prior to use. Myeloma cells were treated with or without Z-DEVD-FMK for 1 h before treatment with matrine for 48 h. The measurements of activated caspase-3 were performed as described above, and then the most suitable concentration of Z-DEVD-FMK in myeloma cells treated with matrine was decided.

**Effects of matrine on peripheral blood mononuclear cells**

Peripheral blood samples were obtained from five healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll–Hypaque centrifugation. PBMCs with and without stimulation by phytohemagglutinin (PHA, 10 mg/L) were treated with different doses of matrine for 24 h, 48 h, and 72 h. Cell survival and apoptosis were detected by CCK-8 dye and the FITC Annexin V Apoptosis Detection Kit II, respectively.

**Statistical analysis**

Data are representative of at least three different experiments and are expressed as the mean ± SD.

A one-way analysis of variance (ANOVA) was performed to examine the differences of values among the matrine groups with different doses; $p < 0.05$ was considered statistically significant.

**Results**

Matrine inhibited proliferation of myeloma cells

We examined the effects of matrine on the proliferation of myeloma cell lines RPMI8226, U266, and primary MM cells (BMMNCs). The rate of CD138 expression on BMMNCs from five patients with MM was 69.22 ± 8.20%. Because normal lymphocytes are smaller and their cellular granules fewer compared with primary MM cells,
Figure 2. Matrine-induced apoptosis of myeloma cells. Apoptosis was analyzed by FCM using annexin V–FITC binding and PI staining method. (A) Myeloma cell lines (RPMI8226, U266) and primary MM cells (BMMNCs) were treated by different doses of matrine (0–3 g/L) for 48 h. (B) Representatives of myeloma cells were treated by 1.5 g/L matrine for 48 h. Both annexin V- and PI-negative (bottom left quadrant), annexin V-positive and PI-negative (bottom right quadrant), and annexin V- and PI-positive (top right quadrant) cells were considered as viable, early-phase apoptotic, and late-phase apoptotic/necrotic cells, respectively. The percentage (%) of each cell is described in each quadrant. (C) Myeloma cells were treated by 1.5 g/L matrine for 24 h, 48 h, and 72 h. The values represent the mean ± SD of at least three independent experiments. *p < 0.05 vs. the respective control.

Figure 3. Effect of matrine on changes of ΔΨm in myeloma cells for 24 h. Loss of ΔΨm was analyzed by FCM using Rhodamine 123 dye. (A) Representatives of myeloma cells were treated by 3.0 g/L matrine for 24 h. M1 peak represents the area of lost ΔΨm, which means the percentage of cells with low ΔΨm. (B) Myeloma cells were treated by different doses of matrine for 24 h. The values represent the mean ± SD of at least three independent experiments. *p < 0.05 vs. the respective control.
primary MM cells could be roughly gated out in BMMNCs using forward-scatter (FSC) and side-scatter (SSC) channels, in which the rates of CD138 expression were approximately 90%. Matrine inhibited the proliferation of myeloma cell lines RPMI8226 and U266 in a dose- and time-dependent manner, with IC_{50} at 48 h of 1.13 and 1.33 g/L, respectively [Figures 1(A) and 1(B)]. Importantly, matrine also inhibited the proliferation of BMMNCs, in which primary MM cells were approximately 70%, with IC_{50} at 48 h of 1.04–1.40 g/L [Figure 1(C)]. The results indicated that matrine could induce cytotoxicity potently in MM cell lines as well as primary MM cells.

**Matrine induced myeloma cell apoptosis**

Matrine induced cell apoptosis of some solid tumor cells, such as A549 cells at a concentration of 0.25 g/L, hepatoma SMMC-7721 cells at a concentration of 1.0 g/L, and A375 cells at a concentration of 1.0 g/L, for 48 h [8,9]. To confirm whether the growth inhibition of myeloma cells induced by matrine was caused by apoptosis, the annexin V–FITC/propidium iodide (PI) double staining method was carried out. After treatment with matrine for 48 h, the maximal apoptosis rates were 71.05% at the concentration of 2.0 g/L in U266 cells, 32.89% at the concentration of 1.5 g/L in RPMI8226 cells, and 45.05% at the concentration of 1.5 g/L in primary MM cells, respectively. In contrast, fewer than 3% of untreated myeloma cells underwent apoptosis under the same conditions [Figure 2(A)]. The concentration of 1.5 g/L matrine induced apoptosis in approximately 12% of myeloma cells at 24 h and 48% at 48 h, and then the percentage of apoptotic cells decreased [Figure 2(C)]. Analysis of annexin V-positive cells showed that apoptotic cells increased significantly after matrine treatment.

**Matrine disturbed Δψm in myeloma cells**

Mitochondria are key organelles in the regulation of apoptosis. Therefore, we investigated the involvement of mitochondrial dysfunction in matrine-induced apoptosis. The Δψm is a highly sensitive indicator of mitochondrial function. In the present study, we analyzed Δψm after matrine treatment by FCM using Rhodamine 123. The onset of Δψm collapsed between 1.5 and 3.0 g/L after exposure to matrine for 24 h [Figure 3(B)]. The Δψm was lost in a dose-dependent manner, and approximately 60% of myeloma cell lines and 50% of primary MM cells decreased in Δψm after treatment with 3.0 g/L matrine [Figure 3(A)]. These results indicated that mitochondria-mediated apoptosis in myeloma cells might be induced by high concentrations of matrine.

**Matrine induced cyt c release from mitochondria to cytosol in myeloma cells**

The levels of cyt c were detected in the mitochondrial-free cytosolic fraction and mitochondrial fraction in myeloma cells treated by matrine for 24 h. Levels of cyt c decreased in the mitochondrial fraction and increased in the mitochondrial-free cytosolic fraction in dose-dependent manner (Figure 4). The results showed that matrine could induce cyt c release from mitochondria to cytosol in myeloma cells.

**Matrine down-regulated Bcl-2/Bax ratio in myeloma cells**

Untreated myeloma cells expressed high levels of Bcl-2 protein and low levels of Bax protein [Figure 5(A)]. After treatment with higher concentrations of matrine (0.5, 1.0, 1.5, 2.0, and 3.0 g/L) for 48 h, the expression of Bcl-2 decreased and that of Bax increased in myeloma cells. Thus, ratios of Bcl-2/
Bax were down-regulated [Figure 5(B)]. After treatment with 1.5 g/L matrine for 48 h, the expression of Bcl-2 protein decreased about 50% in RPMI8226 cells, while there was a small decrease in U266 cells and primary MM cells, with a concomitant increase in Bax protein levels (about 20%) in myeloma cells [Figure 5(A)].

Matrine induced apoptosis of myeloma cells via activating caspase-3

We investigated the effect of matrine on the activity of caspase-3, in order to research whether matrine-induced apoptosis of myeloma cells was dependent on activation of the caspase family. The levels of activated caspase-3 expression markedly increased in myeloma cell lines (RPMI8226, U266) and primary MM cells (BMMNCs) treated with high concentrations of matrine (1.0, 1.5, 2.0, and 3.0 g/L) for 48 h [Figure 6(B)]. After U266 cells were exposed to 1.5 g/L matrine for different times (0, 24, 48, 72 h), the levels of activated caspase-3 expression changed from $7.82 \pm 1.46\%$ to $13.96 \pm 1.84\%$, $52.34 \pm 4.95\%$, and $46.62 \pm 3.81\%$, respectively, in the cell population [Figure 6(C)]. These results showed that matrine could induce caspase-3 activation in U266 cells in a time-dependent manner within 48 h, then decreased the level of activated caspase-3 expression after 48 h. In addition, it was examined whether apoptosis induced by matrine was affected by Z-DEVD-FMK, a caspase-3 inhibitor. When U266 cells were pretreated with 5 μM Z-DEVD-FMK, activation of caspase-3 induced by matrine was completely inhibited [Figure 6(D)]. Matrine-induced apoptosis in myeloma cells was partially diminished by the addition of Z-DEVD-FMK [Figure 6(E)], which implied the participation of caspase-3.

Slight side effects of matrine on PBMCs

The results showed that lower concentrations of matrine (0.25, 0.5, 1.0, 1.5 g/L) had no effects on the proliferation of PBMCs and higher concentrations of matrine (2.0, 3.0, 5.0 g/L) showed suppression of the proliferation of PBMCs within 72 h, and the inhibition rates of PBMCs with and without stimulation by PHA were no more than 50% by treatment with 5 g/L matrine for 72 h (Figure 7). The 0.5, 1.5, 3.0, and 5.0 g/L concentrations of matrine had no effects on the induction of apoptosis of PBMCs with and without PHA for 48 h (Figure 8).
Discussion

Until now, the anti-tumor effects of matrine have remained as laboratory research, and have not been confirmed clinically. Matrine inhibited the growth of A549 and SMMC-7721 cells by reducing the ratio of Bcl-2/Bax protein [9]. In addition, matrine suppressed A549 cell migration by reducing the secretion of vascular endothelial growth factor A [9]. Matrine could inhibit cell proliferation and induce apoptosis of SGC-7901 cells by up-regulating Fas/FasL expression and activating the caspase-3 enzyme [15]. Matrine inhibited the invasiveness and metastasis of A375 cells by the inhibition of cellular proliferation, induction of apoptosis, and down-regulation of heparanase mRNA and protein expression [8]. Matrine could inhibit the proliferation of murine hepatocellular carcinoma H22 cells in in vitro and in vivo models [16]. In the present study, it was demonstrated that matrine could inhibit cell proliferation and induce apoptosis of myeloma cell lines and primary MM cells via the mitochondria-mediated pathway.

The proliferation of myeloma cell lines RPMI8226 and U266 was markedly inhibited by matrine. RPMI8226 and U266 cells belong to different varieties of MM; the former secretes Ig lambda light chain, the latter secretes IgE. RPMI8226 cells were more sensitive to the cytotoxic effects of matrine than U266 cells, with IC_{50} at 48 h of 1.13 and 1.33 g/L, respectively. Matrine also showed remarkable cytotoxicity in BMMNCs from five patients with MM,
which included about 70% primary MM cells in our research. These results suggested that matrine could inhibit cell proliferation of myeloma cells.

Activation of apoptosis pathways is a key mechanism by which cytotoxic drugs kill tumor cells. The induction of apoptosis may be one of the main mechanisms of the cytotoxicity of matrine. After treatment by matrine, the maximal apoptosis rates of RPMI8226, U266, and primary MM cells were approximately 33%, 71%, and 45%, respectively. The optimal concentration and treatment time of matrine were 1.5 g/L and 48 h. If the concentration was too high or the treatment time was too long, necrosis of myeloma cells would happen as well as apoptosis.

There are two pathways that initiate apoptosis: one is mediated by the activation of so-called death receptors such as extracellular ligands [17]; the other is mediated by loss of mitochondria [18]. Both pathways involve the activation of a cascade of enzymes called caspases, a family of cysteine proteases that cleave cellular substrates and lead to the biochemical and morphological changes characteristic of apoptosis [19]. Much evidence has confirmed that mitochondria play a key role in cell death as an organelle involved in signal transduction and amplification of the apoptotic response. Mitochondria dysfunction is characterized by an increase in mitochondrial membrane permeability and loss of Δψm, and the mitochondrial permeability transition pore (MPTP) complex takes part in this process [20]. MPTP is controlled by pro- and anti-apoptotic members of the Bcl-2 family of proteins [21]. Our results showed that RPMI8226, U266, and primary MM cells had a reduction of the ratio Bcl-2/Bax protein after treatment with matrine. Bcl-2 inhibits apoptosis, which displays on the outer membranes of mitochondria in healthy cells. Matrine might have caused Bax to migrate from cytosol to the surface of the mitochondria, where it inhibited the protective effect of Bcl-2 and inserted itself into the outer mitochondrial membrane, punching holes in it and causing Δψm loss and cyt c leak-out. Cytosolic cyt c can bind to Apaf-1, a cytosolic protein containing a caspase-recruitment domain, and caspase-9 to form a complex, which activates procaspase-3 [22]. Subsequently, the activated caspase-3 effect on a target cell leads to final destruction of the cell. Caspase-3 is the primary activator of apoptotic DNA fragmentation [23]. In our research, it was found that the activated caspase-3 induced by matrine increased in a dose-dependent manner, and the expression of activated caspase-3 in U266 cells reached a maximum level at 48 h. Matrine-induced apoptosis occurred concomitantly with activation of caspase-3 in myeloma cells, which demonstrated that the activation of caspase-3 might lead to apoptosis. This view was further supported by results obtained by the addition of Z-DEVD-FMK, a caspase-3 inhibitor. When myeloma cells were co-treated with 1.5 g/L matrine and 5 μM Z-DEVD-FMK, the activated caspase-3 was completely inhibited and apoptosis was partially blocked. These results indicated that matrine-induced apoptosis in myeloma cells was partially dependent on the mitochondria-mediated pathway: decrease of Bcl-2/Bax ratio → loss of Δψm → release of cyt c → activation of caspase-3 → apoptosis.

In the past decade, there have been major therapeutic advances in the treatment of MM. Thalidomide, bortezomib, and lenalidomide have emerged as highly active agents in the treatment of MM [2,3,24]. Mechanisms of these agents have been identified, including thalidomide and lenalidomide as inhibitors of tumor necrosis factor production, and bortezomib as a proteasome inhibitor. Higher
response rates have been observed, but MM remains largely incurable as disease relapse is inevitable. Meanwhile, these agents have brought about a few side effects such as peripheral neuritis [25]. In contrast with those agents, matrine confirmed its anti-tumor activity against MM in vitro, and the maximal concentration of matrine showed slight side effects on PBMCs. Next, we need to research the effects of matrine in an MM mouse model so as to investigate its role in vivo.

In conclusion, matrine exhibited a strong inhibitory effect on the proliferation of myeloma cells in vitro. The anticancer activity of matrine could be attributed to its inhibition of proliferation and induction of apoptosis of cancer cells through the mitochondria-mediated pathway. Given our finding that matrine inhibits proliferation and induces apoptosis in multiple myeloma cell lines and primary MM cells, matrine may be a useful candidate as a chemotherapeutic agent against MM.

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References


