Matrine Inhibits Proliferation and Induces Apoptosis of Pancreatic Cancer Cells in Vitro and in Vivo

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Matrine, an alkaloid extracted from a Chinese herb, Sophora flavescens Arr., has exhibited anti-proliferative and pro-apoptotic abilities against various types of cancer cells. This study aims to investigate its anti-cancer activity and underlying mechanisms in human pancreatic cancer cells in vitro and in vivo. Human BxPC-3 and PANC-1 pancreatic cancer cells, and human HL-7702 liver cells were incubated with matrine at different concentrations. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and cell apoptosis, by flow cytometry. Subcutaneous BxPC-3 xenograft tumors were established in nude BALB/c mice, and matrine was intraperitoneally (i.p.) administered. The tumors were monitored and harvested. Tumor sections were immunostained with an anti-Ki-67 antibody (Ab) to examine cell proliferation, or stained with terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) to evaluate in situ cell apoptosis. The expression of proliferating cell nuclear antigen (PCNA) and several apoptosis-related proteins in cells and tumor tissues were evaluated by Western blot analysis. In in vitro assays, matrine inhibited cell viability by downregulating the expression of PCNA, and induced cell apoptosis by reducing the ratio of Bcl-2/Bax, upregulating Fas, and increasing activation of caspases-8,-3 and -9, in a dose-dependent manner. Administration of matrine inhibited tumor growth in a dose-dependent manner, and regulated tumoral gene expression consistent with the in vitro results. But matrine had no significant effects on the viability of HL-7702 cells or the bodyweight of mice compared to controls. These results indicate matrine may be a potential and promising agent of natural resource to treat pancreatic cancer.

Key words matrine; pancreatic cancer; proliferation; apoptosis; caspase

Panicellular cancer is one of the most aggressive and lethal cancers worldwide. In 2008, 37680 estimated new cases were diagnosed in the United States, with a nearly identical rate of estimated deaths (34290) from pancreatic cancer.1) Pancreatic cancer is highly resistant to chemotherapy and other forms of treatment.2) Therefore, novel treatment modalities are worth being investigated.

In seeking novel anti-cancer drugs, matrine (dodecahydro-3a,7a-diaza-benzo[de]anthracen-8-one, molecular formula: C15H24N2O3),3) an alkaloid extracted from a traditional Chinese herb, Sophora flavescens Arr., has drawn attention. Matrine has exhibited multiple pharmaceutical benefits, such as anti-inflammation, anti-virus and anti-fibrosis, in treating viral hepatitis and liver cirrhosis, and protected livers from ischemia reperfusion, without obvious toxicity or side-effects.4) Recently, matrine has displayed anti-tumor activities mainly by inhibiting the proliferation and/or inducing the apoptosis of cells from cervical cancer, leukemia, gastric cancer, hepatocellular carcinoma, lung cancer and breast cancer.5—20) or induced the differentiation of leukemia K-562 cells.12) Furthermore, matrine inhibits the adhesion and migration of cervical cancer HeLa cells,13) the invasion and metastasis of human malignant melanoma A375 cells,15) or the growth of established gastric tumors in mice.11) We have previously reported that matrine inhibits the proliferation and induces the programmed cell death of rat glioma C6 cells in vitro.16) The mechanisms accounting for its anti-cancer activities included regulating apoptosis- and proliferation-related genes or proteins, such as N-ras, p53, c-myc, E2F-1, Apaf-1, Rb, Bcl-2 and caspases,12—16) or regulating the transcriptional activity of eIF4E via dephosphorylation of 4E-BP1,18) or vascular endothelial growth factor (VEGF)-Akt-nuclear factor (NF)-κB signaling pathways.19) However, little is known about its effects on pancreatic cancer. Therefore, the present study aims to investigate its anti-cancer activity and underlying mechanisms in human pancreatic cancer cells in vitro and subcutaneous xenograft tumors in mice in vivo.

MATERIALS AND METHODS

Mice, Cell Lines and Reagents Male nude BALB/c mice, 6—8 weeks old, were obtained from the Animal Research Center, the First Affiliated Hospital of Harbin Medical University, China. The Human pancreatic cancer cell lines BxPC-3 and PANC-1 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). The normal human liver cell line, HL-7702, was obtained from the Cell Bank of Chinese Academy of Sciences. The cells were cultured in RPMI 1640 medium as described previously.21) Matrine was purchased from Shaanxi Huike Botanical Development Co., Ltd., Xi’an, China (Batch No.: MA20070302, 98% purity). The antibodies against Bcl-2, Bax, Fas, cleaved caspase-8, cleaved caspase-9, cleaved caspase-3, PCNA (proliferating cell nuclear antigen) and β-actin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. The anti-Ki67 antibody (Ab) was purchased from Abcam.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay BxPC-3 (2×104), PANC-1 (4×104) and HL-7702 (4×103) cells were seeded in 200 μl of RPMI 1640 medium into 96-well plates, and cultured overnight. Then the medium was replaced with fresh RPMI 1640 or the same media containing different concentrations of matrine (0.25—2.0 mg/ml). After a further incubation for 72 h, MTT (5 mg/ml, 20 μl) was added to each well, followed by a 4 h
When tumors reached 1 mm in diameter, the cell viability index was calculated according to the formula: experimental OD value/control OD value × 100%. The experiments were repeated thrice.

**In Vitro Cell Apoptosis Assay** The cells were incubated with matrine as above for 72 h and harvested. $1 \times 10^5$ cells were suspended in 100 μl binding buffer, the 5 μl of Annexin V and 5 μl of propidium iodide (PI) were added. The mixture was incubated for 15 min at room temperature in the dark, according to the manufacturer’s instruction (BD Biosciences, San Jose, CA, USA.). Then the cells were subjected to flow cytometry to measure the apoptosis rate (%) with a Beckman Coulter Epics Altra II cytometer (Beckman Coulter, California, USA.). The experiments were repeated thrice.

**Animal Experiments** All surgical procedures and care administered to the animals were in accordance with institutional guidelines. Tumors were established by subcutaneous injection of $5 \times 10^6$ BxPC-3 cells into the flanks of mice. Tumor volumes were estimated according to the formula: $\pi a^2 b / 6$, where $a$ is the short axis, and $b$ the long axis. When tumors reached ca. 120 mm³ at about 2 weeks, the mice were randomly assigned to 4 groups (each group had 7 mice): control and 3 treatment groups. The mice in the control group received a daily intraperitoneally (i.p.) injection of 100 μl of phosphate buffered saline (PBS), and the mice in the 3 treatment groups received i.p. injections of 100 μl of matrine at doses of 50, 100 or 200 mg/kg, respectively. The mice were closely monitored, weighed, and euthanized 18 d after treatment, and the tumors harvested. Each tumor was split into two parts: one part was fixed with 10% buffered formalin, and the other part was fixed with 100%.

**Terminal Deoxynucleotidyl Transferase Mediated Deoxyuridine Triphosphate (dUTP) Nick-End Labeling (TUNEL) Staining** Tumor sections were stained with the TUNEL agent (Roche, Shanghai, China), and adjacent sections were counterstained with hematoxylin and eosin. The TUNEL positive cells were counted in 10 randomly selected high-power fields under microscopy. The apoptosis index was calculated according to the following formula: the number of apoptotic cells/total number of nucleated cells × 100%.

**Western Blotting Analysis** The methodology has been described previously. Briefly, Tumor sections were blocked with 3% bovine serum albumin (BSA), and then incubated with an anti-Ki-67 Ab. They were subsequently incubated for 30 min with the secondary Ab using the Ultra Sensitive TMS-P kit (Zhongshan Co., Beijing, China), and immunoreactivity was developed with Sigma FAST 3,3′-diaminobenzidine tetrahydrochloride (DAB) and CoCl₂ enhancer tablets (Sigma-Aldrich, Shanghai, China). Sections were counterstained with hematoxylin. The Ki-67 positive cells were counted on 10 randomly selected ×400 high-power fields under microscopy. The Ki-67 proliferation index was calculated according to the following formula: the number of Ki-67 positive cells/total cell count × 100%.

**Statistical Analysis** The results were expressed as mean values±standard deviation (S.D.), and an analysis of variance (ANOVA) and Dunnett t test were used to evaluate statistical significance. A value of less than 0.05 ($p<0.05$) was used for statistical significance.

**RESULTS**

**Matrine Inhibits the Growth of Pancreatic Cancer Cells but Not Normal Liver Cells in Vitro** As shown in Fig. 1, matrine reduced the viability of two types of pancreatic cancer cells in a dose-dependent manner. With a logarithmic regression analysis, the concentration of matrine which resulted in 50% of maximal proliferation inhibition (IC₅₀) of cells was calculated. The IC₅₀ value was 0.73 mg/ml.
Matrine Induces the Apoptosis of Pancreatic Cancer Cells and Regulates Apoptosis-Related Proteins *in Vitro*

BxPC-3 and PANC-1 cells were incubated with matrine at different concentrations (0.25, 0.75 or 1.25 mg/ml) for 72 h. The absence of matrine served as the control. (A) Flow cytometry was performed to measure apoptosis rates. * Indicates a significant difference at \( p<0.05 \), and ** a highly significant difference at \( p<0.001 \), compared with control. (B) Representative dot plots were from cytometrically analyzed BxPC-3 (upper panel) or PANC-1 (lower panel) cells incubated with matrine at different concentrations. The absissa shows the number of Annexin V-positive cells, and the ordinate, PI-negative cells. (C, D) The BxPC-3 (C) or PANC-1 (D) cells were incubated in the absence of matrine (lane 1) or presence of matrine at concentrations of 0.25 (lane 2), 0.75 (lane 3) or 1.25 (lane 4) mg/ml, and then homogenized and subjected to Western blot analysis to detect expression of PCNA, Bcl-2, Bax, Fas, cleaved caspase-8, cleaved caspase-9 and activated caspase-3. β-actin served as an internal control.

Matrine Induces Apoptosis of Pancreatic Cancer Cells *in Vitro*

BxPC-3 and PANC-1 cells were incubated with matrine at different concentrations for 72 h, and flow cytometric analysis was used to measure the apoptosis rates. As shown in Fig. 2A, matrine increased the apoptosis rate in a dose-dependent manner. Matrine at a concentration of 0.25 mg/ml led to an apoptosis rate of BxPC-3 cells at 9.90±0.25 mg/ml, which was significantly higher than that of the untreated cells (\( p<0.05 \)). Furthermore, 0.75 or 1.25 mg/ml of matrine resulted in a highly significantly higher apoptosis rates (33.67±5.42% or 48.83±3.91%, respectively) than the controls (\( p<0.001 \)). Similarly, matrine also increased the apoptosis rates of PANC-1 cells in a dose-dependent manner (Fig. 2A). The representative histograms of flow cytometry for BxPC-3 cells, and 0.91 mg/ml for PANC-1 cells. However, matrine had no significant effect on the viability of normal HL-7702 cells. We also detected the expression levels of PCNA with Western blot analysis, which showed that matrine downregulated the expression of PCNA in a dose-dependent manner, in both BxPC-3 and PANC-1 cells (Fig. 1B).

Matrine Regulates Expression of PCNA and Apoptosis-Related Proteins *in Vivo*

In a *in Vivo* study, tumors of mice treated with 100 mg/kg or 200 mg/kg of matrine were smaller than the control (Fig. 2D, PANC-1 cells). We further examined the expression of apoptosis-related proteins in the two types of cells with Western blot analysis. Matrine treatment upregulated the expression of Bax and downregulated the expression of Bcl-2 (thus reducing the ratio of Bcl-2/Bax), upregulated the expression of Fas, and increased the activation of caspases-8, -9 and -3, in the two types of cells in a dose-dependent manner (Fig. 2C, BxPC-3 cells; Fig. 2D, PANC-1 cells).

Administration of Matrine Inhibits Pancreatic Tumors *in Vivo* without Significant Side-Effects

BxPC-3 tumors were established in the flank of mice. When the tumors reached ca. 120 mm³ in volume, the mice received daily injection of PBS (control), or the equal volume of matrine at the doses of 50, 100 or 200 mg/kg, as indicated. The sizes (mm³) of tumors were monitored and recorded. A significant difference in tumor volume from control is denoted by “∗∗” (B). The bodyweight of mice was measured and recorded. (C) Tumors from the above mice receiving an injection of PBS (lane 1), or matrine at doses of 50 (lane 2), 100 (lane 3) or 200 (lane 4) mg/kg, were homogenized and subjected to Western blot analysis to detect expression of PCNA, Bcl-2, Bax, Fas, cleaved caspase-8, caspase-9 and activated caspase-3. β-actin served as an internal control.

Matrine Regulates Expression of PCNA and Apoptosis-
We further detected tumoral expression of PCNA and apoptosis-related proteins, and found that matrine therapy downregulated the expression of PCNA and Bcl-2, and upregulated the expression of Bax and Fas, thus reducing the ratio of Bcl-2/Bax, and also increased the activation of caspase-9, -8 and -3, in a dose-dependent manner, in accordance with the findings in vitro (Fig. 3B).

**Matrine Inhibits Cell Proliferation in Situ** We further detected tumoral expression of PCNA and apoptosis-related proteins, and found that matrine therapy downregulated the expression of PCNA and Bcl-2, and upregulated the expression of Bax and Fas, thus reducing the ratio of Bcl-2/Bax, and also increased the activation of caspase-9, -8 and -3, in a dose-dependent manner, in accordance with the findings *in vitro* (Fig. 3B).

**Matrine Induces Cell Apoptosis in Situ** Tumor sections from above were stained with the TUNEL agent to examine apoptotic cells. A small number of apoptotic cells were detected in tumors in the control group (Fig. 5A), whereas a greater number of apoptotic cells were detected in tumors in all three matrine treatment groups at doses of 50 (Fig. 5B), 100 (Fig. 5C), or 200 (Fig. 5D) mg/kg. The apoptotic cells in sections were counted to record the apoptosis index. As shown in Fig. 5E, the effects of matrine on the apoptosis index were dose-dependent. Matrine therapy at the dose of 50 mg/kg resulted in an apoptosis index of 10.29±2.06%, which is significantly ($p<0.05$) higher than that in the control group (4.43±0.85%). Furthermore, matrine treatments at doses of 100 and 200 mg/kg led to apoptosis indexes of 19.8±4.16% and 24.17±4.90%, respectively, which were highly significantly ($p<0.001$) higher than that in the control group (Fig. 5E).

**DISCUSSION**

The present study has demonstrated that matrine exhibited therapeutic effects against human pancreatic cancer cells *in vitro* and subcutaneous xenograft tumors in mice. Its anti-proliferative activity may rely on the downregulation of PCNA expression, and pro-apoptotic activity, reduction of the ratio of Bcl-2/Bax, upregulation of Fas expression, and increased activation of caspase-8, caspase-9 and caspase-3.
Since the discovery of matrine as a potential anti-cancer drug, many lines of studies have examined the therapeutic effects of matrine including inhibiting cell proliferation, and inducing cellular differentiation and apoptosis, in various types of cancer cells. However, to our knowledge, the effect of matrine on pancreatic cancer has not been reported previously; and most of those studies examined its anti-tumor activity in cultured cancer cells.

Uncontrolled proliferation is one of the major biological features of cancer cells, and inhibiting cell proliferation could achieve the arrest of tumor growth. The present study has shown that matrine inhibited the proliferation of pancreatic cancer cells in vitro and in vivo. PCNA is a 36 kDa, highly conserved nuclear protein required for DNA synthesis via DNA polymerases δ and ε. PCNA is also required for post-replicative DNA repair through interactions with MSH2 and MLH1. By downregulating PCNA expression, matrine could inhibit DNA synthesis and repair, thus inhibiting cell proliferation.

Cellular apoptosis can be triggered by the death receptor extrinsic pathway and/or the mitochondrial intrinsic pathway. In the mitochondrial pathway, the released cytochrome c from mitochondria binds to Apaf-1, resulting in proteolytic processing and activation of caspase-9, initiating a cascade of additional caspase activation that culminates in apoptosis. The members of the Bcl-2 family are the most prominent regulators of apoptosis. Bcl-2, located on the membrane of mitochondria, is an anti-apoptotic protein, while Bax is a pro-apoptotic one by inhibiting the function of Bcl-2. Matrine has been shown to induce the apoptosis of gastric cancer MKN45 cells via increasing pro-apoptotic molecules of the Bcl-2 family. Matrine triggers the apoptosis of leukemia K562 cells primarily through the mitochondrial pathway, including upregulation of Apaf-1, Bax translocation, cytochrome c release and activation of caspase-9 and -3. The regulation of Bcl-2/Bax expression and caspase-3 activation has also been observed in matrine-induced apoptosis of angiotensin II-stimulated hyperplasia of cardiac fibroblasts, breast cancer cells, and human lung cancer and hepatoma cells. The present study has demonstrated that matrine-induced apoptosis was accompanied by a decrease in Bcl-2 and a concomitant increase in Bax in pancreatic cancer cells, and increased the activation of caspase-9 and -3 in a dose-dependent manner, indicating that mitochondrial pathway may be involved in matrine-induced apoptosis of pancreatic cancer cells. We have previously reported that matrine induced apoptosis by upregulating Bcl-2 at mRNA levels in a cDNA-microarray. We propose that matrine regulates the expression of other molecules involved in the death receptor pathway.

To summarize, the present study, to our knowledge, is the first one to investigate the anti-cancer effects of matrine and the underlying mechanisms in pancreatic cancer cells in vitro and in vivo. Compared to conventional chemotherapeutic agents, matrine, of natural resource, is of less cost, and exhibits no serious adverse cytotoxicity. All these features have made matrine a potential and attractive anti-cancer agent in treating pancreatic cancer. Furthermore, matrine has displayed synergistic effects with a chemotherapeutic drug, 5-fluorouracil, in treating implanted human gastric tumors in mice, and also with other agents including celecoxib, trichostatin and rosiglitazone in inhibiting the proliferation of human breast cancer cells. These studies imply that matrine might have synergistic effects with gemcitabine, a current first-line treatment for pancreatic cancer, though further investigation is required.

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