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Matrine induces programmed cell death and regulates expression of relevant genes based on PCR array analysis in C6 glioma cells

Shujun Zhang · Jiping Qi · Libo Sun · Binglin Cheng · Shangha Pan · Min Zhou · Xueying Sun

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Abstract Matrine, one of the main components extracted from *Sophora flavescens Ait*, has a wide range of pharmacological effects including anti-tumor activities on a number of cancer cell lines. This study has investigated whether matrine could also display anti-tumor action on rat C6 glioma cells. Exposure of C6 cells to matrine resulted in inhibition of proliferation and induction of apoptosis in a dose-dependent manner, as measured by the MTT assay and Flow cytometry. The Annexin V/PI staining further detected the apoptotic cells at both early and late phases of apoptosis. We used AO/EB staining to examine the programmed cell death of matrine-treated C6 cells, and showed that the death rate detected by AO/EB staining was higher than the apoptosis rate measured by Annexin V/PI staining, suggesting that autophagy, the Type II programmed cell death, may be involved in matrine-induced cell death, which was further confirmed by electronic microscopy. To explore the molecular mechanism, an apoptosis real-time PCR array was performed, which has demonstrated that 57 genes were at least 2-fold upregulated, and 11 genes were at least 2-fold downregulated in matrine-treated C6 cells, compared with untreated cells. However, the gene expression profiles could only partly and roughly explain molecular mechanisms of apoptosis and autophagy in matrine-treated C6 cells, thus further investigations are required to confirm the specific molecular pathways and related molecules responsible for the programmed cell death.

Keywords Matrine · Glioma · Apoptosis · PCR array · Programmed cell death

S. Zhang · J. Qi · M. Zhou
Department of Pathology, The First Clinical Medical School of Harbin Medical University, Harbin 150012, China

L. Sun · S. Pan · X. Sun (✉)
The Hepatosplenic Surgery Center/Department of General Surgery, The First Clinical Medical School of Harbin Medical University, Harbin 150012, China
e-mail: kevsun88@hotmail.com

B. Cheng
Department of Traditional Chinese Medicine, The First Clinical Medical School of Harbin Medical University, Harbin 150012, China

Introduction

Gliomas rank the first place and are the major lethal tumours in the central nervous system [1]. The mean survival time for patients with glioma is only 1 year from the time of diagnosis [2], as the current treatments are ultimately ineffective in curing gliomas, due to the radioresistance of glioma cells and the difficulty of achieving complete tumor resection. There is therefore an urgent need to seek novel therapeutic drugs to combat gliomas.

Matrine is one of the main components extracted from a traditional Chinese herb, *Sophora flavescens Ait*, with a molecular formula of $C_{15}H_{24}N_2O$ [3]. Matrine has displayed a wide range of pharmacological effects including anti-fibrotic activities [4–7], and not shown obvious toxicity or side effects [7]. Recently, it has been reported that matrine exhibits anti-tumor effects by inhibiting proliferation and inducing apoptosis of cancer cells from cervical cancer, leukemia, gastric cancer, hepatocellular carcinoma, breast cancer, and lung cancer [8–14], or inhibiting adhesion and migration of cervical cancer HeLa cells [12], or stimulating differentiation of leukemia K-562 cells [13]. Matrine is also able to inhibit the growth of establish gastric tumors in mice [11]. The anti-cancer

activity of matrine may rely on its effects on apoptosis- and proliferation-related genes or proteins including N-ras, p53, c-myc, E2F-1, Apaf-1, Rb, Bcl-2 family, and caspases [12–14]. However, the precise molecular mechanisms involved in matrine-induced cell death remain unclear. Therefore, we designed this study to investigate whether matrine could induce death of C6 glioma cells, and used a PCR array to obtain an expression profile of apoptosis related genes.

Materials and methods

Cell line and reagents

The rat C6 glioma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Matrine was purchased from Shaanxi Huike Botanical Development Co., Ltd, Xi'an, China (Batch No. MA20070302, 98% purity).

MTT assay

5×10^4 C6 cells were seeded in 200 μl of DMEM (Dulbecco's modified Eagle's medium) (Life technologies, China) supplemented with 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C in the CO_2 incubator. The cells were cultured for 24 h, and the medium was replaced with fresh DMEM or the same media containing different concentrations of matrine. After a further incubation for 24, 48 or 72 h, 20 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added to each well followed by incubation for 4 h. The medium was discarded and 150 μl of DMSO was added into each well, and incubated for 20 min. The OD_{570nm} was measured and the proliferation inhibition rate (%) was calculated according to the formula:(1 − experimental OD value/control OD value) × 100. The experiments were repeated thrice.

Flow cytometric analysis

The C6 cells were incubated with matrine at different concentrations as above for 24 h, harvested, washed, fixed with ice-cold 75% ethanol, and the numbers of cells were counted. 5×10^5 cells were suspended in 100 μl PBS, 5 μl of Annexin V and 10 μl of propidium iodide (PI) (Jingmei Biotech Co. Ltd., Shenzhen, China) were added, and incubated for 15 min at room temperature in dark. The apoptosis rate (%) was measured with a Beckman Coulter Epics Altra II cytometer (Beckman Coulter, California, USA). The experiments were repeated thrice.

Annexin V/PI staining

5×10^4 C6 cells were incubated in DMEM for 24 h. After removing the supernatants, the fresh DMEM or same medium containing 0.67 mg/ml matrine was added and cells cultured for further 24 h, followed by staining with Annexin V/PI for 15 min at room temperature in dark. The cells were viewed under a laser scanning confocal microscope (LSM-510, Carl Zeiss Jena GmbH, Jena, Germany). The apoptosis index (%) was calculated as the percentage of apoptotic cells, namely number of apoptotic cells × 100/total number of nucleated cells. The experiments were repeated thrice.

AO/EB staining

The C6 cells were treated with matrine for 24 h as above. Acridine orange and ethidium bromide (AO/EB) (Sigma) were freshly mixed at 0.01/100 (v/v) in dark, and one drop of mixture solution was added on the cells, which were immediately viewed using a fluorescence microscope. The cells that were stained green or red with condensed fragmented nuclei were defined as dead cells. The death rate (%) was calculated as the percentage of positively stained cells, namely number of cells undergoing programmed cell death (PCD) × 100/total number of cells. The experiments were repeated thrice.

Electronic microscopy

The C6 cells were treated with matrine for 24 h as above, harvested after trypsinization, and washed with PBS thrice. Then the cells were fixed with 2.5% glutaraldehyde solution for 1 h, washed twice with PBS, followed by a further fixation with 1% Osmic acid for 1 h, gradient dehydration with ethanol, embedded, and sectioned. The sections were stained with Uranium Acetate and Lead Citrate, and observed under a transmission electronic microscope (JEN-M1220, Toshiba, Japan).

RNA preparation

The cells were incubated with 0.67 mg/ml matrine for 24 h as above. After removing supernatant, RNA was extracted by addition of TRIzol (Invitrogen life Technologies, Shanghai, China) followed by chloroform according to the manufacturer's protocol. Samples were vigorously shaken for 15–20 s and incubated for 2–3 min at room temperature, followed by centrifugation at 12,000*g* for 15 min to allow separation of aqueous layer, which was transferred to a fresh tube. RNA was precipitated by cold isopropanol, followed by a final wash in cold 70% ethanol. RNA pellets were air-dried and resuspended in diethyl pyrocarbonate-treated

water. RNA quality was determined by running a sample with an RNA loading dye (Sigma-Aldrich) on a 1% agarose gel and inspecting for distinct 18S, 28S and total RNA bands, indicating lack of degradation; quantity was determined by A_{260} measurement. Samples were frozen at −80°C.

RT^2 Profiler™ PCR array

The first strand cDNA synthesis was performed with 2 μg of total RNA and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. The diluted first strand cDNA synthesis reaction was mixed with SuperArray PCR master mix, and loaded onto the 96-Well RT^2 Profiler™ PCR Array (PARN-012, rat apoptosis, SuperArray, Inc., Frederick, MD, USA). Real-time PCR detection was performed by heating the plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The data were analyzed with ΔΔCt method according to the manufacturer's manual. Quality control was performed with genomic DNA, reverse transcription and positive PCR controls, and the data were normalized to the housekeeping gene β-actin. Changes in gene expression were illustrated as a fold increase/decrease. The cut-off determining expression was ≥2.0 or ≤−2.0 fold changes. Genes which met both above criteria were considered to be upregulated or downregulated. The experiments were repeated thrice.

Statistical analysis

The half maximal inhibitory concentration (IC_{50}) was calculated with a simple linear regression analysis. The real-time PCR array data were analyzed with ΔΔCt method. All the other data were expressed as mean values ± standard deviation (SD), and a Student's t test was used for evaluating statistical significance. A value less than 0.05 ($P < 0.05$) was used for statistical significance.

Results

Matrine inhibits proliferation of C6 cells in a dose-dependent manner

The proliferation inhibition effect of matrine on C6 cells was determined with MTT assay. As shown in Fig. 1, matrine inhibited the proliferation of C6 cells incubated with matrine for 24 h in a dose-dependent manner, compared with untreated cells. Prolongation of incubation time to 48 or 72 h had little effect on proliferation inhibition of C6 cells, compared to the incubation for 24 h. Therefore, incubation with matrine for 24 h was selected for the following experiments. With a simple linear regression analysis, the IC_{50} was calculated to be 0.67 mg/ml when cells were incubated with matrine for 24 h (Fig. 1), and this concentration was used for cell staining and PCR array experiments.

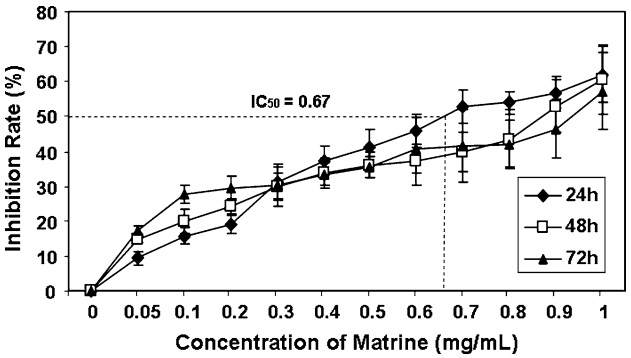

Fig. 1 Matrine inhibits the proliferation of glioma cells in vitro. C6 cells were incubated in the absence or presence of matrine at different concentrations, and harvested at different time points. The proliferation of C6 cells was assessed by the MTT method to calculate the proliferation inhibition rate (%). The dotted lines show the concentration of matrine, which resulted in 50% of maximal proliferation inhibition (IC_{50}) of C6 cells

Flow cytometric assay for apoptosis

C6 cells were incubated with matrine at different concentrations for 24 h, and flow cytometric analysis was used to measure the apoptosis rates. As shown in Fig. 2a, matrine induced apoptosis of C6 cells in a dose-dependent manner. When the concentration of matrine reached 0.3 mg/ml, the apoptosis rate of C6 cells was significantly higher than that of untreated cells ($P < 0.05$). Furthermore, 0.7 or 0.9 mg/ml of matrine resulted in a highly significantly difference in apoptosis rate between matrine-treated and untreated cells ($P < 0.001$). As shown in the representative histograms of flow cytometry, the apoptosis rates of C6 cells were 0.55, 1.15, 9.45 or 19.95% when they were treated with matrine at concentrations at 0, 0.1, 0.5, 0.9 mg/ml, respectively (Fig. 2b–e).

Cell apoptosis by Annexin V/PI staining

To confirm cell apoptosis induced by matrine, we further used Annexin V/PI method to stain the C6 cells, and examined them under a laser confocol microscope. As shown in Fig. 3a, only a small number of apoptotic cells were detected among the untreated cells, and most of them were at the early phase of apoptosis. The early-staged apoptotic cells were stained green by Annexin-V, as these cells display phosphatidylserine on their outer surface membranes, which is readily detectable by Annexin V. However, among the matrine-treated C6 cells, we detected

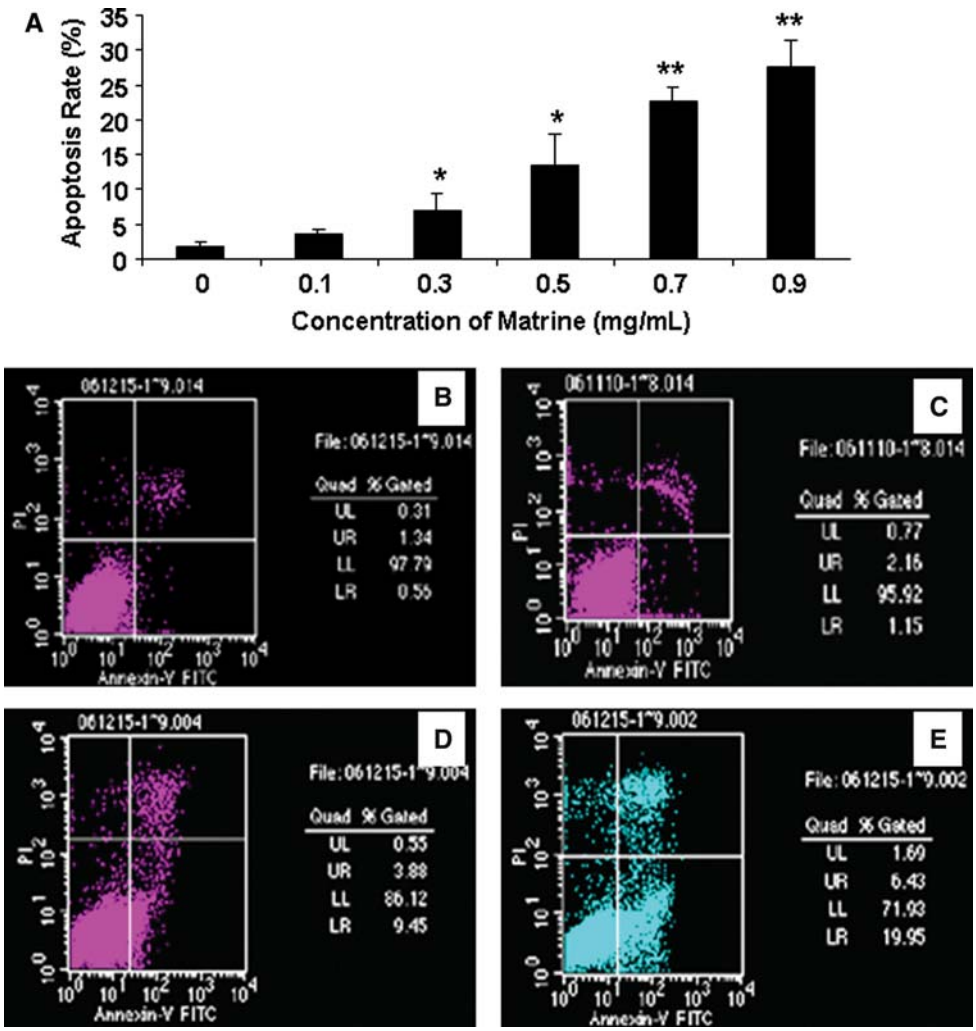

Fig. 2 Apoptosis by cytometric analysis. C6 cells incubated with different concentrations of matrine for 24 h were harvested for cytometric analysis to measure apoptosis rates. Untreated cells served as control. The apoptosis rates were shown in (**a**). * Indicates a significant difference at $P < 0.05$, and ** a highly significant difference at $P < 0.001$, compared with from control. (**b–e**) Representative histograms are from cytometrically analyzed C6 cells incubated with different concentrations [0(**b**), 0.1(**c**), 0.5(**d**), or 0.9(**e**) mg/ml] of matrine

more apoptotic cells including late-staged apoptotic cells, which had their nuclei stained red by PI as plasma membrane becomes increasingly permeable during the later stage of apoptosis, allowing PI to move across the cell membrane and bind to DNA (Fig. 3b). We further counted the apoptotic cells and calculated apoptosis indexes. The cells treated with matrine had an apoptosis index of 13.8 ± 2.3%, which was significantly higher than that of untreated cells (2.4 ± 1.5%, $P < 0.05$) (Fig. 3e).

PCD by AO/EB staining

We further stained the cells with AO/EB to detect PCD. As shown in Fig. 3c, only a small number of cells undergoing PCD were detected among the untreated cells, and most of them were at early phase of PCD, which had their condensed fragmented nuclei stained green with by AO. However, among the matrine-treated C6 cells, we detected more cells undergoing PCD at both early and late phases (Fig. 3d). The C6 cells at late phase of PCD had their nuclei stained red by EB. We further counted the cells undergoing PCD and calculated death rates. The matrine-treated cells had a death rate of 19.8 ± 2.7%, which was significantly higher than that of untreated cells (5.4 ± 2.2, $P < 0.05$) (Fig. 3f). We also compared the death rate detected by AO/EB staining and the apoptosis index measured by Annexin V/PI staining in the same batch of cells, and found that the death rate (19.8 ± 2.7%, Fig. 3f) was significantly higher than the apoptosis index (13.8 ± 2.3%, Fig. 3e), indicating that another form of PCD besides apoptosis, possibly autophagy, may be involved in matrine-induced cell death in C6 cells.

Autophagy detected by electronic microscopy

To further clarify autophagy in matrine-treated C6 cells, we used electronic microscopy to examine the autophagic cells. As shown in Fig. 4a, the untreated C6 cells displayed normal nuclear and cytoplasmic morphology. The most prominent morphological change in autophagic cells is the membrane enclosed vesicles, called autophagic bodies, in the cytoplasm that engulf portions of cytoplasm or organelles such

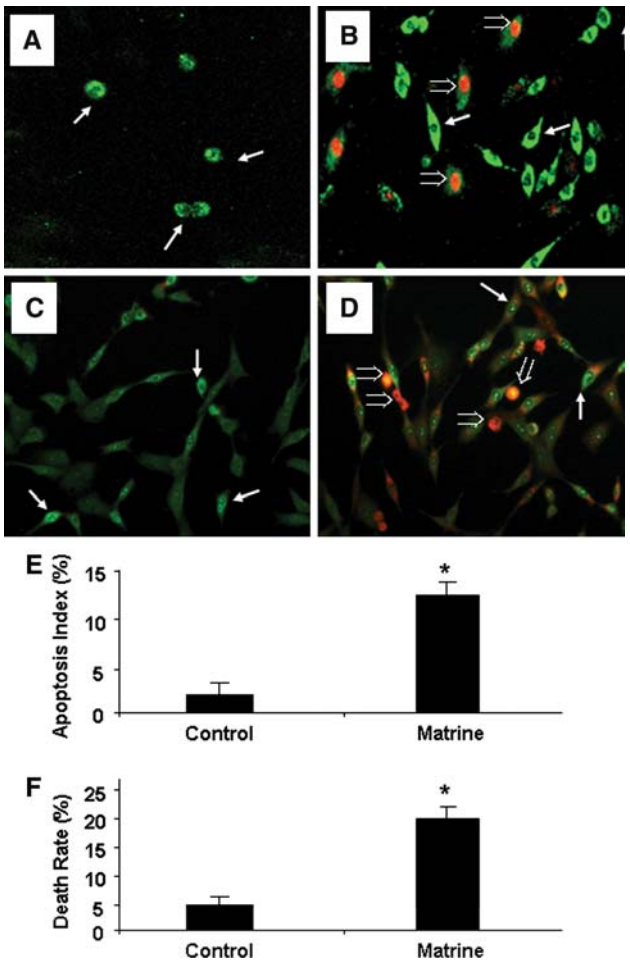

Fig. 3 Programmed cell death induced by matrine. C6 cells were incubated in DMEM (**a**, **c**) or DMEM containing 0.67 mg/ml matrine (**b**, **d**) for 24 h. Illustrated are representative photographs (×200 magnification) for cells stained with Annexin V/PI and examined under a laser scanning confocal microscope for apoptosis (**a**, **b**), or stained with AO/EB and examined under a fluorescence microscope(**c**, **d**) for programmed cell death. Untreated cells served as control. The cells at early phase of apoptosis or programmed cell death were pointed by "→", and late phase by "⇒". (**e**) Apoptotic cells from Annexin V/PI staining were counted to record the apoptosis index, and (**f**) cells undergoing programmed cell death were counted to record the death rate. "*" indicates significant difference between control and matrine-treated cells

as mitochondria and endoplasmic reticula [15]. These vesicles fuse with lysosomes and deliver their cargo for degradation by lysosomal enzymes of cells. As shown in Fig. 4b, several autophagic bodies (pointed by "→") and one engulfed mitochondrion (pointed by "⇒") were observed in matrine-treated cells.

Gene expression profile induced by matrine

Given that matrine treatment resulted in proliferation inhibition and programmed cell death including apoptosis in C6 cells, we further investigate the gene expression profile with a PCR array. The cells were incubated with matrine for 24 h, and their gene expression profiles were analyzed using a 96-Well RT^2 Profiler™ PCR Array containing 87 key apoptosis related genes, as compared with untreated C6 cells. RT-PCR was performed to detect expression of several representative genes, showing the consistency of the assays (data not shown).

The detailed changes of gene expression were shown in Table 1. Fifty-seven genes were at least 2-fold upregulated, and 11 genes were at least 2-fold downregulated, in matrine-treated C6 cells. BIRC3, the most upregulated gene by matrine for 85.6 folds, belongs to IAP family; and BAG3, the most downregulated gene by matrine for 11.24-fold, belongs to Bcl-2 family. The upregulated genes by matrine belong to Bcl-2, TNF ligand, TNF receptor, CIDE domain, IAP, caspase, CARD, IGF, TRAF, BNIP, DNA damage/p53-ATM pathway gene families, and the down-regulated genes belong to Bcl-2, CIDE domain, IAP, TRAF and other gene families (Table 2).

Discussion

The present study has demonstrated that matrine inhibits proliferation and induces programmed cell death of rat glioma C6 cells in vitro. Similarly, Deng et al. [16] has shown that matrine inhibited proliferation of C6 cells and downregulated expression of proto-oncogene C-myc. The key findings of the present study are that matrine induced two types of programmed cell death, i.e. apoptosis and autophagy, in C6 cells, and regulated most of the apoptosis related genes in C6 cells based on a PCR array analysis.

Apoptosis, the best-described form of programmed cell death, involves the activation of catabolic enzymes in signaling cascades, which leads to the rapid demolition of cellular structures and organelles [17, 18]. Classical apoptosis is characterized morphologically by cell shrinkage, chromatin condensation, nucleosomal DNA degradation and finally, fragmentation of the cell into apoptotic bodies [15]. Matrine induced apoptosis of C6 cells in the present study, in accordance with several previous reports, where matrine was shown to induce apoptosis of gastric cancer MKN45 cells [14], leukemia K562 cells [9, 19], and leukemia U937 cells [8]. Apoptosis can be initiated through two alternative pathways: death receptors and intrinsic mitochondrial pathways. The first pathway involves ligand-induced activation of death receptors including Fas/Fas ligand and TNF/TNFR, which leads to activation of caspase-8 [20], followed by sequential cleavage of pro-caspase-3, resulting in activation of the apoptotic process [21]. Here we have shown that matrine upregulated expression of 14 members of TNF/TNFR gene family and almost all the

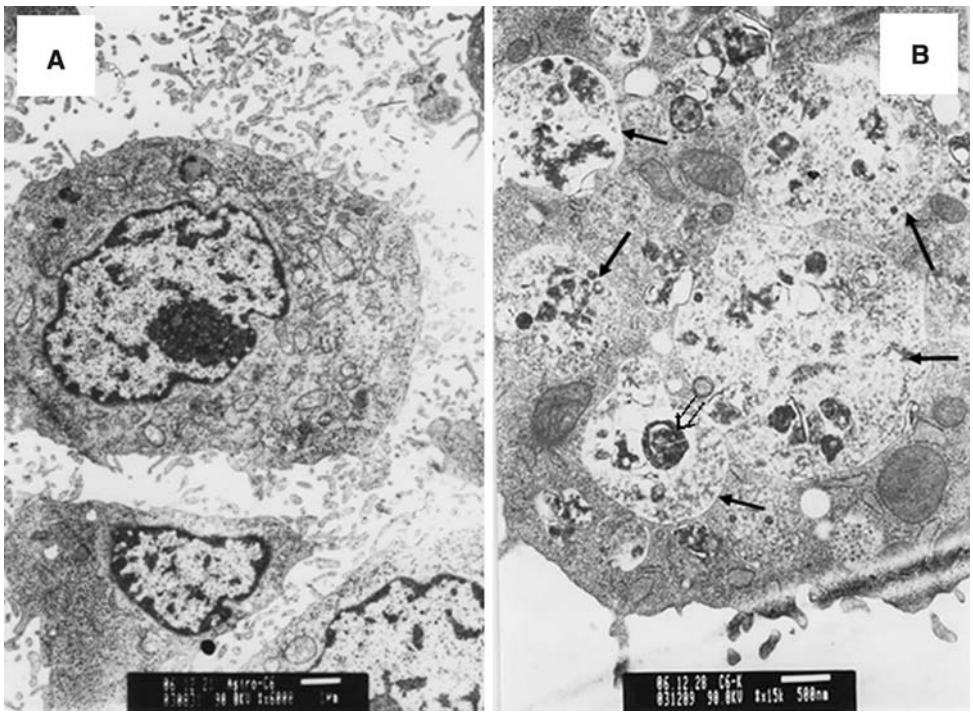

Fig. 4 Cell autophagy detected by electronic microscopy. C6 cells were incubated with 0.67 mg/ml matrine for 24 h, and examined under a transmission electronic microscope. Illustrated are representative photographs from untreated cells (**a**, ×6,000 magnification) or matrine-treated cells (**b**, ×15,000 magnification), where the autophagic bodies are pointed by "→", and an engulfed mitochondrion in an autophagic body is pointed by "⇒"

caspase family genes in the PCR array, implying that the death receptor pathway may be involved in matrine-induced cell apoptosis. It has also been shown that matrine triggers apoptosis via the mitochondrial pathway by upregulating cell cycle protein E2F-1 in K562 cells [9], or pro-apoptotic molecules of Bcl-2 family in MKN45 cells [14], or reducing Bcl-2/Bax ratio and upregulating cleaved caspase-3 activity in cardiac fibroblasts [3]. In the mitochondrial pathway, mitochondria are induced to release cytochrome *c*, which binds to Apaf-1 and pro-caspase-9. Active caspase-9 in turn directly activates pro-caspase-3, initiating a cascade of additional caspase activation [22]. Besides the effects of matrine on Apaf-1 and caspases, here we could also demonstrate the effects of matrine on Bcl-2 family evidenced by upregulation of Bad, Bag1 and 4, Bak1, Bid, BcL10, Bcl2L1, BclAF1 and HRK, and downregulation of Bag3, Bcl2L11, Bcl2L2 and Bax. Given the fact that the complex interaction of these molecules remains unclear, the present study could only provide a clue like the tip of the iceberg, but it did demonstrate that matrine has effects on expression of the Bcl-2 family involved in intrinsic mitochondrial pathway of apoptosis.

Accumulating data have provided evidence that besides the caspase-dependent apoptosis (Type I PCD), autophagic machinery can be also recruited to kill cells generating a caspase-independent form of PCD, named autophagy or Type II PCD. The most prominent morphological change in autophagy is the appearance of double- or multiple- membrane enclosed vesicles, called autophagic bodies, in the cytoplasm that engulf portions of cytoplasm and organelles [15]. We compared the death rate detected by AO/EB staining and the apoptosis index measured by Annexin V/PI staining in the same batch of cells, and found that the death rate was significantly higher than the apoptosis index (19.8% vs. 13.8%), indicating 6% of total cells undergoing PCD were not apoptotic cells. To further examine these cells, we used electronic microscopy and found so-called autophagic bodies that contained engulfed mitochondria in matrine-treated cells, indicating matrine also induce autophagic cell death. It has been demonstrated that autophagic cell death is induced by well-established death promoting proteins such as BNIP3 [23], death-associated protein kinase DRPK1/DAPK2 [24], or suppressed by death protective proteins including Bcl-2 [23–27]. DAPK1 has been shown to modulate the apoptotic death, but their overexpression in HEK293 and HeLa cells induced autophagic cell death [24, 28]. In the present study, BNIP1, BNIP2, NNIP3 and DRPK1 were all upregulated by matrine, supporting the finding of autophagy by electronic microscopy. Despite the fact that apoptosis and autophagy proceed via independent mechanisms, several lines of evidence point out the existence of crosstalk between the two pathways, and the two types of PCD may also be activated simultaneously [15]. Gene expression profiling studies of steroid-triggered development in *Drosophila* revealed that several apoptosis-related genes were upregulated together with autophagy-related genes [28, 29], supporting the findings in the present study that both apoptosis and autophagy were detected in matrine-treated C6 cells. The present study has also shown that matrine upregulated expression of FADD, a molecule that triggers apoptosis and stimulates autophagy [30].

Table 1 Changes of gene expression induced by matrine in C6 cells

Unigene No.	Gene symbol	Fold change	Unigene No.	Gene symbol	Fold change
Hs.523309	BAG3	−11.24	Hs.431048	ABL1	7.52
Hs.469658	BCL2L11	−10.85	Hs.405153	NOD1	7.52
Hs.552567	APAF1	−7.84	Hs.516966	BCL2L1	7.73
Hs.410026	BCL2L2	−6.92	Hs.144873	BNIP3	8.11
Hs.249129	CIDEA	−6.06	Hs.356076	BIRC4	8.57
Hs.103755	RIPK2	−4.89	Hs.131226	BNIP3L	8.57
Hs.412707	HPRT1	−4.63	Hs.329502	CASP9	8.75
Hs.550061	BRAF	−3.89	Hs.80409	GADD45A	9.45
Hs.159428	BAX	−3.32	Hs.194726	BAG4	9.78
Hs.522506	TRAF2	−3.07	Hs.408312	TP53	10.56
Hs.150107	BIRC6	−2.14	Hs.443577	TNFRSF21	10.63
Hs.150749	BCL2	−1.30	Hs.138378	CASP4	11.47
Hs.192132	TP73	−1.12	Hs.193516	BCL10	13.74
Hs.200242	CARD6	1.12	Hs.368982	CASP2	14.03
Hs.591630	CASP8	1.12	Hs.503704	BIRC2	14.22
Hs.193418	TNFRSF9	1.21	Hs.244139	FAS	14.32
Hs.2007	FASLG	1.23	Hs.521456	TNFRSF10B	14.32
Hs.355307	CD27	1.43	Hs.510528	TRAF3	14.62
Hs.43555	BFAR	1.64	Hs.525622	AKT1	14.83
Hs.558359	NAIP	1.96	Hs.86131	FADD	15.35
Hs.227817	BAL2A1	1.97	Hs.643120	IGF1R	15.56
Hs.283672	BCL2L10	1.97	Hs.1116	LTBR	16.00
Hs.475055	BIK	1.97	Hs.377484	BAG1	16.22
Hs.348263	BIRC8	1.97	Hs.646490	BNIP2	16.45
Hs.466057	CASP14	1.97	Hs.484782	DFFA	16.68
Hs.213327	CASP5	1.97	Hs.279594	TNFRSF1A	17.15
Hs.592244	CD40LG	1.97	Hs.485139	BAK1	17.39
Hs.87247	HRK	1.97	Hs.38533	CRADD	17.39
Hs.36	LTA	1.97	Hs.370254	BAD	18.13
Hs.501497	CD70	1.97	Hs.534255	B2M	18.38
Hs.241570	TNF	2.04	Hs.486542	BCLAF1	18.64
Hs.494901	TNFSF8	2.19	Hs.141125	CASP3	18.64
Hs.380277	DAPK1	2.23	Hs.81791	TNFRSF11B	18.77
Hs.642693	CIDEB	2.66	Hs.523968	TP53BP2	18.77
Hs.591834	TNFRSF10A	3.32	Hs.546356	RPL13A	18.77
Hs.632486	MCL1	3.34	Hs.390736	CFLAR	19.43
Hs.8375	TRAF4	3.58	Hs.462529	TNFRSF25	21.41
Hs.2490	CASP1	3.71	Hs.460996	TRADD	23.59
Hs.389452	CASP6	4.50	Hs.446146	CARD8	25.11
Hs.478275	TNFSF10	4.56	Hs.591054	BID	26.35
Hs.5353	CASP10	5.54	Hs.145726	BNIP1	28.84
Hs.472860	CD40	5.58	Hs.9216	CASP7	29.86
Hs.513667	NOL3	5.86	Hs.127799	BIRC3	85.63
Hs.499094	PYCARD	7.46			

In conclusion, the present study has demonstrated that matrine inhibits proliferation and induces PCD of C6 glioma cells in vitro. Two types of PCD were detected in matrine-treated C6 cells, but the precise molecular mechanisms remain unclear as the preliminary results are only able to provide a clue on expression of related genes at

Table 2 Functional gene families regulated by matrine in C6 cells

Functional gene family	Upregulated gene	Downregulated gene
Bcl-2	BAD, BAG1, BAG4, BAK1, BID	BAG3, BCL2L11
	BCL10, BCL2L1, BCLAF1, HRK	BCL2L2, BAX
TNF ligand	TNF, TNFSF8, TNFSF10, FAS	
TNF receptor	TNFRSF10A, TNFRSF10B, TNFRSF11B	
	TNFRSF1A, TNFRSF21, TNFRSF25	
	FADD, LTBR, MCL1, CD44	
CIDE domain	CIDEB, DFFA, DAPK1, CRADD	CIDEA
IAP	BIRC2, BIRC3, BIRC4	BIRC6
Caspase	CASP1, CASP2, CASP3, CASP4, CASP6	
	CASP7, CASP9, CASP10	
CARD	CARD8, NOL3, PYCARD	
IGF	IGF1R	
TRAF	TRAF3, TRAF4, TRADD	TRAF2
BNIP	BNIP1, BNIP2, BNIP3, BNIP3L	
DNA damage/P53-ATM pathway	GADD45A, TP53, TP53BP2	
Oncogene	ABL1, AKT1	BRAF
Others	CFLAR, B2M, RPL13A, NOD1	APAF1, RIPK2, HPRT1

mRNA level. Further investigations are definitely required to clarify the specific molecular pathways especially PCD related molecules at protein levels affected by matrine, and the answers to these questions will improve our understanding of the molecular mechanisms of matrine on C6 glioma cells. Furthermore, matrine is advantageous as it does not show obvious toxicity [7], compared with cytotoxic drugs, which are currently used to treat gliomas. The present study, along with a previous report [16], supports the possibility of matrine to be used to treat gliomas, alone or in combination with other toxic drugs.

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