Matrine, a novel autophagy inhibitor, blocks trafficking and the proteolytic activation of lysosomal proteases

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Autophagy has been referred to as a double-edged sword in tumorigenesis and tumor progression. Emerging evidence suggests that pharmacological modulation of autophagy is a promising therapeutic strategy for cancer. However, few autophagy-modulating compounds are currently approved for clinical use in humans. Matrine is a natural compound extracted from traditional Chinese medicine that is widely used for treatment of a variety of diseases without any obvious side effects. Recently, matrine has been reported to induce autophagy and autophagic cell death in cancer cells, although the underlying mechanisms have yet to be elucidated. Here, we systematically examined the autophagic events induced by matrine in SGC7901 cells. The accumulation of autophagic vacuoles in matrine-treated cells was verified by the conversion of microtubule-associated protein light chain 3 as well as confocal and transmission electron microscopy. Furthermore, we demonstrated that matrine blocked autophagic degradation by impairing the activities of lysosomal proteases. Moreover, confocal microscopy and gradient ultracentrifugation revealed that the trafficking processes and proteolytic activation of cathepsins were inhibited by matrine. Using a pH sensor probe, we found elevated pH values in endosomes/lysosomes in response to matrine treatment. Therefore, matrine seems to be a novel autophagy inhibitor that can modulate the maturation process of lysosomal proteases.

Introduction

Matrine (C₃₅H₆₀N₆O₁₂) is a quinolizidine alkaloid compound that is extracted from the herb root of a traditional Chinese medicine, Sophora flavescens Ait (1). It has been reported that matrine has promising pharmaceutical efficacies for many diseases without any obvious side effects, such as viral hepatitis, liver cirrhosis, cardiac arrhythmia and skin inflammations (2–5). Importantly, matrine has been approved as an adjuvant drug for preventing cachexia in China, and clinical studies have demonstrated that the immune function and quality of life of cancer patients were greatly improved by combining standard therapies with the use of matrine (6,7).

Due to its therapeutic significance, the mechanism responsible for the pharmacological effects of matrine has received considerable interest for the development of novel anticancer drugs. It is well documented that matrine is able to inhibit proliferation and metastasis and induce apoptosis in a variety of malignant cells (1,8,9). Matrine also reduces the occurrence of multidrug-resistant tumor cells induced by chemotherapy and displays synergistic effects with anticancer agents (10,11). Moreover, in vitro and in vivo studies have revealed that matrine inhibits the production of the proinflammatory cytokines TNF-α and IL-6 and prevents cachexia-related symptoms in tumor-bearing mice (12). Recent evidence has indicated that matrine induces autophagy in cancer cells. HepG2 cells treated with matrine exhibited remarkable morphological changes, including abundant autophagic vacuoles (AVs) of varied sizes. In addition, 3MA, a class III PI3K inhibitor that blocks autophagic sequestration, prevented the accumulation of AVs induced by matrine (13). After treatment with matrine, transmission electron microscopy (TEM) examination revealed massive AVs in the cytoplasm of C6 glioma cells and SGC7901 gastric cancer cells (14,15). However, the mechanisms by which matrine induces autophagy remain largely unknown.

Macroutrophagy (hereafter referred to as autophagy) is an evolutionarily conserved catabolic process in which proteins, organelles and intracellular pathogens are engulfed by autophagosomes and self-consumed by lysosomes. A growing body of evidence demonstrates a role for autophagy in the pathogenesis of diverse diseases, such as neuronal degeneration, pathogen infection, myopathy, aging and cancer (16). The role of autophagy in cancer is dependent on the tumor type, stage and genetic context. Autophagy can favor tumor cell survival under stresses such as hypoxia, nutrient limitation and certain forms of therapy. On the contrary, a persistent and excessive autophagic response may lead to a form of non-apoptotic cell death, termed autophagic cell death or type II programmed cell death (PCD), under certain circumstances (17,18). Due to its critical functions in regulating cell survival and cell death, autophagy has become one of the most attractive therapeutic targets for cancer treatment. However, few autophagy-modulating compounds have been approved for cancer therapies. Moreover, the dual role of autophagy in promoting both survival and death presents the dilemma of how to manipulate the autophagic response for cancer therapy. Therefore, tracing the molecular events and determining the mechanism by which increased AVs, initiated by addition of the autophagy-related drugs, cause cell death is essential to further our understanding of the role of autophagy in cancer therapy. In this regard, by systematically studying matrine-induced alterations in autophagy, we are able to characterize one potential autophagy modulator and explore the roles of autophagy-related drugs in cancer therapy.

In this study, we carefully evaluated the autophagic events induced by matrine in a gastric cancer cell line, SGC7901. For the first time, we observed that matrine promoted the accumulation of AVs accompanied by the attenuation of proteinase activity in lysosomes. Furthermore, we revealed that matrine altered the pH environment of lysosomes, thereby resulting in the inhibition of trafficking and proteolytic activation of lysosomal enzymes. Thus, our data indicate that matrine is a novel inhibitor of autophagy that blocks autophagic degradation.

Materials and methods

Chemicals

Matrine and oxymatrine (purity >99%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China. Rapamycin, nigericin and baflomycin A1 (BAF) were obtained from Fermentek Co., Ltd.

Abbreviations: AVs, autophagic vacuoles; BAF, Baflomycin A1; CQ, chloroquine; DMEM, Dulbecco’s modified Eagle’s medium; EBSS, Earle’s balanced salt solution; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; LC3, microtubule-associated protein light chain 3; LTR, Lysotracker Red DND-99; M6PR, mannose 6-phosphate receptor; MDC, monodansylcadaverine; PBS, phosphate-buffered saline; PCD, programmed cell death; TEM, transmission electron microscopy.
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(Jerusalem, Israel). Monodansylcadaverine (MDC), chloroquine diphosphate salt (CQ) and monensin was purchased from Sigma–Aldrich (St Louis, MO). Antibodies against LC3, Atg5, cathepsin B and cathepsin L were purchased from Sigma–Aldrich (St Louis, MO). Antibodies against β-actin, procathepsin D (mouse and rabbit), PDI and GRP78 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against LAMP-1, Rab5 and 58K were obtained from Abcam (Cambridge, UK). Antibodies against p-56 and Rab7 were obtained from Cell Signaling Technology (Danvers, MA). The LTR antibody was purchased from BD Pharmingen (Heidelberg, Germany). The cathepsin D antibody was obtained from Beijing Protein Innovation (Beijing, China).

Cell culture

SGC7901 and BGC823 cells were obtained from Peking University School of Oncology, AGS and HeLa were obtained from the American Type Culture Collection. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 µg/ml streptomycin. To induce autophagy, SGC7901 cells were treated with 20 mM rapamycin or incubated in Earle’s balanced salt solution (EBSS) for the last hour of treatment as indicated. For autophagy treatment experiments, matrine dissolved in the physiological saline was added into culture medium for various doses and time periods. Addition of physiological saline was as vehicle. A lysosomal inhibitor, BAF, was used to inhibit the autophagic flux.

Plasmids construction

The full length of cathepsin D was amplified by PCR using forward (5′-CCAAGTTATAGGCGGTCCAGCTTGC-3′) and reverse (5′-TTCCCTCCGGAAGGCGGCCAGCTTCGG-3′) primers, and the SGC7901 cDNA library as template. The PCR fragment of cathepsin D digested with HindIII and Sall was cloned into pEGFP-N2 plasmid (Clontech, Palo Alto, CA) for generation of transfection vector cathepsin D–enhanced green fluorescent protein (EGFP). A plasmid that encodes LC3 tagged with EGFP (EGFP-LC3) was constructed into pEGFP-C2 plasmid (Clontech) at EcoRI and BamHI sites using the specific forward (5′-CGGATATCTACGCAATCCATCCCCG-3′) primers. All constructs were verified by sequencing.

Immunofluorescence

Subconfluent SGC7901 cells growing on glass coverslips were incubated with vehicle or 2 mM matrine for 3 h. The cells were fixed in 3.7% paraformaldehyde and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) at room temperature. For blockage of non-specific binding sites, the cells were incubated with 5% normal goat serum in PBS for 2 h at room temperature. The indicated primary antibodies diluted in PBS containing 1% bovine serum albumin and 0.3% Triton X-100 were incubated with the treated cells at 4°C overnight. The secondary anti-IgG–FITC, goat anti-rabbit IgG–fluorescein isothiocyanate (Santa Cruz, CA), were incubated with the cells for 1 h at room temperature. 4′,6-Diamidino-2-phenylindole was used to stain nuclei.

Live-cell imaging

ForMDC staining, SGC7901 cells were trypsinized and labeled with 0.05 mM MDC in PBS at 37°C for 10 min. For transient transfection, constructs were verified by sequencing.

Assay of cathepsin B/D enzymatic activities

Briefly, SGC7901 cells were lysed in the assay buffer provided in the kits, and assayed kits according to manufacturer’s instructions. Briefly, SGC7901 cells were lysed in the assay buffer provided in the kits, and equal amounts of cell lysate from the control and matrine-treated group were incubated with 200 µM Ac-Arc-Arc-APC, a cathepsin B substrate, for 1 h at 37°C or 20 µM MCA-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-NH2 trifluoroacetate salt, a cathepsin D substrate, for 30 min at 37°C. The lysates containing 20 µM cathepsin B inhibitor Z-Phe-Phe-FCMK or 200 µg/ml cathepsin D inhibitor pepstatin A were used as negative controls. Free amino-4-trifluoromethylcoumarin or 7-methoxycoumarin–4-acetyl released by the cleavage were measured in a fluorometer reader at an excitation of 400 nm and emission of 505 nm or excitation of 328 nm and emission of 393 nm, respectively. The protein concentrations of cell lysates were determined using the Bradford Assay Kit (Bio-Rad, Hercules, CA), and the cathepsin B/D activities were normalized to the protein concentration.

Subcellular fractionation on Percoll gradient

The subcellular fractionation was performed as described previously (19). Briefly, cells were scraped into PBS and sedimented at 300g for 5 min followed by resuspension in a solution containing 250 mM sucrose, 2 mM ethylendiaminetetraacetic acid and 3 mM imidazole at pH 7.2. The cell suspension was homogenized by repeated passage through a 3 mm needle syringe. A postnuclear supernatant obtained after 800 g centrifugation was loaded over 8 ml of 27% Percoll (GE Healthcare, Little Chalfont, Buckinghamshire) in a solution containing 250 mM sucrose, 3 mM imidazole and 1 mg/ml bovine serum albumin at pH 7.2 on a 0.5 ml cushion of 2 M sucrose and sedimented at 20 000g for 40 min in a Beckman ultracentrifuge. The fractions were collected at 0.6 ml/fraction from top to bottom. The organelle markers and cathepsin D in each fraction were evaluated by western blotting.

Western blotting

Cells lysates were prepared by extracting proteins with lysis buffer [8M urea, 4% (3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate, 2 mM ethylenediaminetetraacetic acid, 20 mM Tris–HCl (pH 8.5) and 10 mM dithiothreitol] supplemented with protease inhibitors. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20, the membranes were probed with designated first and second antibodies. The immunoreactive signals were stained with chemiluminescent reagents kit (Millipore, Schwalbach). The chemical illuminances were detected by ImageQuant ECL (GE Healthcare). The images of western blotting were processed using ImageJ open source software (Wayne Rasband, NIH, Bethesda, MD).

Lysosomal pH measurements

Lysosensor Yellow/Blue DND-160 (Molecular Probes) was used as a lysosomal pH indicator. The pH calibration curve was generated according to a previous report (20). Briefly, SGC7901 cells were trypsinized and labeled with 5 µM Lysosensor Yellow/Blue DND-160 for 5 min at 37°C in complete DMEM medium without serum, and excessive dye was washed out using ice-cold PBS. The labeled cells were treated for 2 min with 10 µM monensin and 10 µM 5-iodoacetamido-4-trifluoromethylcoumarin or 7-methoxycoumarin–4-acetyl released by the cleavage were measured in a fluorometer reader at an excitation of 370 nm and emission of 400 nm or excitation of 328 nm and emission of 393 nm, respectively. The protein concentrations of cell lysates were determined using the Bradford Assay Kit (Bio-Rad, Hercules, CA), and the cathepsin B/D activities were normalized to the protein concentration.

Estimation of the distribution of matrine using mass spectrometry

SGC7901 cells with and without matrine treatment were fixed for 24 h were homogenized, and the crude organelles were prepared by differential centrifugation as

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of LC3-positive puncta are positively correlated with the amount of gel electrophoresis. During autophagy, the diffuse cytosolic form of (LC3-II), when resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. During autophagy, the diffuse cytosolic form of LC3-II is converted into the lipidated (phosphatidylethanolamine-conjugated) form, LC3-II, which aggregates onto the membranes of AVs (21). Therefore, the abundance of LC3-II and the number of LC3-positive puncta are positively correlated with the amount of intracellular AVs. As shown in Figure 1A and 1B, the treatment of SGC7901 cells with matrine resulted in a significant increase in the abundance of LC3-II protein and LC3 puncta staining was apparent, suggesting that the number of AVs increased in response to matrine. This observation was further supported by TEM analyses showing that the number of AVs was markedly increased in the cytoplasm of SGC7901 cells following treatment with matrine (Figure 1C). In addition, matrine treatment altered the LC3-II protein levels in a dose- and time-dependent manner (Figure 1D). To determine whether these changes in the autophagic process were specifically induced by matrine, we treated SGC7901 cells with either matrine or oxymatrine, an analog of matrine, in parallel experiments. As shown in Figure 1E, the LC3-II levels were significantly increased in response to the matrine treatment, but no obvious changes were observed after treatment with oxymatrine. Furthermore, three additional cell lines, HeLa (cervix cancer cell line), BGC823 and AGS (gastric cancer cell lines), displayed a similar increase in the abundance of LC3-II following treatment with matrine (Figure 1F). Taken together, these results indicate that matrine treatment results in the accumulation of AVs in tumor cells.

Matrine blocks autophagic degradation

Autophagy is a dynamic process consisting of five basic steps: initiation, elongation, closure, maturation and degradation (22). The accumulation of AVs in cells can occur due to either an increase in their rate of formation or a reduction in their rate of degradation. To distinguish between these two events, we examined the effects of matrine on LC3-II levels in SGC7901 cells in the presence or absence of BAF, a proton-ATPase inhibitor that blocks autophagic degradation but does not affect autophagosome formation (23). If matrine enhances the autophagy flux, a considerable increase in LC3-II would be expected upon treatment with matrine in the presence of BAF. However, compared with BAF treatment alone, dual treatment with matrine and BAF did not show any significant changes in the abundance of LC3-II (Figure 2A). In contrast, the induction of autophagy in response to nutrient starvation, which was induced by culturing cells in EBSS, the addition of BAF resulted in a significant increase in endogenous LC3-II levels (Figure 2B). This result suggests that matrine exerted different effects on the autophagic process induced by nutrient starvation conditions and probably plays a role as an autophagy inhibitor. In addition, we also examined the effects of matrine on LC3-II degradation under EBSS culture conditions. As shown in Figure 2C, matrine treatment resulted in a significant accumulation of LC3-II, which was similar to the result caused by BAF. Furthermore, we measured the autophagic flux by detecting the abundance of p62, an LC3 binding protein that is degraded via the autophagy pathway. Treatment with rapamycin, which is known to induce autophagy by inhibiting the mammalian target of rapamycin (mTOR), was used as a positive control (24). As shown in Figure 2D, the p-S6 (which is used as a readout for mTORC1 activity) levels were dramatically reduced by rapamycin treatment, but were not obviously affected by matrine or BAF; the LC3-II levels were slightly increased in rapamycin-treated cells, but were obviously increased in the cells treated with matrine or BAF; the p62 levels were decreased in response to rapamycin treatment, but were markedly increased in the cells treated with matrine or BAF. These data indicate that matrine functions are similar to BAF by blocking p62 degradation, whereas matrine functions are different from rapamycin by promoting p62 degradation. Furthermore, in SGC7901 cells with or without matrine treatment, we detected the abundances of Beclin-1 and ATG5-ATG12 conjugates, which have been reported to be upregulated after the induction of autophagy (25). However, neither Beclin-1 nor ATG5-ATG12 conjugates were significantly changed in response to matrine treatment (Figure 2E). Together, the evidences presented in this study suggest that the matrine-induced accumulation of AVs occurs due to the inhibition of autophagic degradation rather than the promotion of AV formation.
procathepsins, and then the procathepsins are proteolytically activated within endosomes or lysosomes (29). We chose cathepsin D as an indicator to evaluate whether the trafficking and maturation processing of cathepsins were impacted by matrine. SGC7901 cells with or without matrine treatment were homogenized and fractionated by ultracentrifugation through a Percoll gradient. A total of 16 gradient fractions were collected and analyzed by western blotting using antibodies against cathepsin D, GRP78 (ER marker), and \( \beta \)-actin as a loading control.

**Fig. 1.** Autophagic processes are affected by matrine treatment. (A) Accumulation of LC3-II in cells treated with matrine. SGC7901 cells that were treated with 2 mM matrine or vehicle (physiological saline) for 24 h were examined by Western blotting with the LC3 antibody, and \( \beta \)-actin was used as a loading control. (B) Immunofluorescence staining of LC3. SGC7901 cells with or without 2 mM matrine treatment for 48 h were stained with LC3 antibody. The distribution of endogenous LC3 was examined by confocal microscopy. Bar, 25 \( \mu \)m. A total of 200 cells from each group were randomly selected, and cells with LC3 punctate staining were counted for the evaluation of autophagosome formation. A cell with more than 10 LC3 spots was defined as an autophagic cell. The ratio of autophagic cells in the population of two hundred cells is shown in the right panel (\( n = 3, *** P < 0.001; \) Student's t-test). (C) Representative TEM images depicting ultrastructures of SGC7901 cells with or without matrine treatment for 24 h are presented at three magnifications (8 000×, 20 000× and 40 000×). N, nuclear material; M, mitochondrion; AV, autophagic vacuole. Bars, 1 \( \mu \)m. (D) Matrine increases the accumulation of LC3-II in a dose- and time-dependent manner. SGC7901 cells that were treated with increasing amounts of matrine or in a certain concentration of matrine over time were analyzed by western blotting for endogenous LC3. (E) Comparison of the effects of matrine and oxymatrine on the abundance of LC3-II in SGC7901 cells. (F) HeLa, BGC823 and AGS cancer cell lines were treated with or without matrine for 24 h, and the conversion of LC3-I to LC3-II was monitored by western blotting.

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Rab5 (early endosome maker), Rab7 (late endosome marker) and LAMP-1 (lysosome marker). Organelles, such as the ER and early and late endosomes, were mainly located in the light fractions (1–6; Figure 4A), and lysosomes were mainly distributed in the dense fractions (13–15; Figure 4B). These observations were in agreement with previous finding (19). In untreated cells, only a slight procathepsin D signal was detected in the light fractions, whereas a majority of cathepsin D was processed into a mature form and located in the dense fractions, suggesting that the delivery and maturation of cathepsin D was normal in control cells. When treated with matrine, procathepsin D was significantly increased in the light fractions, and mature cathepsin D was almost completely absent from the dense fractions (Figure 4C), implying that proteolytic cleavage steps during the maturation of cathepsin D and its delivery to the lysosomes were impaired by matrine in SGC7901 cells. To confirm that matrine affected the lysosomal targeting of procathepsin D, we compared the colocalization of cathepsin D with lysosomes using immunofluorescent staining in the SGC7901 cells with or without matrine treatment. As seen in Figure 4D, cathepsin D was frequently colocalized with LAMP-1 in control cells, whereas the majority of cathepsin D was not colocalized with LAMP-1 in the matrine-treated cells. In addition, we also evaluated the lysosomal targeting of newly synthesized cathepsin D-EGFP chimeras in living SGC7901 cells labeled with LTR. As expected, cathepsin D-EGFP was highly colocalized with LTR in the control cells, whereas the colocalization patterns were nearly abolished in the matrine-treated cells (Figure 4E). Collectively, these data demonstrate that the lysosomal targeting and proteolytic activation of cathepsin D are disrupted following matrine treatment.

We also examined the distribution of LC3 in these organelle preparations, as shown in Figure 4E. Compared with LC3-I, LC3-II was found obviously upregulated in both light fractions (2–6) and dense fractions (13–15) after matrine treatment, which further supporting the conclusion that matrine blocks the degradation of LC3-II. More importantly, LC3-II accumulation was even observed in lysosomal fractions (13–15) in matrine treated cells, suggesting that autolysosome formation remained unaffected following matrine treatment. This is in complete agreement with our previous results showing the colocalization of EGFP-LC3 and LTR (Figure 3A).

**Fig. 2.** Autophagic degradation in SGC7901 cells is inhibited by matrine. (A) The conversion of LC3-I to LC3-II was assayed in the matrine-treated cells with or without BAF. SGC7901 cells were cultured in DMEM complete medium with or without 2 mM matrine in the presence or absence of 200 nM BAF for 2h. Western blotting was used to monitor LC3 levels. (B) SGC7901 cells were cultured in DMEM complete medium or EBSS nutrient starvation medium in the presence or absence of 200 nM BAF for 4h, and the corresponding changes in LC3 were monitored by western blotting. (C) SGC7901 cells cultured in DMEM complete medium or EBSS nutrient starvation medium were cultured with or without 2 mM matrine for 2h. The conversion and degradation of LC3 were examined by western blotting. (D) The accumulation of p62 in cells treated with autophagy-modulating compounds. SGC7901 cells were treated with 2 mM matrine, 20 nM rapamycin or 200 nM BAF for 24h as indicated, and western blotting was used to analyze the status of p62, p-S6 and LC3. All the data for comparison of the intensities of western blotting in the figures above were statistically estimated and represented as means ± SEMs of three independent experiments. N.S., not significant; *P < 0.05; Student’s t-test. (E) The effect of matrine on the abundances of ATG5-ATG12 conjugates and Beclin-1 in SGC7901 cells.
Procathepsin D is trapped in the Golgi apparatus in response to matrine treatment

Matrine treatment impairs the delivery of the majority of procathepsin D to the lysosome; therefore, we sought to determine the distribution of procathepsin D among subcellular organelles. Considering that trace amounts of mature cathepsin D were observed in the matrine-treated cells, we used anti-procathepsin D antibodies to address the above question. As observed by confocal microscopy (Figure 5), the level of procathepsin D was markedly increased in matrine-treated cells relative to the controls. An overlay of these images revealed that procathepsin D was only partially colocalized with Rab7 or PDI regardless of the matrine treatment (Figure 5A and 5B), whereas the colocalization of procathepsin D with Golgi 58K was significantly enhanced following matrine treatment (Figure 5C). Thus, the elevated level of procathepsin D was not retained within the late endosome or ER after matrine treatment but was trapped within the Golgi apparatus.

Fig. 3. The effects of matrine on autolysosome formation and lysosomal hydrolase maturation. (A) Colocalization analysis of EGFP-LC3 and LTR. SGC7901 cells were transiently transfected with EGFP-LC3 and cultured in complete medium with or without 2 mM matrine for 24 h. The fluorescent signals of EGFP-LC3 and LTR were sequentially scanned with different channels using confocal microscopy. Bar, 7.5 µm. (B) Analyzing the autolysosome formation through MDC staining. SGC7901 cells treated with vehicle, 2 mM matrine or 200 nM BAF for 24 h were stained by MDC, and delivered to confocal microscope for image acquisition. Bar, 25 µm. (C) The enzymatic activities of cathepsin B and cathepsin D in SGC7901 cells with or without matrine treatment. Lysates extracted from SGC7901 cells treated with or without matrine were incubated with the corresponding peptides conjugated with fluorescent probe as substrates. The fluorescent signals at different wavelengths were quantitatively acquired for the estimation of enzyme activities. The results from three independent experiments are expressed as the mean ± SD. (***P < 0.001; Student’s t-test). (D) The maturation state of the cathepsins in SGC7901 cells. SGC7901 cells with or without matrine treatment for 48 h were analyzed by western blotting for the processing of endogenous precursor forms of cathepsins B, D and L to their mature forms. p, precursor; i, intermediate; m, mature. β-actin was used as a loading control. (E and F) The abundance of procathepsin D was regulated by matrine in a dose- and time-dependent manner. The SGC7901 cells were treated with various doses of matrine over time, and the corresponding changes in procathepsin D abundance were examined by western blotting.
Fig. 4. Effects of matrine on lysosomal targeting and the proteolytic activation of cathepsin D. (A–C) The postnuclear supernatants from SGC7901 cells with or without matrine treatment were fractionated using a Percoll gradient ultracentrifuge, as described in the Materials and methods. The forms of cathepsin D and organelle markers were verified by western blotting in each fraction. (A) Subcellular distributions of Rab5 (early endosome), GRP78 (ER) and Rab7 (late endosome). (B) Subcellular distributions of LAMP-1 (lysosome). (C) Subcellular distributions of various isoforms of cathepsin D. (D) Subcellular distributions of endogenous cathepsin D and LAMP-1. SGC7901 cells with or without matrine treatment were fixed and incubated with cathepsin D and LAMP-1 antibodies. The immunofluorescent signals were acquired by confocal microscopy and analyzed using LAS AF software. Bars, 25 μm. (E) Subcellular distributions of lysosomes and newly synthesized cathepsin D-EGFP. Living SGC7901 cells were transiently transfected with cathepsin D-EGFP, treated with or without matrine, stained with LTR and delivered to the confocal microscopy facility for image acquisition. The yellow signals are indicative of colocalization. Bars, 7.5 μm. (F) Subcellular distribution of endogenous LC3-I/II.
Fig. 5. Procathepsin D is trapped in the Golgi apparatus in response to matrine treatment. SGC7901 cells with or without matrine treatment for 48h were fixed, permeabilized and analyzed using monoclonal or polyclonal antibodies against procathepsin D to detect endogenous cathepsin D, polyclonal antibodies against Rab7 (A) and PDI (B), and a monoclonal antibody against 58K (C) to recognize subcellular organelles. Goat anti-mouse antibodies labeled with TRITC and goat anti-rabbit antibodies labeled with fluorescein isothiocyanate were used as secondary antibodies. Cells were visualized by confocal microscopy. Yellow signals are indicative of colocalization. Bars, 25 µm.
Matrine inhibits the acidification of the endosome and lysosome in SGC7901 cells

It has been demonstrated that an acidic pH in the lumina is required for effective trafficking and proteolytic activation of lysosomal proteases. Most lysosomal proteases, including cathepsins, are sorted and delivered into endosomes and lysosomes through the mannose 6-phosphate receptor (M6PR) system in the trans-Golgi network. An acidic pH in the lumina is required for the dissociation of the proenzymes of the M6P-M6PR complex and retrograde transport of M6PR from endosomes to the Golgi network (29, 30). Because matrine is a weakly basic compound, we postulated that matrine traps protons in acidic cellular compartments, such as endosomes and lysosomes, thereby elevating the intracellular pH. To test this hypothesis, we analyzed the intracellular distribution of matrine in SGC7901 cells using differential centrifugation and mass spectrometry identification. As shown in Figure 6A, among five separated fractions, matrine showed the greatest accumulated in the crude lysosomal fraction. To further monitor alterations in the lysosomal pH caused by matrine accumulation, we utilized an acidotropic fluorescence probe, LysoSensor Yellow/Blue DND-160, to selectively label the acidic organelles of living cells. A lysosomotropic agent, CQ, which inhibits lysosome acidification, was used as positive control to elevate the lysosomal pH. A calibration curve (Figure 6B) revealed that the intensity ratios of fluorescence, acquired at 535 nm emission with 340 and 380 nm excitation, were directly proportional to the lysosomal pH obtained using MES buffers. The lysosomal pH in SGC7901 cells treated with matrine (pH 5.58) was significantly higher than in the control (pH 4.78) (Figure 6C). Moreover, the pH shift in response to matrine was similar to the lysosomal pH change in the CQ-treated cells (pH 5.34) (Figure 6C). Thus, these data strongly support our hypothesis that the effect of matrine on the pH of the endosome and lysosome inhibits the trafficking and proteolytic activation of lysosomal proteases.

Discussion

In this report, we systematically studied the effect of matrine treatment on AV accumulation, the inhibition of posttranslational transport and the maturation of the lysosomal proteases, and the elevation of the lysosomal pH. We propose that the entrapment of matrine in the endosomes and lysosomes elevates their pH, which disturbs the trafficking of lysosomal proteases from the Golgi apparatus to endosomes and blocks their proteolytic activation. Therefore, matrine is capable of causing lysosome dysfunction and the inhibition of autophagic degradation.

The accumulation of AVs has been observed by TEM in several cancer cell lines treated with matrine. Based on these observations, most investigators have concluded that matrine induces autophagy and triggers autophagic cell death (13–15). However, the accumulation of AVs is not a unique feature that indicates the enhancement of autophagosome formation, but could also be due to the blockage of autophagic degradation, or both. zVAD, a pan-caspase inhibitor, was reported to induce autophagic cell death in L929 cells because massive AVs were found in dying cells after zVAD treatment (17). However, recent studies have demonstrated that zVAD triggered necrosis by inhibiting apoptosis and autophagic flux, indicating that this chemical not only inhibited caspases but also suppressed the activity of lysosomal proteases, which subsequently blocked autophagic degradation (31). Imitinib, an ALK kinase inhibitor, is regarded as an inducer of autophagy in a variety of mammalian cells (32). However, Yogalingam et al. (33) found that imatinib promoted the accumulation of autophagosomes by impairing the function of lysosomal hydrolases and the subcellular trafficking of lysosomes. In this study, we demonstrate that the accumulation of AVs in matrine-treated SGC7901 cells was due to the inhibition of autophagic degradation rather than the enhancement of autophagic flux. This conclusion is supported by several important observations: (i) the degradation of LC3-II was impaired, (ii) a substrate of autophagy was notably accumulated, (iii) the enzymatic activities of several important lysosomal hydrolases were markedly attenuated, and (iv) the pH of endosomes and lysosomes was significantly elevated in response to matrine treatment.

Based on the morphologic appearances of the dying cells, there are two types of PCD: apoptotic cell death (Type I) and autophagic cell death (Type II) (34). Whether Type II PCD is cell death with autophagy or cell death by autophagy remains poorly defined. As described above, matrine does not induce autophagosome formation but blocks autophagic degradation. Thus, autophagy is not activated by matrine treatment and probably does not directly trigger cell death in this case. In fact, it has been reported that the activation of both apoptotic pathways, i.e. the intrinsic mitochondria-mediated pathway and the extrinsic death receptor-mediated pathway, was detected during matrine-induced cell death (1, 35). Is it possible that the blockage...
of autophagic degradation triggers the apoptotic procedure? It has been demonstrated that inhibiting the formation of autolysosome in starved HeLa cells by LAMP2 knockdown caused AV accumulation and subsequent apoptosis (36). Similarly, Boya et al. (37) found that AV accumulation induced by hydroxychloroquine triggered mitochondrial-mediated apoptosis in HeLa cells. Moreover, Hou et al. (38) recently demonstrated that TRAL-mediated autophagic response counter-balances the TRAL-mediated apoptotic response by sequestration of large caspase-8 subunit in autophagosomes and its subsequent degradation in lysosomes. Therefore, inhibition of autophagic response would sensitize the cells to apoptosis. In the study, we also observed that 24 h incubation of matrine caused G1/G0 cell cycle arrest in SGC7901 cells, whereas prolong incubation (72 h) with matrine resulted in Bax mitochondrial translocation and cytochrome c releasing (data not shown).

Taken together, these findings suggest that the blockage of autophagic degradation by matrine induces apoptotic death. However, the reciprocal influence of AV accumulation and apoptotic cell death in matrine-treated cells requires further investigation.

Recent reports have demonstrated that the genetic or pharmacological inhibition of autophagy can sensitize cancer cells or tumors to various cancer therapies, such as DNA-damaging agents, anthor-mones and radiation (39). Therefore, the inhibition of autophagy is therapeutically beneficial for anticancer therapies. Currently, there are nearly 20 clinical trials registered with the National Cancer Institute (http://www.cancer.gov/clinicaltrials) that are evaluating whether the inhibition of autophagy has synergistic effects in a variety of human cancers (22). However, few autophagy inhibitors have been approved by the Food and Drug Administration. Hydroxychloroquine, a derivative of CQ, can inhibit autophagic degradation and is the most frequently used agent in registered trials. However, the long-term use of CQ and its derivate cause ocular toxicities, such as retinopathy (40). Thus, novel inhibitors of autophagy with a lower toxicity and a better therapeutic index are needed. Our findings demonstrate that matrine functions as an inhibitor of autophagy by blocking autophagic degradation. Matrine has also been widely used in clinical settings to treat different diseases; therefore, the clinical information regarding therapeutic efficacy, toxicity and dosage of matrine is available. Importantly, the curative effects of matrine in cancer have recently been acknowledged in China. This information endows matrine as a promising anticancer drug in the near future. Furthermore, it is worth noting that autophagy levels are tumor type dependent. A high basal level of autophagy is detected in cancer cells with activated Ras mutations and is required for pancreatic cancer growth (41,42). As a potent autophagy inhibitor, matrine is expected to have therapeutic effects on cancers with high basal autophagy addiction.

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References

Matrine blocks autophagic degradation

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