Original Article

Inhibition of autophagy induced by proteasome inhibition increases cell death in human SHG-44 glioma cells

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Aim: The ubiquitin-proteasome system (UPS) and lysosome-dependent macroautophagy (autophagy) are two major intracellular pathways for protein degradation. Recent studies suggest that proteasome inhibitors may reduce tumor growth and activate autophagy. Due to the dual roles of autophagy in tumor cell survival and death, the effect of autophagy on the destiny of glioma cells remains unclear. In this study, we sought to investigate whether inhibition of the proteasome can induce autophagy and the effects of autophagy on the fate of human SHG-44 glioma cells.
Methods: The proteasome inhibitor MG-132 was used to induce autophagy in SHG-44 glioma cells, and the effect of autophagy on the survival of SHG-44 glioma cells was investigated using an autophagy inhibitor 3-MA. Cell viability was measured by MTT assay. Apoptosis and cell cycle were detected by flow cytometry. The expression of autophagy related proteins was determined by Western blot.
Results: MG-132 inhibited cell proliferation, induced cell death and cell cycle arrest at G_2/M phase, and activated autophagy in SHG-44 glioma cells. The expression of autophagy-related Beclin-1 and LC3-I was significantly up-regulated and part of LC3-I was converted into LC3-II. However, when SHG-44 glioma cells were co-treated with MG-132 and 3-MA, the cells became less viable, but cell death and cell numbers at G_2/M phase increased. Moreover, the accumulation of acidic vesicular organelles was decreased, the expression of Beclin-1 and LC3 was significantly down-regulated and the conversion of LC3-II from LC3-I was also inhibited.
Conclusion: Inhibition of the proteasome can induce autophagy in human SHG-44 glioma cells, and inhibition of autophagy increases cell death. This discovery may shed new light on the effect of autophagy on modulating the fate of SHG-44 glioma cells.

Keywords: proteasome inhibitors; autophagy; cell death

Introduction

In eukaryotic cells, proteins are degraded by two major pathways, the ubiquitin proteasome system (UPS), for the disposal of short-lived proteins, and the lysosomal system, for the degradation of intracellular and extracellular proteins[1]. The proteasome is involved not only in protein degradation, but also in cellular differentiation, antigen presentation and cell cycle modulation[2]. Therefore, inhibition of proteasome activity has become a new chemotherapy strategy and many chemicals and natural compounds have proven to be effec-

tive at inducing tumor cell death by inhibiting proteasome activity[3–6]. Autophagy, as part of the lysosomal system, is an evolutionarily conserved cellular strategy to engulf and degrade long-lived cytosolic proteins and organelles to provide substrates for energy metabolism and to recycle amino acids, fatty acids, and nucleotides for the biosynthetic needs of cells[7]. Although autophagy can be induced in many different tumor cell lines, including malignant glioma cells, by different ways such as chemical agents or ionizing radiation[8–10], the impact of autophagy on the death or survival of tumor cells is still unclear. Generally, autophagy plays dual roles in cellular death or survival; one is to induce type II programmed cell death, which is different from apoptosis and is often termed autophagic cell death, whereas the other is to recycle cellular components to sustain metabolism and to prevent the accu-

mulation of damaged, toxic proteins and organelles during stress[11]. A recent study reported that impairment of the UPS induced autophagy[12], whereas genetic ablation of autophagy results in the accumulation of ubiquitinated proteins[13], indicating that the UPS and autophagy serve as two complementary, reciprocally regulated protein degradation systems.

Malignant gliomas account for approximately 70% of the 22,500 new cases of malignant primary brain tumors that are diagnosed in adults in the United States each year[14]. Although relatively uncommon, malignant gliomas are associated with high morbidity[15]. It is very difficult to eliminate malignant glioma cells, because they cannot be completely removed surgically and they are resistant to postoperative radiotherapy and chemotherapy. Recent findings show that induction of autophagy suppresses the proliferation and tumorigenicity of human glioma cells[16–18]. However, other reports show that autophagy makes glioma survive extracellular stress[19, 20]. Because the proteasome has become a target for tumor therapy, in this study we sought to investigate whether inhibition of the proteasome can induce autophagy and the effects of autophagy on the fate of human SHG-44 glioma cells.

Materials and methods
Reagents
RPMI-1640 medium was from Gibco (Rockville, MD, USA), fetal bovine serum (FBS) was from Life Technologies (Grand Island, NY, USA) and MG-132 was from Calbiochem (San Diego, CA, USA). MG-132 was dissolved in PBS to a storage concentration of 50 µmol/L. Antibodies against Beclin-1 and LC3 were from Cell Signaling Technology (Beverly, MA, USA). Actin-β and other reagents were from Sigma Company (St Louis, MO, USA).

Cell line and culture
Human SHG-44 glioma cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). SHG-44 cells were cultured in RPMI-1640 medium supplemented with 10% (*v/v*) heat inactivated fetal calf serum, 2 mmol/L glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL), and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. Cells in the mid-log phase were used in the experiments.

Cell viability assay
SHG44 glioma cells were seeded onto 96-well microplate (3×10^5 cells/well) and cultured overnight. The cells were divided into two groups; one group was treated with 1.5, 3.0, and 6.0 µmol/L MG-132 for 48 h at 37 °C, and the other group was treated with 10, 20 and 30 mmol/L 3-MA under the same conditions. In another experiment, SHG-44 cells were also divided into two groups, with group one treated with 6.0 µmol/L MG-132 for 12 h, 24 h, and 48 h and group two co-treated with 10 mmol/L 3-MA for the same duration. Cell viability was assessed by MTT assays. The absorbance value (*A*) at 570 nm was read using an automatic multi-well spectro-

photometer (Bio-Rad, Richmond, CA, USA). Each concentration included four wells and each test was repeated four times.

Proteasome chymotrypsin-like activity assay
After SHG-44 cells were cultured and treated with 6.0 µmol/L MG-132 for 12, 24, or 48 h at 37 °C in six-well plates, the cells were thoroughly scraped from the culture dishes with a cell scraper and washed with cold PBS. After centrifugation for 10 min at 10 000×*g*, the cell pellets were suspended in ice-cold buffer containing 50 mmol/L Tris-HCl, pH 7.5, 20 µmol/L ATP, 5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, and 20% glycerol and homogenated with a glass Pyrex micro-homogenizer (20 strokes). The homogenate was centrifuged at 15 000×*g* for 10 min at 4 °C to obtain the supernatant. Protein concentration was determined using protein assay kits (Bio-Rad Laboratories). Ten microliters (1 µg/µL) of each freshly made supernatant was incubated in a 96-well plate at 37 °C for 30 min with 10 µL of 300 µmol/L of Succinyl-LLVY-AMC (Calbiochem, San Diego, CA, USA) and 85 µL of assay buffer (20 mmol/L Tris-HCl, pH 7.5, and 20% glycerol). Release of fluorescent AMC was measured with a spectrofluorometer (Perkin-Elmer Life and Analytical Sciences, Inc, Wellesley, Mass) at 440 nm with an excitation wavelength of 380 nm.

Detection of apoptosis and cell cycle
After 12 h of starvation in serum serum-free DMEM/F12, SHG44 cells were incubated with 6.0 µmol/L MG-132 or co-treated with 10 mmol/L 3-MA for 48 h at 37 °C, both attached and floating cells were harvested using 0.25% trypsin, washed with phosphate-buffered saline (PBS), counted and adjusted to 1×10^6 cells/mL. The cells were fixed in 70% ethanol at 4 °C overnight, treated with 100 mg/L RNase at 37 °C for 30 min and stained with 50 mg/L propidium iodide for 30 min. The cells were analyzed using flow cytometry (FAC2Scan, Bection Dickinson, San Jose CA, USA). The rate of apoptosis and cell cycle were analyzed using CELLquest software (Bection Dickinson). Data acquisition was conducted by collecting 20 000 cells per tube and the number of viable and apoptotic cells was determined for each experimental condition.

Transmission electron microscopy
After SHG-44 cells were cultured in 6-well plates (3×10^5 cells/well) and incubated with 6.0 µmol/L MG-132 for 48 h, they were harvested using 0.25% trypsin, washed with phosphate-buffered saline (PBS) and collected by centrifugation for 10 min at 10 000×*g*, followed by treatment as described by Watkins and Cullen[21]. Briefly, the cells were fixed in ice-cold 2.5% glutaraldehyde in PBS (pH 7.3), rinsed with PBS and postfixed in 1% osmium tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30%−90%), and embedded in Epon (Energy Beam Sciences, Agawam, MA, USA). Semi-thin (300 nm) sections were cut using a Reichart Ultracut, stained with 0.5% toluidine blue, and examined under a light microscope. Ultrathin sections (65 nm) were stained with 1% uranyl acetate and 0.1% lead citrate, and examined on a JEM2000EX transmission electron micro-

scope (JEOL, USA).

Detection of AVOs

SHG-44 cells (3×10^5) were plated on coverslips and allowed to attach for 24 h. Following treatment with 6.0 µmol/L MG-132 or co-treatment with 10 mmol/L 3-MA for 48 h, the cells were stained with 1 µg/mL acridine orange for 15 min, washed with PBS, and examined under an Olympus fluorescence microscope at ×20 objective lens magnification.

Preparation of subcellular fractions

SHG-44 cells were cultured in six-well plates (3×10^5 cells/well) for 24 h and treated with 6.0 µmol/L MG-132 or co-treated with 10 mmol/L 3-MA for 48 h at 37 °C. Cells were thoroughly scraped from the culture dishes with a cell scraper and washed with cold PBS. After centrifugation for 10 min at 2000 r/min, the cell pellets were suspended in ice-cold buffer containing 15 mmol/L Tris, pH 7.6, 250 mmol/L sucrose, 1 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 1 mmol/L EGTA (ethylene glycol-*bis* (beta-aminoethyl ether) tetraacetic acid), 1 mmol/L dithiothreitol, 1.25 mg/mL pepstatin A, 10 mg/mL leupeptin, 2.5 mg/mL aprotinin, 1.0 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.1 mmol/L Na_3VO_4, 50 mmol/L NaF, and 2 mmol/L $Na_4P_2O_7$ and homogenated with a glass Pyrex microhomogenizer (20 strokes). Homogenates were centrifuged at $800\times g$ at 4 °C for 10 min to obtain P1 pellets (containing the heaviest cellular components, including the nuclei and secondary lysosomes) and supernatants (S1). The S1 was further centrifuged at $10\,000\times g$ at 4 °C for 10 min to obtain crude cell membrane mitochondrial pellet (P2) and its supernatant (S2). The S2 fraction was centrifuged again at $165\,000\times g$ at 4 °C for 1 h to obtain the cytosol S3 and the microsomal pellet P3, containing intracellular membrane structures such as the endoplasmic reticulum and Golgi. All pellet fractions were suspended in homogenization buffer containing 0.1% Triton X-100. The protein content of each fraction was determined using Bio-Rad protein assay reagent.

Gel electrophoresis and Western blotting

Equal protein amounts were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to PVDF membranes. The membranes were blocked with 3% bovine serum albumin in TBS for 30 min and then incubated overnight at 4 °C with the following primary antibodies: Beclin-1(1:1000) and LC3 (1:1000). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Cell signaling technology) for 90 min at room temperature. The blots were developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway NJ, USA) and developed on Kodak X-omat LS film (Eastman Kodak Company, New Haven CT, USA). Densitometry was performed with Kodak ID image analyses software (Eastman Kodak Company).

Statistical analysis

All data represent at least 4 independent experiments and are expressed as means±SD. Statistical comparisons were made using Student's *t*-test. *P*-values of less than 0.05 were considered to represent statistical significance.

Results

MG-132 inhibits cell proliferation and proteasome activity

After SHG-44 cells were treated with MG-132 or 3-MA at the indicated concentrations for 48 h, cell viability was assessed by MTT assay. As shown in Figure 1, SHG-44 cell growth was effectively inhibited by MG-132 or 3-MA in a dose dependent manner. There was no significant effect of 10 mmol/L 3-MA on the proliferation of SHG-44 glioma cells, whereas the greatest inhibition of cell proliferation was achieved with 6.0 µmol/L MG-132. Moreover, 6.0 µmol/L MG-132 inhibited proteasome activity in a time-dependent manner.
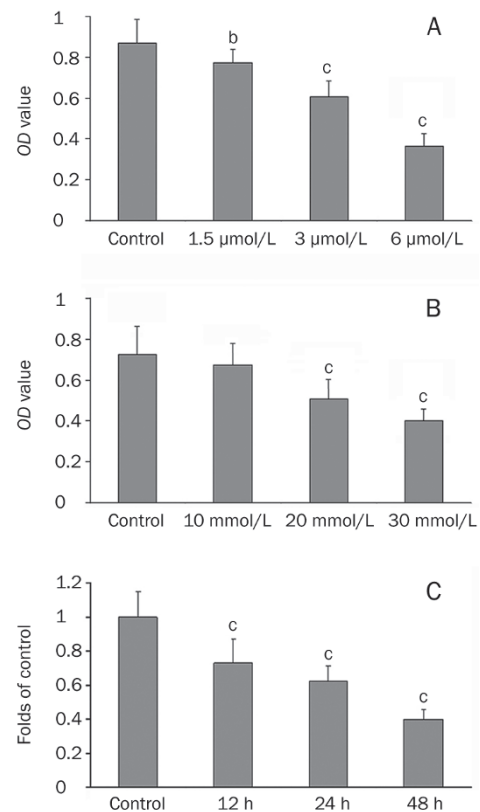

Figure 1. MTT assay and proteasome activity assay. (A) MTT assays of MG-132. (B) MTT assay of 3-MA. (C) Proteasome activity after MG-132 6.0 µmol/L treatment. $^{b}P<0.05$, $^{c}P<0.01$ compared with control group.

MG-132-induced autophagy is reversed by 3-MA

To determine the effect of proteasome inhibition on autophagy, transmission electron microscopy was performed. As shown in Figure 2A, many vesicles including double-membraned autophagosomes and single-membraned autophagolysosomes, containing entrapped cytoplasm or entire organelles, were induced by treatment of cells with MG-132. Lamellar structures were also observed in the cytoplasm. In contrast,
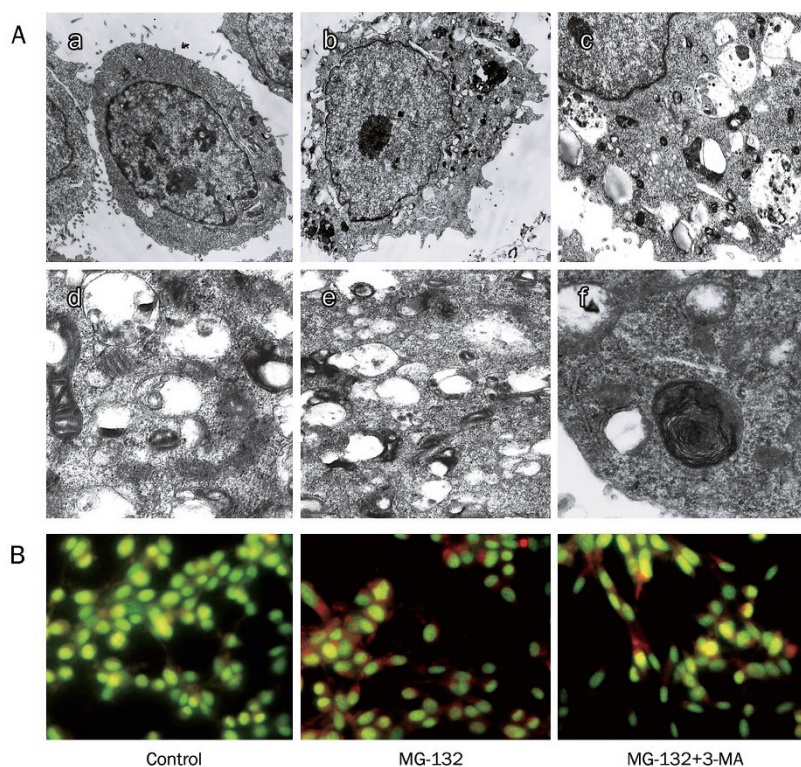
Figure 2. Morphological alterations in autophagy. (A) Observation under TEM. (a) Control group. (b) MG-132 group, vesicles formed in cytoplasm. (c) Vesicles in cytoplasm containing cytoplasm or organelles. (d) and (e) Autophagosomes and autolysosomes. (f) Lamellar structure. (B) Detection of autophagy by acridine orange. In the MG-132 group, SHG-44 cells emitted bright red fluorescence, which was counteracted by 3-MA.

there were relatively few vesicles induced in control cells.

For further detection of autophagy, we used the lysosomotropic agent acridine orange, which emits bright red fluorescence in acidic vesicles but fluoresces green in the cytoplasm and nucleus[22, 23]. As shown in Figure 3B, vital staining of SHG-44 cells with acridine orange showed the accumulation of AVO in the cytoplasm of cells exposed to 6.0 µmol/L MG-132, but this was counteracted by addition of 3-MA (10 mmol/L).

To obtain clues as to the mechanism by which the proteasome inhibitor MG-132 induces autophagy, Western blot analysis was performed. Beclin-1 is a Bcl-2-interacting protein that promotes autophagy, associated with inhibition of cellular proliferation and tumorigenesis[24]. As shown in Figure 3A, MG-132 significantly up-regulated the expression of Beclin-1. However, after 3-MA was added into the culture medium, the increase of protein Beclin-1 was inhibited. Microtubule-associated protein light chain 3 is another marker of autophagy and it is synthesized as a proform that is cleaved by a protease to become LC3-I[25]. LC3-I conjugates covalently with a neutral lipid PE (phosphatidylethanolamine) to form lipidated LC3-II, which has a faster mobility on immunoblots. Thus, LC3 antibody was able to recognize both the upper LC3-I band and the lower LC3-II. As shown in Figure 3B, after a 48-h incubation with MG-132, the LC3-I protein level was significantly increased in P2, P3, and S3, but the LC3-II protein level was significantly increased in the pellet fractions, which indicated that MG-132 up-regulated the expression of LC3-I and part of LC3-I was converted into LC3-II. However, after the addition of 3-MA, compared with the MG-132 group, no significant alteration in the expression of LC3-I was found, but the con-

version of LC3-I to LC3-II was significantly decreased.

3-MA increases MG-132 induced cell death and cell cycle arrest at G$_2$/M

To investigate the effect of inhibition of autophagy on cell viability, MTT assays were performed. As shown in Figure 4A, 10 mmol/L 3-MA significantly enhanced the inhibition of cell viability induced by MG-132 at 24 h and 48 h, which suggested that inhibition of autophagy effectively inhibited cell viability.

For further study, flow cytometry with PI staining was used to analyze the rate of apoptosis and cell cycle progression, based on the principle that after DNA fragmentation, permeabilized cells exhibit a reduced chromatin stainability and accessibility to fluorochromes[26, 27]. As illustrated in Figure 4, compared with the MG-132 group, the apoptosis rate of the SHG-44 cells co-treated with 3-MA significantly increased from 19.34% to 28.65% ($P<0.05$) and the percentage of cells in G$_2$/M phase increased from 23.65% to 27.75% ($P<0.05$), accompanied by a decrease in cells found in G$_1$ phase.

Discussion

Supporting previous work showing that the impairment of proteasome activity induces apoptotic tumor cell death and cell cycle arrest at G$_2$/M phase[4], we prove in this study that inhibition of the proteasome by MG-132 decreases cell proliferation, induced cell death and G$_2$/M arrest and activates autophagy in SHG-44 glioma cells. Furthermore, we found that 6.0 µmol/L MG-132 effectively inhibited proteasome activity and induced cell death and cell cycle arrest in SHG-
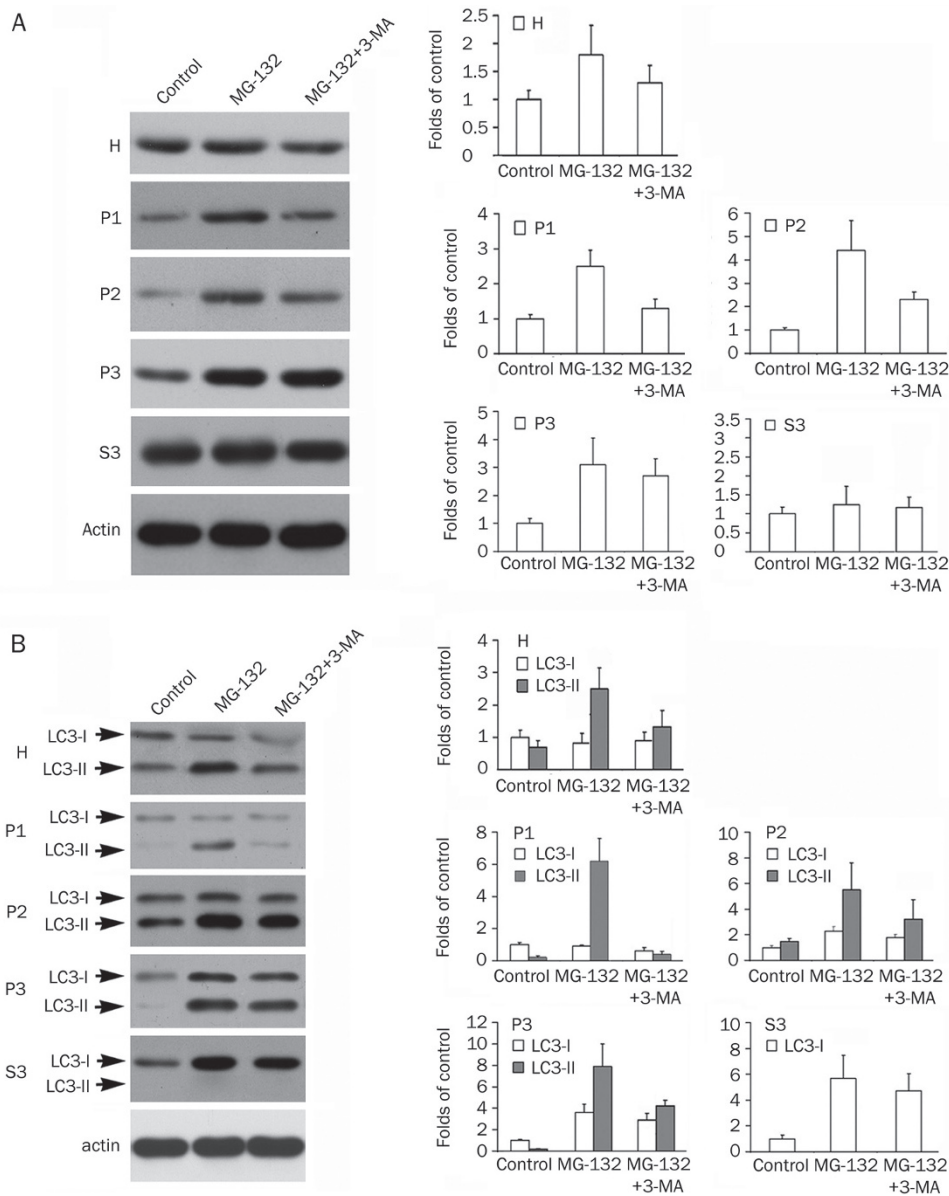
A

B

Figure 3. The expression of autophagy related proteins. (A) Beclin-1 was up-regulated in each cellular fraction in the MG132 group. However, 3-MA counter-acted the up-regulation of Beclin-1 in P1 and P2. (B) LC3-1 was also up-regulated in P2, P3, and S3 fraction and LC3-II increased in Homogenate and Pellets. After co-treatment with 3-MA, LC3-I decreased and the conversion of LC3-I to LC3-II was also reduced.

44 glioma cells, which was consistent with a previous report[5]. Moreover, we found that autophagy was activated after SHG-44 glioma cells were incubated for 48 h with 6.0 µmol/L MG-132.

The proteasome and autophagy are regarded as complementary systems for protein degradation[28]. It was reported that impairment of the UPS led to accumulation of misfolded proteins, which triggered endoplasmic reticulum stress and activated the downstream IRE1/c-Jun NH$_2$-terminal kinase pathway to induce autophagy[29]. In malignant glioma cells, autophagy can be induced by different means, such as temozolomide, or by the activation of the neurotrophin receptor TrkA[30–32]. In this study, autophagy was demonstrated first by TEM, which is considered the standard for the morphological evaluation of autophagy[25]. As our results shown, double-membraned autophagosomes and single-membraned autophagolysosomes containing entrapped cytoplasm and organelles were observed under TEM. Furthermore, autophagy was shown at the molecular level by biochemical methods. Our results showed that the autophagy-related proteins Beclin-1 and LC3-I were significantly up-regulated after a 48 h incubation with MG-132, in parallel with the conversion of LC3-I into its active form, LC3-II.

Studies have reported that activated autophagy allows tumor cells to survive extracellular stress. Rapamycin-induced autophagy, for example, can protect various tumor cell lines against apoptosis induced by general apoptotic stimuli[19], and DNA damaging agents temozolomide and Etoposide can induce an autophagy-associated ATP surge and protect malignant glioma cell lines[20]. However, other reports have shown that inducing autophagy can cause cell death in tumors[33]. Thus, the effect of autophagy on cellular survival or death is
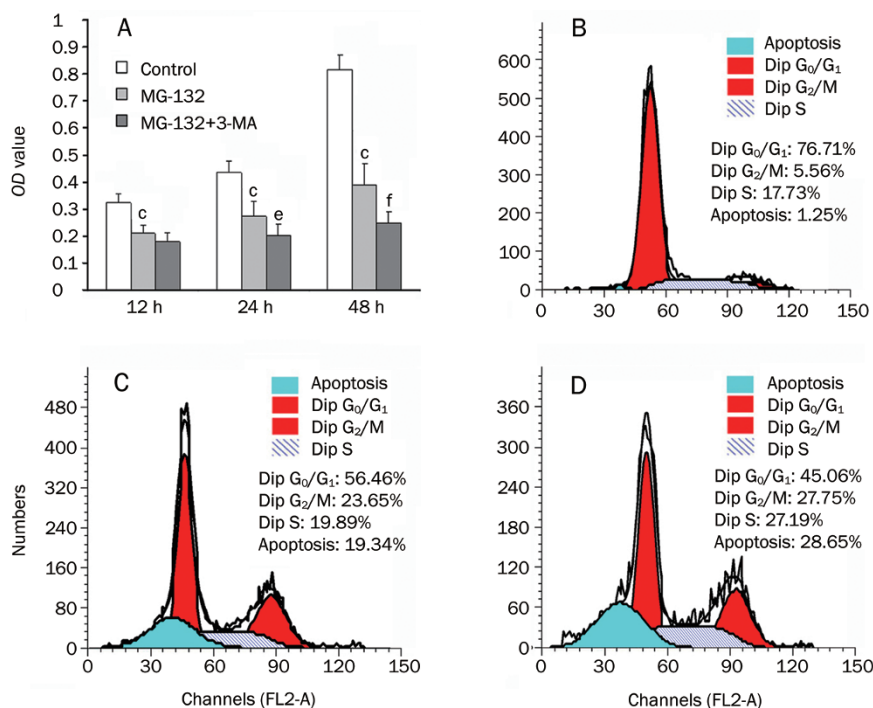
Figure 4. 3-MA enhanced the effects of MG-132 on cell viability and cell death. (A) results of MTT assays. Compared with the MG-132 group, cell viability decreased significantly at 24 h and 48 h when co-treated with 3-MA. (B, C, and D) are the flow cytometry results of the control group, MG-132 group and MG-132+3-MA group, respectively. Compared with the MG-132 group, the apoptosis rate of the SHG-44 cells that were co-treated with 3-MA significantly increased from 19.34% to 28.65% ($P<0.05$) and the percentage of cells in G_2/M phase increased from 23.65% to 27.75% ($P<0.05$), which was accompanied by a decrease in cells at G_1 phase ($P<0.05$). [c]$P<0.01$, compared with control group at each time point; [e]$P<0.05$, compared with MG-132 group at 24 h; [f]$P<0.01$, compared with MG-132 group at 48 h.

still a matter of dispute. In order to clarify the effect of MG-132-induced autophagy on the fate of SHG-44 glioma cells, we used 3-MA, a class III phosphatidylinositol 3-kinase inhibitor and generally accepted as a specific inhibitor of autophagy[34]. Our results show that 3-MA down-regulates the expression of Beclin-1 and LC3-I and inhibits the conversion of LC3-I into LC3-II, as well as decreasing the formation of acid vesicles in the cytoplasm of SHG-44 glioma cells. Moreover, 3-MA enhances MG-132-induced cell death and the number of cells in G_2/M phase, indicating that autophagy has a protective effect on SHG-44 glioma cells, which is consistent with previous studies reporting that inhibition of autophagy enhances or accelerates cell death in malignant tumors[35, 36] or reverses tumor cells' resistance to apoptosis[37].

In conclusion, we show that the proteasome inhibitor MG-132 produces *in vitro* growth inhibition, G_2/M cell cycle arrest, and cell death in human SHG-44 glioma cell and demonstrate that inhibition of the proteasome activates autophagy. Moreover, we find that cell death increases when autophagy is inhibited. These findings suggest that proteasome inhibitors should be investigated as potential novel chemotherapeutic agents for the adjuvant treatment of human malignant gliomas.

Acknowledgements

Author contribution

Prof Yi-nan LUO and Feng LING designed research; Peng-fei GE, Ji-zhou ZHANG, Xiao-fei WANG, Fan-kai MENG and Wen-chen LI performed research. Yong-xin LUAN analyzed data; Peng-fei GE wrote the paper.

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