

## Full-length article

**Dynamic effects of autophagy on arsenic trioxide-induced death of human leukemia cell line HL60 cells**Ya-ping YANG<sup>2</sup>, Zhong-qin LIANG, Bo GAO, Yan-li JIA, Zheng-hong QIN<sup>1</sup>*Department of Pharmacology and Laboratory of Aging and Nervous Diseases, Soochow University School of Medicine, Suzhou 215123, China***Key words**As<sub>2</sub>O<sub>3</sub>; autophagy; HL60 cells; 3-methyladenine; lysosome; mitochondria<sup>1</sup> Correspondence to Prof Zheng-hong QIN. Phn/Fax 86-512-6588-0406.

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**Abstract**

**Aim:** To evaluate the contribution of an autophagic mechanism to the As<sub>2</sub>O<sub>3</sub>-induced death of human acute myeloid leukaemia cell line HL60 cells. **Methods:** The growth inhibition of HL60 cells induced by As<sub>2</sub>O<sub>3</sub> was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. The activation of autophagy was determined with monodansylcadaverine labeling and transmission electron microscope. The role of autophagy in the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells was assessed using autophagic and lysosomal inhibitors. Immunofluorescence, flow cytometry, and Western blot analysis were used to study the apoptotic and autophagic mechanisms. **Results:** After treatment with As<sub>2</sub>O<sub>3</sub>, the proliferation of HL60 cells was significantly inhibited and the formation of autophagosomes increased. The blockade of autophagy maturation with the autophagy-specific inhibitor 3-methyladenine (3-MA) or the lysosome-neutralizing agent NH<sub>4</sub>Cl 1 h before As<sub>2</sub>O<sub>3</sub> potentiated the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells. In contrast, 3-MA attenuated As<sub>2</sub>O<sub>3</sub>-induced death when administered 30 min after As<sub>2</sub>O<sub>3</sub>. 3-MA and NH<sub>4</sub>Cl also inhibited As<sub>2</sub>O<sub>3</sub>-induced upregulation of microtubule-associated protein 1 light chain 3, the protein required for autophagy in mammalian cells. Following As<sub>2</sub>O<sub>3</sub>, lysosomes were activated as indicated by increased levels of cathepsins B and L. The apoptotic response of HL60 cells to As<sub>2</sub>O<sub>3</sub> was suggested by the collapse of mitochondrial membrane potential, release of cytochrome *c* from mitochondria, and the activation of caspase-3. Pre-treatment with 3-MA prior to As<sub>2</sub>O<sub>3</sub> amplified these apoptotic signals, while post-treatment with 3-MA 30 min after As<sub>2</sub>O<sub>3</sub> attenuated the apoptotic pathways. **Conclusion:** Autophagy plays complex roles in the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells; it inhibits As<sub>2</sub>O<sub>3</sub>-induced apoptosis in the initiation stage, but amplifies the As<sub>2</sub>O<sub>3</sub>-mediated apoptotic program if it is persistently activated.

**Introduction**

Autophagy is a major cellular pathway for the degradation of long-lived proteins and organelles in eukaryotic cells<sup>[1]</sup>. A large number of intracellular/extracellular stimuli, including amino acid starvation and invasion of microorganisms, are able to induce the autophagic response in cells. In addition to the degradation of cellular proteins and organelles, autophagy can serve as a temporary survival strategy to defend against damage caused by environmental changes

through the sequestration of noxious substance (such as certain aggregation-prone proteins or the expression of aggregating mutant variants of specific proteins) and injured organelles. Furthermore, autophagy may also be a strategy for self-destruction through the induction of programmed cell death (PCD), which is different from apoptosis<sup>[2]</sup>.

PCD is an essential and highly orchestrated process, which plays an important role in the development, cellular homeostasis, and prevention of cancer cell growth. There

are five types of PCD that have been described: apoptosis, autophagy, anoikis, amorphosis, and mitotic catastrophe<sup>[3]</sup>. The two main PCD types are referred to as apoptosis (PCD I) and autophagy (PCD II). Apoptosis, or PCD I, is characterized by cell shrinkage, oligonucleosomal DNA fragmentation, chromatin condensation, pyknotic nuclei, and controlled disintegration of the cell into so-called apoptotic bodies. By contrast, autophagy, or PCD type II, is featured by the accumulation of autophagic vacuoles (AV) in the cytoplasm<sup>[4]</sup>.

Many reports have shown that under certain conditions, autophagy and apoptosis appear to interact with each other either positively or negatively<sup>[5]</sup> in the following ways: (i) autophagy may be indispensable for apoptosis occurrence. Autophagy inhibitors, such as 3-methyladenine (3-MA), delay apoptosis while converse, broad-range caspase inhibitors fail to inhibit autophagy<sup>[6,7]</sup>; (ii) autophagy may antagonize apoptosis. The inhibition of autophagy may increase the sensitivity of cells to apoptotic signals<sup>[8]</sup>; and (iii) apoptosis and autophagy may occur independent of each other. The inhibition of apoptosis may convert cell death morphology to autophagic and *vice versa*<sup>[5,9–11]</sup>.

Many studies have shown that the compromise of autophagic activity may contribute to the development of cancers. In cancer cell lines and experimental carcinogenesis in animals, the autophagy activity was often lower than that in their normal counterparts. Moreover, autophagy could be reactivated by several anticancer agents<sup>[12,13]</sup>. As<sub>2</sub>O<sub>3</sub> has been proven to be a useful drug for the treatment of acute promyelocytic leukemia (APL). Apoptosis seems to be a main mechanism underlying its antitumor activity<sup>[14,15]</sup>. Recently, it was shown that As<sub>2</sub>O<sub>3</sub> induced autophagy, but not apoptosis in human malignant glioma cell lines by the upregulation of mitochondrial cell death protein Bcl-2/adenovirus E1B 19 kDa-interacting protein 3<sup>[16,17]</sup>. As<sub>2</sub>O<sub>3</sub> has also been demonstrated to activate autophagy and induce the autophagic death of MoI<sub>t</sub>-4 cells through the upregulation of beclin 1<sup>[18]</sup>. We have previously found that autophagy plays different roles in the crotoxin-induced death of chronic myeloid leukemia cell line K562 cells and human breast cancer cell line MCF-7 cells<sup>[19,20]</sup>. In the present study, we investigated the role of autophagy in the As<sub>2</sub>O<sub>3</sub>-induced death of human acute myeloid leukaemia cell line HL60 cells and its interactions with the apoptotic cascade. The results showed that blocking autophagy before and after As<sub>2</sub>O<sub>3</sub> exposure had opposite effects on the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells, suggesting that autophagy plays different roles in the different stages of apoptosis.

## Materials and methods

**Cell culture** HL60 leukemia cells were purchased from

the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials, Hangzhou, China) and 0.03% *L*-glutamine (Sigma, St Louis, MO, USA) and incubated in a humidified 5% CO<sub>2</sub> incubator at 37 °C. The cells in the mid-log phase were used in the experiments.

**Quantitative analysis of the viability of HL60 cells after exposure to As<sub>2</sub>O<sub>3</sub>** As<sub>2</sub>O<sub>3</sub> (0.625, 1.25, 2.5, 5, 10, and 20 μmol/L; Sigma, USA) was added to the culture medium, and HL60 cell viability was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 12, 24, 48, and 72 h after exposure to As<sub>2</sub>O<sub>3</sub> following a previously described method<sup>[21]</sup>. Briefly, MTT (Sigma, USA) solution was added to the culture medium (final concentration = 500 μg/mL) 4 h before the end of the treatment. The reaction was terminated by the addition of 10% acidified SDS (100 μL) to the cell culture. The absorbance value (*A*) was measured at 570 nm using a multiwell spectrophotometer (Bio-Rad, Richmond CA, USA). The percentage of cell death was calculated with the following formula: cell death (%) = (1 - *A* of experiment well / *A* of control well) × 100%.

**Visualization of monodansylcadaverine-labeled AV** AV were detected using a method as previously described<sup>[22]</sup>. The cells were seeded onto 24-well plates and incubated with As<sub>2</sub>O<sub>3</sub> (10 μmol/L) for 3, 6, and 12 h. The cells were then incubated with monodansylcadaverine (MDC; 50 μmol/L) in RPMI-1640 at 37 °C for 10 min. After incubation, the cells were washed three times with phosphate buffered saline (PBS) and immediately analyzed with a fluorescence microscope (Eclipse TE 300; Nikon, Japan) equipped with a filter system (V-2A excitation filter: 380/420 nm, barrier filter: 450 nm)<sup>[23]</sup>. Images were captured with a charge-coupled device (CCD) camera and imported into Adobe Photoshop (Adobe, Japan).

**Transmission electron microscopic analyses of autophagosomes** HL60 cells were cultured in 60 mm dishes and treated with As<sub>2</sub>O<sub>3</sub> (10 μmol/L) for 6, 12, and 24 h. After treatment, the cells were fixed in ice-cold 2.5% glutaraldehyde in 0.1 mol/L PBS and preserved at 4 °C for further processing. When processing resumed, the cells were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in graded alcohols, embedded in Epon 812, sectioned with ultramicrotome, and stained with uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (TEM; Technai 10; Philips, the Netherlands).

**Cytotoxicity assay** It has been reported that lactose dehydrogenase (LDH) leakage not only occurs during

necrosis, but also during the process of apoptosis<sup>[24,25]</sup>. Since 3-MA interferes with the MTT assay, LDH leakage was assessed as an index of cell death after the cotreatment of As<sub>2</sub>O<sub>3</sub> and 3-MA or NH<sub>4</sub>Cl. To examine the contribution of autophagy to the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells, the cells were treated with the specific autophagy inhibitor 3-MA (Sigma, USA; 10 mmol/L) or lysosome-neutralizing agent NH<sub>4</sub>Cl (10 mmol/L) 1 h before As<sub>2</sub>O<sub>3</sub> and LDH leakage was measured 24 h after As<sub>2</sub>O<sub>3</sub>, or the cells were treated with 3-MA (10 mmol/L) 5 min to 12 h after As<sub>2</sub>O<sub>3</sub> (10 μmol/L) and LDH leakage was measured 24 h after As<sub>2</sub>O<sub>3</sub>. Briefly, after drug treatment, the supernatant of the cell culture was reserved. The cells were rinsed with PBS and lysed with 1% Triton X-100 at 37 °C for 45 min. Then samples of supernatants and cell lysates were prepared following the manufacturer's instructions for the LDH test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance value (A) at 400 nm was determined with an automatic multiwell spectrophotometer (Bio-Rad, USA). LDH leakage was calculated as follows: LDH leakage (%)=(A positive/A positive blank)/(A negative/A negative blank)×100%<sup>[26]</sup>.

**Immunofluorescence detection of microtubule-associated protein 1 light chain 3** Briefly, the HL60 cells were incubated in 6-well plates and the specific autophagy inhibitor 3-MA (10 mmol/L) or lysosome-neutralizing agent NH<sub>4</sub>Cl (10 mmol/L) was added 1 h before As<sub>2</sub>O<sub>3</sub> (10 μmol/L); or cells were treated with 3-MA (10 mmol/L) 30 min after As<sub>2</sub>O<sub>3</sub> (10 μmol/L). After being incubated with As<sub>2</sub>O<sub>3</sub> for 24 h, the cells were washed with PBS and then fixed with paraformaldehyde (4% w:v). After rinsing in PBS, the cells were blocked with 0.1% Triton X-100 and 1% bovine serum albumin in PBS for 1 h. This was followed by incubation in goat polyclonal antibody against microtubule-associated protein 1 light chain 3 (LC3; 1:100, sc-16756; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h at 4 °C in a humidified chamber. After 3 washes for 10 min in PBS, the cells were incubated in donkey anti-goat immunoglobulin G-fluorescein-isothiocyanate (1:400, sc-2024; Santa Cruz, USA) for 1 h at 4 °C. Finally, cells were rinsed in PBS and examined with a fluorescence microscope (Eclipse TE 300, Nikon, Japan).

**Cytofluorometric assessment of mitochondrial membrane potential** To measure the mitochondrial membrane potential ( $\Delta\Psi_m$ ), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), a sensitive fluorescent probe for  $\Delta\Psi_m$  was used<sup>[27]</sup>. The HL60 cells were cultured in 24-well plates and treated as follows: control group, 3-MA group, As<sub>2</sub>O<sub>3</sub> group, As<sub>2</sub>O<sub>3</sub>+3-MA (pretreatment) group, and As<sub>2</sub>O<sub>3</sub>+3-MA (post-treatment) group. After exposure to the

As<sub>2</sub>O<sub>3</sub> (10 μmol/L) for 24 h, the cells were harvested and stained with 1 mL 10% RPMI-1640 medium containing 5 μmol/L JC-1 (Molecular Probes, USA) for 30 min at 37 °C. Then the cells were rinsed with ice-cold PBS twice, resuspended in 1 mL ice-cool PBS, and immediately assessed for red and green fluorescence with flow cytometry<sup>[28]</sup>.

JC-1 has been reported to differentially label mitochondria with high and low membrane potential<sup>[27]</sup>. JC-1 exists as a monomer and emits green fluorescence ( $\lambda=527$  nm) at low membrane potential. However, at higher potentials, JC-1 forms "J-aggregates", which emit red fluorescence ( $\lambda=590$  nm). For the assessment of individual samples for JC-1 staining, a total of 10000 gated events were analyzed per sample using a flow cytometer (EPICS-XL, Beckman, USA). A 488 nm filter was used for the excitation of JC-1. Emission filters of 535 and 595 nm were used to quantify the population of mitochondria with green (JC-1 monomers) and red (JC-1 aggregates) fluorescence, respectively<sup>[28]</sup>. Frequency plots were prepared for FL1 (green) and FL2 (red) to determine the percentage of the mitochondria stained green (low membrane potential) and red (normal membrane potential).

**Subcellular fractionations** The HL60 cells were cultured in 60 mm dishes and divided into 5 treatment groups, as described earlier. After exposure to As<sub>2</sub>O<sub>3</sub> (10 μmol/L) for 24 h, the cells were harvested and rinsed with ice-cold PBS twice. The isolation of mitochondria was performed as described previously<sup>[29]</sup>. The cells were suspended in buffer A (250 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 50 mmol/L Tris-HCl, 1 mmol/L DL-dithiothreitol (DTT), 1 mmol/L Phenylmethanesulfonyl fluoride (PMSF), 1 mmol/L benzamidine, 0.28 U/mL apotinin, 50 μg/mL leupeptin, and 7 μg/mL pepstatin A, pH 7.4) and homogenated with a glass Pyrex microhomogenizer (30 strokes). The homogenate was centrifuged at 1000×g at 4 °C for 10 min, and the resultant supernatant was transferred to a new Eppendorf tube and centrifuged at 10 000×g at 4 °C for 20 min to obtain the mitochondrial pellet and supernatant. The supernatant was transferred to a new tube and centrifuged at 100000×g for 1 h at 4 °C to generate the cytosolic fraction. The mitochondrial pellet was washed 3 times in buffer B (250 mmol/L sucrose, 1 mmol/L LEGTA, 10 mmol/L Tris-HCl, 1 mmol/L DTT, 1 mmol/L PMSF, 1 mmol/L benzamidine, 0.28 U/mL apotinin, 50 μg/mL leupeptin, and 7 μg/mL pepstatin A, pH 7.4), spun at 10000×g at 4 °C for 10 min, and then lysed in Western blot lysing buffer.

**Protein preparation and immunoblotting** The cells were harvested and rinsed with ice-cold PBS twice. Five volumes of Western blot lysing buffer for each volume of cell pellets was added and the mixture was sonicated on ice (1 s/mL per

sonicate, resting 30 s between intervals, a total of 5 times). The sample was centrifuged at  $10000\times g$  at  $4^{\circ}\text{C}$  for 10 min and the supernatant was preserved at  $-70^{\circ}\text{C}$ . Before immunoblotting, the protein concentrations were determined with a BCA detection kit (Pierce, USA) and adjusted to equal concentrations across different samples. The proteins were separated by 10% or 12% SDS-PAGE gel, transferred to nitrocellulose membranes, and immunoblotted with a goat polyclonal antibody against cathepsin B (1:100; sc-6493; Santa Cruz, USA), a goat polyclonal antibody against cathepsin L (1:100; sc-6499; Santa Cruz, USA), a mouse monoclonal antibody against cytochrome *c* (cyto-*c*) 1:1000; 7H8.2C12, PharMingen, San Diego, CA, USA) or rabbit polyclonal antibody against caspase-3 (1:200; H277; Santa Cruz, USA) at  $4^{\circ}\text{C}$  overnight. The reactions of the primary antibodies were detected using horseradish peroxidase-conjugated antigoat, antimouse, or antirabbit antibodies (Sigma, USA) and were used at a 1:5000 dilution in blocking solution for 1 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and visualized by autoradiography. Protein  $\beta$ -actin (1:5000; A5441; Sigma, USA) and heat shock protein 60 (HSP-60) (1:2000; H3524; Sigma, USA) were used as the loading controls.

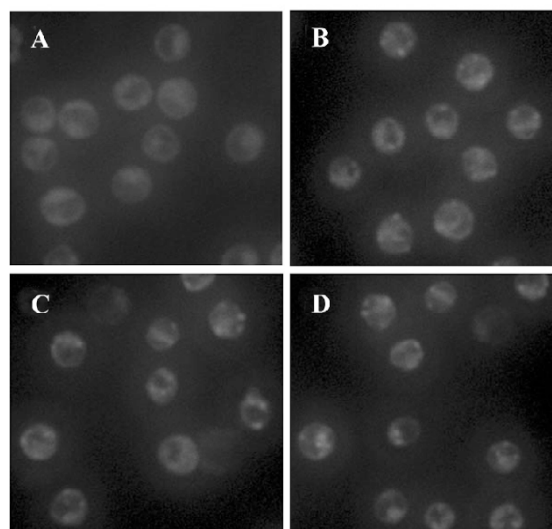
**Statistical analysis** All data are presented as mean $\pm$ SD. The statistical analysis was carried out by ANOVA followed by a Student's *t*-test with  $P<0.05$  representing significance.

## Results

**Increased AV following  $\text{As}_2\text{O}_3$  treatment**  $\text{As}_2\text{O}_3$  inhibited the viability of the HL60 cells in a time- and dose-dependent fashion. After treatment with  $20\ \mu\text{mol/L}$   $\text{As}_2\text{O}_3$  for 12, 24, 48, and 72 h, the viability of the HL60 cells was significantly decreased ( $P<0.05$ ). The rate of the inhibition of the viability of HL60 was  $47.09\%\pm 2.01\%$  ( $P<0.05$ ) at 24 h,  $72.18\%\pm 1.23\%$  at 48 h, and  $93.13\%\pm 0.04\%$  ( $P<0.05$ ) at 72 h, respectively, after  $\text{As}_2\text{O}_3$  ( $10\ \mu\text{mol/L}$ ) treatment (results not shown).

The autofluorescent substance MDC has been recently shown to be a marker for AV. When the cells were viewed with a fluorescence microscope, the AV labeled by MDC appeared as distinct dot-like structures distributed in the cytoplasm or in the perinuclear regions. We found that there was an increase in the number of MDC-labeled vesicles starting at 3 h and peaking at 6–12 h after  $\text{As}_2\text{O}_3$  treatment, suggesting an induction of AV formation by  $\text{As}_2\text{O}_3$  (Figure 1).

**Apoptosis and autophagy are induced in HL60 cells exposed to  $\text{As}_2\text{O}_3$**  The ultrastructural analysis of the control cells showed a round shape and contained normal looking

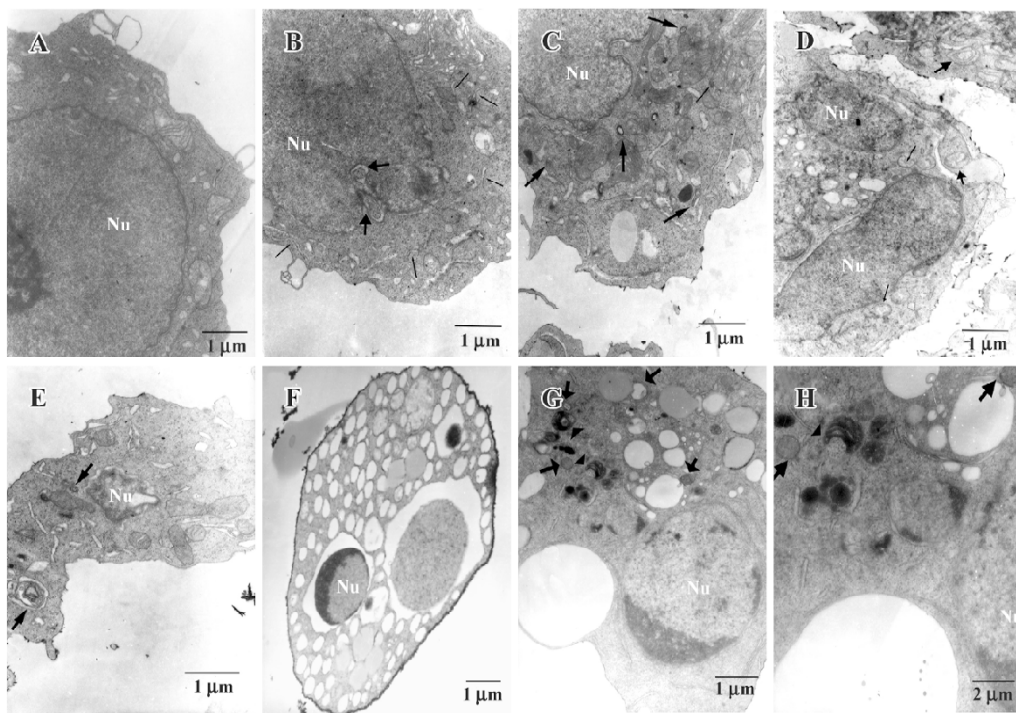


**Figure 1.** Monodansylcadaverine (MDC)-labeled vesicles were induced after  $\text{As}_2\text{O}_3$  treatment. Human acute myeloid leukaemia cell line HL60 cells (HL60) were incubated with  $\text{As}_2\text{O}_3$  ( $10\ \mu\text{mol/L}$ ) for the indicated time and stained with MDC ( $50\ \mu\text{mol/L}$ ). Fluorescence particles in the cytoplasm indicate autophagic vacuoles. (A) control, (B) 3 h, (C) 6 h, (D) 12 h after  $\text{As}_2\text{O}_3$  treatment. Microphotographs were shown as representative results from 3 independent experiments. Magnification  $\times 400$ .

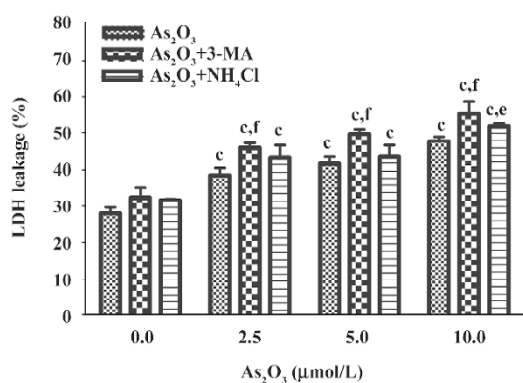
organelles, nuclei, and chromatin. While after  $\text{As}_2\text{O}_3$  treatment, cells were found to contain many vesicles with the typical morphological features of autophagosomes, a number of isolated double-membrane structures were observed in the cytoplasm. These membrane structures engulfed cytoplasm fractions and organelles to form double or multimembrane autophagosomes. Mitochondria were swollen and lysosomal staining darkened, indicating the injury of mitochondria and the activation of lysosomes. Meanwhile, the morphological features of apoptosis, such as chromatin condensation and margination, were also observed (Figure 2).

**Effects of 3-MA and  $\text{NH}_4\text{Cl}$  on the cytotoxicity of  $\text{As}_2\text{O}_3$  in HL60 cells** When the specific autophagy inhibitor 3-MA or the lysosome-neutralizing agent  $\text{NH}_4\text{Cl}$  were added 1 h before  $\text{As}_2\text{O}_3$ , LDH leakage significantly increased, suggesting that autophagy inhibits the cytotoxicity of  $\text{As}_2\text{O}_3$  in HL60 cells (Figure 3). In contrast, after the treatment of HL60 cells with 3-MA 30 min after  $\text{As}_2\text{O}_3$  ( $10\ \mu\text{mol/L}$ ), LDH leakage significantly decreased. The inhibitory effects of 3-MA on LDH leakage were observed when it was added to the culture 5 min–12 h after  $\text{As}_2\text{O}_3$ , suggesting that persistent autophagy activation enhances the cytotoxicity of  $\text{As}_2\text{O}_3$  in HL60 cells (Figure 4).

**LC3 activity assay** LC3 is considered as a marker of cellular autophagosomes, and was observed as green punctu-



**Figure 2.** Ultrastructural changes in the  $As_2O_3$ -treated HL60 cells. Electron microscopy images showing ultrastructural features of a control cell (A) and coexistence of morphological features of autophagy and apoptosis in HL60 cells exposed to  $As_2O_3$  (10  $\mu mol/L$ ) (B–H). (A) untreated cells exhibited the normal appearance of the cytoplasm and nucleus. (B,C) treatment with  $As_2O_3$  for 6 h. HL60 cells exhibited the characteristic ultrastructural morphology of autophagy: isolated double-membrane and double-membrane autophagosomes, which engulfed cytoplasm fraction and organelles were seen distributing throughout the cytoplasm. (D,E) treatment with  $As_2O_3$  for 12 h. Less frequency of isolated double-membrane and autophagosomes was seen than that of 6 h after  $As_2O_3$ . (F,G) Treatment with  $As_2O_3$  for 24 h. Morphological features of apoptosis and autophagy coexisted in HL60 cells; autolysosomes also appeared in the cytoplasm. (H) the double magnified picture of (G). Nu, nucleus. Thin arrows represent isolated membranes; thick arrows represent autophagosomes; arrow heads represent autolysosomes.

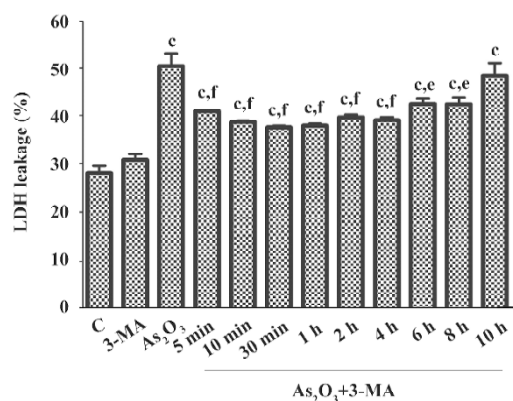


**Figure 3.**  $As_2O_3$ -induced lactose dehydrogenase (LDH) leakage of HL60 cells was enhanced by pretreatment with 3-methyladenine (3-MA) and  $NH_4Cl$ . Cells were pretreated with 3-MA (10  $mmol/L$ ) or  $NH_4Cl$  (10  $mmol/L$ ) 1 h before various doses of  $As_2O_3$  treatment and harvested 24 h later for a LDH assay. Supernatant and cell lysates were prepared as described in Materials and methods. LDH leakage was detected with an LDH assay kit according to the manufacturer’s protocol. Results are presented as mean $\pm$ SD of 3 independent experiments. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  compared to the control group; <sup>b</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$  compared to the  $As_2O_3$  alone treatment group.

ated labeling distributed in the cytoplasm after treatment with  $As_2O_3$  (10  $\mu mol/L$ ). The negative control cells only showed diffuse immunostaining; pretreatment with the lysosome-neutralizing agent  $NH_4Cl$  attenuated the immunoreactivity of LC3 induced by  $As_2O_3$ ; treatment with the specific autophagy inhibitor 3-MA before or after  $As_2O_3$  inhibited the immunoreactivity of LC3 completely (Figure 5).

**Effects of 3-MA on  $As_2O_3$ -induced activation of cathepsins B and L** The Western blot analysis revealed that  $As_2O_3$  increased the protein levels of cathepsins B and L. 3-MA alone had no effect on the protein levels of cathepsins B and L. Post-treatment with 3-MA 30 min after  $As_2O_3$  (10  $\mu mol/L$ ) lessened  $As_2O_3$ -induced increases in cathepsin L protein levels, while pretreatment with 3-MA 1 h before  $As_2O_3$  enhanced  $As_2O_3$ -induced increases in cathepsin L protein levels. The same trend was seen on  $As_2O_3$ -induced increases in cathepsin B, although it was less robust (Figure 6).

**Effects of 3-MA on  $As_2O_3$ -induced  $\Delta\Psi m$  collapse** When  $\Delta\Psi m$  was low, JC-1 existed mainly in a monomeric form, which emits green fluorescence. As shown in Figure 7, after expo-

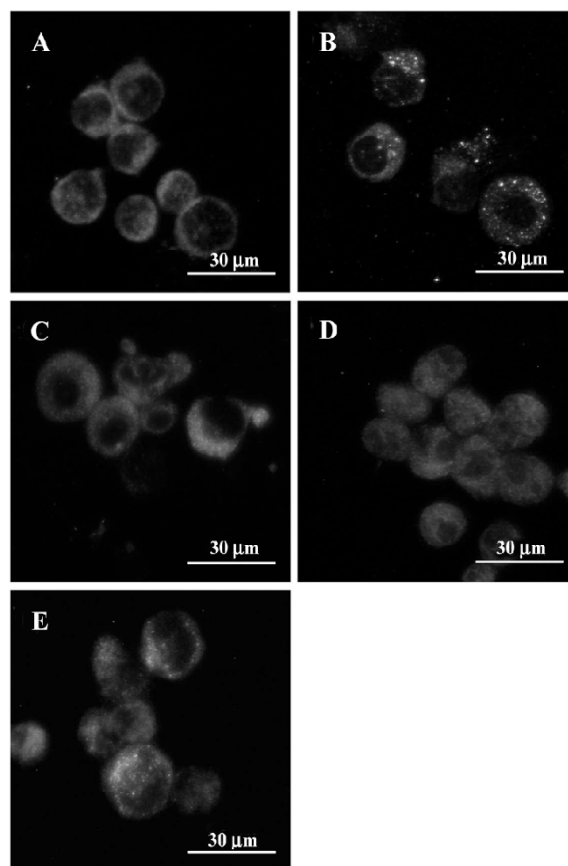


**Figure 4.** As<sub>2</sub>O<sub>3</sub>-induced lactose dehydrogenase (LDH) leakage of HL60 cells was decreased by post-treatment with 3-methyladenine (3-MA). Cells were post-treated with 3-MA (10 mmol/L) 5 min–12 h after As<sub>2</sub>O<sub>3</sub> (10 μmol/L), and the cells were harvested 24 h later for assay of LDH leakage. Supernatant and cell lysates were prepared as described in Materials and methods. LDH leakage was detected with a LDH assay kit according to the manufacturer's protocol. Results are presented as mean±SD of 3 independent experiments. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 compared to the control group; <sup>e</sup>*P*<0.05, <sup>f</sup>*P*<0.01 compared to the As<sub>2</sub>O<sub>3</sub> alone treatment group.

sure to As<sub>2</sub>O<sub>3</sub> for 24 h, an increase in the population of mitochondria with collapsed membrane potential was observed. When autophagy was inhibited with 3-MA 30 min after As<sub>2</sub>O<sub>3</sub> treatment, the decrease in ΔΨ<sub>m</sub> induced by As<sub>2</sub>O<sub>3</sub> was slightly attenuated, but it had no significant difference compared to As<sub>2</sub>O<sub>3</sub> alone. However, when autophagy was blocked 1 h before As<sub>2</sub>O<sub>3</sub> treatment, the collapse of ΔΨ<sub>m</sub> induced by As<sub>2</sub>O<sub>3</sub> was significantly exacerbated (Figure 7).

**Effects of 3-MA on As<sub>2</sub>O<sub>3</sub>-induced cyto-*c* redistribution and caspase-3 activation** Cyto-*c* release from mitochondria into the cytoplasm is a critical event in apoptosis. The Western blot analysis results demonstrated that the release of cyto-*c* took place when the HL60 cells were exposed to As<sub>2</sub>O<sub>3</sub> for 24 h. As<sub>2</sub>O<sub>3</sub>-induced cyto-*c* release was significantly inhibited by post-treatment with 3-MA 30 min after As<sub>2</sub>O<sub>3</sub> treatment. In contrast, As<sub>2</sub>O<sub>3</sub>-induced cyto-*c* release was enhanced by pretreatment with 3-MA 1 h before As<sub>2</sub>O<sub>3</sub> (Figure 8).

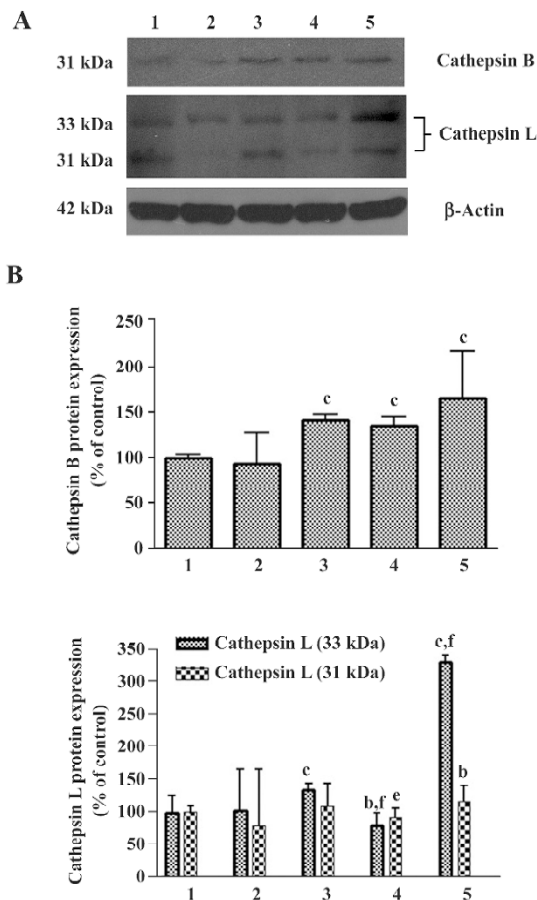
The redistribution of cyto-*c* could initiate a cascade of caspase activation. As seen in Figure 9, after treatment with As<sub>2</sub>O<sub>3</sub> for 24 h, caspase-3 was activated, as revealed by the production of 20 kDa active caspase fragments. The blockade of autophagy 30 min after As<sub>2</sub>O<sub>3</sub> treatment with 3-MA significantly suppressed the As<sub>2</sub>O<sub>3</sub>-induced activation of caspase-3, while blockage of autophagy with 3-MA 1 h before As<sub>2</sub>O<sub>3</sub> treatment potentiated the As<sub>2</sub>O<sub>3</sub>-induced activation of caspase-3 (Figure 9).



**Figure 5.** Microtubule-associated protein 1 light chain 3 (LC3) activation assay. Cells were pretreated with 3-methyladenine (3-MA; 10 mmol/L) or NH<sub>4</sub>Cl (10 mmol/L) 1 h before As<sub>2</sub>O<sub>3</sub> (10 μmol/L) treatment or post-treated with 3-MA (10 mmol/L) 30 min after As<sub>2</sub>O<sub>3</sub> (10 μmol/L) and then harvested 24 h later for a LC3 immunofluorescence assay. (A) control; (B) As<sub>2</sub>O<sub>3</sub>; (C) As<sub>2</sub>O<sub>3</sub>+3-MA (30 min after As<sub>2</sub>O<sub>3</sub>); (D) As<sub>2</sub>O<sub>3</sub>+3-MA (1 h before As<sub>2</sub>O<sub>3</sub>); (E): As<sub>2</sub>O<sub>3</sub>+NH<sub>4</sub>Cl (1 h before As<sub>2</sub>O<sub>3</sub>).

## Discussion

Extensive research has revealed that As<sub>2</sub>O<sub>3</sub> exerts potent antitumor effects against several types of cancers, including leukemia<sup>[17,30-32]</sup>, and it has been recognized as being highly effective in clinics for the treatment of APL<sup>[33,34]</sup>. It is believed that the antitumor efficacy of As<sub>2</sub>O<sub>3</sub> involves several actions, including the inhibition of proliferation, induction of incomplete differentiation, and promotion of apoptosis of tumor cells<sup>[35-37]</sup>, but the underlying molecular mechanisms of its antitumor effects still remain elusive. Autophagy is a gatekeeping mechanism for stabilizing cell homeostasis<sup>[38]</sup>. Studies have shown that autophagy plays important roles in physiology and pathophysiology<sup>[39]</sup>. There is a potential link between autophagy and a number of human diseases, such



**Figure 6.** Effects of autophagy inhibitor 3-methyladenine (3-MA) on the protein levels of cathepsins B and L in HL60 cells. HL60 cells were pretreated or post-treated with 3-MA (10 mmol/L), and total proteins were extracted 24 h later for As<sub>2</sub>O<sub>3</sub> (10 μmol/L) for the Western blot analysis. (A) lane 1: control; lane 2: 3-MA; lane 3: As<sub>2</sub>O<sub>3</sub>; lane 4: As<sub>2</sub>O<sub>3</sub>+3-MA (30 min after As<sub>2</sub>O<sub>3</sub>); lane 5: As<sub>2</sub>O<sub>3</sub>+3-MA (1 h before As<sub>2</sub>O<sub>3</sub>). (B) results (mean±SD) from 3 independent experiments were quantitatively analyzed with an image analyzer. <sup>b</sup>P<0.05, <sup>c</sup>P<0.01 compared to the control group; <sup>e</sup>P<0.05, <sup>f</sup>P<0.01 compared to the As<sub>2</sub>O<sub>3</sub> alone treatment group.

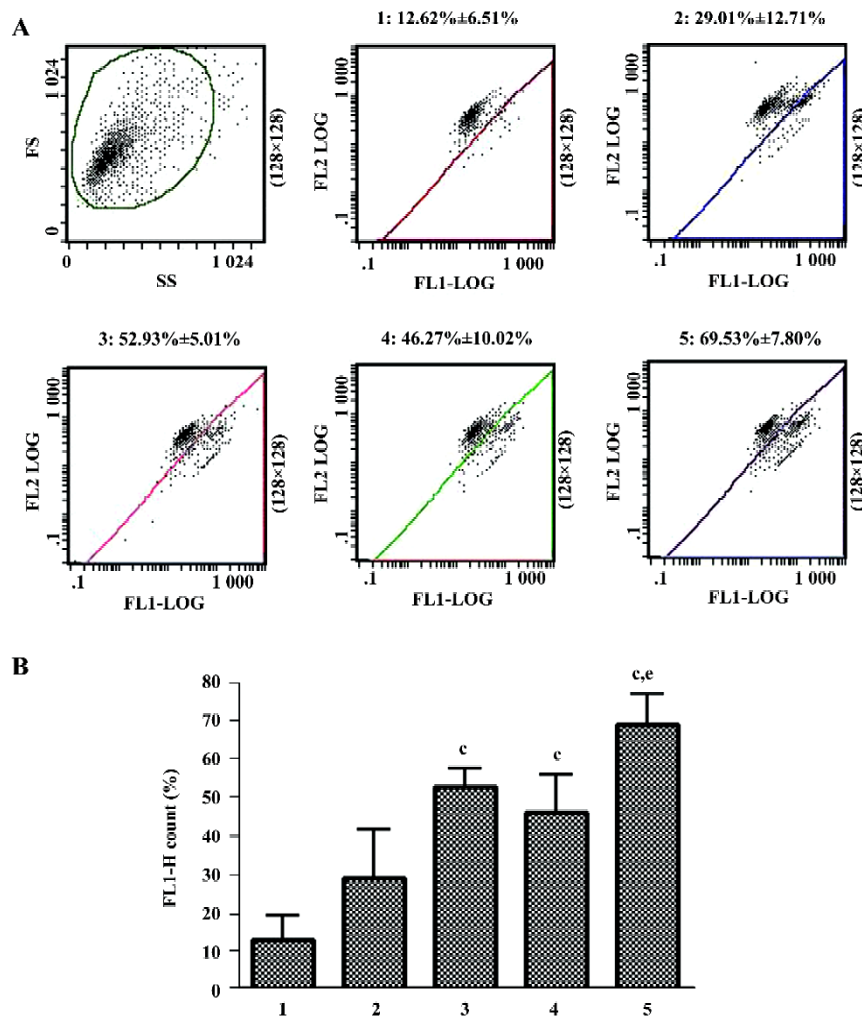
as cancer, cardiomyopathy, and neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's<sup>[40]</sup>. Therefore, the study of the involvement of autophagy in the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells would result in a better understanding of the molecular mechanisms by which As<sub>2</sub>O<sub>3</sub> inhibits tumor cells and would also result in proposals for new therapeutic approaches for leukemia.

In the present study, it was found that the viability of HL60 cells was significantly inhibited by As<sub>2</sub>O<sub>3</sub> and vacuoles labeled by MDC increased in the cytoplasm, suggesting the induction of autophagy in HL60 cells after As<sub>2</sub>O<sub>3</sub> administration. At present, transmission electron microscopic

examination is still the most reliable means to detect the induction of autophagy and the dynamic morphological changes of autophagosomes and autolysosomes. After exposure to As<sub>2</sub>O<sub>3</sub> for 3 h, a number of isolated double-membrane structures and autophagosomes could be identified in HL60 cells with TEM. When the duration of As<sub>2</sub>O<sub>3</sub> treatment was prolonged to 24 h, coexistence of apoptotic features, such as cell shrinkage, chromatin condensation, and margination were observed.

LC3 is an autophagosomal ortholog of yeast Atg8, and is considered an autophagosomal marker. LC3 modification is essential for the autophagic process. When the autophagosomes were induced, LC3 was cleaved to a smaller protein (LC3-II). Cleaved LC3 is redistributed from the cytoplasm to the membranes of pre-autophagosomes and autophagosomes, thus the pattern of immunostaining of LC3 will be altered from a diffuse to punctate pattern<sup>[41]</sup>. After treatment with As<sub>2</sub>O<sub>3</sub>, we observed green punctate immunostaining of LC3 scattered in the cytoplasm, suggesting that As<sub>2</sub>O<sub>3</sub> activated autophagy. 3-MA is a class III phosphatidylinositol 3-kinase inhibitor<sup>[42]</sup>. Early in 1982, it was found that 3-MA inhibits the formation of autophagosomes<sup>[43]</sup>, and now it is generally accepted as a specific inhibitor of autophagy. In our study, it was shown that pre- or post-treatment with 3-MA completely inhibited the activation of LC3 induced by As<sub>2</sub>O<sub>3</sub>, indicating that autophagy maturation was fully suppressed by 3-MA. The lysosome is an important organelle in autophagy. In the last step of autophagy, the autophagosome targets the lysosome, where its outer membrane fuses with the lysosomal membrane; the inter sac (autophagic body) enters the lysosome/vacuole and is then degraded in the lysosome/vacuole so the carrying constituent components can be recycled<sup>[1]</sup>. As shown in the results of immunofluorescence, lysosome-neutralizing agent NH<sub>4</sub>Cl attenuating the expression of LC3 induced by As<sub>2</sub>O<sub>3</sub> due to injury to the lysosomal function, suggesting it could partly inhibit autophagy.

To determine the contribution of autophagy to the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells, the present study examined the effects of the inhibition of autophagy on the cytotoxicity of As<sub>2</sub>O<sub>3</sub>. In this work, 10 mmol/L 3-MA (an effective dose for the inhibition of autophagy, as reported by many investigators) was shown to potentiate the cytotoxicity As<sub>2</sub>O<sub>3</sub> in HL60 cells when it was administered 1 h before As<sub>2</sub>O<sub>3</sub>. The present study also found that the same dose of 3-MA weakened the cytotoxicity of As<sub>2</sub>O<sub>3</sub> when it was administered after As<sub>2</sub>O<sub>3</sub> (10 μmol/L) treatment. These data raise the possibility that autophagy might have dynamic effects on the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells. In the early stage, autophagy can be a survival pathway representing a defense



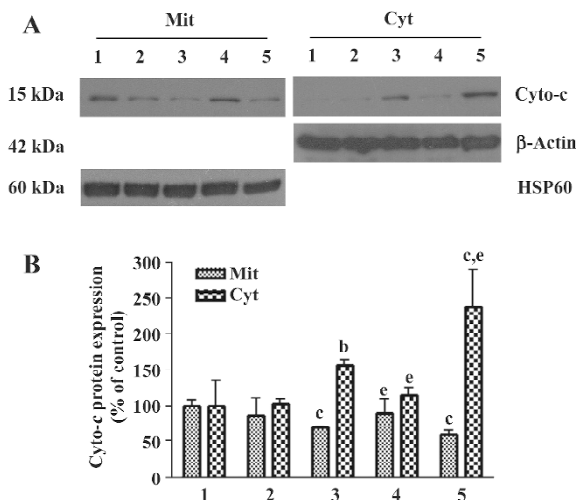
**Figure 7.** Effects of 3-methyladenine (3-MA) on alterations in mitochondria membrane potential. Mean percentage of HL60 cells stained green (FL 1-H) in the total cells was determined by flow cytometry. HL60 cells were treated with As<sub>2</sub>O<sub>3</sub> (10 μmol/L) or pretreated or post-treated with 3-MA (10 mmol/L). (A) lane 1: control; lane 2: 3-MA control; lane 3: As<sub>2</sub>O<sub>3</sub>; lane 4: As<sub>2</sub>O<sub>3</sub>+3-MA (30 min after As<sub>2</sub>O<sub>3</sub>); lane5: As<sub>2</sub>O<sub>3</sub> +3-MA (1 h before As<sub>2</sub>O<sub>3</sub>). (B) results (mean±SD) from 3 independent experiments were quantitatively analyzed with an image analyzer. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 compared to the control group; <sup>e</sup>*P*<0.05 compared to the As<sub>2</sub>O<sub>3</sub> alone treatment group.

mechanism against As<sub>2</sub>O<sub>3</sub>-induced toxicity in HL60 cells, and in the later stage, autophagy can also be a death pathway leading HL60 cells to apoptotic or autophagic cell death.

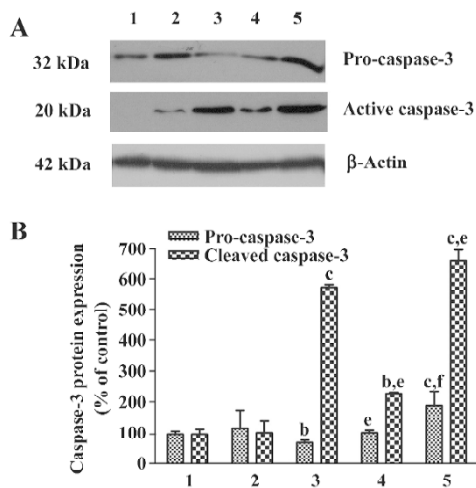
Recently, the role of autophagy in cell survival and death has become an important subject of research. However, the significance of autophagy in various diseases remains to be determined<sup>[40,44–46]</sup>. In our previous study for a role of autophagy in the snake venom crotoxin-induced death of tumor cell lines, we found that autophagy played different roles in the crotoxin-induced death of K562 cells and MCF-7 cells. In K562 cells, autophagy delayed crotoxin-induced cell death, while in MCF-7 cells, which are deficient in caspase-3 activity, autophagy promoted crotoxin-induced cell death<sup>[19,20]</sup>. The

present findings further suggest that autophagy has different effects on cell survival depending on the stages of cell death process. Autophagy activation shortly after exposure of HL60 cells to As<sub>2</sub>O<sub>3</sub> exerted protective effects. However, persistent activation or overactivation of autophagy exerted destructive effects. This raises the possibility that in the early stage, when the death signals induced by As<sub>2</sub>O<sub>3</sub> were relatively weak, autophagy is activated as an adaptive response to maintain HL60 cell survival under stress conditions via increasing protein catabolism; the elimination of excess or damaged organelles like peroxisomes, mitochondria, and the endoplasmic reticulum; and sequestering noxious substances, thus delaying the induction of apoptosis. In





**Figure 8.** Effects of autophagy inhibitor 3-methyladenine (3-MA) on the release of mitochondria cytochrome *c* (cyto-*c*) in HL60 cells. HL60 cells were pretreated or post-treated with 3-MA (10 mmol/L), and the mitochondrial and cytosolic proteins were prepared 24 h after As<sub>2</sub>O<sub>3</sub> (10 μmol/L). Western blot analysis was used to detect cyto-*c* levels in cytosolic (Cyt) and mitochondrial (Mit) fractions. (A) lane 1: control; lane 2: 3-MA; lane 3: As<sub>2</sub>O<sub>3</sub>; lane 4: As<sub>2</sub>O<sub>3</sub>+3-MA (30 min after As<sub>2</sub>O<sub>3</sub>); lane 5: As<sub>2</sub>O<sub>3</sub>+3-MA (1 h before As<sub>2</sub>O<sub>3</sub>). (B) results (mean±SD) from 3 independent experiments were quantitatively analyzed with an image analyzer. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 compared to the control group; <sup>e</sup>*P*<0.05 compared to the As<sub>2</sub>O<sub>3</sub> alone treatment group.



**Figure 9.** Effects of autophagy inhibitor 3-methyladenine (3-MA) on the activation of caspase-3 in HL60 cells. HL60 cells were pretreated or post-treated with 3-MA (10 mmol/L), and total proteins were extracted 24 h after As<sub>2</sub>O<sub>3</sub> (10 μmol/L) for the Western blot analysis. (A) lane 1: control; lane 2: 3-MA; lane 3: As<sub>2</sub>O<sub>3</sub>; lane 4: As<sub>2</sub>O<sub>3</sub>+3-MA (30 min after As<sub>2</sub>O<sub>3</sub>); lane 5: As<sub>2</sub>O<sub>3</sub>+3-MA (1 h before As<sub>2</sub>O<sub>3</sub>). (B) results (mean±SD) from 3 independent experiments were quantitatively analyzed with an image analyzer. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 compared to the control group; <sup>e</sup>*P*<0.05, <sup>f</sup>*P*<0.01 compared to the As<sub>2</sub>O<sub>3</sub> alone treatment group.

contrast, in the late stage, with the continuous presence of As<sub>2</sub>O<sub>3</sub>, cell death signals are reinforced to cause the aggravated damage of HL60 cells, and then the defense mechanism of autophagy ultimately fails in its mission to preserve cell viability and turns to induce an autophagic cell death or promote the apoptotic cascade.

There is complex interplay between autophagic and apoptotic pathways, but the details of its mechanisms and molecular switching points between these 2 pathways in tumor cells have still not been fully elucidated. It is speculated that mitochondria may be the critical organelle that integrate the 2 types of cell death. In the apoptotic process, the mitochondrial permeability transition (MPT) would initiate the release of mitochondrial proapoptotic proteins, such as cyto-*c* and apoptosis-inducing factor, from the intermembrane space. These proteins activate a family of proteolytic enzymes called caspases and other downstream events in apoptotic cell death<sup>[47–49]</sup>. Now it has been proposed that MPT might account for the induction of autophagy<sup>[50]</sup>. Studies have revealed that a good gatekeeper protein of autophagy, the mammalian target of rapamycin, existed in mitochondria, and the damaged mitochondria could initiate autophagy<sup>[51–53]</sup>. Damaged mitochondria could be selectively sequestered into autophagosomes and delivered to lysosomes for degradation<sup>[54]</sup>. The genetic data from mammals revealed that the deletion of autophagy-specific gene *Atg7* resulted in the accumulation of mitochondria with abnormal morphology<sup>[55]</sup>. In conclusion, apoptosis and autophagy may be the different results caused by the unified regulation of mitochondria<sup>[56,57]</sup>. In the present study, we found that following treatment with As<sub>2</sub>O<sub>3</sub>, ΔΨ<sub>m</sub> collapsed, cyto-*c* was released from mitochondria to the cytoplasm, and caspase-3 was activated. Moreover, pretreatment with the autophagy-specific inhibitor 3-MA prior to As<sub>2</sub>O<sub>3</sub> amplified the above-mentioned apoptotic signaling pathways, while it inhibited these signaling pathways when 3-MA was administered post-As<sub>2</sub>O<sub>3</sub> insult. We speculate that in the earlier period, autophagy induced by As<sub>2</sub>O<sub>3</sub> could delete impaired mitochondria and sequester certain proapoptotic molecules, such as cyto-*c*, resulting in the blockage or retardation of apoptosis. However, in the late period, As<sub>2</sub>O<sub>3</sub>-induced autophagy could cause autophagic death or amplification of the mitochondria-mediated apoptotic signaling pathway possibly through the lysosomal pathway.

Another organelle that plays a causal role in both autophagy and apoptosis is the lysosome, a major organelle where most proteins are broken down by proteinases<sup>[1,58]</sup>. There are many different hydrolases in lysosomes<sup>[59–61]</sup>. Among them, cysteine proteinases such as cathepsins B and L are the major lysosomal proteases, but their exact roles

are still unknown. A wide variety of death stimuli can induce partial lysosomal membrane permeabilization and release cathepsins into the cytosol. The leakage of active cathepsins triggers caspase- and mitochondrion-independent programmed cell death<sup>[62–65]</sup>. It has also been documented that cathepsins could activate the classic apoptosis pathway possibly by cleaving the proapoptotic Bcl-2 family member Bid<sup>[66]</sup>. Autophagic cargos, including cytoplasmic soluble constituents and cellular organelles (such as mitochondria and peroxisomes) engulfed by autophagosomes would eventually be delivered to the lysosomal lumen and degraded by numerous hydrolases, while leupeptin, a potent inhibitor of cysteine proteinases, has been shown to increase the density of lysosomes due to the accumulation of undergraded proteins sequestered by autophagy and/or heterophagy<sup>[67–69]</sup>. In this study, we found that cathepsins B and L were activated after exposure to As<sub>2</sub>O<sub>3</sub>. Pretreatment with 3-MA before As<sub>2</sub>O<sub>3</sub> increased the expression of cathepsin L, while post-treatment with 3-MA after As<sub>2</sub>O<sub>3</sub> depressed it. We speculate that cathepsins, especially cathepsin L, might also be involved in the interaction of autophagy and apoptosis.

In summary, this study suggests that autophagy and apoptosis could be triggered by As<sub>2</sub>O<sub>3</sub> in HL60 cells. The data also suggest that autophagy has differential effects on the As<sub>2</sub>O<sub>3</sub>-induced death of HL60 cells: it may be a protective mechanism against apoptosis in the earlier period of As<sub>2</sub>O<sub>3</sub> treatment, while it promotes apoptosis and/or leads to autophagic death in the later period of As<sub>2</sub>O<sub>3</sub> treatment. Autophagy regulated cell survival may through interfering with the mitochondria-mediated apoptotic pathway.

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