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Protection against arsenic trioxide-induced autophagic cell death in U118 human glioma cells by use of lipoic acid

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Abstract

Arsenic is an environmental toxicant found naturally in ground water. Epidemiological studies have suggested a correlation between chronic arsenic exposure and potential brain tissue damage in clinical case and animal experiments. Lipoic acid (LA) is a thiol-compound naturally occurring in plants and animals, which is thought to be a strong antioxidant and possess neuroprotective effects. The objective of this study was to determine if the AS_2O_3-induced glial cell toxicity could be prevented by LA. The human malignant glioma cell (U118) was selected as a research model. By using acridine orange staining and flow cytometry analysis, we found that autophagic, but not apoptotic, cell death was significantly induced by AS_2O_3 in U118 cells, and that AS_2O_3-mediated autophagic cell death was nearly completely attenuated by LA. Down-regulation of p53 and Bax proteins and the up-regulation of Bcl-2 and HSP-70 proteins were observed by western blot in AS_2O_3-mediated autophagic cell death. Our results implied that LA completely inhibited U118 cells autophagic cell death induced by AS_2O_3. We suggested that LA may emerge as a useful protective agent against arsenic-induced glial cell toxicity and reversing arsenic-induced damage in human brain.
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Keywords: Lipoic acid; Arsenic trioxide; Neurotoxicity; Autophage

1. Introduction

Higher doses or chronic exposure to arsenic is still a global health problem affecting many millions of people (Ratnaike, 2003). Arsenic at a nonlethal level in drinking water consumed over a period of time may result in the manifestations of toxicity in practically all systems of the body (Hantson et al., 2003). Neurotoxic effects have been reported in clinical cases and animal experiments with chronic exposure to arsenic (Rao and Avani, 2004). Epidemiological studies also have suggested a correlation between arsenic exposure and potential neurotoxicity (Hall, 2002). For example, higher concentrations of AS_2O_3 were detected in the plasma and cerebrospinal fluid of Alzheimer's (Basun et al., 1991), mental health burden (Fujino et al., 2004), and Parkinson's disease (Larsen et al., 1981) patients.

Lipoic acid (LA) is a thiol-compound naturally occurring in plants and animals (Sohal et al., 1994). It is consumed in the daily diet, absorbed through the blood-brain barrier, and taken up and transformed in cells and tissues into

Abbreviations: AS_2O_3; arsenic trioxide; DHLA, dihydrolipoic acid; ERK, extracellular regulated kinases; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSPs, heat shock proteins; JNK, c-jun terminal kinase; LA, lipoic acid; MAP, mitogen-activated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; ROS, reactive oxygen species; (ST), staurosporine.

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dihydrolipoic acid (DHLA) (Packer et al., 1997). Both LA and DHLA are thought to be strong antioxidants. Aside from acting as a potent antioxidant, LA increases or maintains levels of other low molecular weight antioxidants such as ubiquinone, glutathione, and ascorbic acid (Kozlov et al., 1999). Therefore, it appears that LA could be a potential agent in the prevention of different diseases that may be related to an imbalance of the oxidoreductive cellular status. This occurs in cases of neurodegeneration, ischemia-reperfusion, polyneuropathy, diabetes, AIDS, and hepatic disorder status (Packer et al., 1995).

Human fetal brain explants exposed to arsenic in tissue culture showed the characteristics of cell death, neuronal network damage, loss of ground matrix, cell loss and apoptosis in isolated brain cells and neighboring cells. The arsenic toxicity appears to act through interference the tissue homeostasis in the brain rather than only affect neuron cells (Chattopadhyay et al., 2002). Other studies also indicated that neurological system is the major target of toxic effects of heavy metals such as arsenic (Lee et al., 2001). Pathological alterations of glial cells have also been indicated associated with brain disease. However, the exact mechanism of the toxicity of arsenic in glial cells is not well studied. The objective of this study was to determine if the AS_2O_3-induced glial cell toxicity could be prevented by LA. The human malignant glioma cell (U118) was selected as a research model. We found that autophagic, but not apoptotic, cell death was significantly induced by AS_2O_3 in U118 cells, and that AS_2O_3-mediated autophagic cell death was nearly completely attenuated by LA. Our results provide the molecular basis for the LA prevention of AS_2O_3-induced cell death in U118 cells; such observations may have significance in clinical application.

2. Material and methods

2.1. Cell line and cell culture

Human glioblastoma cell lines U118MG (ATCC HTB-15) and U937 cells, a human pre-monocytic leukemia cell line, were obtained from the American Type Culture Collection (ATCC). U937 cells were cultured in RPMI 1640 medium supplemented with antibiotics containing 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technology, Grand Island, NY), and 10% heat-inactivated fetal calf serum (FCS) (HyClone, South Logan, UT, USA). U118 cells were cultured in DMEM containing supplement as well as U937 cells with additional nonessential amino acids and MEM sodium pyruvate (Gibco, Grand Island, NY). Incubation both cells were performed in a humidified atmosphere containing 5% CO_2 at 37 °C. Exponentially growing cells were detached by 0.05% trypsin-EDTA (Gibco) in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics (Life Technologies, Grand Island, NY).

2.2. Determination of cell viability

Human U118 cells were treated with AS_2O_3 (1–50 μM) in the presence or absence of LA (50–100 μM) for 24 h. For the time-dependent study, U118 cells were treated with AS_2O_3 (5–10 μM) for 24, 48 and 72 h. Cell viability was determined at the indicated times based on a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and allowed to adhere overnight. After removing the medium, 200 μL of fresh medium per well, containing 10 mmol/L Hepes (pH 7.4), was added. Then, 50 μL of MTT was added to the wells and the plate was incubated for 2–4 h at 37 °C in the dark. The medium was removed, and 200 μL DMSO and 25 μL Sorensens's glycine buffer was added to the wells. Absorbance was measured using an ELISA plate reader at 570 nm.

2.3. Western analysis

Proteins isolated from the U118 cells were loaded at 50 μg/lane on 12% (w/v) sodium dodesylsulfate–polyacrylamide gel electrophoresis, blotted, and probed using antibodies, including anti-caspase-3 (E8), anti-caspase-8 (E20), anti-caspase-9 (H170), cyclin E, p53, p21/Cip1, Bax, Bcl-2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (Santa Cruz, Inc. CA), cyclin A, cyclin E, cyclin B, and HSP70 (Transduction Laboratories, Lexington, KY). Immunoreactive bands were visualized by incubation with colorigenic substrates, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co., St. Louis, MO). The expression of GAPDH was used as the control for equal protein loading.

2.4. Determination of apoptosis

Apoptosis was judged by the following criteria: (a) Cell morphology as described previously (Ho et al., 1996). (b) Translocation of phosphotidyl serine to the cell surface detected by an Annexin V-FITC apoptosis detection kit (Calbiochem, Bad Soden, Germany), according to our previous paper (Liu et al., 2003). The U937 cells were selected as a positive control, and treated with either staurosporine (ST) (1 μM) or AS_2O_3 (20 μM) for 24 h, then harvested for Annexin V staining assay. (c) The presence of a sub-G1 peak detected by flow cytometry and measured using a fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany) (Lee et al., 2003). (d) The appearance of DNA fragmentation analyzed by the method described previously (Ho et al., 1996).

2.5. ROS production measurement

ROS production was monitored by flow cytometry using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), as described by Zegura (Zegura et al., 2004). This dye is a stable nonpolar compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. Hydrogen peroxide or low molecular weight peroxides produced by the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF); thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. The cells were incubated with 20 μM DCFH-DA. After 30 min, DCFH-DA was removed and the cells were treated with AS_2O_3 (5 μM) in PBS for 0–60 min. H_2O_2 was added to the U118 cells and incubated for 60 min as a positive control.

2.6. Supravital cell staining with acridine orange for autophagy detection

Cell staining with Acridine orange (Sigma Chemical Co.) was performed according to published procedures (Kanzawa et al., 2003; Traganos and Darzynkiewicz, 1994), adding a final concentration of 1 mg/ml for a period of 20 min. AS_2O_3 (10 μM) was dissolved in DMSO and added to the cells 30 min before the addition of acridine orange. Photographs were obtained with a fluorescence microscope (Axioscop) equipped with a mercury 100-W lamp, 490-nm band-pass blue excitation filters, a 500-nm dichroic mirror, and a 515-nm long-pass barrier filter. Flow cytometric analysis is also available to detect AVO percentage (Kanzawa et al., 2003).

2.7. Statistical analysis

Values are expressed as the mean ± S.E. The significance of the difference of the respective controls for each experimental test condition was

assayed using an unpaired Student's t-test comparing each data point to independent groups. A p value <0.05 was considered to be significant.

3. Results

In this study, U118 cells were treated with AS_2O_3 at different concentrations (1–50 μM) for 24 h, and the viability of the cells was determined. Fig. 1a showed that the viability of U118 cells was less than 60% at 24 h after exposure of the cells to AS_2O_3 (5 μM). In Fig. 1b, the cytotoxic effects of the exposure of U118 cells to 5 and 10 μM AS_2O_3 were examined in a time-dependent manner. Fig. 1b also indicated that, As_2O_3 inhibited the viability of cells in a dose- and time-dependent manner. The number of viable cells treated with As_2O_3 (5 or 10 μM) for 24–72 h decreased to a level below the initial cell number (5×10^3) (Fig. 1b). These results indicate that treatment with As_2O_3 not only inhibited cell viability, but also induced cell death in U118 cells.

The morphological changes of the U118 cells treated with AS_2O_3 (5–25 μM) in the presence or absence of LA (50 μM) were illustrated in Fig. 2. Under stimulating condition, the morphology of U118 cells becoming rounded. Interestingly, the morphological changes of the AS_2O_3-induced cytotoxic effects were nearly completely reversed by LA treatment in the U118 cells (Fig. 2a). The viability assays revealed that the 50% mortality of the AS_2O_3-treated cells that died within 24 h was reversed by 50 μM LA (Fig. 2b). The LA (>50 μM) treatment promoted long-term survival in cells treated with AS_2O_3 (5 μM), and LA provided more than 80% protection during days 2–3.

In Fig. 3a, DNA smearing instead of fragmentation was observed in human U118 cells, using AS_2O_3 treatment in a dose-dependent manner. Flow cytometric analysis was further performed to confirm the apoptotic cells with Annexin V-PI staining. Our results demonstrated that apoptosis was not induced even by higher dose of AS_2O_3 (50 μM) treatment in U118 cells (Fig. 3b). However, significant apoptosis were induced in U118 cells treated with apoptosis inducer staurosporine (ST), a highly potent, nonspecific inhibitor of PKC, which degrades DNA to oligonucleosomal fragments for 24 h. The model of U937 cells treated with AS_2O_3 (Nolte et al., 2004) and ST (Grant et al., 1994) was served as apoptosis positive controls, further confirmed that AS_2O_3 could induce apoptosis in U937 cells but not in U118 cells (Fig. 3c).

As described in the previous report, significant ROS production was the major mechanism for the induction of apoptosis in different types of human cancer cells (Chun et al., 2002; Gao et al., 2002; Maeda et al., 2001; Shen et al., 2003). To determine whether ROS production could be induced by AS_2O_3 in U118 cells, the ROS level was then detected at different doses (5–25 μM) and time periods (0–120 min). Our results demonstrated that the ROS level in human U118 cells did not change in response to AS_2O_3 within 2 h (Fig. 3d). It has been demonstrated that AS_2O_3-induced apoptosis in U937 cells was mediated through the production of H_2O_2 (Park et al., 2003). Thus, the U118 cells treated with H_2O_2 were adapted as a positive control for the determination of ROS (Woo et al., 2002).

The results in Fig. 4a revealed that AS_2O_3-induced DNA degradation was nearly completely reversed by LA pretreatment. Flow cytometric analysis was further performed to evaluate the percentage of DNA-damaged (sub-G1) cells induced by AS_2O_3, and the protective effects of LA during a 24–48 h exposure (Fig. 4b). Our results showed that AS_2O_3-induced sub-G1 cell formation was significantly attenuated, from $19.78 \pm 0.18\%$ to $4.34 \pm 0.23\%$, in response to 50 μM LA for 24 h. Similar results were also observed in a long-term (48 h) experiment (Fig. 4b).

To determine the regulatory mechanisms of AS_2O_3-induced cytotoxicity, U118 cells were treated with AS_2O_3 for 24 h in a dose-dependent (1–25 μM) manner. The regulatory proteins which were related to cell cycle and apoptosis were then determined by immunoblotting analysis (Fig. 5). As described previously, the AS_2O_3-induced apoptosis was seen as an important mechanism for cancer chemotherapeutic purposes when applied in different human cancer cells. The p53 (Filippova and Duerksen-Hughes, 2003), Bax (Karlsson et al., 2004), and caspases 3, 8, and

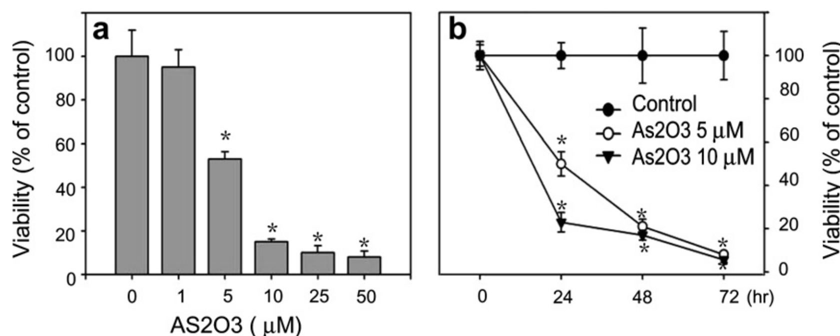

Fig. 1. AS_2O_3 is cytotoxic to U118 cells. (a) For a determination of the dose-dependent cytotoxic effects of AS_2O_3 in U118 cells, cells were treated with different concentrations (1–50 μM) of AS_2O_3 for 24 h. The viability of cells under different treatments was detected by MTT assay, and expressed as the percentage of control (survival of control). (b) Time-dependent effects of the AS_2O_3-induced cytotoxicity in U118 cells. Cells were treated with AS_2O_3 (5 and 10 μM) for 24, 48 and 72 h, and the viability of cells was analyzed. Data were expressed as the mean ± S.E. $^{*}p < 0.05$ indicated a significant difference in AS_2O_3 treated groups compared with untreated group.

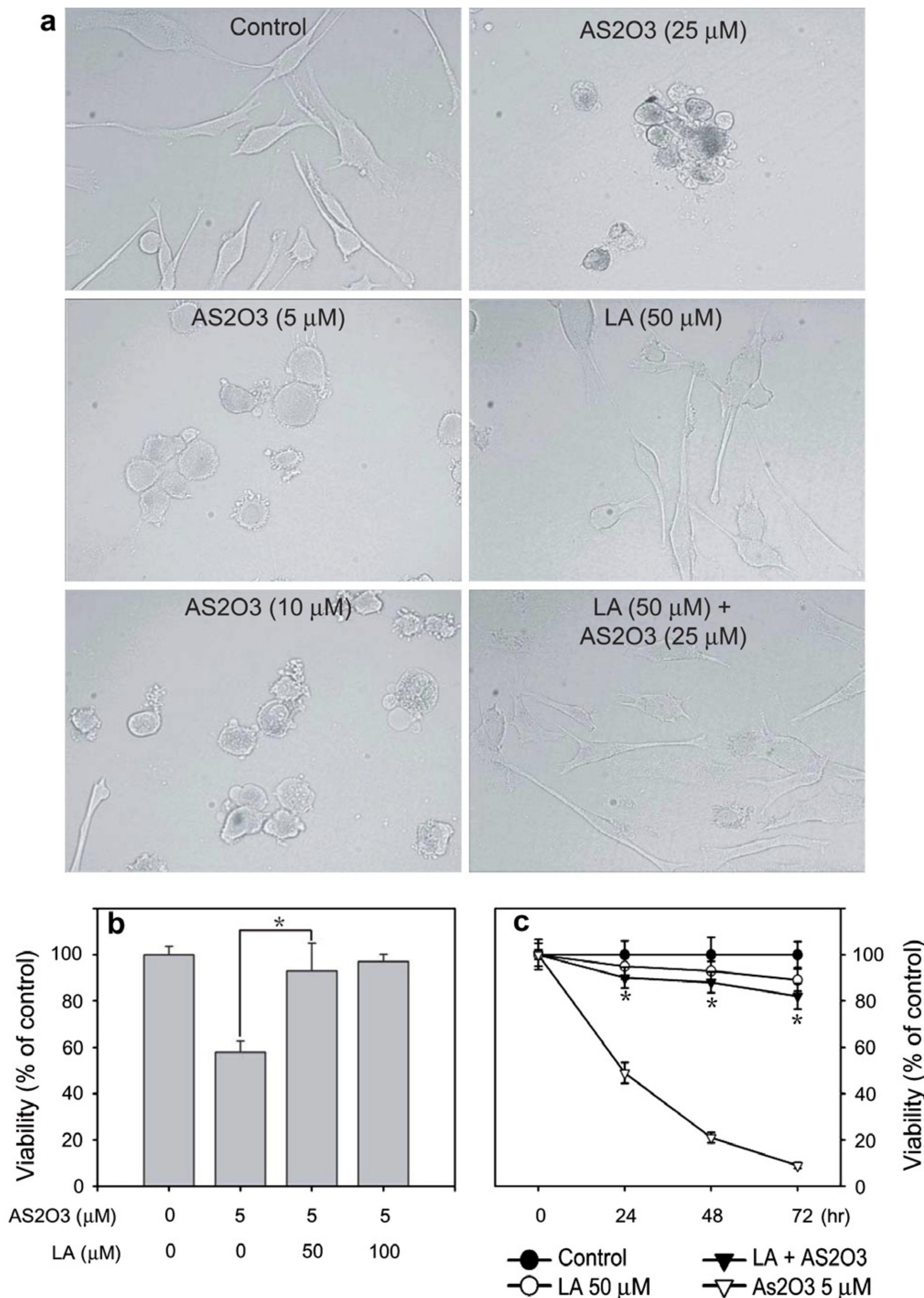

Fig. 2. LA protection of U118 cells from AS_2O_3-induced cell death. (a) Phase contrast photomicrograph observations on the morphological changes of U118 cells. Human U118 cells were treated with AS_2O_3 (5–25 μM) in the presence or absence of LA (50 μM) for 24 h, whereas the cells treated with DMSO (0.05%) were the control. (b) LA inhibition of AS_2O_3-induced cell death by MTT assay. Cells were treated with 5 μM of AS_2O_3 in the presence of LA (50 and 100 μM) treatment for 24 h. The cellular viability was detected by MTT assay as described in Section 2. (c) The AS_2O_3-induced cell death was attenuated by LA treatment in a time-dependent manner. Cells were treated with AS_2O_3 (5 μM) in the presence or absence of LA. The cellular viability was detected by MTT assay at the indicated time points. Data are expressed as the mean ± S.E. $^{*}p < 0.05$ indicated a significant difference between AS_2O_3-treated and combine-treated groups, as analyzed by unpaired Student's *t*-test.

9 (Jiang et al., 2001) were induced in AS_2O_3-induced apoptosis. However, in our study, the p53 and Bax proteins were down-regulated while Bcl-2 was up-regulated in the U118 cells treated with AS_2O_3 in a dose-dependent manner (Fig. 5). In addition, caspases 3, 8 and 9 were not activated by AS_2O_3, although PARP, the substrate of caspase 3, was degraded in the 25 μM AS_2O_3-treated cells. A previous study demonstrated that the cell cycle arrest of the G0/G1 and G2/M phases was induced by AS_2O_3 (2 μM, for 72 h) in human myeloma cells. The expression level of cyclin A was inhibited, whereas the CDK inhibitor p21 was induced (Park et al., 2000). In this study, the protein

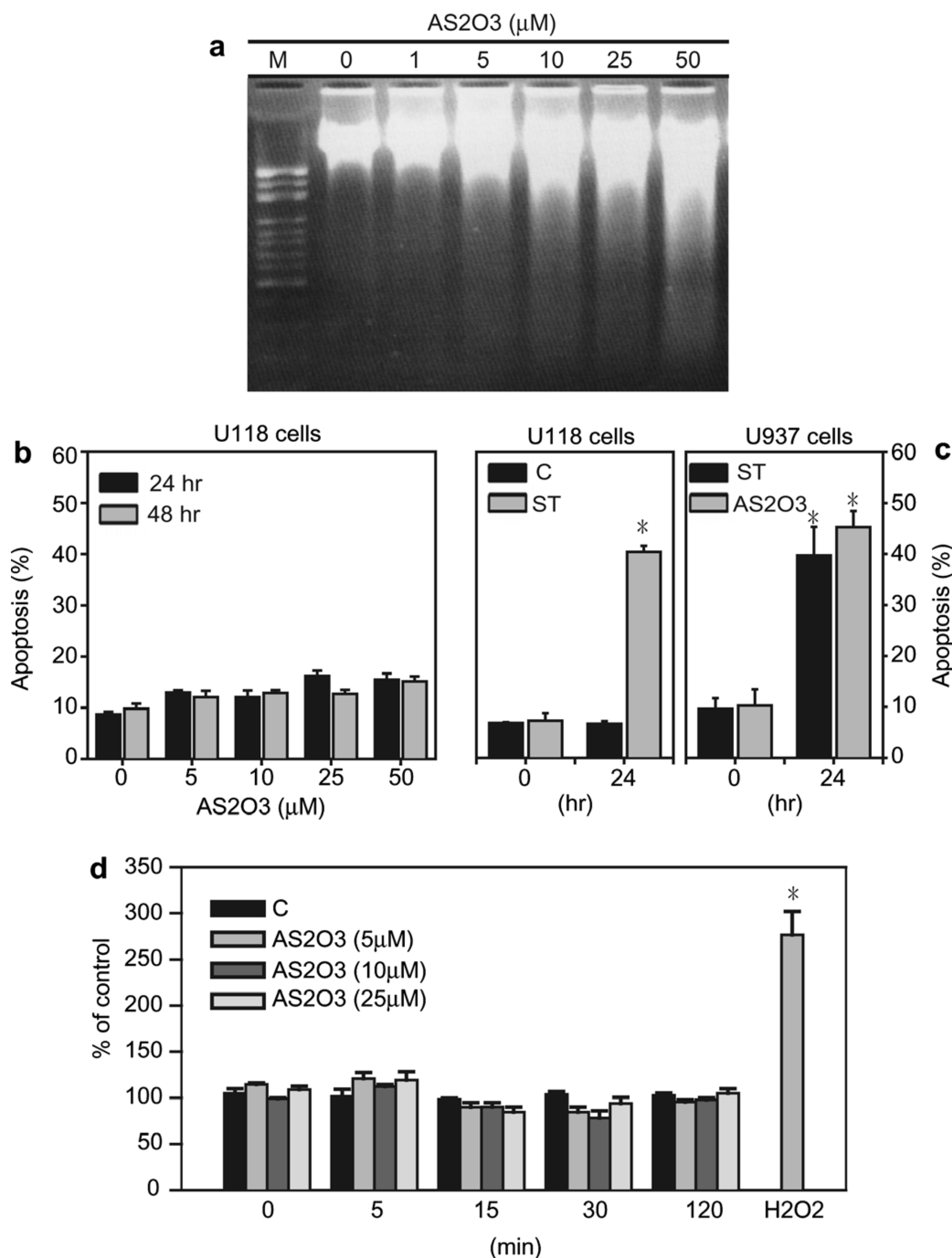

Fig. 3. Apoptotic cell death and intracellular peroxide levels were not induced by AS_2O_3 in U118 cells. (a) U118 cells were treated with AS_2O_3 (1–50 μM) for 24 h. Cells were harvested and DNA degradation was determined by gel electrophoresis (b). (c) The apoptotic effect of the U118 cells treated with AS_2O_3 (5–50 μM) or staurosporine (ST) for the indicated time points was monitored by flow cytometric analysis with Annexin V-FITC apoptosis detection kit. Human U937 cells were treated either with 10 μM AS_2O_3 (Nolte et al., 2004) or 50 nM ST (Grant et al., 1994) for 24 h as a positive control. (d) U118 cells were treated with AS_2O_3 at difference doses (5–15 μM) and time period (0–120 min). For a positive control (Gamalei et al., 1998), H_2O_2 (400 μM) was added to the culture medium for 60 min. The level of intracellular peroxide in cells was measured by flow cytometric analysis using DCHF-DA as a fluorescence dye. Data are derived and counted from three independent experiments. $^*p < 0.05$.

levels of cyclins A, E, and p21 were not changed, even by treatment with 25 μM AS_2O_3, but cyclin B1 was decreased in a dose-dependent manner.

As described above, the cytotoxicity induced by AS_2O_3 was nearly completely prevented by LA (Figs. 2 and 4). To further investigate the molecular mechanisms of the LA affect on AS_2O_3-induced cytotoxicity, U118 cells were treated with AS_2O_3 (5 μM) in the presence of LA (50 μM) for 24 h. The cell lysates were isolated, and Western blotting analysis was then performed (Fig. 6). Our results, as presented in Fig. 5, revealed that Bcl-2 and HSP70 were up-regulated in the AS_2O_3 (5 μM)-treated

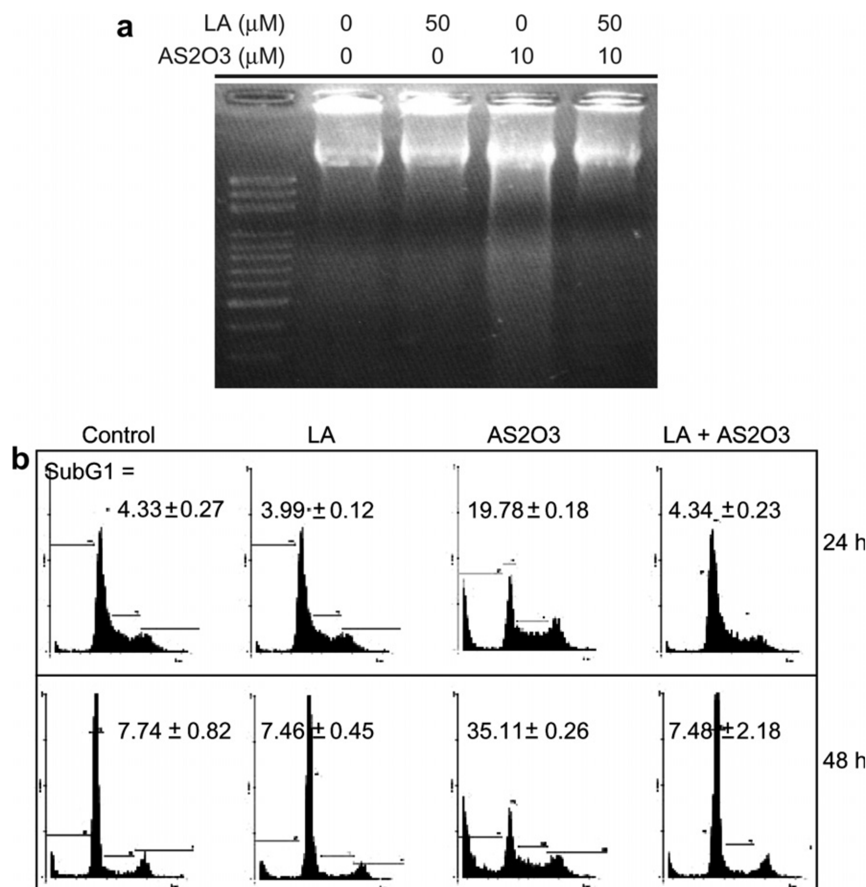

Fig. 4. LA attenuation of AS_2O_3-induced cell death was not through apoptosis inhibition. (a) AS_2O_3-induced DNA degradation was completely prevented by LA treatment in U118 cells. Cells were treated with AS_2O_3 (10 μM) with or without LA (50 μM) for 24 h. DNA integrity in cells was analyzed by agarose electrophoresis. (b) The DNA degradation effect on the cells treated with AS_2O_3 was also monitored by flow cytometric analysis. Cells were treated with AS_2O_3 (5 μM) in the presence or absence of LA (50 μM) for either 24 or 48 h. Cells were then harvested for determination of the subG1 population (representing the apoptotic or necrotic cells). The percentage of cells in the subG1 phase of the cell cycle was determined using established CellFIT DNA analysis software, as shown at the bottom. Results are shown by the means ± SD of three independent experiments. $^{*}p < 0.05$.

U118 cells. The increased Bcl-2 and HSP70 level was significantly reversed by LA (50 μM) treatment when U118 cells were exposed to AS_2O_3 (Fig. 5). These results differed from those of a previous report which indicated that HSP70 was inhibited in AS_2O_3-induced apoptosis in human U937 premonocytic cells in which the down-regulated-HSP70 was attenuated by Bcl-2 over-expression (Ramos et al., 2005). In addition, Fig. 5 demonstrated that p53 and Bax protein expressions were inhibited by AS_2O_3 treatment in U118 cells. Such effects were also reversed by LA treatment when U118 cells were exposed to AS_2O_3. Moreover, pretreatment with protein synthesis inhibitor cyclohexamide (CXM) blocked the autophagy indicated the requirement of the protein synthesis in the induction of AVO induced by AS_2O_3 (Fig. 6b).

However, in this study, apoptosis was judged by four independent criteria, including (a) cell morphology, as described previously (Fig. 2a) (Ho et al., 1996), (b) translocation of phosphotidyl serine to the cell surface, as detected by an Annexin V-FITC apoptosis detection kit (Fig. 3b), (c) the presence of a sub-G1 peak, as detected by flow cytometry (Fig. 4b), and (d) the appearance of DNA fragmentation analyzed by the method previously described (Fig. 3a) (Ho et al., 1996). Our results indicated that apoptosis was observed in only 14.8% of U118 cells when treated with high-dose AS2O5 (50 μM) for 48 h. And, in the 10 μM AS_2O_3-treated U118 cells, supravital cell staining with acridine orange revealed the induction of acidic vesicle organelles (Fig. 7b), which were completely blocked by LA (50 μM) (Fig. 7d), suggesting the involvement of autophagy. Calculation of the acridine orange positive-stained cells demonstrated that AS_2O_3-induced autophagic cell death was increased in the U118 cells in a dose-dependent manner (Fig. 7f). To further confirm arsenic induced autophagy was not mainly through ROS generation. One of the antioxidant DMSA (Dimercaptosuccinic acid) was used to test this hypothesis. Fig. 7e showed that the ROS scavenger DMSA could not reverse arsenic induced autophagy in U118 cells analyzed by flow cytometry. However, AS_2O_3-induced autophagy in U118 cells was significantly attenuated by LA treatment (Fig. 7f). Our results suggested that arsenic mediated autophagic cell death was not mainly through

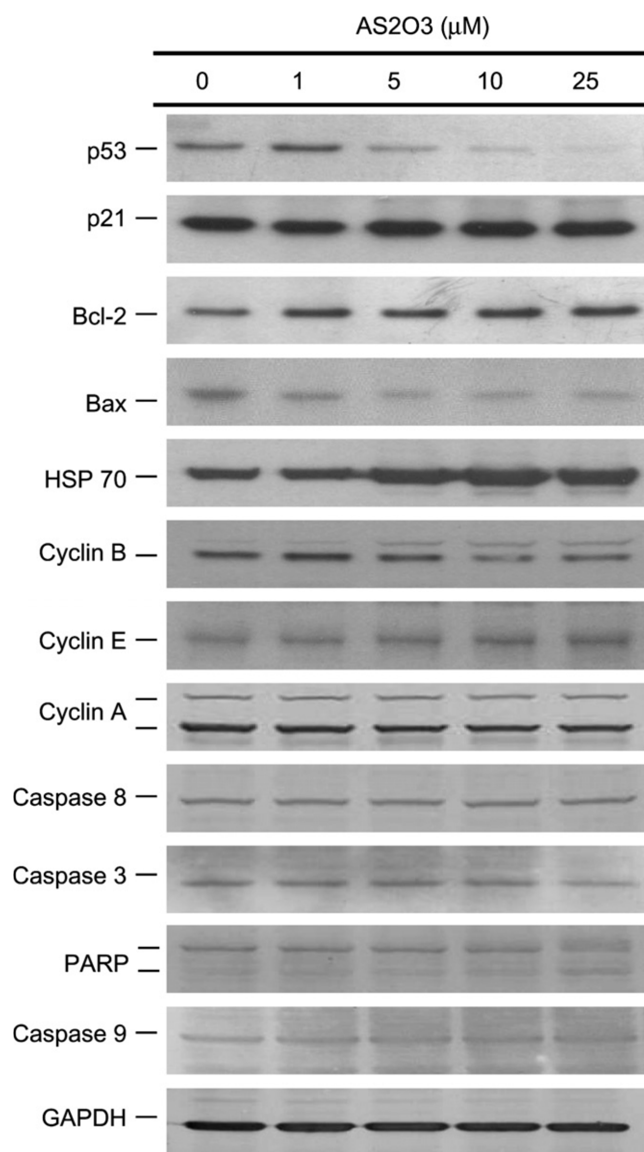

Fig. 5. The dose-dependent effect of the cell growth and apoptosis-associated regulatory proteins expression in U118 cells treated with AS_2O_3. U118 cells were treated with AS_2O_3 (1–25 μM) for 24 h, and the expression levels of cell cycle- and cell death-associated proteins were detected by Western blot analysis. Cells were mock-treated with DMSO (0.05%) as a control group. The expression level of GAPDH protein was examined and served as a loading control.

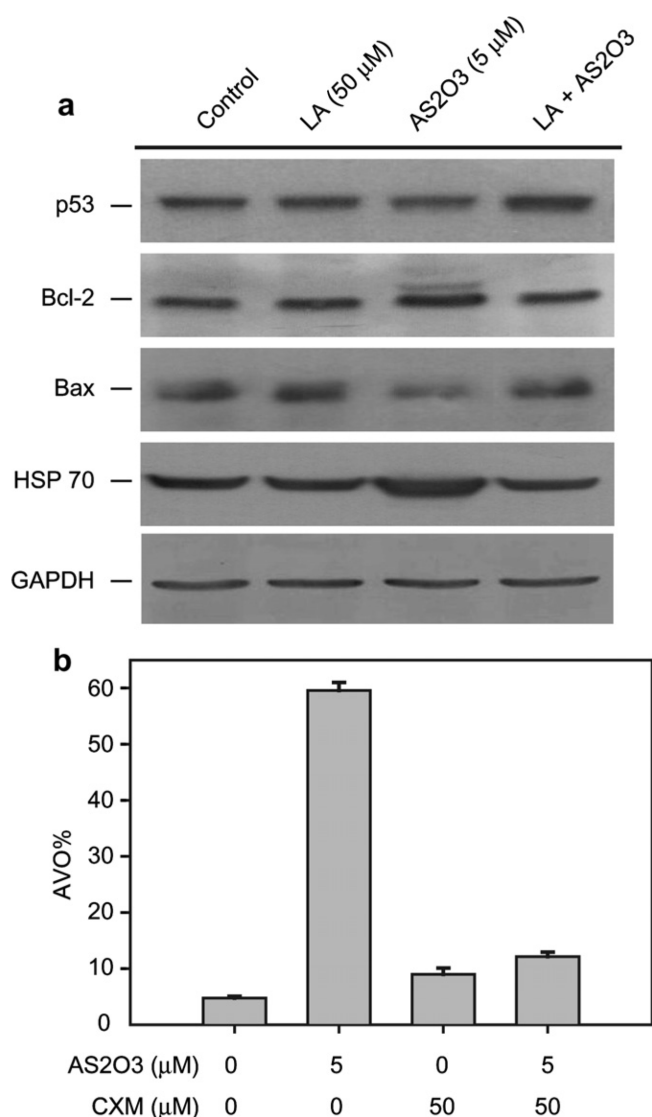

Fig. 6. The AS_2O_3-regulated protein expression was reversed by LA treatment in U118 cells. (a) U118 cells were treated with LA (50 μM), AS_2O_3 (5 μM), or combine-treated with LA plus AS_2O_3 for 24 h. Cells were then harvested and the protein expression was detected by Western blot analysis. Cells were mock-treated with DMSO (0.05%) as a control group. The expression level of GAPDH protein was examined and served as a loading control. (b) U118 cells were treated with AS_2O_3 5 μM in the presence or absence of protein synthesis inhibitor cyclohexamide (CXM) 50 μM for 24 h. Cells were stained with acridine orange and the percentage of AVO occurred was detected by flow cytometry.

ROS generation, and the protective effect of LA was not attributed to its antioxidant activity only.

4. Discussion

Increasing evidence indicated that glial cells play critical roles in numerous brain functions such as migration, axonal growth, and terminal differentiation of different neuronal subsets, through the release of soluble factors and cell-cell contact. Pathological alterations of glial cells have also been indicated associated with brain disease that impaired normal functions of neuron (Kim and de Vellis, 2005). The main purpose of this study was to investigate the mechanisms of glial cell toxicity caused by arsenic and to test the protective effects of LA. Human glioblastoma cell line U118 cells were used as the research model to study arsenic-induced toxicity in glial cells. Although the use of cell lines for *in vivo* human disease fills a necessary gap, there is an implicit assumption that these tumor cell lines offer a representation differ from primary cultured cells. However, tumor cell lines are still good models widely used for studying the mechanisms, functions of the cells not restricted in the oncology field.

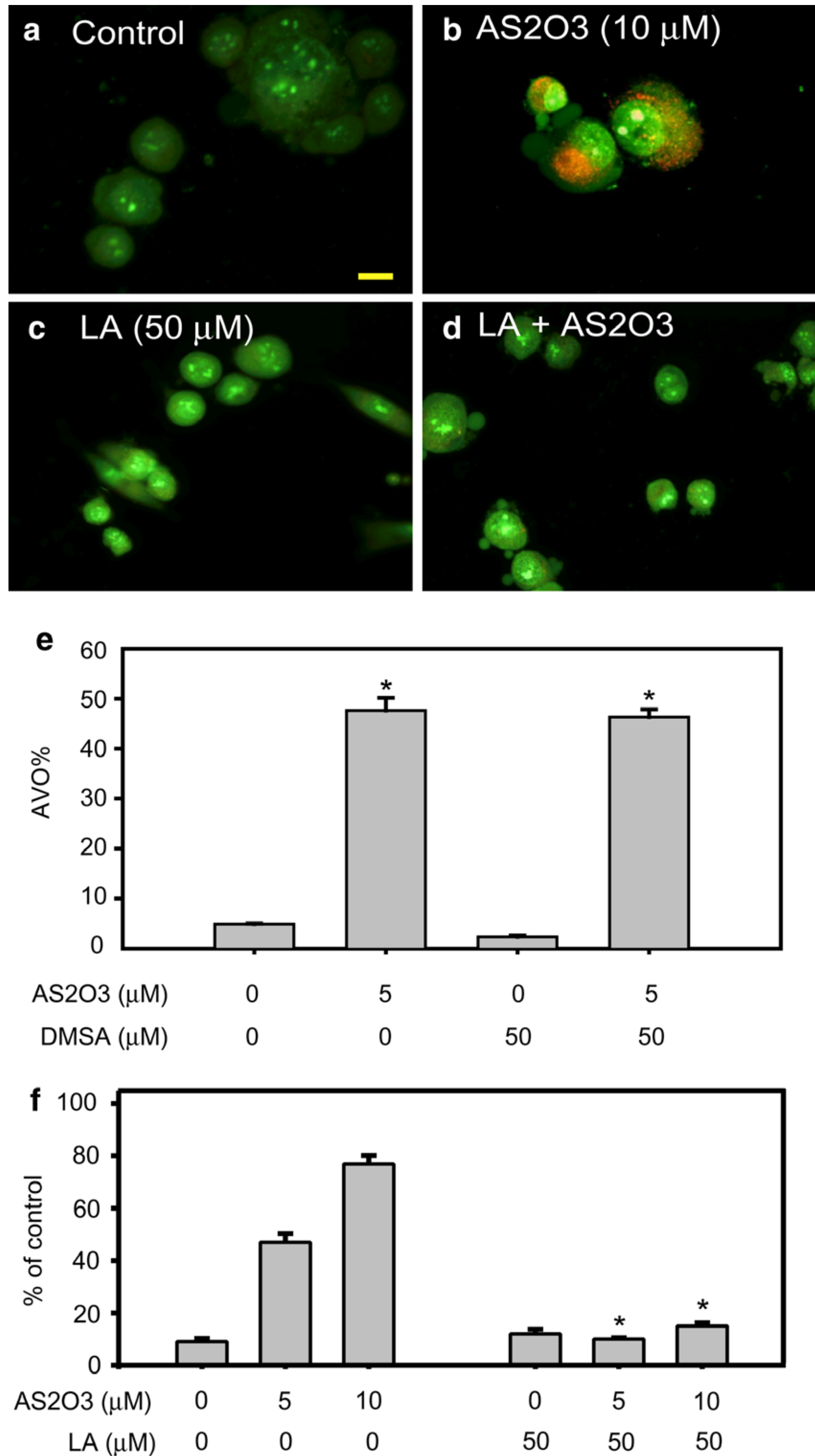

Fig. 7. Microphotograph using supravital cell-stain acridine orange in AS_2O_3-treated U118 malignant glioma cells. (a) control; (b) 10 μM AS_2O_3-treated cells; (c) 50 μM LA-treated cells; (d) 10 μM AS_2O_3 and 50 μM LA-treated cells. Note the large amount of acidic vesicular organelle (AVOs) after treatment with AS_2O_3 (b). This is consistent with the autophagic changes described in the previous paper. Acidification of AVO was inhibited by LA (d). In control cells, the cytoplasm and nucleus basically revealed the green fluorescence, but a small accumulation of the acidic component was occasionally observed (a). The bar appearing in (a) is 10 μM. (e) U118 cells pretreated with antioxidant DMSA 50 μM following AS_2O_3 5 μM did not show any inhibition on AVO induction. The mean ± S.E. $^*p < 0.05$ indicated a significant difference in AS_2O_3 alone or combined DMSA groups compared with control group, as analyzed by unpaired Student's *t*-test. (f) The AVOs positive cells were counted from 10^3 cells and counted from three independent experiments, and each value was presented as the mean ± S.E. $^*p < 0.05$ indicated a significant difference between AS_2O_3 alone groups and combined LA groups, as analyzed by unpaired Student's *t*-test.

AS_2O_3-induced apoptosis was a general mechanism found in most of clinical applications for cancer chemotherapy (Park et al., 2003, 2000; Woo et al., 2002). Apoptotic neuronal cell death was also suggested as a general

mechanism underlying arsenic neurological toxicity. However, in this study, when U118 cells were exposed to higher doses of AS_2O_3 (>5 μM, Figs. 1 and 7), autophagy (programmed cell death type II), but not apoptosis (programmed cell death type I) was induced in U118 cells as evidenced by AVOs assays. Similar results were reported in recent studies. Kanzawa et al. demonstrated that AS_2O_3 induced autophagic cell death, but not apoptosis, in several human malignant glioma cell lines, where U118 cells were not included (Kanzawa et al., 2003, 2005). Autophagy is a process in which subcellular membranes undergo dynamic morphological changes for the degradation and turnover of cytoplasmic organelles (Mizushima et al., 1998). Excessive autophagy may contribute to the pathogenesis of some neurodegenerative disorders such as Alzheimer's disease by altering the processing of mutant forms of amyloid precursor proteins (Meijer and Codogno, 2004). However, the precise mechanisms regulating autophagic cell death in glial cells remain unclear.

AS_2O_3-induced apoptosis has been observed in different types of human cancer cells and in brain tissue cultures (Shila et al., 2005). In these models, increased intracellular oxidative stress was demonstrated to be one of the important mechanisms that lead to AS_2O_3-induced cell death (Chun et al., 2002; Liang et al., 2003; Samuel et al., 2005; Shen et al., 2003; Shila et al., 2005; Tong et al., 2001; Wang et al., 2005; Woo et al., 2002). The present results reveal that ROS generation was not induced by 5 μM AS_2O_3 within 2 h in U118 cells (Fig. 3d), suggesting the possibility that AS_2O_3-induced autophagic cell death in U118 cells may not occur through the ROS-mediated signaling pathway. Our results postulated that specific regulatory mechanisms of AS_2O_3-induced cytotoxicity must occur in U118 cells. Previous studies indicated that autophagic cell death and apoptosis are pathways to the same end; a functional connection between both forms of cell death is likely to be operative. Both forms of cell death can act as backup mechanisms of each other, under conditions where cell death is imperative (Ng and Huang, 2005). As described previously, the apoptosis-associated regulatory proteins, including p53, Bax, and caspases 3, 8, and 9, were induced in AS_2O_3-mediated apoptosis (Liang et al., 2003) and AS_2O_3-induced apoptosis was more easily observed in p53 wild-type glioblastoma cells (U87MG), when compared to p53-mutated cells (T98G) (Zhao et al., 2002). The p53 status in U118 cells has been described as a mutated type in a previous report (Ohneseit et al., 2005). Interestingly, we also found that caspases 3, 8, and 9 remained unchanged, and p53 and Bax proteins were significantly down-regulated in the U118 cells treated with AS_2O_3 in a dose-dependent manner (Fig. 5). Such results implied that sufficient or intact p53 protein functions are needed to induce apoptosis in response to AS_2O_3 in human glioma cells. Thus, arsenic induced U118 cells autophagy maybe occurred when the p53 mediated apoptotic pathway of U118 cells was blocked after arsenic exposure. However, the precise mechanisms regulating autophagic cell death in glial cells remain unclear.

While some heat shock proteins (HSPs) are constitutively expressed, others are induced by various stress agents. Induction of HSP-70 has been demonstrated in C6 rat glioma cells treated with 100 μM sodium arsenite for 1 h (Kato et al., 1997). It is suggested that HSP expression is generated by abnormally folded, nonnative proteins produced by stress capable of triggering the oxidation of nonprotein thiols and leading to heat shock transcription factor activation. A previous report indicated that one of the three types of autophagy is chaperone mediated autophagy, in which the heat shock protein families (Hsc-73) bind to soluble proteins and initiate their transport to prelysosomal or lysosomal compartment leading to complete degradation of the proteins (Ng and Huang, 2005). In our current study, we found that HSP-70 expression was increased in arsenic-induced U118 cell autophagy and attenuated by LA treatment. These results implicate that arsenic-induced autophagic cell death mechanisms in glial cells may related to the heat shock response. Arsenic is capable of inducing HSPs of various sizes, from the smaller proteins (HSP-25, HSP-27, and HSP-30) to the larger HSP species, such as HSP-105 depends on cell type, dose, and metabolic state of the cell. However, the detailed mechanisms of Hsp-70 in AS_2O_3 induced autophagy need to be further investigated.

Current evidence supports the role of reactive oxygen species in a number of types of acute and chronic pathologic conditions in the brain and neural tissue in response to arsenic. The study of Shila et al. also confirmed that arsenic reduced the activities of antioxidants enzymes in the rat brain region. Simultaneously treatment of LA along with arsenic may against arsenic-induced decline in the antioxidants enzymes, increased oxidants and lipid peroxidation (Shila et al., 2005). Samuel et al. further suggested the protective effects of LA was due to its antioxidant activity to directly react with various ROS species, regulate the reproductive nature of the system to prevent oxidation of protein thiols and prevent arsenic from binding to thiols (Samuel et al., 2005). The original purpose of this study was to test the hypothesis that LA prevents AS_2O_3-induced autophagy through the scavenging of ROS generation. Induction of oxidative stress, including ROS generation, lipid peroxidation, and glutathione reduction in U118 cells treated with mycotoxin fumonisin B1 has been reported before (Samuel et al., 2005). However, our result reveal that ROS generation was not induced by AS_2O_3 within 2 h in U118 cells (Fig. 3d), suggesting the possibility that AS_2O_3-induced autophagic cell death in U118 cells may not occur through the ROS-mediated signaling pathway. On the one hand, AS_2O_3-mediated autophagic cell death was proved to be nearly completely attenuated by LA, but not by the other antioxidant such as DMSA (Fig. 7e). Thus, we suggested that the protective effect of LA was not attributed to its antioxidant activity only and we strongly believe that other regulating pathways could dominate the direct effects of ROS in AS_2O_3-induced autophagy in U118 cells. It is suggested that LA may

activate certain as-yet-uncharacterized signaling intermediates by inducing intramolecular disulfide bond formation, and these intermediates in turn trigger the activation of transcription factors which promote the expression of several genes that can protect U118 cells from autophagy (McCarty, 2001; Prestera and Talalay, 1995; Talalay et al., 1995). Moreover, the binding of arsenic to critical thiol groups in proteins is one of its toxic mechanisms. Since LA itself is a thiol compound, direct interaction between LA and arsenic may occur. Thus, the protective effect of LA against AS_2O_3-induced glial cells from autophagy may be attributed to the direct chelating of the compound or through inducing uncertain signaling pathways.

From oncologic aspect, glioblastoma is a highly malignant glioma and possesses resistant to many treatments, including radiotherapy, chemotherapy, and adjuvant therapy (Haga et al., 2005). Recently, clinical trial results have suggested that AS_2O_3 has potential effectiveness in patients with other solid tumors, including melanoma (Kim et al., 2005), hepatocellular carcinoma (Zhang et al., 2003), and renal cancer (Vuky et al., 2002). Previous reports and the current study have demonstrated that arsenic trioxide could induce autophagy in malignant glioma cells (Kanzawa et al., 2003, 2005). These suggested a clinical benefit by using AS_2O_3 as anticancer agent in the treatment of glioma. Furthermore, the current study also found that LA could reverse AS_2O_3-induced autophagic cell death of U118 cells. It has been reported that treatment of antioxidants can protect cells against radiation and chemotherapy (Greggi Antunes et al., 2000; Sonneveld, 1978; Witenberg et al., 1999). Theses results implied that antioxidants might protect cancer cells, thereby reducing the oncologic effectiveness or cytotoxicity therapy. Although the protective effects of LA in inhibiting U118 cells from autophagy were not mainly through antioxidant property, we suggested that patients should avoid LA supplements during arsenic based chemotherapy or arsenic combined radiotherapy.

Taken together, the present study demonstrated for the first time that AS_2O_3-induced glial cells toxicity could be completely attenuated by LA. U118 cells, autophagic, but not apoptotic, cell death was induced by AS_2O_3, and the AS_2O_3-mediated autophagic cell death may not occur mainly through the ROS-triggered signal pathway. Down-regulation of p53 and Bax proteins and up-regulation of Bcl-2 and HSP-70 proteins may involve in AS_2O_3-mediated autophagic cell death. Even the detailed mechanisms of LA against AS_2O_3-induced glial cells autophagy are still unclear and need to be further investigated. We concluded that LA could be a useful protective agent against arsenic-induced glial cells toxicity and reversing arsenic-induced damage in human brain.

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