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N-acetyl-L-cysteine reduces arsenite-induced cytotoxicity through chelation in U937 monocytes and macrophages

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Abstract. In the present study, in order to clarify the preventive mechanism of *N*-acetyl-L-cysteine (NAC) on arsenite-induced apoptosis in U937 cells, which lack functional p53, the cytotoxicity among U937 cells [monocytes and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated macrophages] receiving NAC treatment at different times post arsenite treatment was examined. TPA-treated macrophages were more resistant to arsenite-induced apoptosis than monocytes, which may be associated with the induction of Bcl-2 expression. Pretreatment with 20 mM NAC prior to arsenite exposure suppressed apoptosis up to 75% in the monocytes and 100% in the macrophages. However, 6-h NAC pretreatment and subsequent washing out of NAC from the culture medium prior to arsenite treatment did not inhibit the arsenite-induced apoptosis. Post-treatment by NAC up to 1 h following arsenite exposure almost completely inhibited the cytotoxic effects of arsenite in U937 monocytes and macrophages. The results of the current study indicate that the preventive mechanism of NAC on arsenite-induced apoptosis in U937 monocytes and macrophages mainly involves chelation of arsenite in culture medium.

Introduction

Arsenic is recognized as a serious threat to public health in numerous Asian countries (1-4). In Pakistan, for example, there are still a number of regions in which the arsenic concentrations of drinking water exceed the World Health Organization standard of 10 ppb (μg/l), and arsenic levels in the large water reservoirs have been demonstrated to be as high as 600-700 μg/l (2-5). The majority of arsenic compounds in drinking water are inorganic arsenite or arsenate (6).

Chronic exposure to inorganic arsenite induces various biological effects in tissues and cells, including cancer development at various sites (7), an immunosuppressive status (8) and apoptosis (9). In particular, one study demonstrated that the impaired immune function of macrophages contributed to immune suppression in arsenic-exposed individuals (10). Luna *et al* (11) reported an association between arsenic exposure and the increase of nitric oxide and the superoxide anion in peripheral blood mononuclear cells and monocytes, suggesting that arsenic induced oxidative stress in circulating blood cells. Another study indicated increased production of reactive oxygen species (ROS), which mediate p53 activation and apoptosis, during the metabolism of inorganic arsenic (12).

The U937 cell line is an established model for monocyte/macrophage differentiation, and the absence of functional p53 in U937 monocytes is well established (13). The treatment of U937 monocytes with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces differentiation along the macrophage pathway (14,15), and TPA-induced differentiation has been suspected to modify pharmacological effects, for example, offering resistance to apoptosis induced by a series of anticancer drugs (16,17). A number of studies have examined the difference in the cytotoxicity of arsenite between TPA-treated and non-treated human myeloid cells, but the results have been inconsistent: Sordet *et al* (18) reported that TPA-differentiated cells exhibited increased susceptibility to apoptosis induced by arsenite but the results of others implied that TPA-treated U937 macrophages were more resistant to the arsenite-induced apoptosis than untreated cells (19,20).

N-acetyl-L-cysteine (NAC), a widely-used antioxidant, is a precursor of L-cysteine and glutathione (GSH) (21). NAC treatment has been demonstrated to ameliorate mortality rates in arsenic-treated mice and induce a reduction in the levels of arsenic-induced oxidative stress in the tissues (22). Reddy *et al* (23) indicated the beneficial role of NAC in counteracting arsenic-induced oxidative stress. NAC has also been reported to act as a chelator (24).

To clarify the preventive mechanism of NAC on arsenite-induced apoptosis in U937 cells, the cytotoxicity

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among U937 cells (monocytes and TPA-treated macrophages) receiving NAC treatment at different times post arsenite treatment was examined.

Materials and methods

Cells and chemicals. The human U937 monocytic leukemia cell line (Dainippon Sumitomo Pharma, Osaka, Japan) was maintained in suspension culture of RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA) at 37˚C in a humidified 5% CO_2 atmosphere.

U937 monocytes (initial cell concentration: $2x10^5$ cells/ml) were differentiated to macrophages (attached to the bottom of plates) by the addition of TPA (Wako Pure Chemical Industries, Osaka, Japan) into the regular medium at a final concentration of 10 nM for 48 h.

TPA stock solution (10 mM) was dissolved in dimethylsulfoxide (DMSO) and stored at -80˚C. Further dilutions of TPA were prepared in regular medium immediately prior to use. The final DMSO concentration did not exceed 0.1% (v/v).

Sodium arsenite (Sigma-Aldrich, Tokyo, Japan) was dissolved in distilled water to form a 100 mM stock solution, and was diluted with regular medium immediately prior to use.

NAC (1 M; Wako Pure Chemical Industries) was dissolved in regular RPMI-1640 medium without FBS, and the pH of the medium was adjusted to 7.4 with NaOH prior to use.

Arsenite and/or NAC treatments, and cytotoxicity determination. An assessment of the cytotoxicity of arsenite was performed with an initial cell concentration of $2x10^5$ cells/ml (100 ml/well) in 96-well plates. The U937 monocytes and macrophages were treated with arsenite (1-100 μM) for 24 h. Cell proliferation was determined by the trypan blue exclusion assay for U937 monocytes (25) using 0.2% trypan blue solution (Life Technologies, Tokyo, Japan) in phosphate-buffered saline (PBS) and the WST-8 assay for U937 macrophages using CCK-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the manufacturer's instructions. The cytotoxicity of NAC in U937 monocytes and macrophages was also determined, by treating cells with a range of concentrations of NAC (0, 1, 2, 5, 10 and 20 mM) for 25 h.

The preventive effects of NAC on arsenite-induced cytotoxicity were then examined. The U937 cells were treated with each concentration of NAC (0, 1, 2, 5, 10 or 20 mM) for 1 h, and then incubated in the presence of 50 μM arsenite for 24 h.

To determine whether arsenite-induced cytotoxicity can be prevented by the pretreatment of cells with NAC and subsequent wash-out, U937 monocytes were incubated with 20 mM NAC in a 12-well plate for 6 h, followed by washing twice with regular medium and centrifugation (500 x g for 3 min), prior to incubation with 50 μM arsenite in a 96-well plate for 24 h. Macrophages were incubated with 20 mM NAC in a 96-well plate for 6 h, followed by washing twice with regular medium, prior to incubation with 50 μM arsenite for 24 h.

To determine the time-course dependence for the preventive effects of NAC on arsenite-induced cytotoxicity, NAC (final concentration: 20 mM) was added to U937 monocytes and macrophages at 0, 1, 3, 6 or 12 h following exposure to 50 μM arsenite.

Apoptosis detection. To evaluate the levels of apoptosis of U937 cells in the presence of arsenite, U937 monocytes with an initial concentration of $2x10^5$ cells/ml (100 μl/well) were plated in 8-well Lab-Tek Permanox Chamber slides (Nalge Nunc International, Rochester, NY, USA) with TPA for 48 h and then treated with arsenite for a further 24 h. Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using an *in situ* Apoptosis Detection kit according to the manufacturer's instructions (Takara Bio, Otsu, Japan). It is established knowledge that ultraviolet (UV) treatment induces apoptosis in U937 cells (26). Therefore, U937 monocytes were exposed to a 302 nm M-15 UV transilluminator (UVP, Upland, CA, USA) from below for 5 min at room temperature and used as a positive control for apoptosis detection in U937 cells.

Immunocytochemistry. U937 monocytes and macrophages were transferred to the glass slides by a Cytospin 4 Cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 452.79 g for 5 min. Slides were dried at room temperature and cells were fixed by dipping slides in 4% paraformaldehyde for 30 min at room temperature. After rinsing with PBS (pH 7.4), non-specific antibody binding was reduced by incubating the cells with horse serum in PBS for 30 min. The cells were then incubated for 1 h at 4˚C with a rabbit polyclonal antibody for Bcl-2 (N-19, 1:50 dilutions; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following a thorough PBS wash, the slides were incubated with biotinylated rabbit antibody IgG (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature, and then with a 1:100 dilution of the avidinbiotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Inc.) for an additional 30 min at room temperature. The peroxidase signal was visualized by treatment with DAB Substrate-Chromogen system (Dako, Carpinteria, CA, USA) for 10 min. The cells were then stained lightly with hematoxylin. Cytoplasmic staining was considered to indicate Bcl-2 expression. The cells were viewed with a Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany) and images were captured using a Pixera Viewfinder 3.0.1 (Pixera Corporation, Los Gatos, CA, USA).

Statistical analysis. All values are expressed as the mean ± standard error. Statistical analysis was conducted using the Mann-Whitney U test. All P-values presented are two-sided and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cytotoxicity of arsenite in U937 monocytes and macrophages. The LD_{50} values of arsenite in monocytes and macrophages were 10 μM and 20 μM, respectively. Arsenite at 50 μM led to 95% and 85% cell death in monocytes and macrophages, respectively (Fig. 1A). Levels of apoptotic cell death were confirmed by TUNEL assay. Following treatment of macrophages with 20 μM arsenite, positive signs of apoptosis were clearly observed in the nuclei of the cells (data not shown).

Determination of the toxicity of NAC in U937 monocytes and macrophages. The cell viabilities of the two types of U937 cell

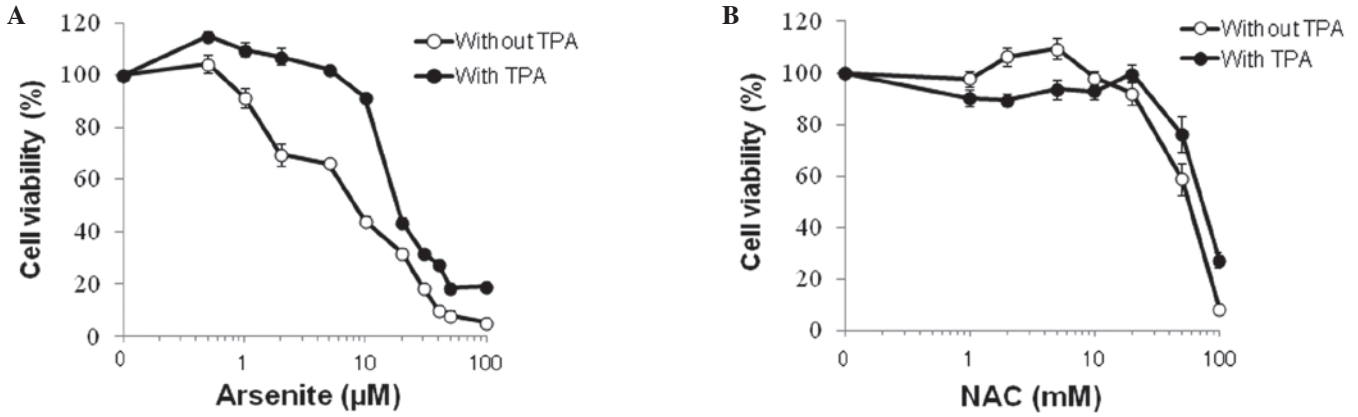

Figure 1. Cytotoxicity of arsenite and *N*-acetyl-L-cysteine (NAC) in U937 monocytes and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated U937 macrophages. (A) Treatment with 0-100 μM arsenite for 24 h. (B) Treatment with 0-100 μM NAC for 25 h. Values represent the mean ± standard error of five experiments.

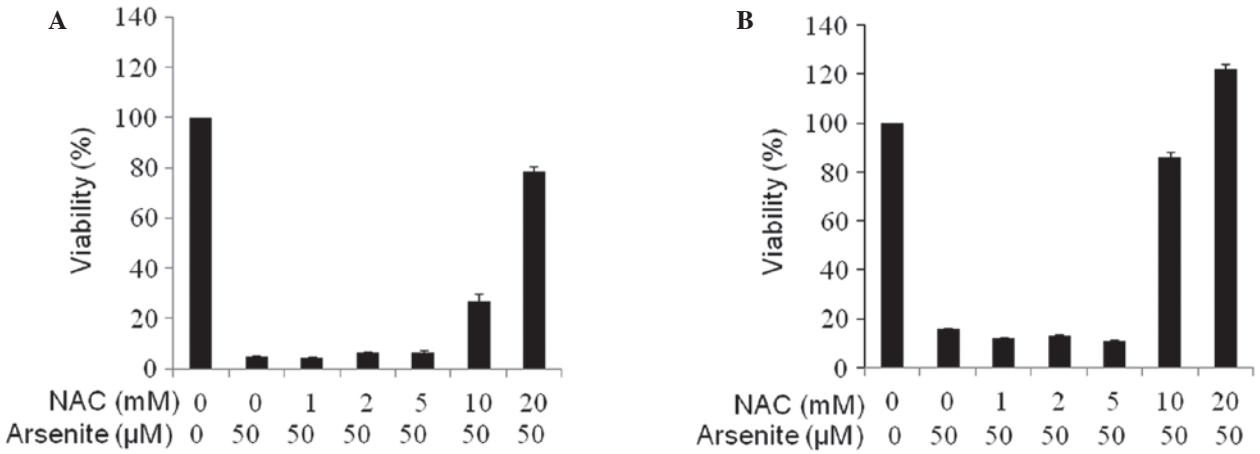

Figure 2. Effect of pretreatment with a range of concentrations (0, 1, 2, 5, 10 and 20 mM) of *N*-acetyl-L-cysteine (NAC) on arsenite-induced cytotoxicity in (A) U937 monocytes and (B) 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated U937 macrophages. Values represent the mean ± standard error of five experiments.

were not altered following NAC treatment of ≤20 mM, then significantly declined following NAC incubation at 50 μM, for 25 h (P=0.016 and P=0.026 for monocytes and macrophages, respectively; Fig. 1B). Therefore, the concentration of 20 mM NAC was selected for use in further experiments.

Effect of NAC on arsenite-induced cytotoxicity in U937 monocytes and macrophages. Preventive effects of NAC on the arsenite-induced apoptosis were examined. The cells were incubated with NAC-containing medium 1 h prior to arsenite administration. The 50-μM arsenite treatment alone caused 95% cell death in U937 monocytes without NAC, while only 20% cell death was observed in the presence of 20 mM NAC (Fig. 2A). The treatment with 20 mM NAC completely prevented arsenite-induced cell death in U937 macrophages (Fig. 2B). The effect of NAC on arsenite-induced apoptosis in macrophages was greater than that in monocytes (P<0.001). In U937 monocytes and macrophages pretreated with 10 mM NAC, 27% and 86% of cells were viable in comparison with the viability of the untreated cells, respectively (Fig. 2A and B).

Effect of NAC pretreatment and wash-out on arsenite-induced cytotoxicity in U937 monocytes and macrophages. To examine the effects of NAC pretreatment and subsequent wash-out on arsenite-induced cytotoxicity, cells were incubated with 20 mM NAC for 6 h, and then washed twice with medium prior to administration of 50 mM arsenite. The apoptosis-preventive effect of NAC was not observed in U937 monocytes or macrophages when NAC was washed out prior to arsenite administration (Fig. 3A and B). This difference in cytotoxicity between washed out and non-washed out cells was significant in both U937 monocytes and macrophages (P<0.001 for both types of cell).

Effects of NAC treatment following arsenite administration in U937 monocytes and macrophages. To examine the effect of NAC treatment following arsenite administration on arsenite cytotoxicity, NAC was added to the culture medium 0, 1, 3, 6, and 12 h post arsenite administration. In monocytes and macrophages, the arsenite-induced apoptosis was almost completely blocked by 20-mM NAC treatment if applied ≤1 h post arsenite administration (Fig. 4A and B).

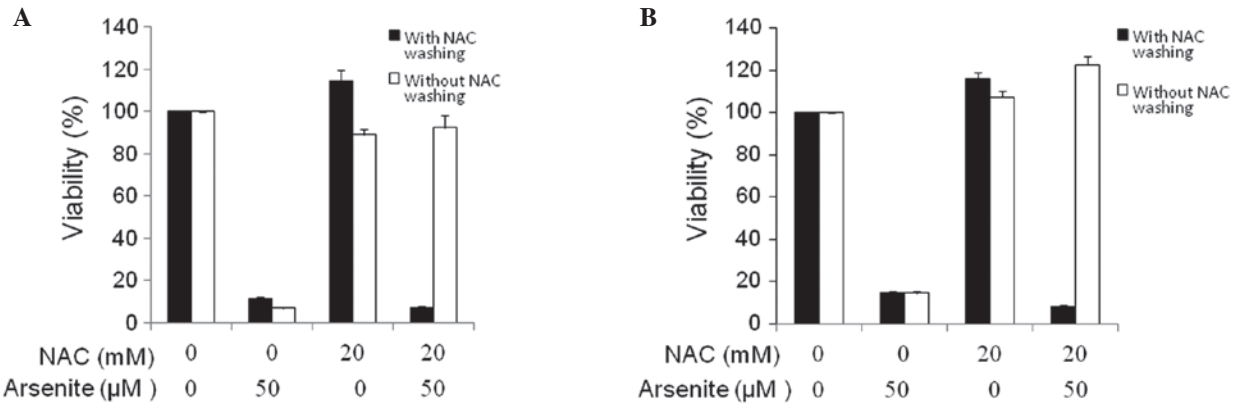

Figure 3. Effect of *N*-acetyl-L-cysteine (NAC) wash-out prior to arsenite treatment in (A) U937 monocytes and (B) 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated U937 macrophages. Values represent the mean ± standard error of five experiments.

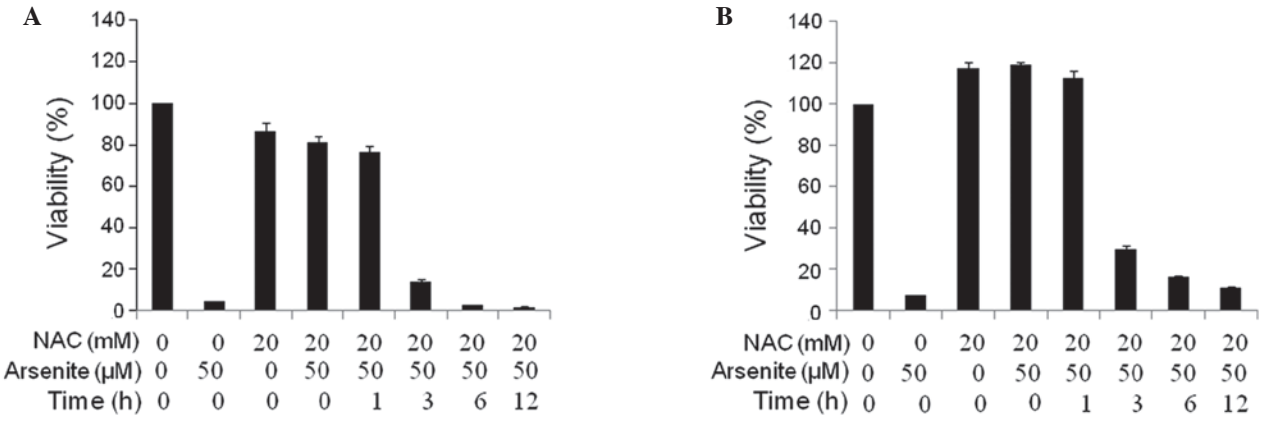

Figure 4. Time course dependence for post-treatment of *N*-acetyl-L-cysteine (NAC) in (A) U937 monocytes and (B) 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated U937 macrophages. NAC (20 mM) was added to cultured cells at 0, 1, 3, 6 or 12 h following exposure to 50 μM arsenite. Values represent the mean ± standard error of five experiments.

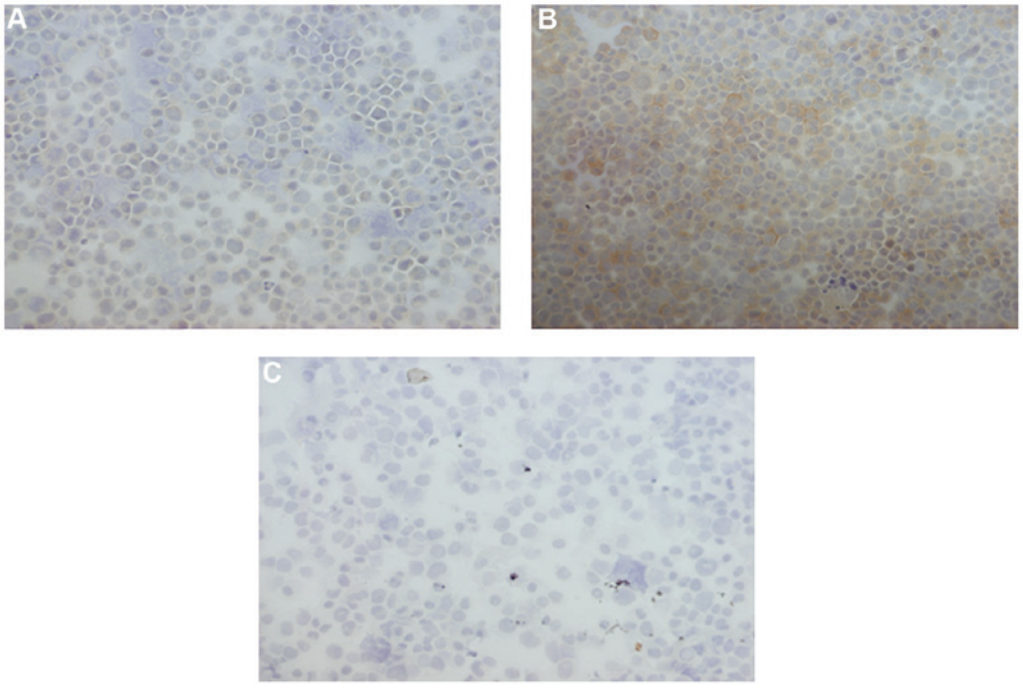

Figure 5. Bcl-2 expression in U937 monocytes and macrophages. Bcl-2 expression was examined by immunocytochemistry in (A) U937 monocytes and (B) 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated U937 macrophages. (C) U937 macrophage without Bcl-2 antibody was used as a negative control. Cytoplasmic staining indicates Bcl-2 expression. The cells were stained with hematoxylin and DAB. Magnification, x200 was used for all images.

Immunocytochemistry for Bcl-2. To examine the mechanism of resistance to arsenite treatment in U937 macrophages, Bcl-2 expression was examined using immunocytochemical assay. Bcl-2 expression was activated in TPA-treated U937 macrophages, but no activation of Bcl-2 expression in U937 monocytes was observed (Fig. 5).

Discussion

In the current study, arsenite-induced apoptosis was suppressed in the presence of 20 mM NAC in U937 monocytes and macrophages. This preventive effect was not observed when NAC was washed out from the medium prior to arsenite treatment, indicating that NAC in the medium can protect arsenite-induced apoptosis. According to a previous study, NAC can act as a chelator of metals, including chromium, lead, and boron (24). Findings of the present study indicated that the preventive mechanism of NAC in the cytotoxicity of arsenite on U937 monocytes and macrophages mainly involves chelation of arsenite in culture medium, although other studies reported non-chelation antioxidative roles of NAC in other cell lines (24).

Previous studies have indicated that the preventive effects of NAC on arsenic-induced cytotoxicity may be predominantly due to antioxidative stress occurring by increasing cellular GSH levels (21). In a study in rat heart microvessel endothelial (RHMVE) cells, the effect of NAC on arsenic-induced cytotoxicity was due to the antioxidative role of non-protein thiols and not due to chelation of arsenic in the culture medium (27). The effects of extracellular NAC on the cellular levels of GSH appear to depend on cell types. For example, A549 human epithelial cells do not take up a significant amount of the NAC or GSH in the medium, while mouse glial cells are able to take up extracellular GSH (28). Furthermore, the addition of NAC to the culture medium did not increase, if any, the intracellular GSH concentration in RHMVE cells (29).

Post-treatment of NAC within 1 h of arsenite administration completely inhibited the apoptosis in U937 monocytes and macrophages in the present study. It is possible that a small portion of arsenite in the medium is taken up by the U937 cells in 1 h, but it may not reach the threshold for toxicity. In U937 cells, histone deacetylase inhibitor MS275-induced ROS production has been demonstrated to be blocked by 20 mM NAC treatment for 1 h (30). Thus, it may be possible for NAC to be used to reduce the acute toxicity of arsenite. Martin *et al* (31) reported a clinical application of NAC for a case in which a patient ingested a potentially lethal dose of sodium arsenate (900 mg) in a suicide attempt. In addition to the demercaprol, NAC (4 g) was administered intravenously every 4 h in total of 18 doses, and the clinical condition of the patient improved markedly within 24 h.

The results of the present study clearly indicate that the preventive effect of NAC on the cytotoxicity of arsenite in U937 cells mainly acts through chelating arsenic in the culture medium. The Environmental Protection Agency (EPA) denotes an arsenic level of <10 ppb in drinking water as safe (32). Since the cytotoxicity of 50 μM (3,750 ppm=75 g/mol x 50 μM) arsenite was almost completely inhibited by NAC as low as 20 mM, 20 μM NAC may inhibit the cytotoxic effect of arsenite at the concentration of 3.75 ppm, which is 375 times higher than the EPA standard, and even higher than the arsenic concentration of water in the areas with serious arsenic contamination of water. Supplementary intake of NAC may be valuable to prevent arsenic-induced cytotoxic effects among the inhabitants of the arsenic-contaminated regions.

In experiments of the current study, the LD_{50} values of arsenite in U937 monocytes and macrophages were approximately 10 μM and 20 μM, respectively, indicating that U937 macrophages were more resistant to arsenite-induced apoptosis. This tendency was also observed in the presence of NAC. A number of studies have examined the difference in the cytotoxicity of arsenite between TPA-treated and non-treated human myeloid cells, but the results have been controversial. Sordet *et al* (18) reported that TPA-differentiated cells exhibited increased susceptibility to apoptosis induced by arsenite, which may be associated with a reduction of intracellular GSH. However, others demonstrated that TPA-treated U937 macrophages were more resistant to the arsenite-induced apoptosis than untreated cells (19,20). Although TPA has the ability to decrease intracellular GSH in U937 monocytes, this ability was not observed following TPA-induced differentiation (20). Similarly, the resistance to apoptosis by other cytotoxic agents among differentiated myeloid cells was also reported in other studies (16,17,33). These findings indicate that there may be factors other than intracellular GSH involved in the resistance to arsenic-induced apoptosis of TPA-treated U937 macrophages, under certain circumstances.

Bcl-2, one of the key proteins in the regulation of apoptosis, is an integral membrane protein located mainly on the outer membrane of mitochondria. Overexpression of Bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli directly on mitochondria by inhibition of cytochrome *c* translocation and subsequently blocking caspase activation (34,35). In the present study, activation of Bcl-2 expression was observed in TPA-treated U937 macrophages but not in U937 monocytes. These findings are in accordance with those of a previous study indicating the upregulation of anti-apoptotic proteins, including Bcl-2, in TPA-treated U937 macrophages (36). Other studies did not demonstrate the upregulation of Bcl-2 protein by TPA treatment in U937 cells (17,37). Since p53 expression in U937 cells was not induced by TPA treatment in a previous study (38), the effect of TPA treatment on the pathway downstream of p53 may be involved in the sensitivity to stimuli.

In conclusion, the present study demonstrated that NAC treatment prevents the arsenite-induced apoptosis mainly by acting as a chelating agent in U937 monocytes and macrophages. In addition, Bcl-2 expression may be involved in the differences between the sensitivity of U937 monocytes and macrophages to arsenite exposure.

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