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Arsenic trioxide induces apoptosis and G2/M phase arrest by inducing Cbl to inhibit PI3K/Akt signaling and thereby regulate p53 activation

Yingchun Li¹, Xiujuan Qu¹, Jinglei Qu, Ye Zhang, Jing Liu, Yuee Teng, Xuejun Hu, Kezuo Hou, Yunpeng Liu*

Department of Medical Oncology, The First Hospital, China Medical University, No. 155, North Nanjing Street, Heping District, Shenyang City 110001, China

ABSTRACT

Arsenic trioxide (ATO) strongly induces apoptosis in acute promyelocytic leukemia (APL), but it induces cell cycle arrest in most solid tumors. In this study, we investigated the mechanism of ATO action on APL-derived NB4 cells and gastric cancer cell lines. ATO decreased the viability of both cell lines, but gastric cancer cells were much less susceptible. ATO-induced G2/M phase arrest and p53 degradation in gastric cancer MGC803 cells. In contrast, ATO-induced apoptosis in NB4 cells without degradation of p53. Both processes were accompanied by transient activation of Akt. The PI3K/Akt inhibitor LY294002 significantly increased the amount of p53 protein and ATO-induced apoptosis in both cell lines and decreased G2/M phase arrest of MGC803 cells. In addition, ATO up-regulated the expression of Cbl proteins in both cell lines. Inhibition of Cbl with the proteasome inhibitor Ps341 decreased apoptosis in NB4 cells and increased the G2/M phase arrest of MGC803 cells, and it also prolonged the activation of PI3K/Akt by ATO. Consistent results with those in MGC803 cells were showed in gastric cancer cell BGC823 and SGC7901 after ATO treatment. These results demonstrate that inhibition of PI3K/Akt signaling by Cbl is involved in both ATO-induced apoptosis of NB4 cells and ATO-induced G2/M phase arrest of gastric cancer cells. Cbl achieved these effects probably via its regulating PI3K/Akt pathway, and thereby modulated p53 activation.

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1. Introduction

Arsenic trioxide (As2O3, ATO) is very effective in the treatment of patients with acute promyelocytic leukemia (APL). APL is characterized by the PML/RARα fusion protein, a product of the t (15:17) translocation [1]. Relatively low concentrations (≤2 μM) of ATO induce apoptosis and downregulate the PML–RARα fusion protein [2]. ATO also has potential anticancer activity against other kinds of cells which lack the PML–RARα fusion protein [3–15]. In most solid tumor cells, however, ATO induces arrest in the G1 or G2/M phases of the cell cycle rather than apoptosis [4–14]. This different mechanism of ATO action on solid tumor cancer cells and APL cells is not well understood. p53 is a critical determinant in controlling both cell cycle arrest and apoptosis [16,17]. In several different types of cancer cells, the functional status of p53 determines the cellular response to ATO [7–13]. Liu qun et al. reported that multiple myeloma cells with normal p53 were resistant to ATO-induced apoptosis and were arrested in G1 phase, while cells lacking functional p53 were sensitive and were arrested in G2/M phase [13]. Similar results were also shown in certain AML cells [15] and solid tumor cells [7–9]. In response to DNA damage, normal p53 accumulates and leads cells into G1 phase arrest for DNA repair or apoptosis [13,16–19]. Cancer cells lacking normal p53 function lack this G1 checkpoint; in these cells, ATO-induced apoptosis is highly dependent on a functional G2
[9–13,15] and spindle checkpoints [7,20], p53-defective cells thus tend to be preferentially arrested in G2/M phase. ATO induces DNA damage, causing the accumulation and activation of p53 [8,10,23] through the ATM/ATR pathway [23]. The promyelocytic leukemia protein (PML) protects p53 from degradation by Mdm2 [18,19,24]. In contrast, the phosphatidyl inositol 3 kinase (PI3K)-Akt/ PKB pathway decreases p53 protein expression and inhibits the functions of p53 by enhancing the function of the phosphatidyl inositol 3 kinase (PI3K)-Akt/PKB pathway decreases p53 protein expression and inhibits the functions of p53 by enhancing the function of leukemia cells [28,29], and its inhibitors enhance the apoptotic action of ATO [30]. These results indicated that inhibition of p53 by PI3K/Akt signaling might be critical for ATO action.

The casitas B-lineage Lymphoma (Cbl) family of ubiquitin ligases is a negative regulator of PI3K/Akt signaling in several cell types, including osteoblast and T cells [31–34]. Cbl proteins can interact with the p85 regulatory subunit of PI3K, resulting in PI3K ubiquitination and degradation [35,36]. However, it is not clear whether Cbl family members are involved in ATO action by regulating PI3K/Akt signaling.

In this study, we investigated the different mechanism of ATO action on gastric cancer cells and NB4 cells. The results showed that ATO up-regulated the expression of Cbl proteins, and Cbl is involved in ATO action by inhibiting PI3K/Akt signaling.

2. Materials and methods

2.1. Reagents and antibodies

Anti-Bcl-2, anti-Tubulin, anti-Cbl-b and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-AKT (Ser-473) and anti-AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-c-Cbl antibody was from Transduction Laboratories (Lexington, KY, USA). Arsenic trioxide and 2-phenylindole were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human gastric adenocarcinoma MGC803, BGC823 and SGC7901 cells were established in China and were cultured in our laboratory [21,22]. Gastric cancer cell lines and APL-derived NB4 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under an atmosphere of 95% air and 5% CO2. Cells were routinely sub-cultured every 2–3 days and cell samples used were all in the logarithmic growth phase.

2.3. Cell viability assay

The effect of ATO on NB4 cell proliferation was measured using the trypsin blue exclusion assay. At assay time, NB4 cells were collected, mixed with an equal volume of PBS containing 0.4% trypsin blue dye, and manually counted. Actual cell numbers were calculated by multiplying diluted times compared with initial cell numbers. Cell viability % = viable cell numbers/total (viable + dead) cell numbers × 100%. The effect of ATO on MGC803 cell proliferation was measured using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded at 5 × 104 cells/well in 96-well plates and incubated overnight, and then different concentrations of ATO were added and further incubated for the indicated time. Thereafter, 20 μl of MTT solution (5 mg/ml) was added to each well and the cells were incubated for another 4 h at 37°C. After removal of the culture medium, the cells were lysed in 150 μl of dimethylsulfoxide and then the optical density (OD) was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The following formula was used: cell viability % = (OD of the experimental sample/OD of the control group) × 100%.

2.4. Cell cycle phase analysis

Phase distributions of the cell cycle and hypodiploid DNA were determined by flow cytometry. Cells were treated with 1–20 μM of ATO for 4–24 h and then collected and washed twice with phosphate-buffered saline (PBS). After fixing in ice-cold 70% ethanol for 12 h, samples were washed twice with PBS and then incubated with 20 μg/ml RNase A and 10 μg/ml propidium iodide (PI) for 30 min in the dark. Finally, samples were evaluated by flow cytometry and data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA, USA). The experiment was repeated three times.

2.5. Western Blot analysis

Western Blotting was performed using standard techniques as previously described. Briefly, cells were washed twice with PBS buffer and lysed in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris–Cl pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na3VO4, 1 mM PMSF, and 2 μg/ml aprotinin) on ice. Protein concentration was determined by Lowry method. Total proteins (30–50 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (Immoblin–P, Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk in TBST (10 mM Tris, pH 7.4, 150 mM NaCl and 0.1% Tween 20) at room temperature for 2 h and incubated with the indicated primary antibodies at 4°C overnight. After washing with TBST, the membrane was reacted with the appropriate horseradish peroxidase–conjugated secondary antibodies for 30 min at room temperature. After extensive washing with TBST, proteins were visualized by the enhanced chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA).

2.6. Statistical analysis

Data are expressed as mean ± SD. Differences between two groups were evaluated by Student's t-test. p < 0.05 was considered to be statistically significant.
3. Results

3.1. Growth inhibition of NB4 and MGC803 cells by ATO

The effect of ATO on the viability of NB4 and MGC803 cells was examined by the trypan blue exclusion assay and MTT assay. Dose-dependent inhibition of cell growth was observed in both cell lines (Fig. 1). The IC50 at 24 h was 1.91 μM in NB4 cells and 23.8 μM in MGC803 cells.

3.2. Induction of apoptosis in NB4 cells and G2/M phase arrest in MGC803 cells by ATO

Upon observation under the microscope, NB4 cells appeared to undergo apoptotic changes after treatment with ATO but MGC803 cells remained viable and rounded up and detached from the culture dishes (data not shown). Flow cytometry analysis showed that ATO increased the apoptotic (sub-G1) population in a dose-dependent manner at 24 h in NB4 cells, but did not increase cells in the G2/M phase. The sub-G1 population was about 13.5% and 24.79% at 1 μM and 2 μM of ATO, respectively (Fig. 2A). In contrast, treatment with ATO induced a significant G2/M phase arrest, but not an increase in the sub-G1 population, in MGC803 cells. When we increased the dose of ATO we saw a parallel increase in the population of G2/M phase cells. At 20 μM ATO the G2/M phase population started to decrease, but the sub-G1 phase population slightly increased (Fig. 2B). Treatment with 10 μM of ATO for different times showed that the population of G2/M phase cells peaked at 16 h, up to approximately 55.85%, but that of sub-G1 phase was less than 5% (Fig. 2C). These results indicated that ATO primarily induced apoptosis in NB4 cells but G2/M phase arrest in MGC803 cells.

3.3. Effects of ATO on activation and expression of p53 and Akt proteins

In order to elucidate the molecular mechanisms of action of ATO on NB4 and MGC803 cells we investigated the changes in important signal transduction pathways.
transduction proteins during treatment. As shown in Fig. 3, NB4 and MGC803 cells had a relatively high basal level of p53 expression. 10 μM ATO arrested MGC803 cells in G2/M, and this concentration decreased p53 expression in this cell line in a time dependent manner. A significant decrease of p53 appeared as early as 4 h. 1 μM of ATO-induced apoptosis of NB4 cells, but did not affect p53 expression. Phospho-Akt was strongly increased after 4 h ATO treatment in both cell lines, while it was decreased to less than basal level after 24 h in NB4 cells and after 16 h in MGC803 cells. The levels of Akt were unchanged upon ATO treatment in both cell lines. We used LY294002 to inhibit PI3K/Akt signaling. 25 μM of LY294002 significantly elevated the percentages of the sub-G1 population in ATO-treated NB4 cells, from approximately 15.01% up to 48.9% (Fig. 4A). In MGC803 cells, 25 μM of LY294002 elevated the percentages of the sub-G1 cells up to 18.1% at 16 h (Fig. 4B) and markedly reduced G2/M phase cells from 55.21% to 32.65%. These results indicate that the activation of PI3K/Akt was critical for the action of ATO. Next, we analyzed the effects of LY294002 on the expression of p53 protein. The combination of LY294002 and ATO significantly increased the expression of p53 protein in MGC803 cells (Fig. 4D). An increase of p53 protein was seen in MGC803 cells at 16h. Slight up-regulation of p53 by LY294002 was seen in NB4 cells (Fig. 4C). But phospho-Akt did not show this transient increase at 4 h, and descended to much lower at 24 h or 16 h (Fig. 4C and D). These data supported the possibility that ATO-induced apoptosis of NB4 cells and G2/M phase arrest of MGC803 cells depends on the level of p53 expression, which can be inhibited by transient activation of PI3K/Akt.

3.4. Up-regulation of the Cbl family of ubiquitin ligases by ATO

To explore the mechanism of regulation of PI3K/Akt by ATO we examined the expression levels of c-Cbl and Cbl-b and their functions in both cell lines. Treatment with ATO significantly increased the levels of Cbl-b and c-Cbl proteins in both cell lines starting at 4 h, with maximal expression observed at 24 h in NB4 cells and 16 h in MGC803 cells (Fig. 5). Flow cytometry analysis revealed that treatment with 10 nM Ps341 significantly reduced ATO-induced apoptosis of NB4 cells at 24 h (Fig. 6A) and reduced the percentage of sub-G1 phase cells from 17.5% to 12.3% (p < 0.05). Ps341 significantly elevated ATO-induced G2/M phase arrest of MGC803 cells at 16 h, causing the percentage of G2/M phase cells to rise from 52.09% up to 64.4% (p < 0.05) (Fig. 6B). These results indicate that Cbl might impact the action of ATO. To further confirm this presumption, we assessed the level of phospho-Akt after treatment with the combination of Ps341 and ATO (Fig. 6C and D). In both NB4 cells and MGC803 cells, Ps341 alone did not affect the basal level of phospho-Akt. But Ps341 combined with ATO persistently increased the level of phospho-Akt at 24 h in NB4 cells and at 16 h in MGC803 cells. So Ps341 markedly prolonged the activation of PI3K/Akt by ATO, which suggests that Cbl inhibi-

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**Fig. 3.** Effects of ATO on the activation and expression of p53 and Akt proteins in ATO-treated NB4 and MGC803 cells. Exponentially growing cells were treated with the indicated concentrations of ATO for the indicated times. The total proteins or the proteins in cytosolic fractions were isolated for measuring p53, phospho-Akt, Akt and β-tubulin levels by Western Blotting as described in Section 2. (A) NB4 cells and (B) MGC803 cells.

**Fig. 4.** Effects of the PI3K/Akt inhibitor LY294002 on the action of ATO in NB4 cells in MGC803 cells. Exponentially growing cells were treated with LY294002 (25 μM) in addition to ATO for the indicated times. The changes in cell cycle phase distribution were assessed by DNA flow cytometric analysis and the expression of p53 and p-Akt proteins were analyzed by Western Blotting as described in Section 2. (A, C) 1 μM of ATO in NB4 cells (B, D) 10 μM of ATO in MGC803 cells. Data are means ± SD of three independent experiments. *p < 0.05 compared to the control group cells. **p < 0.05 compared to cells treated with only ATO.
ated the activation of PI3K/Akt by ATO. In addition, Ps341 combined with ATO further decreased the expression of p53 in MGC803 cells, but not in NB4 cells (Fig. 6C and D).

3.5. Effects of ATO on gastric cancer cell BGC803 and SGC7901

ATO also inhibited the growth of BGC823 and SGC7901 cells in a dose-dependent way (data not shown). As shown in Fig. 7, consistent results with those in MGC803 cells were observed in BGC823 and SGC7901 cells. 10 μM ATO significantly induced G2/M phase arrest and transient activation of PI3K/Akt at 4 h (Fig. 7A and C). Significant down-regulation of p53 protein and up-regulation of Cbl proteins were also shown (Fig. 7B). Furthermore, LY294002 partly reversed ATO-induced G2/M phase arrest and increased apoptosis, while pretreatment with Ps341 partly increased ATO-induced G2/M phase arrest (Fig. 7C).

4. Discussion

In this study, ATO inhibited the proliferation of NB4 and gastric cancer cell lines. However, gastric cancer cells were much less susceptible to ATO than NB4 cells. It was similar to that in other solid tumors such as pulmonary adenocarcinoma cell lines A549 and Calu-6 [4], human epidermoid carcinoma cell line A431 [14] and so on. Treatment with ATO induced apoptosis in NB4 cells, similar to the results reported by Chen et al. [2]. Zhang et al. reported that MGC803 cells treated with ATO showed apoptosis and partial G2/M phase arrest [6]. In our observations ATO primarily induced G2/M phase arrest and seldom apoptosis in MGC803 cells as well as BGC823, SGC7901 cells.

In many reports, ATO induced apoptosis by accumulation and activation of p53 [8,10,23]. In our study, treatment with ATO resulted in degradation of p53 in gastric cancer cell lines but no change of p53 in NB4 cells. It was reported that MGC803, BGC823 and SGC7901 cells had no detectable mutation in exon2, 5, 7, 8 of the p53 gene and that the expression of ATM was lower in MGC803 and SGC7901 cells than that in BGC823 cells [21,22]. Though ATM deficiency might impact the accumulation and activation of p53, ATO-induced degradation of p53
and G2/M phase arrest in BGC823 cells were consistent with the results in MGC803 and SGC7901 cells. In NB4 cells, there was also no mutation in exon 2, 5, 7, 8 of the p53 gene [18] and no obvious change in p53 mRNA expression after ATO treatment [2]. But ATO-treatment restored PML and PML nuclear bodies (PML–NBs) by degradation of PML/RARα, and subsequently protected the function of p53 [19]. Joe Y et al. also demonstrated that ATO-induced apoptosis in NB4 cells through a p53-associated pathway [18]. These results indicated that p53 was inhibited by some factor during its activation in response to stimulation by ATO.

In leukemia cells, ATO-induced apoptosis was correlated with inactivation of PI3K/Akt. High PI3K/Akt activity conferred ATO-resistance to these cells [28–30]. The PI3K/Akt pathway is also correlated with chemoresistance of gastric cancer cells to etoposide and doxorubicin [37], and inhibition of this pathway enhanced an ethanol extract of Rhus verniciflua Stokes-induced apoptosis in gastric cancer cells [38]. However, in our study, PI3K/Akt was transiently activated by ATO in both NB4 and gastric cancer cells and then rapidly inactivated. Treatment with the PI3K/Akt inhibitor LY294002 strongly increased ATO-induced apoptosis in both cell lines and decreased ATO-induced G2/M phase arrest of gastric cancer cells. LY294002 also increased the amount in p53 protein of both cell lines. These results support the idea that transient activation of PI3K/Akt is accompanied by ATO-induced accumulation and activation of p53. This transient activation of PI3K/Akt by ATO might be the most important factor by which it inhibits the accumulation and activation of p53. p53 was significantly decreased in MGC803, BGC823 and SGC7901 cells, probably because it is both inhibited by PI3K/Akt and not activated in these ATM deficient cells [21]. But in NB4 cells a normal ATM/ATR pathway and the restoration of PML ensure the activation of p53. So the final activation status of p53 determines the different effects ATO has on different cell types. If p53 remains active ATO induces apoptosis; if not, it induces G2/M arrest. Thus, transient activation of PI3K/Akt by ATO plays a critical role in regulating ATO action on NB4 cells and gastric cancer cells. But the final inactivation of PI3K/Akt by ATO was also critical for cells, leading to death [28–30]. When we prolonged the time of ATO treatment or elevated the concentration of ATO we observed that induction of apoptosis was enhanced not only in NB4 cells, but also in MGC803 cells, and induction of G2/M phase arrest decreased in MGC803 cells.

In addition, our investigation showed that ATO markedly up-regulated c-Cbl and Cbl-b proteins in NB4 and gastric cancer cell lines. Inhibition of the function of c-Cbl and Cbl-b proteins with the proteasome inhibitor Ps341 decreased apoptosis in NB4 cells and increased G2/M phase arrest in gastric cancer cells, indicating that Cbl-b and c-Cbl might be involved in ATO-induced apoptosis and G2/M phase arrest. Notably, Ps341 also prolonged the time of activation of PI3K/Akt by ATO in NB4 and MGC803 cells, which suggests that Cbl prevents excessive activation of
PI3K/Akt by ATO. These results support the idea that ATO exerts its action by up-regulating Cbl and inhibiting PI3K/Akt signaling.

In summary, the present results demonstrate that ATO-activated p53 was accompanied by transient activation of PI3K/Akt, and this activated PI3K/Akt subsequently inhibited the activation of p53. ATO up-regulated Cbl in order to inhibit excessive activation of PI3K/Akt. The final activation status of p53 determined that ATO-induced apoptosis in NB4 cells and G2/M phase arrest in gastric cancer cells.

At present, the antitumor effects of ATO were widely investigated in malignant hematologic tumors. And it is hardly presumable to be utilized for the treatment of solid tumors, such as gastric cancer. We first reported that Cbl involved in the antitumor activity of ATO via its regulating PI3K/Akt pathway, independently of the model considered, and therefore also independently on the primary mechanism of action of the drug. This is worthy of further study.

Conflict of interest
None declared.

References


