A proteomics research of apoptosis of leukemia cells induced by arsenic trioxide

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Abstract: Leukemia is a malignant hyperplasia disease of hematopoietic system caused by a kind of hematopoietic cells detaining at a certain stage. In this study, we used HL-60 cell line to detect the effect of apoptosis induction by various concentrations of As₂O₃. Percentage of apoptotic cells was increased in the presence of As₂O₃ detected through flow cytometry. Mass spectrometric identification indicated that there were 102 differential proteins before and after intervention. Among them, expression of CH60, AT1A1, ANXA6, and EF2 increased and expression of ATPA, MYO1G, RS3 decreased. Those genes played roles in promoting HL-60 cell apoptosis induced by As₂O₃. This study could lay the foundation of deciphering mechanisms of HL-60 cell apoptosis and give a new possible target to the clinical application for arsenic trioxide treatment for leukemia.

Key words: HL-60 cells; Arsenic trioxide; Apoptosis; Differences protein.

Introduction

Leukemia is a malignant hyperplasia disease of hematopoietic system caused by a kind of hematopoietic cells detaining at a certain stage (1). In China, the most common malignant tumors in children were leukemia, among children with leukemia, 97% were acute leukemia. In the past 20 years, with the development of new drugs and treatments, the prognosis of leukemia has been significantly improved (2, 3). What's more it was prone to relapse or turn into refractory leukemia (4, 5). However, though numerous studies reported searches on leukemia, the pathogenesis of leukemia was not yet fully understood.

As₂O₃ is the main component of arsenic, a traditional Chinese medicine, as a white powder, odorless, tasteless, and soluble in water, alcohol, acids and bases, smelling garlic when heating (6-8). As₂O₃ has strong toxic, which can damage cellular respiration enzymes in vivo, strongly stimulate the gastrointestinal mucosa to induce gastric mucosa ulceration and bleeding, liver and blood vessels damage, even leading to death due to respiratory and circulatory failure (9). However, in recent years, it has been reported that As₂O₃ could kill a variety of malignant tumors, reverse drug resistance of tumor cells, inhibit cell proliferation, arrest cell cycle, induce cell differentiation and promote cell apoptosis (10-12). The main mechanism might be related to mitochondrial pathway, reactive oxygen pathway, the increase of p53 gene and down-regulation of bcl-2, etc. (13, 14). It also has been reported that As₂O₃ could induce HL-60 cell apoptosis, which is an AML cell line (15-17). Although As₂O₃ had the strong cytotoxicity on the malignant cells, researcher found that As₂O₃ had the relatively limited adverse effect on the normal cells. It was reported that As₂O₃ induced changes in (Ca²⁺) in HEK cells (18).

Also, As₂O₃ had cytotoxic effects in malignant cells but not in human embryonic pulmonary cells (19).

Proteome refers to all proteins which are expressed by all cell and tissue genome. Several differentially expressed proteins existed in HL-60 cells before and after treatment of dimethylsulfoxide, in which 21 proteins (including Galectin-1) were up-regulated and 6 proteins were down-regulated or absent (20). These proteins might be associated with AML. Hofmann et al established differentiation of HL-60 induced by all trans-retinoic acid (ATRA), and found that 25 protein spots were only found in undifferentiated gel spectrum and 15 protein spots were only found in differentiated cell protein spectrum (21). In this study, we studied the differential protein expression in HL-60 cells before and after the intervention of As₂O₃, analyzed the role of some proteins in apoptosis, which could provide the theoretical basis for the clinical treatment of leukemia with As₂O₃.

Materials and Methods

Cell culture and interventions

HL60 cells were cultured in RPMI 1640, plus 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin, Sigma-Aldrich). HL-60 cells were treated with 0, 0.1, 1, 2, 4, 8 μmol/L As₂O₃ for 72 h. Triplicates were set up for each concentration.

Annexin V/Propidium Iodide Apoptosis assay

The protocol was described previously (22). The cells were collected and resuspended in binding buffer. Annexin V-FITC and Propidium Iodide were added into the cell suspension, and kept in room temperature for 15 min. The percentage of apoptotic cells were measured by flow cytometry, and the fluorescence intensity...
was adapted and compensated by the cells in the control group.

**Mass spectrometry**

All samples under concentration of $\text{As}_2\text{O}_3$ 4 μmol/L were dissolved and analyzed by two-dimensional liquid chromatography-mass spectrometry, each sample was repeated. The peptide eluting by reversion phase chromatography were detected with Q Exactive mass spectrometer. The m/z range was 300-1800 amu. The secondary spectrum scan was performed with data-dependent manner (10 times of secondary scan were performed after full scan, parent ion m/z width was 3 amu, 35% standard collision energy, dynamic exclusion time was 1.5 min).

**Bioinformatics analysis**

The differential proteins before and after intervention were analyzed with SwissProt Human Fasta database (PROTEOME Discoverer 1.3). The protein function was searched in two websites, Uniprot and David, to analyze the effects of $\text{As}_2\text{O}_3$ on HL-60 cell apoptosis.

**Results**

**Cell morphology was influenced by $\text{As}_2\text{O}_3$ application**

After 72 h, the cell morphology showed no significant changes in the control group by Wright-Gimsa staining (Fig.1A, B), while significant morphological changes appeared in cells treated with $\text{As}_2\text{O}_3$ (Fig.1C-E). Compared with the control group, cell size in the $\text{As}_2\text{O}_3$-treated group was smaller, and nuclei to cytoplasm ratio shrank. Nuclear condensation and fragmentation were observed, and the chromatin was migrated to nuclear membranes to form peripheral type, crescent shape, spot and plaque type (Fig.1C). Some cell membrane showed pseudopodia-like protrusions (Fig.1D), and apparent and typical apoptotic bodies were observed (Fig.1E).

**Cell apoptosis influenced by $\text{As}_2\text{O}_3$**

The number of apoptotic cells was increased with the increasing $\text{As}_2\text{O}_3$ concentration, as shown in Table 1. The cell apoptotic rate was shown in Table 1 after treatment of 0, 0.1, 1, 2, 4, 8 μmol/L $\text{As}_2\text{O}_3$ for 72 h. There were significant differences in all six treatment groups compared to the controls (P<0.05), suggesting that the apoptosis of HL-60 cells showed a dose-dependent relationship to the concentrations of $\text{As}_2\text{O}_3$.

**Differential protein analysis and classification**

The proteins were detected by liquid chromatography-mass spectrometry, and 1058 proteins were identified from the control group while 1972 proteins were identified from experimental group. 102 differentially expressed protein spots were screened, in which 75 were up-regulated and 27 were down-regulated in HL-60 cells (partially summarized in Fig.2, and complete database was shown in Supplementary figure 1). These proteins were mainly metabolic enzymes, or related to cell cycle regulation, cell proliferation and apoptosis, signal transduction and DNA repair. In which, the expression of poly (ADP-ribose) polymerase (PARP), ATP enzyme (ATPase) and elongation factor 2 (EF-2) promoted HL-60 cell apoptosis treated with $\text{As}_2\text{O}_3$ (Table.2). The differentially proteins were performed with mass spectrometry analysis. Each protein was identified for three peptides. The secondary spectrum results are shown in Fig. 3.

**Discussion**

In this study, we detected differentiation and apoptosis of HL-60 cells treated with $\text{As}_2\text{O}_3$ by Swiss staining and flow cytometry, and found that $\text{As}_2\text{O}_3$ could inhibit HL-60 cell proliferation and induce cell apoptosis, which was confirmed by our cell morphology observation. Low concentrations of $\text{As}_2\text{O}_3$ (0.1 μmol/L, 1 μmol/L) mainly induced cell differentiation, including chromatin agglomeration; however, high concentration of $\text{As}_2\text{O}_3$ (8 μmol/L) could significantly induce cell apoptosis, including nuclear condensation and fragmentations.

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**Table 1.** Effect of $\text{As}_2\text{O}_3$ on the cell apoptosis of HL-60 cells determined by flow cytometry.

<table>
<thead>
<tr>
<th>$\text{As}_2\text{O}_3$ (μmol/L)</th>
<th>Apoptotic cell ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.80±0.19</td>
</tr>
<tr>
<td>0.1</td>
<td>14.08±0.45</td>
</tr>
<tr>
<td>1</td>
<td>14.64±0.52</td>
</tr>
<tr>
<td>2</td>
<td>14.85±0.48</td>
</tr>
<tr>
<td>4</td>
<td>15.56±0.42</td>
</tr>
<tr>
<td>8</td>
<td>17.63±0.51</td>
</tr>
</tbody>
</table>

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**Figure 1.** The morphological change of HL-60 cells through the application of $\text{As}_2\text{O}_3$. (A) PBS control, (B-E) Application of 0.1, 1, 2, 4, and 8μmol/L $\text{As}_2\text{O}_3$.

**Figure 2.** The apoptosis of HL-60 cells with the treatment of $\text{As}_2\text{O}_3$ analyzed by flow cytometry. (A) PBS control, (B-E) Application of 0.1, 1, 2, 4, and 8μmol/L $\text{As}_2\text{O}_3$. (F) Statistical analysis of the apoptotic cells. Significance was determined by t-tests; *p<0.05.
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A variety of functional proteins were involved in HL-60 cell apoptosis induced by As$_2$O$_3$, which could provide important information for treatment of leukemia in the future.

In summary, our study proved that As$_2$O$_3$ could affect the apoptosis on HL-60 cells with a concentration-dependent manner. A total of 102 differentially expressed proteins were identified, including 75 of up-regulation and 27 of down-regulation. All these proteins played roles in apoptosis of HL-60 cells, which provide the basis for the study of apoptotic mechanism of leukemia cells.

Acknowledgements
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References
Figure 3. The secondary spectrometry map and amino acid sequence for AT1A1 (A), ATPA (B), RS3 (C), EF2 (D), and CH60 (E).