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Original Research

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 As_2O_3. Percentage of apoptotic cells was increased in the presence of As_2O_3, 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 As_2O_3. 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.

As_2O_3 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). As_2O_3 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 As_2O_3 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 As_2O_3 could induce HL-60 cell apoptosis, which is an AML cell line (15-17). Although As_2O_3 had the strong cytotoxicity on the malignant cells, researcher found that As_2O_3 had the relatively limited adverse effect on the normal cells. It was reported that As_2O_3 induced changes in (Ca^{2+}) in HEK cells (18). Also, As_2O_3 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 As_2O_3, analyzed the role of some proteins in apoptosis, which could provide the theoretical basis for the clinical treatment of leukemia with As_2O_3.

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 As_2O_3 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 As_2O_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 As_2O_3 on HL-60 cell apoptosis.

Results

Cell morphology was influenced by As_2O_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 As_2O_3 (Fig.1C-E). Compared with the control group, cell size in the As_2O_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).

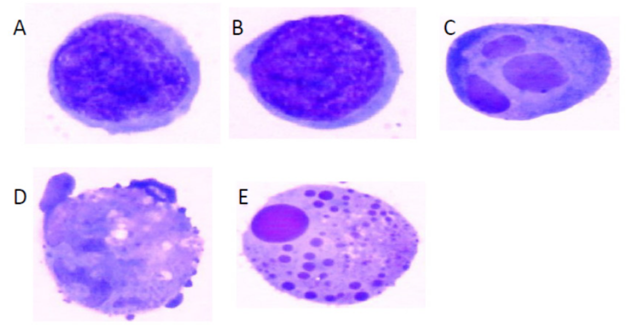

Figure 1. The morphological change of HL-60 cells through the application of As_2O_3. (A) PBS control, (B-E)Application of 0.1, 1, 2, 4, and 8μmol/LAs_2O_3.

Table 1. Effect of As_2O_3 on the cell apoptosis of HL-60 cells determined by flow cytometry.

As_2O_3	Apoptotic cell ratio (%)
0	9.80±0.19
0.1 umol/L	14.08±0.45
1umol/L	14.64±0.52
2umol/L	14.85±0.48
4umol/L	15.56±0.42
8 umol/L	17.63±0.51

Cell apoptosis influenced by As_2O_3

The number of apoptotic cells was increased with the increasing As_2O_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 As_2O_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 As_2O_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 As_2O_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 As_2O_3 by Swiss staining and flow cytometry, and found that As_2O_3 could inhibit HL-60 cell proliferation and induce cell apoptosis, which was confirmed by our cell morphology observation. Low concentrations of As_2O_3 (0.1 μmol/L, 1 μmol/L) mainly induced cell differentiation, including chromatin agglomeration; however, high concentration of As_2O_3 (8 μmol/L) could significantly induce cell apoptosis, including nuclear condensation and fragmen-

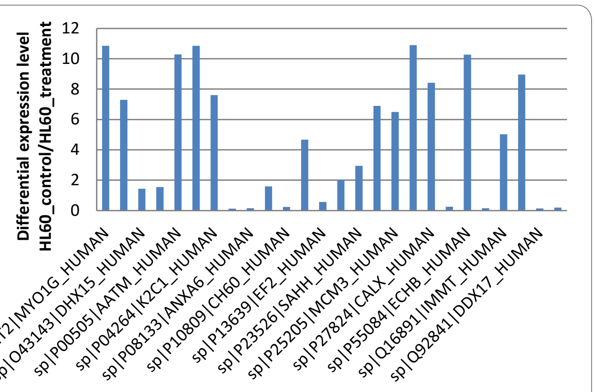

Figure 2. The apoptosis of HL-60 cells with the treatment of As_2O_3 analyzed by flow cytometry. (A) PBS control, (B-E) Application of 0.1, 1, 2, 4, and 8μmol/L As_2O_3. (F) Statistical analysis of the apoptotic cells. Significance was determined by t-tests; $^*p<0.05$.

Table 2. The proteins identified by Mass spectrometry.

Protein No.	Protein Name	Amino Acid Sequence	Molecular Weight	Function
P05023	Na^+/K^+-ATPase	AVAGDASESALLK	112896	Metabolism
		SPDFTNENPLETR		
		VDNSSLTGESEPQTR		
P25705	ATPsynthase	HALIIYDLSK	59751	Metabolism
		EAYPGDVFYLHSR		
		ILGADTSVDLEETGR		
P23396	ribosomal protein S3	AELNEFLTR	26688	Metabolism
		ELAEDGYSGVEVR		
		IMLPWDPTGK		
P13639	EF-2	IMGPNYTPGK	95338	Proliferation
		KEDLYLKPIQR		
		YEWDVAEAR		
P10809	Heat shock protein 60	GYISPYFINTSK	61055	Metabolism
		ISSIQSIVPALEIANAHR		
		LVQDVANNTNEEAGDGTTTATVLAR		

tation.

Our results also showed Na^+-K^+-ATPase was the differentially protein. It has been proved that decrease of Na^+-K^+-ATPase could activate caspase-9 and promote myocardial cell apoptosis through inhibition of mammalian target of rapamycin (mTOR) (22). Heat shock protein 60 (HSP60) was closely associated with cell apoptosis. HSP60 could inhibit the activation of mitochondrial apoptotic pathway, and combined with Bax and Bak to form polymer complex to block apoptosis(23). Therefore, the low expression of HSP60 promoted HL-60 cell apoptosis.

In recent years, studies have shown that ribosomal protein S3a prevented colon cancer through increase of tumor cell apoptosis (24). Thereby S3a could promote cell apoptosis. ATP synthase could activate cysteine protease family (Caspase), then Caspase-9 and Fas-FasL mediated Caspase-8 to generate apoptotic downstream signal. Caspase was the downstream effectors of apoptosis induced by As_2O_3, activation of Caspase could cause tumor cell apoptosis. As_2O_3 could induce cell apoptosis through up-regulation of Caspase-3 in xenografts and acted as an anti-cancer agent. Studies have found that $CD8^+$ T cells of minor histocompatibility antigen HA-2 were detected in peripheral blood of relapsed patients who accepted lymphocyte infusion for 5-7 weeks (25-27). During the appearance of cytotoxic T lymphocytes (CTLs), there was complete remission of malignancy and 100% donor chimerism; in vitro experiments have shown that the CTLs could dissolve leukemia cells and inhibit cell colony formation, suggesting that HA-2 was associated with cell apoptosis. Our results showed that a variety of functional proteins were involved in HL-60 cell apoptosis induced by As_2O_3, which could provide important information for treatment of leukemia in the future.

In summary, our study proved that As_2O_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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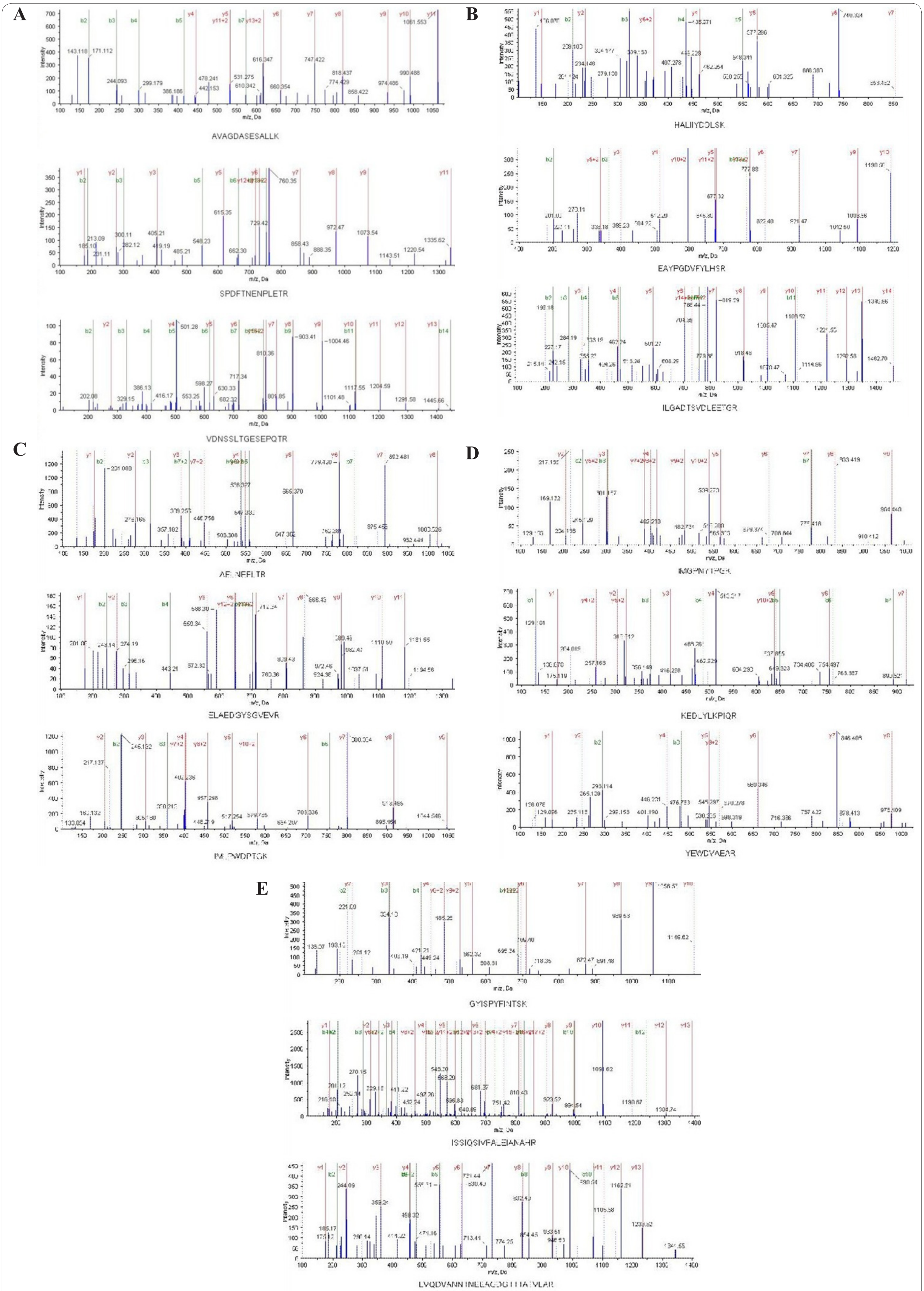

Figure 3. The secondary spectrometry map and amino acid sequence for AT1A1 (A), ATPA (B), RS3 (C), EF2 (D), and CH60 (E).

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