L-asparaginase induces in AML U937 cells apoptosis via an AIF-mediated mechanism

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1. ABSTRACT

Acute Myeloid Leukemia (AML), a cancer of the myeloid line of blood cells, progresses rapidly and is typically fatal within weeks or months if left untreated. Asparaginases are a class of enzymatic anti-leukemia agents that induce apoptosis in leukemia cell lines; however, the role of L-asparaginase in the induction of apoptosis in AML cells has not been investigated. In this study, we investigated the apoptosis-inducing effect of L-asparaginase and its underlying mechanism in AML U937 cells. The results showed that L-asparaginase significantly inhibited the proliferation of U937 cells by inducing apoptosis. Furthermore, the low baseline expression level of asparaginase synthase (ASNS) demonstrated the sensitivity of U937 cells and AML M5, a rare subtype of AML, to L-asparaginase. Apoptosis induced by L-asparaginase is mediated by apoptosis-inducing factor (AIF). Our findings show the potential of L-asparaginase as an effective approach in treating AML via the induction of apoptosis mediated by AIF.

2. INTRODUCTION

Acute myelogenous leukemia (AML) has several subtypes, M_0–M_7. AML is characterized by aberrant accumulation of immature myelocytes in the bone marrow, which interferes with the production of normal blood cells (1). AML is an aggressive hematologic malignancy in adults, and its morbidity is closely associated with age. Prognosis of AML in young patients over the past four decades has made significant progress, while prognosis of adult AML patients has lagged behind. Approximately 50–75% of adult patients with AML achieve complete remission (CR) by a combination of cytoarabinoside (Ara-C) and anthracycline, whereas only 20–30% of patients acquire long-term disease-free survival (DFS) (2). Therefore, development of novel drugs for the treatment of AML is urgent.

L-asparaginase, a classic enzymatic reagent for the treatment of leukemia, has been successfully used as a first-line chemotherapeutic defense for children with acute

L-asparaginase induced apoptosis of U937 cells

Table 1. IC_{50} of each cell line to the treatment of L-asparaginase

Cell lines	L-asparaginase response	
	IC50 (U/ml)	Response (S/I/R)
U937	0.001-0.002	S
Jurkat	0.1-1	S
Namalwa	1-10	I
Karpass299	>100	R
K562	>100	R

Abbreviations: S: sensitive; I: immediate response; R: resistant.

lymphoblastic leukemia (ALL) since 1967 (3). L-asparaginase significantly improves the overall outcomes of patients with ALL (4). Although grade I or II neutropenia and thrombopenia can occur during treatment of L-asparaginase, the hematologic toxicity of L-asparaginase is limited and these side effects disappear after cessation of treatment (5). Furthermore, Wu *et al.* have reported that a low-dose of L-asparaginase (1,000 units/m^2) can achieve favorable therapeutic effects in childhood ALL, thus avoiding therapy-related complications such as pancreatitis, hypofibrinogenemia, and coagulation, compared with the standard application of L-asparaginase (6).

The application of L-asparaginase for the treatment of AML has been investigated. Evidence from clinical trials confirms that L-asparaginase reduces the plasma level of asparagine in patients with AML (7), although childhood and adult AML of different subtypes show different *in vitro* sensitivity to L-asparaginase (8,9). Despite the controversy surrounding the sensitivity of AML to L-asparaginase, it has been shown that of all the AML subtypes, AML M5 is the most sensitive to L-asparaginase (10,11).

The anti-leukemic mechanism of L-asparaginase involves apoptosis induced by L-asparaginase in leukemia cells (12). The mechanism by which L-asparaginase induces apoptosis in AML cells, however, has not been investigated. In this study, we investigated the apoptosis-inducing effect of L-asparaginase and the underlying mechanism of apoptosis in U937 cells, an AML cell line.

3. MATERIALS AND METHODS

3.1. Cell lines and culture

The human AML cell line U937, chronic myeloid leukemia (CML) cell line K562, acute lymphoblastic leukemia (ALL) cell line Jurkat, anaplastic large cell lymphoma (ALCL) cell line Karpass-299, and human B lymphoma cell line Namalwa were obtained from the Department of Oncology/Hematology, Shanghai Children's Medical Center (Shanghai Jiaotong University, Shanghai, China). All cell lines were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. L-asparaginase was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), and dissolved in sterile saline for use. Etoposide (VP-16) and Ara-C were purchased from Sigma (St. Louis, MO, USA).

3.2. Cytotoxic assays

The cytotoxic assays were determined using a cell counting kit-8 (CCK-8, Dojindo, Japan). Briefly, Jurkat, K562, Namalwa, Karpas-299 and U937 cells were cultured in 96-well plates at a density of 1×10^5 cells/well overnight. Cells were treated with a series of L-asparaginase concentrations for 48 hours, and then the cytotoxic assay was performed according to the manufacturer's instructions. The absorbance was measured at 450 nm/630 nm using a BIO-TEK ELx800 Universal Microplate Reader (Bio-Tek, Winooski, VT). The cell inhibition rate was calculated using the formula: $[1 - (A_E - A_B) / (A_C - A_B)]$. A_E, A_C, and A_B were defined as the absorbance of experimental samples, untreated samples, and blank controls, respectively. The IC_{50} is the concentration of L-asparaginase where cell inhibition rate is 50%.

3.3. Flow cytometry assays

The cell apoptosis assay was performed using Annexin V/PI double staining. Briefly, U937 cells were seeded in 12-well plates with a density of 2×10^5 cells per well overnight. After treatment with L-asparaginase for the indicated times, U937 cells were harvested, washed with pre-chilled PBS, and resuspended in 200 μl of AnnexinV-FITC binding buffer. Annexin V-FITC (Beckman Coulter, Fullerton, CA, USA) and propidium iodide (PI) (Sigma, St. Louis, MO, USA) were incubated with the cells at room temperature in the dark for 15 minutes. Alteration in mitochondrial membrane depolarization (ΔΨm) was evaluated using double staining with Rh123 and PI. Briefly, U937 cells treated as above were harvested and resuspended in 1 ml of complete medium containing 1.25 μg/ml of Rh123 for 20 minutes at 37 °C. PI was added to the cells, with a final concentration of 1 μg/ml. The expression of the Fas receptor was assayed using CD95-FITC (BD Pharmingen, Sparks, MD, USA). In the pre-treatment assays, 10 μM of general caspase inhibitor (z-VAD-FMK), caspase-9 inhibitor (z-LEHD-FMK), caspase-8 inhibitor (z-IETD-FMK; BD Pharmingen), or 1 μg/ml of anti-Fas neutralizing antibody (ZB-4; Kamiya Biomedical, USA) were incubated with U937 cells for 2 hours before L-asparaginase treatment. Finally, the cells were measured with a Beckman Coulter Cytomics FC 500 flow cytometer (Beckman-Coulter).

3.4. DNA extraction and agarose gel electrophoresis

The cells were washed once with PBS and lysed in lysis buffer (10 mM Tris, 100 mM EDTA, and 0.5% sodium dodecyl sulfate, pH = 8.0) followed by DNA electrophoresis in 1.5% agarose gels. The resulting DNA migration pattern was visualized under UV irradiation.

3.5. Mouse model

Immunodeficient NOD/SCID mice (NOD.CB17-Prkdc scid, 4–6 w, ~20 g) were obtained from the Shanghai SLAC laboratory Animal Co. Ltd. (Shanghai, China) and bred in a pathogen-free (SPF) facility at the Department of Laboratory Animal Science of Shanghai, Jiao Tong University School of Medicine (Shanghai, China). This study was approved by the Institutional Ethics Committee of Shanghai Children's Medical Center of Shanghai, Jiao Tong University School of Medicine (Shanghai, China). All

Table 2. Dosage of chemotherapeutic agents used in the U937-mice model

Groups	Dosage	Dosage of children ALL
Control	-	-
L-Asp	36 U/20g	6000 U/m^2
Ara-C	1.2 mg/20g	200 mg/m^2
VP-16	0.6 mg/20g	100 mg/m^2

experimental procedures were performed in accordance with the guidelines of the Animal Care Committee of Shanghai Jiaotong University School of Medicine (Shanghai, China). For the establishment of AML mouse model, 24 NOD/SCID mice were intraperitoneally transplanted with 5×10^6 U937 cells per mouse. Eight days after inoculation, the mice were randomly assigned to 4 treatment groups (12 per group) (Table 2): intramuscular administration of 1.8 U/g of L-asparaginase every other day for 2 weeks; 0.06 mg/g of Ara-C for 3 consecutive days per week (days 8, 9, 10, 22, 23, and 24); 0.03 mg/g of VP-16 for 2 consecutive days per week (days 8, 9, 22, and 23); and saline control. The general condition and survival of treated mice were routinely monitored during the treatment.

3.6. Western blotting

Western blot analysis was performed using the standard methods provided by Cell Signaling Technology. Briefly, cytosolic and nuclear protein extracts were isolated according to the nuclear fractionation protocol (Abcam, USA), separated using SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes. The AIF, caspase-9 (Cell Signaling Technology, Danvers, MA, USA), BAX, Bcl-2, caspase-3 (BD Transduction Laboratories, San Jose, CA, USA), caspase-8, PARP (Becton Dickinson Pharmingen), and Hsp90 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used as primary antibodies, and horseradish peroxidase (HRP) labeled goat anti-human IgG was used as the secondary antibody. The bands were detected with the Enhanced Chemiluminiscence (ECL) kit (GE Healthcare), visualized with the ChemiDoc XRS system (Bio-Rad), and quantitative analysis of targeted protein expression was performed by normalizing against α-tubulin or HDAC-2 with the QuantityOne software (BioRad, Hemel Hempstead, Herts, UK).

3.7. RNA isolation and real-time polymerase chain reaction (RT-PCR)

Total RNA extracted using the Trizol reagent (Invitrogen, USA). The first-strand complementary DNA (cDNA) was reversely transcribed using the Reverse Transcription System kit (Promega, Madison, WI). Purified cDNA was subjected to PCR amplification with the following primers: asparaginase synthase (ASNS) forward primer 5′-GCAGCTGAAAGAAGCCCAAGT-3′, reverse primer 5′-GAATTCTCATTTCTGGTGGCAGA-3′; GAPDH forward primer 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse primer 5′-GAAGATGGTGATGGGAGGGC-3′. The PCR products were ligated into the pMD-18T plasmid vector (Takara Biotechnology, China) and the plasmid vector containing the PCR products were serially diluted into 8 successive dilutions (10^7–10^0 copies/μl). RT-PCR of the diluted plasmid vector was performed using SYBR Premix EX

TaqII kit (Takara Biotechnology, China), and analyzed by ABI Prism 7000 (Applied Biosystems, USA). The mRNA expression levels, normalized against GAPDH, were calculated and expressed as $2^{C\Delta\Delta CT}$.

3.8. Confocal microscopic studies

The nuclei and AIF expression of treated U937 cells were observed by a confocal microscopic study. Briefly, U937 cells (2.5×10^5 cells per well) were seeded overnight on coverslips in 12-well tissue culture plates. The cells were treated with 0.01 U/ml of L-asparaginase, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton-X100. The cells were incubated with an AIF antibody at a dilution of 1:100 for 2 hours at room temperature, followed by incubation with a Cy2-conjugated secondary antibody (Jackson Immunoresearch Laboratories, USA) for 1 hours. The nuclei of the cells were stained with 4, 6-diamidino-2-phenylindole (DAPI) (Sigma, Fluka Chemie, Buchs, Israel). The AIF expression, nuclei condensation, and fragmentation (indicating apoptosis) were analyzed with a LAS AF2.0 Confocal Microscope (Leica, Microsystems, Wetzlar, Germany).

3.9. Gene expression analysis

Three cohorts of ALL gene expression raw data (GSE643, GSE645 and GSE654) (13), and 4 cohorts of AML gene expression raw data (GSE1729, GSE10358 GSE6891, and GSE1159) (14-17) were obtained from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo). To perform inter-array comparisons, the CEL files were analyzed using Affymetrix MAS 5.0 software. All data were $\log_2$ transformed. Student's non-paired t-tests (two-tailed) were conducted to perform a direct comparison between two groups. P value of <0.1 was considered statistically significant.

3.10. Statistical analysis

Data in this study were analyzed by the statistic package SPSS 13.0 (SPSS Inc., Chicago, IL). For values that were normally distributed, a direct comparison between two groups was conducted by Student's non-paired t-test. The survival rate was analyzed by the Kaplan-Meyer method. P value of <0.05 was considered statistically significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s. represents not significant ($P > 0.05$).

4. RESULTS

4.1. L-asparaginase showed cytotoxic effect towards U937 cells

The cytotoxic effect of L-asparaginase was evaluated in several leukemic cell lines. As shown in Figure 1A, L-asparaginase significantly inhibited the proliferation of U937 at concentrations higher than 0.01 U/ml cells, although the inhibition rate of L-asparaginase was not dose-dependent. The inhibition rate of L-asparaginase was higher at 48 hr than that at 24 hr (Figure 1B). We also used PI staining to determine the viable cells after treatment with L-asparaginase. As shown in Figure 1C, treatment with 0.1 units/ml of L-asparaginase for 24 and 48 hours resulted in a mean viability of 82.98% and 32.02% for U937 cells, respectively. As expected, the representative ALL cell line,

A

B

C
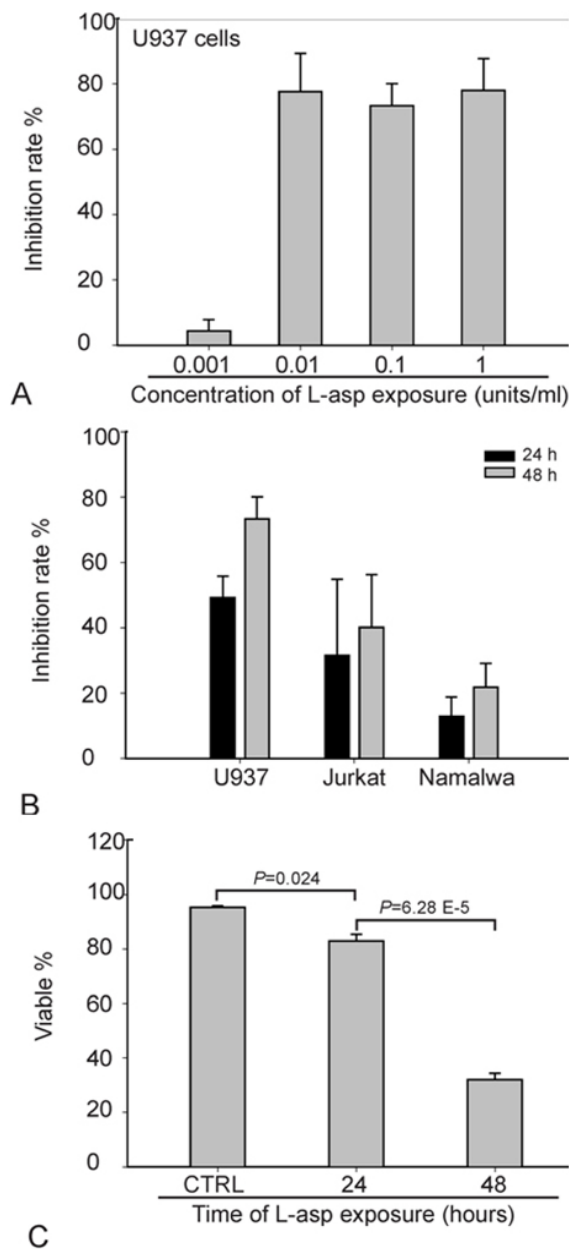
Figure 1. L-asparaginase showed cytotoxic effect towards U937 cells. (A) The cell inhibition rate of a series of concentration of L-asparaginase (0.001, 0.01, 0.1, and 1 units/ml) in U937 cells at 48 hours. Data are expressed as mean ± SD (n = 3). (B) The cell inhibition rate of L-asparaginase (0.1 units/ml) on U937, Jurkat, and Namalwa cells. Data are expressed as mean ± SD (n = 3). (C) The viable U937cells treated by L-asparaginase (0.1 units/ml) at 24 or 48 hours. The percentage of viable cells was measured by PI staining. Data are expressed as mean ± SEM (n = 3).

Jurkat was also sensitive to L-asparaginase with an IC_{50} of 0.1~1 units/ml. However, Namalwa, Karpas-299, and K562 cells showed resistance to L-asparaginase, with higher IC_{50} of 10~100 units/ml (Figure 1B, Figure 2, and Table 1).

These data are consistent with the reports of clinical application (9-11), therefore, 0.1 units/ml of L-asparaginase was chosen as the concentration in the following experiments.

4.2 L-asparaginase induced apoptosis in U937 cells

The apoptosis induced by L-asparaginase was evaluated by Annexin V/PI staining, DAPI staining and DNA electrophoresis. As shown in Figure 3A–3B, 0.1 unit/ml L-asparaginase induced apoptosis in 6.18% and 62.58% of cells at 24 and 48 hours, respectively. As shown in Figure 3C, confocal microscopic studies demonstrated that U937 cells treated with L-asparaginase showed nuclei condensation and rupture. DNA from U937 cells treated with different concentrations of L-asparaginase at the indicated times was extracted and analyzed by electrophoresis (Figure 3D). L-asparaginase induced the formation of a DNA ladder in U937 cells treated with L-asparaginase, demonstrating that L-asparaginase induced apoptosis in U937 cells.

4.3. L-asparaginase improved the survival rate of mice inoculated with U937 cells (U937-mice)

To examine the therapeutic effect of L-asparaginase *in vivo*, we established the AML U937 model by intraperitoneal inoculation of U937 cells in mice (5 × 10^6 per mouse). The successful establishment of AML U937 model was demonstrated by the infiltration of U937 cells into various organs (Figure 4). Next, we examined the therapeutic effect of L-asparaginase *in vivo* (the detailed experimental procedure is seen in Figure 5A). Eight days after the inoculation of U937 cells, the mice were treated with L-asparaginase, Ara-C, or VP-16. L-asparaginase markedly improved the survival rate of mice compared with untreated controls (P = 0.001, Figure 5B and Table 3). No significant differences were found between L-asparaginase-treated and Ara-C- (P = 0.851) or VP-16-treated groups (P = 0.091). In short, L-asparaginase could significantly improve the survival rate of the mice inoculated with U937 cells, and showed similar anti-leukemia effect as Ara-C and VP-16.

4.4. Low expression level of ASNS contributed to the sensitivity of U937 cells to L-asparaginase

To examine the effect of ASNS expression level on the sensitivity of U937 cells to L-asparaginase, we measured the expression level of ASNS in several leukemic cell lines. As shown in Figure 6A, the baseline expression level of ASNS was significantly lower in U937 cells than in the 3 L-asparaginase-resistant cells lines (Namalwa, Karpass-299, and K562 cells). Similar results were obtained at 24 and 48 hours after L-asparaginase treatment (Figure 6B and C and Table 4), suggesting a low expression level of ASNS contributes to the sensitivity of U937 cells to L-asparaginase. We also analyzed the GEO database (GSE643, GSE645, and GSE654) to demonstrate that low ASNS expression level contributes to the sensitivity of ALL to L-asparaginase. As shown in Figure 6C, the expression level of ASNS in L-asparaginase-sensitive ALL was significantly lower than in L-asparaginase-resistant ALL (P = 0.002392 for L-asparaginase-sensitive ALL vs. L-asparaginase-resistant general ALL; P = 0.010505 for L

**Table 3.** The survival rate for U937-mice treated with different chemotherapeutic agents

Group	Control		Ara-C		VP-16		L-Asp	
	X^2	P	X^2	P	X^2	P	X^2	P
Control			6.348	0.012	26.027	0.000	26.027	0.000
Ara-C	6.348	0.012			1.963	0.161	0.059	0.808
VP-16	26.027	0.000	1.963	0.161			2.862	0.091
L-Asp	26.027	0.000	0.059	0.808	2.862	0.091		

* The Kaplan–Meyer method were used to compare the survival rate for U937-mice treated with different chemotherapeutic agents

Table 4. The expression level of ASNS before or after the treatment of L-asparaginase

Cell lines	Before treatment	After treatment	* P values	sensitivity
U937	5.11±6.75	87.72±36.18	0.0045	S
Jurkat	8.44±7.65	152.67±48.58	0.0038	S
Namalwa	159.04±81.81	2347.20±396.82	0.0002	I
Karpas299	306.94±125.51	1005.81±410.55	0.0313	R
K562	1729.61±426.35	5039.79±2231.94	0.0305	R

* Student's non-paired t tests were used to compare the expression levels of ASNS between the two groups before and after treatment for each cell line.
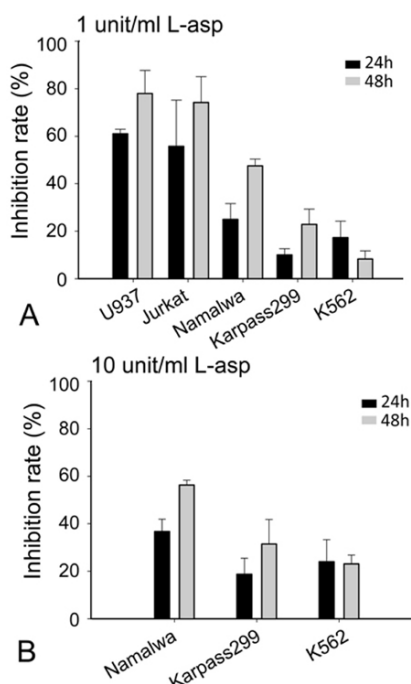
Figure 2. The cell inhibition rate of (A) 1 or (B) 10 unit/ml of L-asparaginase on U937, Jurkat, Namalwa, Karpass 299 and K562 cells at 24 or 24 hours. Data are expressed as mean ± SD (n = 3).

asparaginase-sensitive ALL vs. L-asparaginase-resistant B ALL). Taken together, these results indicate low expression level of ASNS not only contributes to the sensitivity of U937 cells to L-asparaginase, but also contributes to the sensitivity of ALL to L-asparaginase in the clinic.

4.5. AML M5 subtype showed lower expression level of ASNS than other non-M5 subtypes of AML

We analyzed the GEO database (GSE1729,

GSE10358, GSE6891, and GSE1159) to examine the expression level of ASNS in the AML M5 subtypes. First, we used GSE1159 as a database example to measure the expression level of ASNS in all subtypes of AML. The result showed that the AML M5 subtype expression level of ASNS was the lowest among all the subtypes of AML (Figure 7). A lower expression level of ASNS was also found in the AML M5 subtype compared with GSE1159, a non-M5 subtype (P = 8.31 E-10 for non-M5 vs. M5), GSE1159 (P = 0.021 for non-M5 vs. M5), GSE6891 (P = 1.005 E-5 for non-M5 vs. M5), and GSE1729 (P = 0.016 for non-M5 vs. M5). We also compared the expression levels of ASNS between the L-asparaginase-sensitive ALL database (GSE643) and AML M5 subtype databases (GSE1159 and GSE1729). As shown in Figure 8B, the AML M5 subtypes showed similar or lower expression level of ASNS than L-asparaginase-sensitive ALL (P = 0.565 for GSE643 vs. GSE1729; P = 2.1 E-3 for GSE643 vs. GSE1159). Taken together, the AML M5 subtype showed the lowest expression level of ASNS compared to all subtypes of AML. The low expression level of ASNS may contribute to the AML M5 subtype sensitivity to L-asparaginase.

4.6. L-asparaginase induced apoptosis of U937 cells via an AIF-dependent pathway but not a caspase-dependent pathway

Treatment with L-asparaginase resulted in a significant accumulation of cytochrome C in the cytosol and activated caspase-9 in a time-dependent manner (Figure. 9A). Furthermore, the gradual degradation of procaspase-3 at 12 and 48 hours after L-asparaginase treatment was accompanied by PARP cleavage (PARP is a downstream target of activated caspase-3 that is typically cleaved in caspase-mediated apoptosis). To determine the role of caspase in L-asparaginase-induced apoptosis, we pretreated U937 cells with the general caspase inhibitor (z-VAD-FMK), caspase-9 inhibitor (z-LEHD-FMK), and caspase-8 inhibitor (z-IETD-FMK), before L-asparaginase treatment. As shown in Figure 9A (middle panel), L-asparaginase-induced apoptosis was not affected by these caspase inhibitors (P > 0.05). Moreover, L-asparaginase-induced mitochondrial $\Delta\Psi$m collapse was not affected by these caspase inhibitors (Figure 9A, lower panel, P > 0.05). Thus, although L-asparaginase induced caspase-dependent apoptosis in U937 cells, which was not reversed by blocking the activity of caspase, suggests that the activity of caspase is not the dominant factor in L-asparaginase-induced apoptosis. We used western-blot analysis and a confocal microscopy to determine whether AIF plays a role in the apoptosis of U937 cells. As shown in Figure 9B, after L-asparaginase treatment AIF was moderately upregulated and simultaneously translocated into the nucleus, resulting in nuclear condensation in the U937 cells. Fas is a key component of the extrinsic receptor-mediated apoptotic pathway. To determine whether L-asparaginase-induced apoptosis is mediated by Fas, U937 cells were pre-treated for 2 hours using an anti-Fas neutralizing antibody (ZB-4) and subsequently treated with L-asparaginase. After 24–48 hours, apoptosis was examined by flow cytometry (Figure 9C). The suppression of Fas by ZB-4 did not inhibit L-asparaginase-induced apoptosis in U937 cells (data not shown). L-asparaginase consistently showed a minor effect on caspase-8 cleavage (Figure 9C). However, the expression of Fas significantly increased after L-
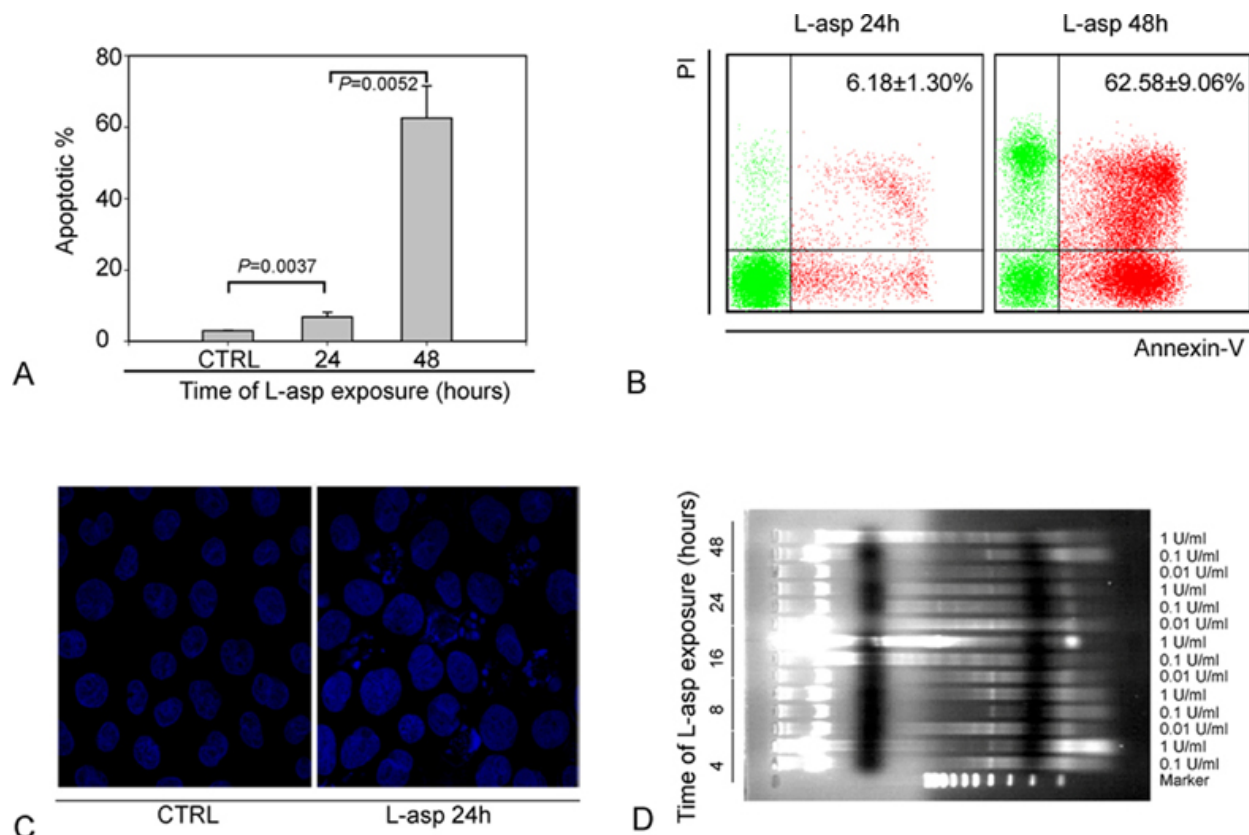
Figure 3. L-asparaginase induces the apoptosis of U937 cells. (A) The percentage of apoptotic cells for U937 cells exposed to 0.1 units/ml of L-asparaginase at the indicated time points. Apoptosis was determined using Annexin V/PI staining by flow cytometry. Data are expressed as mean ± SEM (n = 3). (B) The representative scatter plot of the flow cytometry in (A). (C) Confocal microscopic studies. The representative nuclei staining by DAPI in U937 cells treated with L-asparaginase. (D) DNA electrophoresis of the lysed U937 cells treated by a series of concentration of L-asparaginase at the indicated time points.

asparaginase treatment (Figure 9C). We propose that the upregulation of Fas may be a potential cellular response, but not the cause of apoptosis, as ZB-4 (the anti-Fas neutralizing antibody) failed to rescue U937 cells from apoptosis. Moreover, the expression level of Bcl-2, Bax, and Hsp90 did not change after L-asparaginase treatment (Figure 9D). All of the data suggest that L-asparaginase may induce apoptosis of U937 cells via an AIF-dependent pathway but not a caspase-dependent pathway.

5. DISCUSSION

There is a great need for novel approaches that re-engineer traditional agents to address cancer therapy for several reasons. Unfortunately, there are many obstacles on the road to translational medicine such as long timelines, steep costs, and high failure rates in new drug discovery. In addition, large pharmaceutical companies are inclined to invest prudently to reduce failure rates. There has been great advancement in the exploration of the existing drugs for new applications (2). Conventional chemotherapeutic regimens have been shown to be safe and efficacious in the clinic, with acceptable and reversible side effects. Therefore, there is great potential in the exploration of conventional chemotherapeutic agents that can be modified for new applications to improve leukemia therapy.

In this study, we discovered a new treatment for AML using L-asparaginase, a key chemotherapeutic agent used in the therapy of childhood ALL (28,29). L-asparaginase has been shown to possess potential anti-leukemic activity towards U937 cells *in vitro* and *in vivo*. The mechanism of anti-leukemic activity of L-asparaginase involves AIF-mediated apoptosis, which consists of chromatin condensation, phosphatidylserine externalization, and mitochondrial transmembrane depolarization in U937 cells. It has to be noted that L-asparaginase significantly prolongs the survival of mice inoculated with U937 cells. The analysis of ASNS expression levels in the GEO database and several leukemic cell lines indicate that a low baseline level of ASNS expression contributes to the sensitivity of the AML M5 subtype to L-asparaginase. These results point toward the potential use of L-asparaginase in AML M5 subtype treatment.

Our results demonstrate that L-asparaginase induces apoptosis in U937 cells via an AIF-dependent pathway, but not through the caspase-dependent pathway (Figure 9). Generally, apoptosis is closely associated with the functions of mitochondria and caspases (30-32). Other apoptosis-like cell death pathways involve mitochondria, but rely on alternative and caspase-independent mediators, such as AIF (33) and endonuclease G (34), which are both translocated into the
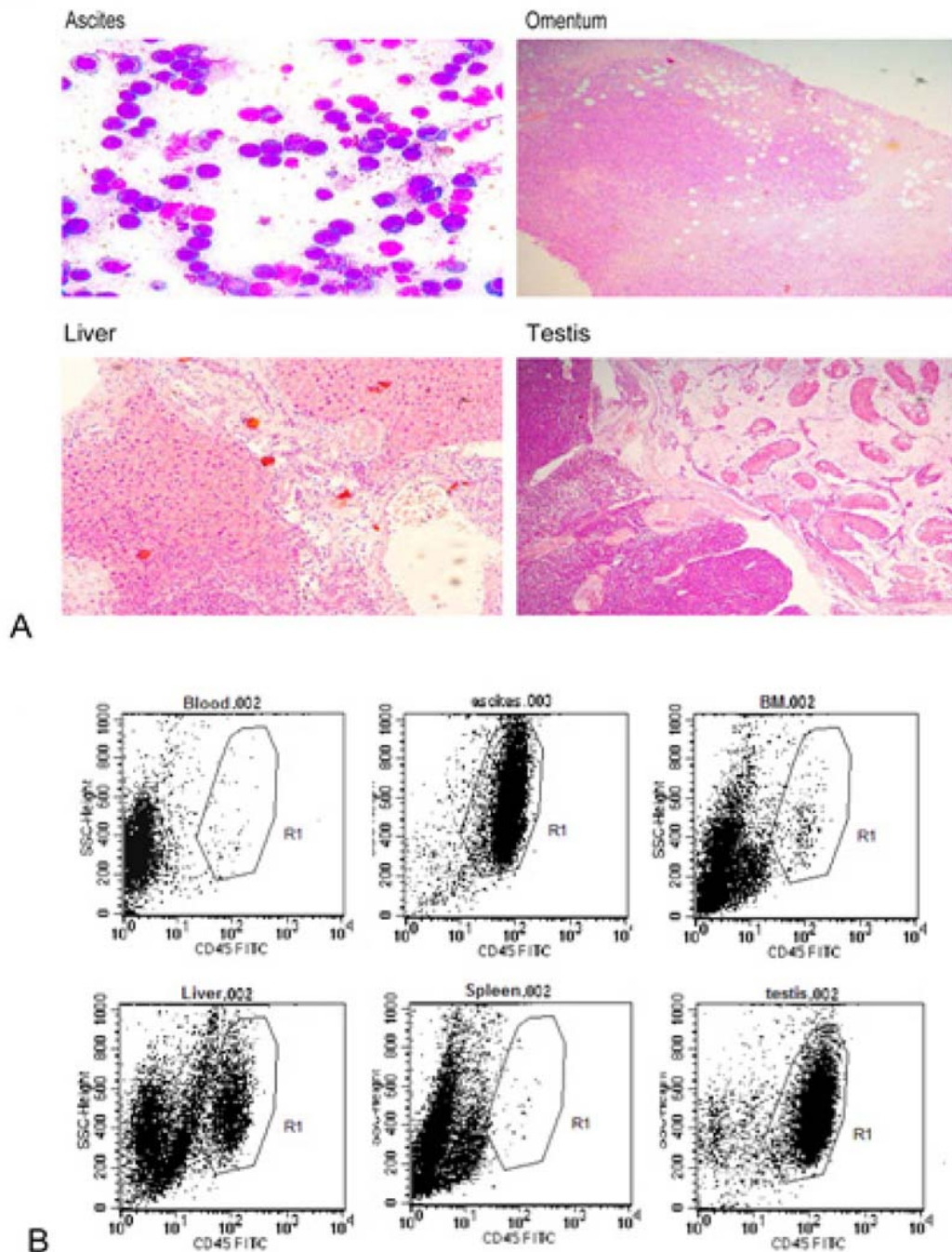
Figure 4. The establishment of AML mouse model by U937 cells. (A) The infiltration of U937 cells into various organs (ascites, omentum, liver, and testis). H&E staining was performed. (B) The infiltration of U937 cells into various organs was demonstrated by flow cytometry. The representative scatter plot was shown.

nucleus to trigger chromatin condensation and apoptosis. AIF homologues have been found in all metazoan phyla, however, no evidence of caspases in plants, fungi, or unicellular organisms have been reported. Increasing evidence from *in vivo* assays suggests that AIF plays an essential role in caspase-independent apoptosis (33, 35, 36).

Thus, AIF is a unique element of apoptotic machinery that obeys the rule of evolution. Our data demonstrate that the nuclear accumulation of AIF accompanies the induction of apoptosis, and the nuclear translocation and accumulation of AIF cannot be reversed by z-VAD-FMK. Taken together, our data shows that AIF is responsible for the L-
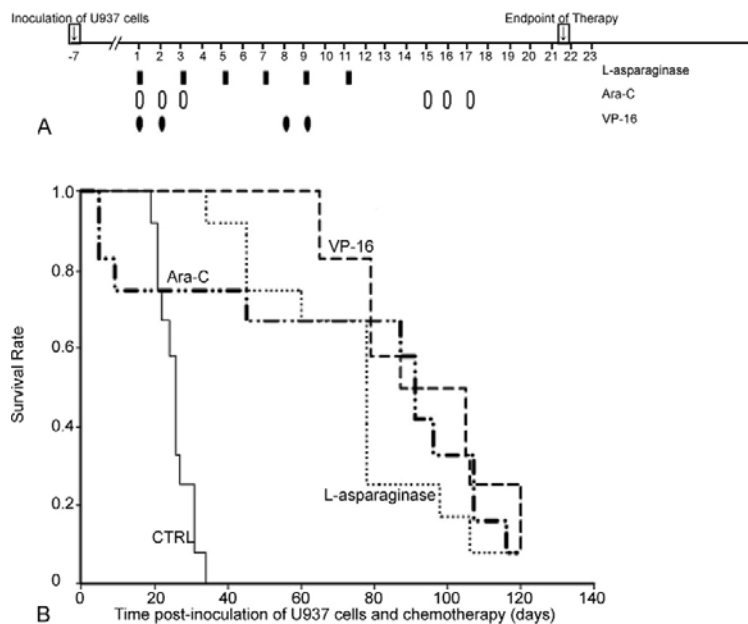
Figure 5. L-asparaginase could significantly improve the survival of the mice inoculated with U937 cells (U937-mice) . (A) The flow chart of administration of various chemotherapeutic agents (L-asparaginase, Ara-C and VP-16) in the U937-mice. (B) The survival rate of the U937-mice treated with various chemotherapeutic agents.
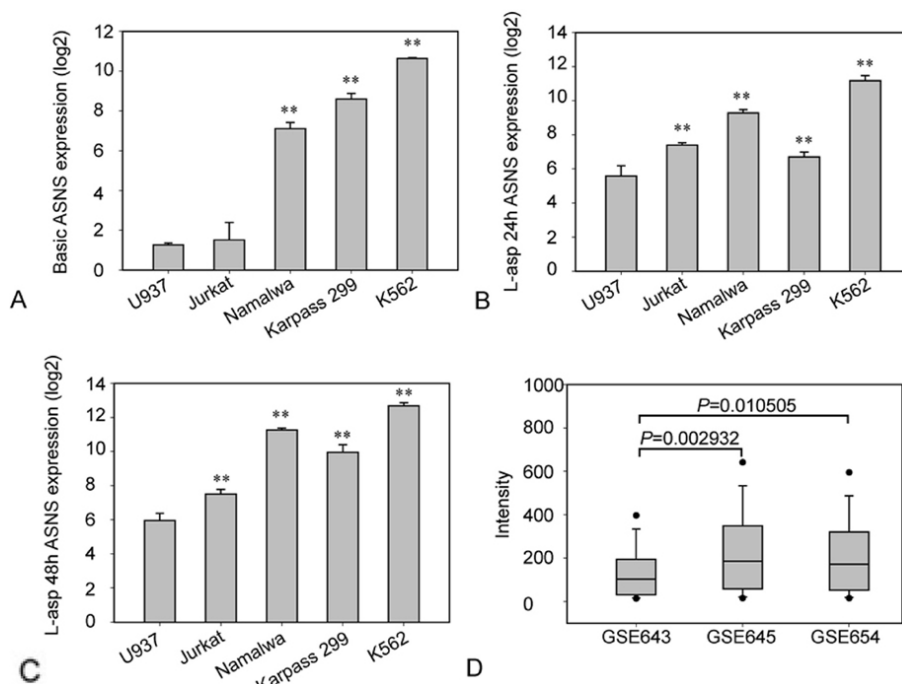

Figure 6. A low expression level of ASNS contributes to the sensitivity of U937 cells to L-asparaginase.(A) The basic ASNS expression of U937, Jurkat, Namalwa, Karpass-299, and K562 cells. The ASNS expression of U937, Jurkat, Namalwa, Karpass-299, and K562 cells 24 (B) or 48 (C) hours after L-asparaginase treatment. Data are expressed as mean ± SEM (n = 3). (D) The relative expression of ASNS in three representative gene expression profiling data(GSE643 (L-asparaginase-sensitive ALL), GSE645 (L-asparaginase-resistant ALL), and GSE654 (L-asparaginase-resistant B-ALL)). Gray boxes indicate the relative expression of ASNS in ALL patients. Top, bottom, and middle lines of each box correspond to the 75th percentile (top quartile), 25th percentile (bottom quartile), and 50th percentile (median), respectively. The whiskers extend from the 10th percentile (bottom decile) to the top 90th percentile (top decile). Dark circles indicate outliers within each group. The differences between the two groups were assessed using Student's non-paired t tests. The *P*-value is shown in the panels.
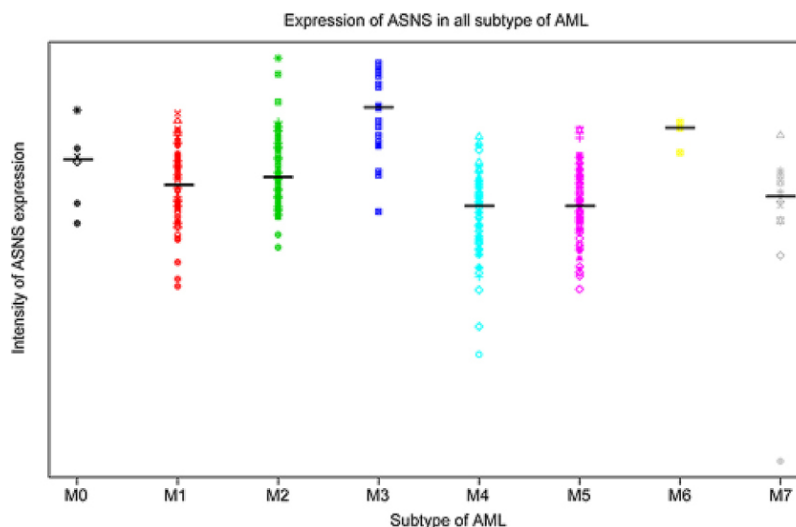
Figure 7. The expression level of ASNS in AML M5 subtype, using GSE1159 as a database. Gray boxes indicate the expression levels of ASNS in AML patients. The middle line of each group represents the 50th percentile.
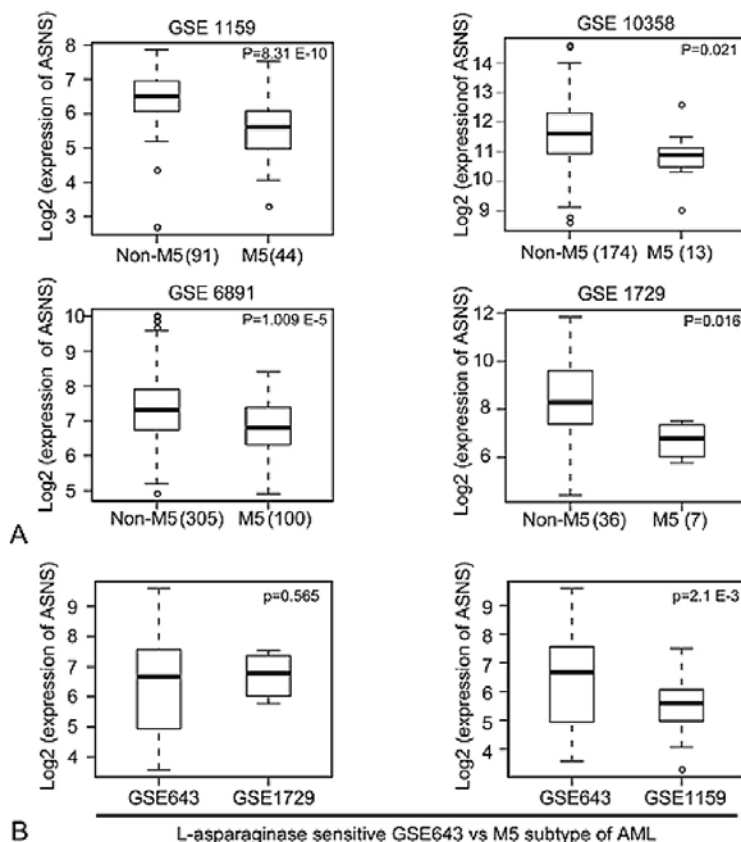
Figure 8. AML M5 subtype showed lower expression level of ASNS than other non-M5 subtypes of AML. (A) The expression level of ASNS of four GEO databases (including GSE1159, GSE10358, GSE6891, and GSE1729) was compared between AML M5 subtype and other non-M5 subtypes of AML. (B) The expression level of ASNS of three GEO databases (including GSE1729, GSE1159 and GSE643) was compared between AML M5 subtype (GSE643) and L-asparaginase-sensitive ALL (GSE1729 and GSE1159). White boxes indicate the levels of expression of ASNS in indicated patients. Top, bottom, and middle lines of each box correspond to the 75th percentile (top quartile), 25th percentile (bottom quartile), and 50th percentile (median), respectively. The whiskers extend from the 10th percentile (bottom decile) to the top 90th percentile (top decile). Open circles indicate outliers within each group. The differences between the two groups were assessed using Student's non-paired t tests. The *P*-value is shown in the panels.
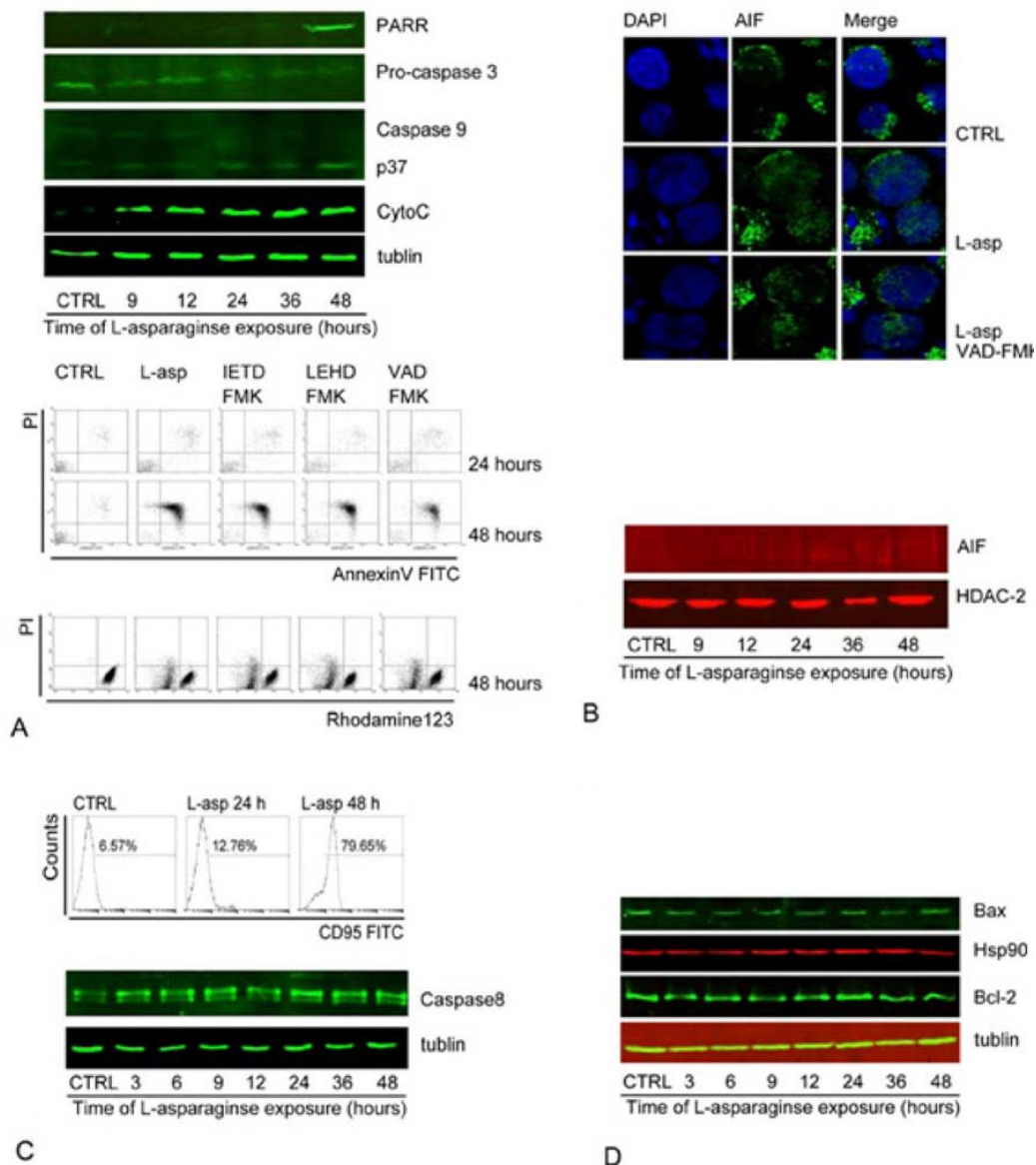
Figure 9. L-asparaginase induced the apoptosis of U937 cells via an AIF-dependent way. (A) L-asparaginase induced the apoptosis of U937 cells in a caspase-independent manner. Total proteins were subjected to Western blot with antibodies specific for PARR, Pro-caspase 3, Caspase 9, p37, Cyto C, using tublin as an internal control. In the pre-treatment assays, general caspase inhibitor (z-VAD-FMK), caspase-9 inhibitor (z-LEHD-FMK) and caspase-8 inhibitor (z-IETD-FMK were added before the treatment of L-asparaginase. After then, the cells were analyzed by Annexin V/PI or Rhodamine 123 staining by flow cytometry. (B) AIF is involved in the apoptosis induced by L-asparaginase-. Confocal microscopic studies and Western blot were used to determine the nucleus translocation of AIF. (C) The expression level of Fas was up-regulated after L-asparaginase treatment, whereas the expression level of caspase 8 expression did not change during the treatment of L-asparaginase treatment. (D) After the treatment of L-asparaginase in U937 cells, total proteins were subjected to Western blot with antibodies specific for Bax, Hsp90, and Bcl-2, using tublin as an internal control.

asparaginase-induced apoptosis in U937 cells. In addition, our study shows that exposure of U937 cells to L-asparaginase induces the release of cytochrome C, collapse of the mitochondria, activation of effector caspase, and upregulation of CD95 whereas the Fas-associated or caspase-dependent apoptosis is not be reversed by caspase inhibitors or the anti-Fas neutralizing antibody. As shown

in Figure 9, no obvious change occurred in the p53-associated apoptotic pathway, due to the p53-deficient nature of U937 cells. However, we cannot completely exclude the role of the mitochondria or Fas in L-asparaginase-induced apoptosis.

An interesting finding of this study was that

lower expression levels of ASNS correlated with chemosensitivity of leukemic cells to L-asparaginase. Evidence from cell lines and a large-scale gene expression profiles confirmed the correlation of the ASNS expression level with L-asparaginase sensitivity. In addition, our in-depth data analysis indicated that the expression level of ASNS in the AML M4 subtype was lower than other subtypes of AML, and similar to that of the AML M5 subtype (data not shown). Our results demonstrate that AML M5 subtype shows a lower expression level of ASNS than other non-M5 subtypes of AML, and the low expression level of ASNS may contribute to the sensitivity of the AML M5 subtype to L-asparaginase. We conclude that the response of AML to L-asparaginase may be predicted in a clinic situation by the examination of ASNS expression levels. Recently, we have found that the mechanism by which ASNS regulates chemoresistance is through an interaction with transporter systems (37).

In conclusion, our results suggest that L-asparaginase is a potential reagent in the treatment of the AML M5 subtype and deserves attention in the treatment of AML due to its ability to improve clinical outcomes. First, the mild cytotoxic effect of L-asparaginase on normal hematopoietic cells assures safe clinical application (38). Although therapy-related pancreatitis and hypofibrinogenemia is usually encountered clinically, it can be prevented and reversed by suitable intervention (8). Second, even though the anti-leukemia effect of L-asparaginase on U937 cells is not superior to standard chemotherapeutic agents, it can be developed into a powerful synergistic agent to improve outcome because of its phenotype specific targeting ability. Third, L-asparaginase is a potential reagent in the treatment of the AML M5 subtype, suggesting that L-asparaginase may be efficacious in leukemic cells with a monocytic phenotype. Given its potential as an active combinative agent, we believe that L-asparaginase will be useful in the treatment of the AML M5 subtype.

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Abbreviations: AML: Acute myelogenous leukemia, CR: complete remission, Ara-C: cytoarabinoside, ALL: acute lymphoblastic leukemia, IC50: half-maximal inhibition of cell viability, SPF: surrounding pathogen-free, DAPI: 4, 6-diamidino-2-phenylindole, AIF: apoptosis-inducing factor, APL: acute promyelocytic leukemia

Key Words: L-asparaginase; Acute Myelomonocytic

L-asparaginase induced apoptosis of U937 cells

Leukemia; Apoptosis; ASNS; AIF

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