Sorting, identification and enrichment of side population cells in THP-1 acute monocytic leukemia cells

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Abstract. The objective of the present study was to examine and determine whether the human acute monocytic leukemia cell line THP-1 contains side population (SP) cells, and, if so, to increase the proportion of SP cells using arabinosylcytosine (Ara-C). Fluorescent microscopy and flow cytometry were employed to detect the percentage of SP cells in THP-1 cells. Then, SP and non-SP (NSP) cell subpopulations were collected and identified. THP-1 cells were incubated with different concentrations of Ara-C for 24 h and the proportion of SP cells was detected. Our results demonstrated that the percentage of SP cells was 1.81±0.99% in THP-1 cells. A majority of the SP cells remained in the G_0/G_1 phase, and the expression of $CD34^+$ and $CD34^+CD38^-$ and the proliferation ability of the SP cells were higher compared to NSP cells ($P<0.05$). The mRNA expression of multidrug resistance genes (*ABCG2* and *ABCB1*), apoptosis regulation genes (*Bcl-2*) and the *Bcl-2*/*Bax* value of SP cells were higher than those of NSP cells. SP cells have been shown to be more tumorigenic than NSP cells. Following co-culture with Ara-C, the proportion of SP cells increased significantly and subsequently the Ara-C concentration increased. These findings suggest that the THP-1 cell line contains SP cells and that SP cells possess certain intrinsic stem cell properties and may contain a larger proportion of leukemia stem cells (LSCs). The concentrations of SP cells can be increased with Ara-C by co-culture, and this technique is a useful and important application for the study of LSCs.

Introduction

Childhood and adolescent acute myeloid leukemia (AML) is one of the most challenging types of childhood cancer to successfully treat (1). The relapse rate remains unacceptably high, with a 5-year event-free survival (EFS) of approximately 50% (2). In addition, successful treatment can only be achieved using highly intensive chemotherapy that results in relatively high rates of treatment-related mortality and significant side-effects (3). Although extensive efforts are being made to eliminate these issues, an efficient and effective method of treating AML has yet to be developed. Novel therapeutic strategies are urgently required to improve the prognosis of this disease.

The cancer stem cells (CSCs) theory postulates the origin of cancer from a new perspective. CSCs are a small subset of cancer cells that possess stem cell-like properties, such as, the ability to self-renew via asymmetric division and to produce differentiated progeny. These cells generally remain in a quiescent state (4) and comprise a small minority of the total tumor population. They have an extensive capacity to proliferate, differentiate, and self-renew, enabling them to repopulate recipients after transplantation (5). This small population of cells within a cancer is responsible for drug resistance and the recurrence of cancer (6). Hence, the specific targeting of CSCs therapeutically must be explored. To date, the possible existence of CSCs has been shown in leukemia (7,8) and in certain solid tumors (9,10). CSCs have also been identified in immortalized cell lines (5), long-term cultured cancer cells (10,11), and patient tumor samples (5), using the side population (SP) technique.

Extensive research has focused on leukemia stem cells (LSCs) in the pursuit of new ideas for targeted therapy. Therefore, sorting, identifying and enriching LSCs has become particularly important in leukemia treatment research. Side population (SP) cells, as defined by Hoechst 33342 exclusion in flow cytometry, represent only a small fraction of the whole cell population (12); their properties occupy an important position in several investigations (13,14). Previous studies have shown that CSCs can be identified by an SP phenotype based on fluorescence-activated flow cytometry. SP cells have been found not only in patient tumor samples but also in immortalized cell lines and long-term cultured cancer cells (15,16); they have demonstrated the capacity to function as stem cells in the tissues from which they were isolated and may be able to transdifferentiate (17). SP cells share the most relevant features of LSCs, i.e., the self-renewal potential and quiescent status, and they contain relatively high concentrations of tumor stem cell indicators (18). Concurrent studies have shown that SP cells in human cancer have various origins, including acute myelogenous leukemia, neuroblastoma, and glioma (10,11,19). These

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studies have also suggested that SP cells may be a source of cancer stem cells (CSCs). SP cells can be sorted using flow cytometry, which is a suitable application for LSC sorting (20).

The human acute monocytic leukemia cell line THP-1, which was originally established from an infant diagnosed with AML (21), provides an experimental model for functional, preclinical therapeutics and target identification studies of AML. In this study, we identified cancer SP cells by isolating them in the THP-1 cell line. In SP and NSP cells, we evaluated the cell cycle, the capacity for self-renewal, the presence of leukocyte surface antigens, and the expression of the multi-drug resistance gene and the apoptosis gene. The aim of this study was to enrich the LSC subpopulation in the THP-1 cell line with arabinosylcytosine (Ara-C) and to study the relationship between SP cells and LSCs.

Materials and methods

Cell line and culture. The human acute monocytic leukemia cell line THP-1 (Shanghai Institute of Cell Biology) was cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin-streptomycin (Invitrogen Life Technologies, Grand Island, NY, USA) at 37˚C under a 5% CO_2 atmosphere.

SP cell analysis using fluorescence microscopy. The SP cells were suspended at $1x10^6$ cells/ml in pre-warmed RPMI-1640 medium containing 2% FBS, 100 U/ml penicillin-streptomycin G, 100 μg/ml streptomycin and 10 mmol/l HEPES buffer. These cells were then incubated at 37˚C for 90 min with 5 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA), protected from light, either alone or in the presence of 50 μmol/l verapamil (Sigma-Aldrich). The cells were placed immediately on ice and were then washed and resuspended in cold phosphate-buffered saline (PBS) containing 1% FBS. Fluorescence microscopy (Leica, Germany) was employed to detect the morphology of SP cells among the THP-1 cells. The cells that were not stained or colored light blue were identified as SP cells.

SP cell analysis and sorting using flow cytometry. Following incubation with Hoechst 33342 at 4˚C, 1 μg/ml propidium iodide (PI) (BD Pharmingen, San Diego, CA, USA) was added to label the dead cells, and the mixture was then filtered through a 40-μm cell strainer (BD Falcon) to obtain a single-cell suspension. Cell analyses and purification were performed using MoFlo carrying a triple-laser (DakoCytomation, Fort Collins, CO, USA). Hoechst 33342 was excited with the UV laser at 350 nm, and fluorescence emission was measured with 405/BP30 (Hoechst blue) and 570/BP20 (Hoechst red) optical filters. PI labeling was measured through the 630/BP30 filter for the discrimination of dead cells. SP and NSP cells in each well were isolated (22).

Cell surface immunophenotyping. Immunophenotyping was conducted using conjugated monoclonal human antibodies reactive to CD34 and CD38 (BD Pharmingen). The staining was performed in the dark at 4˚C for 30 min. Isotype control antibodies and live unstained cells were used to establish gating parameters for positive cells. The percentage of the positive cells was obtained via the CellQuest software (BD Pharmingen).

Table I. Primer sequences for different genes.

Gene	Primer sequences (5'-3')
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA
ABCG2	F: GTCTAAGCAGGGACGAACAATC R: GCCAATAAGGTGAGGCTATCAA
ABCB1	F: TGGTGTTTGGAGAAATGACAGAT R: GAAACCTGAATGTAAGCAGCAAC
Bcl-2	F: GTGGATGACTGAATACCTGAACC R: AGACAGCCAGGAGAAATCAAAC
Bax	F: GGTTGTCGCCCTTTTCTACTT R: GTGAGGAGGCTTGAGGAGTCT

F, forward; R, reverse.

Cell cycle analysis. SP and NSP cells were harvested and then washed twice with PBS. The supernatant was discarded and the pellets were dissolved with 1 ml of 70% cold ethanol. After incubating at 4˚C for at least 12 h, cells were stained with 50 g/ml PI supplemented with 50 g/ml RNase and then incubated in the dark at 21˚C for 30 min. The samples were profiled for DNA content by flow cytometry (BD Biosciences), and 10,000 events were recorded for each sample. The percentages of cells in the G_0/G_1, S and G_2/M phases were obtained by CellQuest software.

Cell proliferation assay. The cells were grown in a 96-well plate, and the relative cell number was determined using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. The SP and NSP cells were plated at a density of $1x10^4$ cells/well in 96-well plates for 0-9 days. After 10 μl CCK-8 solution was added to each well, cells were incubated for a further 4 h at 37˚C, and the absorbance was measured at 450 nm using an automated ELISA reader (BioTek, Winooski, VT, USA).

Validation of gene expression by qPCR. Quantitative-PCR (qPCR) analysis was performed according to the manufacturer's protocol. First, total RNA was extracted from cells with an RNAprep pure Cell kit (Tiangen Biotech Co., Ltd., Beijing, China). One microgram of total RNA was reverse transcribed into cDNA with 1 μl M-MuLV RT (200 μg/μl) using a Single-Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA) and analyzed using an ABI 7900 (Applied Biosystems, Foster City, CA, USA). Specific primers for qPCR of GAPDH (housekeeping gene) and the additional genes of interest were designed using Assay-by-Design primer design software (Applied Biosystems) or were purchased as Assays-on-Demand from Applied Biosystems. The primers for these genes are shown in Table I. The relative amounts of product were calculated using the comparative CT ($2^{-\Delta\Delta Ct}$) method.

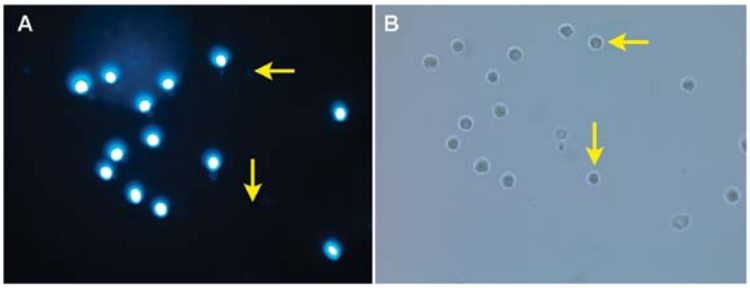

Figure 1. Hoechst 33342 staining of THP-1 cells (magnification, x200). Micrographs of THP-1 cells under a fluorescence microscope. (B) Micrographs of THP-1 cells under a common microscope (magnification, x200). The cells indicated by the arrow are SP cells.

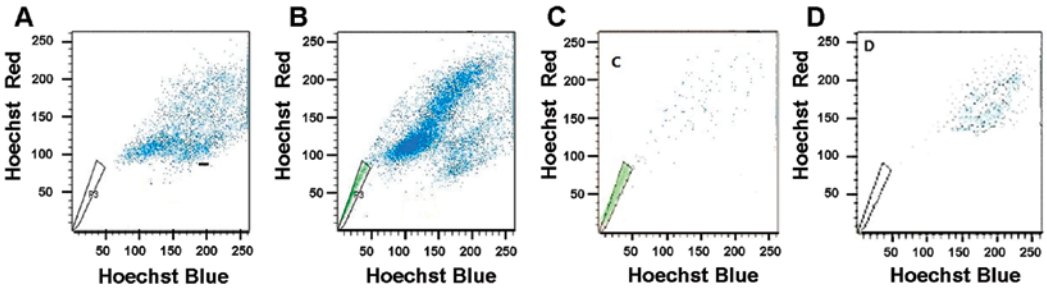

Figure 2. Cell sorting results and sorting purity. (A) The SP cells appear as the Hoechst-resistant fraction capable of pumping out the dye, and typically represent 1.81±0.99% of viable cells from the THP-1 leukemia cell line. The NSP cells that retained high levels of Hoechst staining are the main population of cells. (B) The SP is ablated when verapamil is included in the Hoechst incubation. (C) The sorted SP cells were concentrated in the SP region, the purity reached 96.75±1.55%. (D) The NSP tube cells were concentrated in the subregion of the main group of cells prior to the election, the purity reached 97.03±1.87%.

In vivo tumor formation. All animal studies were performed in compliance with the Guidelines for the Care and Use of the Laboratory Animals in Henan Province, China. Naïve male 6-8-week-old NOD/SCID mice were obtained from Beijing HFK Bioscience Co. (Beijing, China), kept under specific pathogen-free (SPF) conditions and used as tumor transplant recipients. Mice were housed five per cage. Thirty mice were randomly divided into six groups, five mice per group. Growing cells, sorted from the SP cells ($1x10^3$, $1x10^4$ and $1x10^5$ per mouse) and NSP cells ($1x10^4$, $1x10^5$ and $1x10^6$ per mouse) diluted in PBS, were mixed with 50 ml Matrigel (BD Biosciences) and injected intravenously via the tail vein. Three to four weeks later, human AML engraftment ($hCD45^+$/$CD33^+$ cells) was assessed in the peripheral blood and bone marrow by tail bleed and aspiration of the femur, respectively.

Enrichment of LSCs in an SP of THP-1 with Ara-C. THP-1 cells ($1x10^8$ cells/ml) were incubated for 24 h at 37˚C under a 5% CO_2 atmosphere with four different concentrations of Ara-C: 10, 100, 1,000 and 2,000 μg/ml. The cells were then harvested and washed twice with PBS. The proportion of SP cells was detected, respectively, for each Ara-C concentration.

Statistical analysis. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the means ± SD. Differences were determined using the Student's t-test or Fisher's exact test and one-way analysis of variance (ANOVA) followed by Scheffe's *post hoc* test, as indicated in the text. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

Prevalence of SP cells in THP-1 cells. The SP cells overexpressed the multidrug-resistant proteins that allowed them to efflux various drugs and xenobiotics, as well as the Hoechst 33342 dye (23). As shown in Fig. 1, fluorescence microscopy with Hoechst 33342 staining demonstrated that the SP cells were present among the THP-1 cells. Whereas >90% of the THP-1 cells were stained intense blue, a small population of the cells remained unstained (Fig. 1A, arrow). The micrographs of THP-1 SP and NSP cells were observed under visible light. There was no significant difference between SP and NSP cells in morphology (Fig. 1B, arrow).

Flow cytometry analysis with Hoechst 33342 staining demonstrated that the dimly stained Hoechst 33342 cells on the corner of the plot were gated as the SP population and represented a percentage of 1.81±0.99% of total THP-1 cells (Fig. 2A). Since the SP profile was blocked by staining in the presence of verapamil, a calcium channel blocker, the SP cell frequency was practically eliminated in the THP-1 cells stained with Hoechst 33342 and verapamil (Fig. 2B). To determine the sensitivity of our system, the SP and NSP cells in THP-1 cells were sorted and the purity was tested separately. The results showed that the cells of SP tube were still concentrated in the THP-1 SP region and the cells of NSP tube were still concentrated in the subregion of the main group of cells prior to sorting. Purity levels were 96.75±1.55% and 97.03±1.87%, respectively (Fig. 2C and D).

Expression of cell surface markers in SP and NSP cells. To further define the cells within the SP fraction, we used two

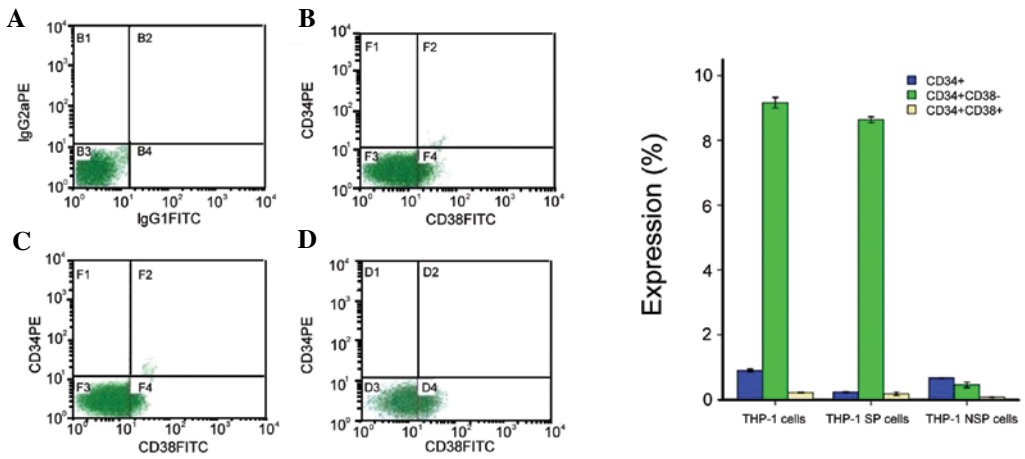

Figure 3. Flow cytometry analysis of CD34/CD38 expression. (A) IgG isotype control in THP-1 cells. (B) CD34/CD38 expression in unsorted THP-1 cells. (C) CD34/CD38 expression in the SP fraction. (D) CD34/CD38 expression in the NSP fraction.

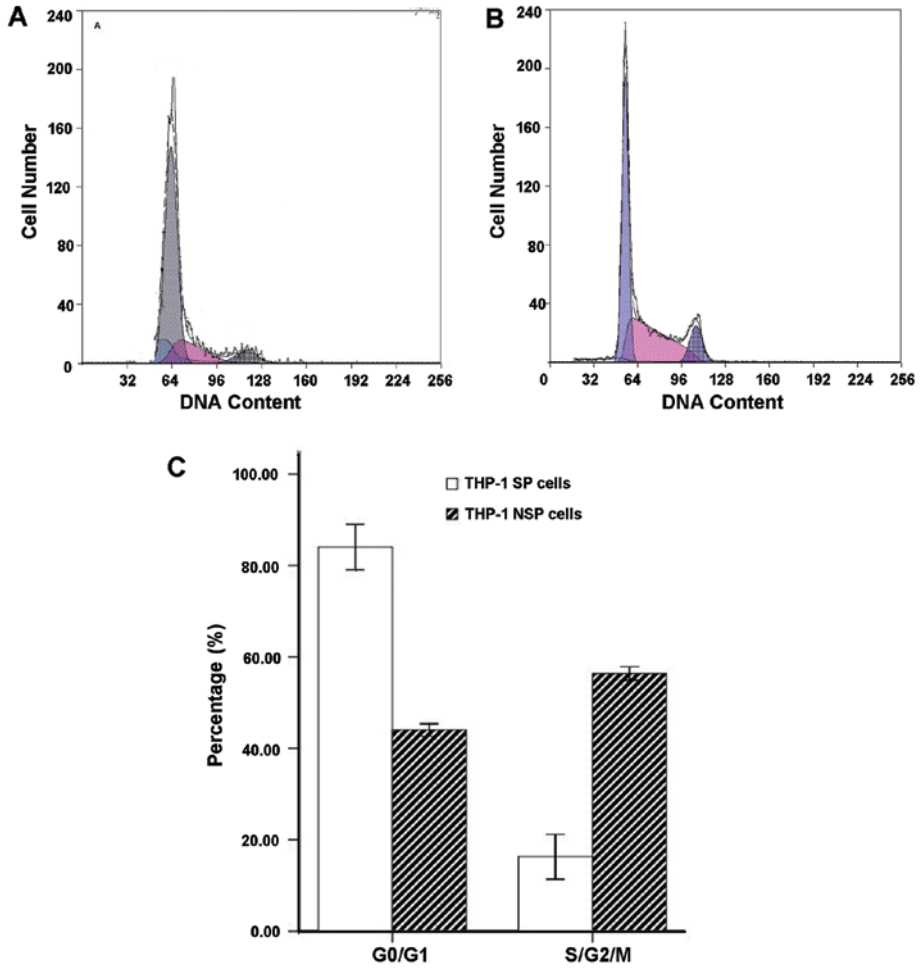

Figure 4. Cell cycle analysis by flow cytometry. (A) Cell cycle distribution in the SP cell groups. (B) Cell cycle distribution in the NSP cell groups. (C) Results of duplicate determinations from three separate experiments. *P<0.05 vs. SP cells.

cell-surface markers (CD34 and CD38) associated with LSCs (24). The analysis of SP and NSP cells revealed that these cells differ significantly in the expression of cell surface markers. The overall percentage of cells positive for CD34 was significantly lower in the NSP compared with the SP cells in all cell lines examined (Fig. 3).

Although the expression of $CD34^+/CD38^-$ in the SP fraction (Fig. 3C) was small, it was statistically significant and substantially higher than that in unsorted THP-1 cells (Fig. 3B) and NSP cells (Fig. 3D). The percentage of $CD34^+$ and $CD38^-$ expression in SP cells was 8.68±0.20%, markedly higher than that in NSP cells (0.16±0.08%) (P<0.05) (Fig. 3).

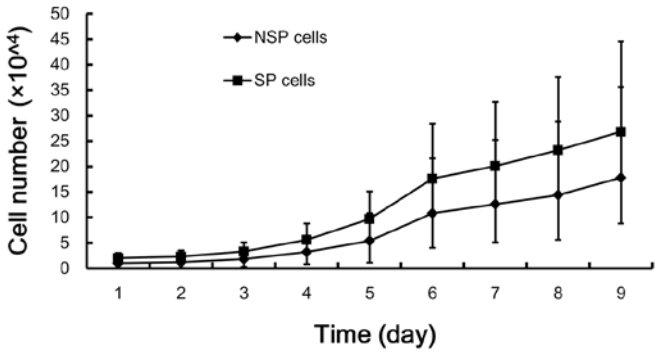

Figure 5. Growth curve of THP-1 SP and NSP cells. The THP-1 SP cells under stem cell-selective conditions grew faster compared with THP-1 NSP cells (P<0.05).

Cell cycle in THP-1 SP and NSP cells. The G_0/G_1 phase cells in the THP-1 SP subpopulation accounted for ~84.04±4.98% of the total cells and more than NSP cells (44.02±1.35%).

THP-1 SP cells resulted in blockage of the cell cycle from the G_0/G_1 phase to the S phase. Compared with NSP cells, the ratio of G_0/G_1 phase cells significantly increased, and the ratio of S-phase cells significantly decreased in SP cells (P<0.05) (Fig. 4).

In vivo growth characteristics of SP and NSP cells. The growth characteristics of the SP subpopulation were consistent with the predicted behavior of primitive precursor cells, including a high proliferative rate and self-renewal capacity. The proliferation of SP cells was significantly higher than that of NSP cells (P<0.05) (Fig. 5).

Analysis of gene expression in SP and NSP cells. We isolated RNA from sorted SP and NSP cells of the leukemia cell line THP-1 and used quantitative real-time RT-PCR amplification analysis to quantify the relative expression of the ABC transporter gene, the *ABCB1* and *ABCG2* gene product currently believed to be most closely associated with the SP phenotype (25). All SP fractions expressed higher levels of the *ABCB1* and *ABCG2* transporter gene than did the NSP fractions (Fig. 6A). We also measured the expression levels of two other cell apoptosis genes, *Bcl-2* and *Bax*; the former gene was clearly expressed at higher concentrations in SP compared with the NSP, whereas the latter gene was not. However, the *Bcl-2* and *Bax* values were significantly higher than in the NSP (P<0.05) (Fig. 6B).

THP-1 SP cells exhibit higher tumorigenicity than NSP cells. To determine whether the SP cells we identified in the THP-1 cell line might also be more tumorigenic, we performed xenograft experiments *in vivo*. Three or 4 weeks after THP-1 SP cells were transplanted intravenously via the tail vein, human AML engraftment ($hCD45^+/CD33^+$) was assessed in the peripheral blood and bone marrow by tail bleed and aspiration of the femur, respectively. The results indicated that the systemic disseminated leukemia model had been established successfully by injecting $1x10^3$ THP-1 SP cells in NOD/SCID mice (Fig. 6A). The SP cells isolated from the THP-1 cells were more tumorigenic than the NSP cells. NSP cells require a quantity of at least $1x10^6$ to establish a tumor model. Statistically, there were significant differences in the incidence of leukemia among different groups (P<0.05). The H&E strain of histologic sections revealed that almost all the leukemia xenotransplants successfully induced leukemia in the mice (Fig. 7B and C).

SP increases in the THP-1 cell line. It has been reported that CSCs can resist apoptosis when they are exposed to apoptosis inducement factors (26). Considering this characteristic of CSCs, we formulated a hypothesis that apoptosis resistance of CSCs can be used to increase the proportion of SP cells. In an apoptosis inducement model, the proportion of SP cells may increase in surviving cells. In this study, SP cells were co-cultured with different concentrations of Ara-C, the proportion of SP cells increased significantly, and the proportion of SP cells increased with the Ara-C concentration (Fig. 8).

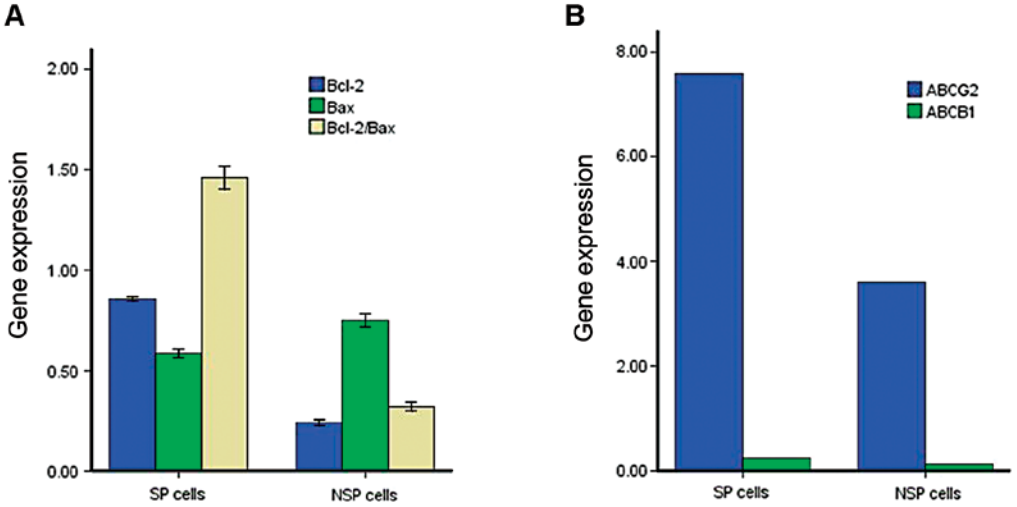

Figure 6. Analysis of ABCB1, ABCG2, Bcl-2 and Bax gene expression.

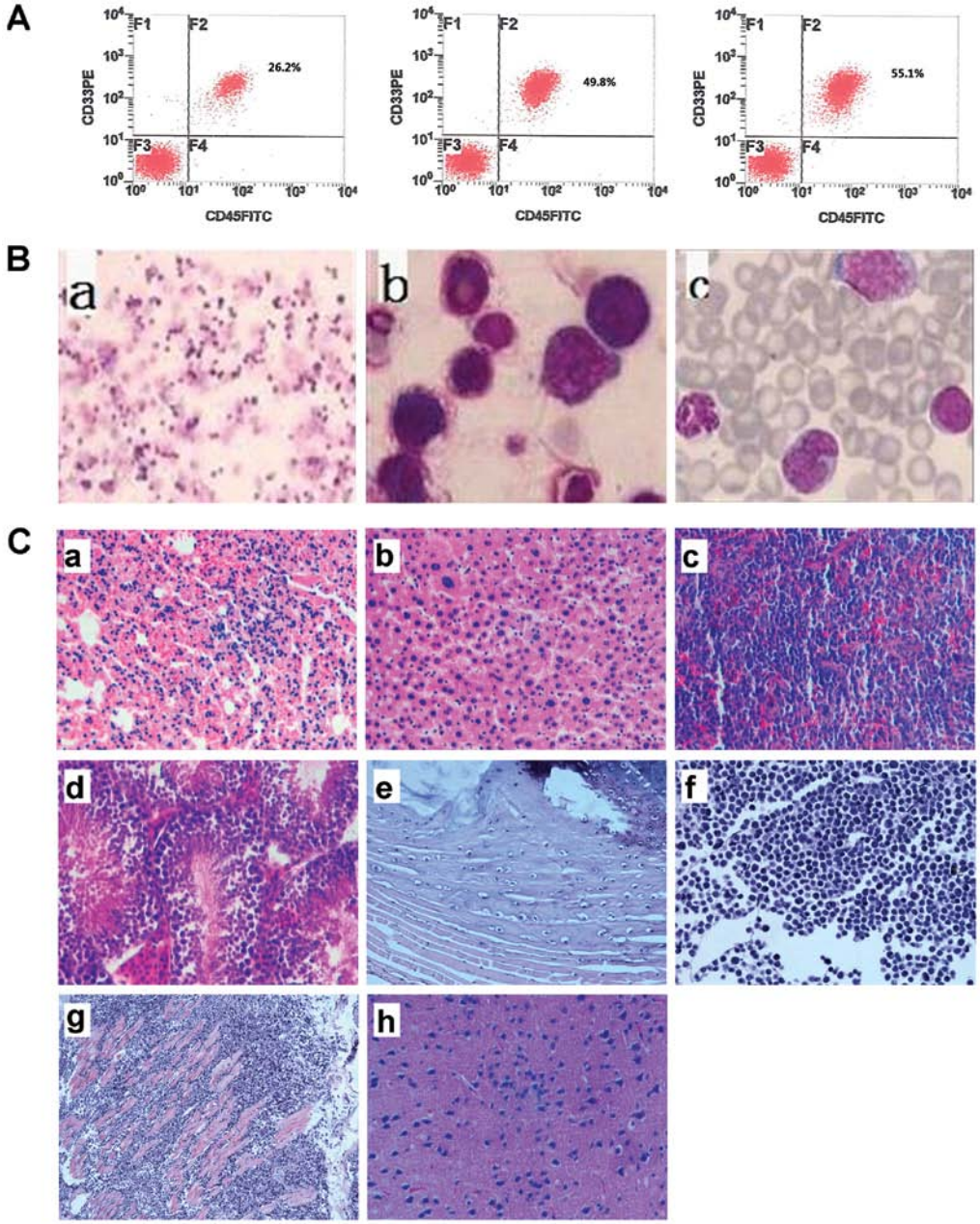

Figure 7. Identification of human THP-1 xenotransplant leukemia models. (A) Human AML engraftment ($hCD45^+CD33^+$ cells) was assessed in the peripheral blood and bone marrow using flow cytometry. (B) Wright's staining of bone marrow of NOD/SCID mice injected with SP cells. (a) magnification, x100, (b) magnification, x1,000 and (c) peripheral blood (magnification, x1,000). (C) Pathological examination of NOD/SCID mice injected with SP cells. Representative H&E staining sections of organs and tissues (H&E magnification, x200). AML cells can be found in (a) lung, (b) liver, (c) spleen, (d) didymus, (e) paravertebral muscles, (f) lymph node, and (g) musculi faciales. (h) Cerebral edema was observed.

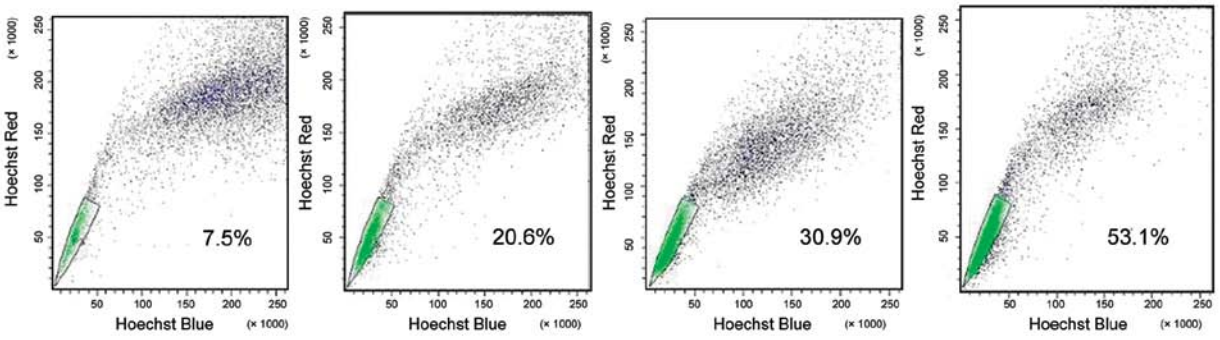

Figure 8. The proportion of SP cells after treatment with different concentrations of Ara-C. Following co-culture with different concentrations of Ara-C, the proportion of SP cells increased significantly and the proportion of SP cells increased with Ara-C concentration.

Discussion

Using the side population (SP) technique, Zheng *et al* (27) reported the SP rate in acute promyelocytic leukemia NB4 cells to be less than 1%. In addition, SP cells possess intrinsic stem cell properties and express some of the characteristic stem cell genes. In the present study, analysis by fluorescence microscopy and flow cytometry demonstrated the presence of SP cells, and we were able to identify a small SP component (1.81±0.99%) of cancer cells from the THP-1 leukemia cell line. The SP cells were practically non-existent in the presence of Hoechst 33342 and verapamil, a calcium channel blocker. The percentages of SP cells detected were similar to those in most previous reports: 0.8-1.9% in human multiple myeloma cell line RPMI-8226 and NCI-H929 (28), 0.47-4% in an adult T-cell leukemia/lymphoma cell line (29), and 0.5-29.9% in human acute myeloid leukemia (AML) (8), but less than the 4-37% noted in neuroblastoma cell lines (15). Furthermore, the SP cells and the majority of non-SP (NSP) cells were indistinguishable morphologically. Therefore, the isolation and identification of cancer stem cells (CSCs) remains very difficult (8). To the best of our knowledge, this is the first described isolation of cancer stem-like cells from the THP-1 cell line.

To date, AML leukemia stem cells (LSCs) are the most well-studied CSCs population (30). AML is typically a disease of stem progenitor cell origin. No special markers have been successfully developed to identify those cells in different tumors; different tumors have different CSCs markers (one or more). Blair *et al* (31) demonstrated that only a small quantity of a defined subset of cells were consistently clonogenic and that all AML LSC subtypes possess the same cell-surface markers (32). As determined by immunophenotyping, SP cells share some phenotypic characteristics with bone marrow, such as the absence of mature hematopoietic lineage markers CD34 and expression of CD38. Bonnet *et al* have identified LSCs in human AML as a common immunophenotype ($CD34^+/CD38^-$) and have demonstrated their self-renewal potential (1). We therefore examined the expression of CD34/CD38 in SP cells, NSP cells, and unsorted THP-1 cells. Our results showed that CD34/CD38 were both expressed at low levels on the membranes of SP and NSP cells. The percentages of $CD34^+$ and $CD34^+/CD38^-$ cells in the SP were higher than those in the NSP and among common THP-1 cells ($P<0.05$). These results suggest that the presence of LSCs in THP-1 cells is rare and that the number of LSCs is quite limited; however, LSCs may be more prevalent in the SP.

LSCs, similar to their normal HSC counterparts, exhibit a range of characteristics that enable their long-term survival. Some of these characteristics also facilitate their escape from the cytotoxic effects of chemotherapy; for example, LSCs are primarily present in a quiescent phase of the cell cycle (33). In the present study, the cell cycles of two cell subsets, SP and NSP of the THP-1 cell line, were analyzed by flow cytometry. A majority of the SP cells remained in the G_0/G_1 phase, and the NSP cells remained in the S or G_2 or M phases. The proliferation of SP cells was significantly higher than that of NSP cells ($P<0.05$).

A study by Hope *et al* (34) showed that AML originates from a hierarchy of LSC classes that differ in self-renewal capacity. Clarke *et al* (35) reported that SP cells produced two to seven times more colonies than NSP cells. In support of their putative stem cell nature, only the SP cells possessed the ability to produce colonies with both myoepithelial and luminal epithelial cell types. In the present study, the cell growth curves showed that the growth rate of SP cells was significantly faster than that of NSP cells; the cells continued to proliferate and the plateau period was not evident. This observation may indicate that SP cells contain more LSCs, and, therefore, cell proliferation is accelerated.

SPs are small subpopulations of cells with enriched stem cell activity that show a distinct 'low' Hoechst 33342 dye-staining pattern. The SP phenotype is mediated by the ATP-binding cassette (ABC) family of transporter proteins. Various types of ABC transporters have been shown to contribute to drug resistance in numerous types of cancer by pumping drugs out of cells (25,36). Notably, some ABC transporters are expressed by several types of stem cells. One of the major mediators seems to be ABCG2 or BCRP (37), which was initially identified in drug-selected MCF7 breast cancer cells and was later found to efflux multiple chemotherapeutic drugs and xenobiotics (38). Other supporting evidence shows that SP cells preferentially express ABCG2 (13,39). SP cells also express other ABC transporters such as MDR-1 (i.e., ABCB1 or P-glycoprotein), suggesting that these latter molecules may also be involved in mediating the SP phenotype (40). We therefore isolated RNA from sorted SP and NSP cells of the THP-1 leukemia cell lines, and we used a real-time RT-PCR assay to quantify the relative expression of the ABCG2 and ABCB1 transporters. The mRNA expression levels of the two genes of SP cells were higher than those among NSP cells. Independent of whether THP-1SP cells are indeed a tumor 'stem cell' population, their high expression of drug efflux transporter genes and their associated high capacity to efflux lipophilic drugs may have a significant effect on treatment outcome (5).

Stem cell resistance to apoptosis through complicated mechanisms (41,42), such as the regulation of Bcl-2 or Bax (43,44), has been proven experimentally. Compared with SP cells, NSP cells in tumors are more susceptible to apoptosis inducement or chemotherapy. We also measured the expression levels of two other apoptosis regulation genes, *Bcl-2* and *Bax*; the former gene was clearly expressed at higher concentrations in SP cells compared with NSP cells, whereas the latter gene was not. There was no difference between SP and NSP cells in *Bax* expression, but the *Bcl-2*/*Bax* values of the SP cells were significantly higher than those of the NSP cells. These results suggest that apoptosis resistance may aid in screening the markers of CSCs.

It is believed that only the CSCs, but not the majority of their remaining descendants, are responsible for tumorigenesis, progression, metastasis, and relapse following treatment (45). Repopulation in recipients after transplantation is the most important characteristic of CSCs. Purified SP cells from certain cell lines were more tumorigenic than the corresponding NSP cells. To evaluate the tumorigenic ability of THP-1 SP cells *in vivo*, NOD/SCID mice were injected with different subpopulations and different quantities of THP-1 cells intravenously by tail vein, and the incidence of leukemia was compared among the groups. As we anticipated, the systemic disseminated leukemia model was established successfully by

injecting $1x10^3$ THP-1 SP cells. Unsorted THP-1 cells require a quantity of at least $1x10^6$ to establish a tumor model.

Isolation and identification of the SP cells is helpful in studying the difference between the CSCs and non-tumorigenic cells in certain aspects. However, the proportion of LSCs is quite limited, and the isolation and identification of CSCs is very difficult. CSCs have been reported to resist apoptosis when they are exposed to apoptosis inducement factors (26). This characteristic of CSCs led us to formulate a hypothesis that apoptosis resistance by CSCs can be used to increase the proportion of SP cells. In an apoptosis inducement model, the proportion of SP cells may increase in surviving cells. Cytarabine (Ara-C) is commonly used for the treatment of acute leukemia. Incorporation of Ara-C into DNA is a key event in the killing of proliferating leukemic cells, but it is relatively ineffective against LSCs, which retain a quiescent status (46). In this study, we used Ara-C to kill common proliferating THP-1 cells. Following co-culture with Ara-C, the proportion of SP cells increased significantly and with Ara-C concentration.

In our study, all the representative LSC markers were significantly increased in SP cells compared with NSP cells; therefore, our results suggest that isolated SP cells could characterize the properties of LSCs. The proportion of SP cells may be increased after they are co-cultured with Ara-C, and this technique can be applied to the study of LSCs.

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