RESEARCH ARTICLE

Senescence Effects of *Angelica sinensis* Polysaccharides on Human Acute Myelogenous Leukemia Stem and Progenitor Cells

Jun Liu, Chun-Yan Xu, Shi-Zhong Cai, Yue Zhou, Jing Li, Rong Jiang, Ya-Ping Wang*

Abstract

Leukemia stem cells (LSCs) play important roles in leukemia initiation, progression and relapse, and thus represent a critical target for therapeutic intervention. Hence, it is extremely urgent to explore new therapeutic strategies directly targeting LSCs for acute myelogenous leukemia (AML) therapy. We show here that *Angelica sinensis* polysaccharide (ASP), a major active component in Dong quai (Chinese *Angelica sinensis*), effectively inhibited human AML $CD34^+CD38^-$ cell proliferation *in vitro* culture in a dose-dependent manner while sparing normal hematopoietic stem and progenitor cells at physiologically achievable concentrations. Furthermore, ASP exerted cytotoxic effects on AML K562 cells, especially LSC-enriched $CD34^+CD38^-$ cells. Colony formation assays further showed that ASP significantly suppressed the formation of colonies derived from AML $CD34^+CD38^-$ cells but not those from normal $CD34^+CD38^-$ cells. Examination of the underlying mechanisms revealed that ASP induced $CD34^+CD38^-$ cell senescence, which was strongly associated with a series of characteristic events, including up-regulation of p53, p16, p21, and Rb genes and changes of related cell cycle regulation proteins P16, P21, cyclin E and CDK4, telomere end attrition as well as repression of telomerase activity. On the basis of these findings, we propose that ASP represents a potentially important agent for leukemia stem cell-targeted therapy.

Keywords: *Angelica sinensis* polysaccharide - leukemia stem/progenitor cells - senescence - telomerase - telomere

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Introduction

Acute myelogenous leukemia (AML) arises from a rare population of leukemia stem cells (LSC), which were initially characterized based on their $CD34^+CD38^-$ immunophenotype and xenotransplantation in NOD/SCID mice (Bonnet et al., 1997). LSC are largely quiescent and refractory to traditional cell cycle-dependent agents contributing to poor long-term survival and continuous relapse. They share properties with normal hematopoietic stem or progenitor cells, such as $CD34^+$, $CD38^-$, $CD71^-$ and HLA-DR-, that make them difficult to exclusively target while sparing normal hematopoietic cells (Blair et al., 1997; Jordan et al., 2000). However, emerging studies demonstrated that LSC can be selectively targeted by some chemical agents or major active constituents of traditional Chinese medicine (Guzman et al., 2005; Guzman et al., 2007; Zhang et al., 2013). As a consequence of these constituents' effects, LSC were exclusively diminished with or without affecting normal cells. Features such as these suggest that LSC-specific targeted therapy has the promise to eradicate AML.

Consistent with normal stem cell, LSC are largely retained in a quiescent state. This allows them to maintain refractory to common drugs such as arabinoside and daunorubicin that interfere with DNA replication and induce cells apoptosis. Moreover, substantial damage to normal tissues is likely to occur in the course of those common drugs treatments. For this reason, an emerging insight that identifying novel AML therapy focused on LSC ageing, contributing to selectively eradicate LSC for AML therapy. Senescence is a specialized form of growth arrest induced by various stressful stimuli including loss of telomere function, reactive oxygen species, some forms of DNA damage and activation of certain oncogenes or reactivation of tumors suppressor genes. Senescence is characterized by several markers such as senescence associated-β-galactosidase, alterations in chromatin structure (senescence associated heterochromatic foci, SAHF) (Dimri et al., 1995). Similar to normal stem cells, cancer stem cells tend to be quiescent and traditionally thought to be immortal and exempt from ageing. However, recent studies demonstrated that stem cells grow old as a result of heritable intrinsic events, as well as extrinsic forces, such as changes in their supporting niches. Mechanisms mainly rely on telomere shortening and DNA damage as well as the activities of p21 and p16INK4a (Janzen et al., 2006; Insinga et al., 2013; Signer et al., 2013).

Accordingly, pharmacological agents are worth exploring based on the critical mechanism of DNA damage and telomere shortening in LSC-targeted ageing therapy. Previous pioneering studies focused on telomere shortening have shown age-related changes in stem cells

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stem (Carnero et al., 2013; Signer et al., 2013). Angelica sinensis polysaccharide (ASP), an acetone extract polysaccharose found as the major active component in Dong quai (Chinese angelica sinensis), is a promising agent that could be used in this regard. Chinese angelica traditionally has been used to treat hematologic and gynecological condition for centuries. More recently, ASP has been found to have several other properties, including antitumor activity (Cao et al., 2010; Cao et al., 2010). In addition, studies show that ASP effectively inhibit malignant brain tumors cells proliferation in a dose dependent manner and arrests cell cycles at the G0/G1 phase by moderating cell cycle-mediated genes expression (Tsai et al., 2005).

In this study, we analyzed the effect of ASP on survival of acute myelogenous leukemia (AML) stem cells enriched $CD34^+CD38^-$ subpopulation (AML LSC-enriched $CD34^+CD38^-$). Our data demonstrate that ASP can effectively inhibit AML $CD34^+CD38^-$ subpopulation proliferation and drive into senescence, which is related with telomere shortening, telomerase activities suppression as well as increase expression on cell cycle-mediated genes p21 and $p16^{INK4a}$. These findings indicate ASP may represent a novel class of agents for targeting myeloid LSC.

Materials and Methods

Reagents

Angelica sinensis polysaccharide (ASP) was purchased from Ci Yuan Biotechnology Co., Ltd. Shanxi (Xi`an, China). It was dissolved in sterile RPMI1640 medium. Serum-free medium was purchased from Stem Cell Technologies. Roswell Park Memorial Institute (RPMI-1640) medium was purchased from Gibco (Gaithersburg, USA); Cell Counting Kit-8 (CCK-8) was purchased from Beyotime (Shanghai, China). Anti-P16, anti-P21, Anti-Cyclin E and anti-CDK4, Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG were from Bioss (Beijing, China). Western blotting kit was from Beyotime (Shanghai, China). SA-β-gal staining kit was purchased from Beyotime Biotechnology (Hangzhou, China). Human leukemia K562 cell line was given as a gift from the college of laboratory medicine of Chongqing medical university.

Cell isolation and Culture

15 AML samples were used in this study including 2 M_0, $3M_1$, $3M_3$, $2M_4$, $2M_5$, $3M_6$ according to French–American–British (FAB) subtype. All samples were obtained from volunteer donors with informed consent or from haematological department of the first affiliated hospital of Chongqing medical university. All Samples were subjected to Ficoll-Paque (Pharmacia Biotech, Piscataway, NY) density gradient separation to isolate mononuclear cells followed by $CD34^+$ CD38- cells separation using high-gradient magnetic-activated cell sorting (MACS) according to the manufacturer's manual (Miltenyi Biotec, German). In some cases the $CD34^+$ CD38- subpopulation were cryopreserved in freezing medium consisting of 90% fetal bovine serum (FBS), and 10% dimethylsulfoxide (DMSO). The cells were maintained in serum-free medium before drug treatment in a humidified atmosphere containing 5% CO_2. Normal $CD34^+$ $CD38^-$ cells were separated from 5 normal bone marrow samples using Ficoll-Paque (Pharmacia Biotech, Piscataway, NY) density gradient separation.

CCK-8 Assays

Viable cells were evaluated using a Cell Counting Kit-8(CCK-8) assay. Briefly, 100 μl of cell suspension (5000 cells/well) was dispensed in a 96-well plate for 24 h in a humidified incubator at 37 °C, 5% CO_2. Add 10 μl of various concentrations of ASP into the culture media in the plate. Incubate the plate for indicated time in the incubator. Add 10 μl of CCK-8 solution to each well of the plate for 1-4 hours in the incubator. Absorbance was measured using a Power Wave X Microplate ELISA Reader (Bio-TeK Instruments, Winoski, VT) at 450 nm. Absorbance of untreated cells was designated as 100%. Data represent the mean ± SD from three independent experiments.

Human Colony-forming Cell Assay

Cells were plated at 10000 cells/ml in the MethoCult®GF H4434 (Stem Cell technologies Vancouver, BC) in the presence of (0, 40, 80, 120) μg/ml ASP. It contains 50 ng/ml rh SCF, 10 ng/ml rh interleukin-3, 10 ng/ml rhGM-CSF and 3 U/ml erythropoietin (Epo). All drug treatments were performed in triplicate. Colonies were maintained at 37°C in humidified incubators at 5% CO_2 and scored after 10 to 14 days of culture under the optical microscope. Single cell sphere with more than 50 cells was taken as one colony.

SA-β-Gal Activity Assay

To determine β-galactosidase expression in AML $CD34^+$ CD38- cells and normal $CD34^+$ CD38- cells after ASP treatment, Cells were treated with various concentrations of ASP for 48. The SA-β-Gal staining assay was carried out using a SA-β-gal staining kit (Beyotime Biotechnology, China). Briefly, cells were washed twice with PBS and fixed with 0.5%glutaraldehyde for 15min. After fixation, cells were then washed with PBS and incubated with SA-β-Gal staining solution 1ml at 37°C separated from CO_2 through over night. The percentage of SA-β-Gal positive cells was determined by counting the number of blue cells and the total number of cells under the same field under a phase-contrast microscope.

Ultra-structure Analyses

To observe the ultra structure changes of AML $CD34^+$ CD38- cells after ASP co-culture, Cells were harvested and washed with PBS after ASP treatment for 48h, and then fixed with 2.5% glutaraldehyde for 6h at 4 °C. After 1% osmium tetroxide fixation for 2h, cells dehydration with ethylalcohol and stained with uranyl acetate and lead nitrate according to standard procedures for ultra structural examination. The cells were observed by H-600 transmission electron microscopy.

Quantitative Real-time RT-PCR

Total RNA was isolated using TRIzol reagent

(Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using Taqman RT reagents (Applied Biosystems), following the manufacturer's protocol. Quantitative real-time PCR was performed using SYBR green Supermix (BioRad) on iCycler Real-Time Detection System (BioRad). Expression level was calculated on the basis of the dissociation curves and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The means ± SD of three independent experiments were determined. PCR primer sequences were lined as follows: human GAPDH, F: 5'-ATGTTCGTCATGGGTGTGAA-3' and R: 5'-TGAGTCCTTCCACGATACCA-3'; human TERT, F: 5'-CGGAAGAGTGTCTGGAGCAA-3' and R: 5'-GGATGAAGCGGAGTCTGGA-3'; human p53, F: 5'-CTCCTCAGCATCTTATCCGAGT-3' and R: 5'-GCTGTTCCGTCCCAGTAGATTA-3'; human $p16^{INK4a}$, F: 5'-CCCAACGCACCGAATAGTTAC-3' and R: 5'-CACGGGTCGGGTGAGAGT-3'; human p21, F: 5'-CCTCTTCGGCCCGGTGGAC-3' and R: 5'-CCGTTTTCGACCCTGAGAG-3' human Rb, F: 5'-TTATCAAAGCAGAAGGCAACTT-3' and R: 5'-TAAGAGGACAAGCAGATTCAAGG-3'.

Western Blotting Analysis

Cells were incubated in various concentrations of ASP for the indicated times. The cells were lysed on ice in 200 μL lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM $MgCl_2$, 0.5 vol% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin,and 50 μg/mL leupeptin) and centrifuged at 10,000 g for 20 min at 4°C. Soluble protein concentrations were determined using the Bradford assay (Bio-RadLaboratories). Lysate supernatants (50 μg) were subjected to SDS-PAGE analysis. Resolved proteins were transferred to PVDF membranes, blocked in 5% nonfat milk overnight, and probed with an appropriate dilution of primary antibody for 1 h at room temperature. Membranes were then washed 3 times with PBS containing 0.1 vol% Tween-20 and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. All proteins were detected using Western Lightning Chemiluminescence Reagent Plus and quantified by densitometry.

Determination of Telomere Length

Cells were digested with Hinf/RsaI enzyme at 37°C for 2 h. Electrophoresis of digested genomic DNA was performed in 0.7% agarose gels for 1 V/cm. After electrophoresis, the gels were denatured and neutralized; DNA was transferred into 20× SSC to nylon membrane filters. The membrane was pre-hybridization for 1 h at 42°C in 10-ml pre-hybridization solution followed by incubated at 42°C overnight after 100 ng/ml telomere probe added. Finally, membrane were washed twice in 2× SSC containing 0.1% SDS for 30 min, blocked for 15 min and then shook in Streptavidin-HRP (1:400) buffer at 37°C for 40 min, after that the membrane were washed in PBS triple followed by 10-min incubation with DAB and photography. The intensity of signals was determined by a computer image system (Alpha Innotech Corporation, St. San Leandro, CA, USA) and the mean lengths of the TRFs were calculated with $L=\Sigma(ODi:1:Li)/\Sigma(ODi)$

Determination of Telomerase Activity

TRAP-PCR assay were performed for changes of telomerase activities. Briefly here, cells were washed once in ice cold buffer and centrifuged at 3 000 rpm for 5 min at 4°C. Precipitation was homogenized with 40μl cold lysis buffer for 30 min on ice and then followed by centrifugation at 13 000 rpm for 30 min at 4°C. The supernatants were collected and added by 50 μl solution (5 μl 10× TRAP buffer, 1μl dNTPs, 1 μl Taq-DNA polymerase, 1μl TS primer, and 2 μl telomerase extraction, 39 uμl DEPC H_2O, and 1μl CX primer) for PCR amplification reaction. TRAP-PCR assay was carried out using the following program: 94°C for 5 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 90 s; 72°C for 10 min. One microliter SYBR Green dye was added to the PCR tube and incubated for 10 min at room temperature. Fluorescence intensity was determined by fluorospectrophotometer. The ratio of telomerase activity=fluorescence intensity/protein concentration.

Statistical Analysis

Data were expressed as means ± S.D. of the mean for a given number of observations. Two-tailed Student's t-tests were used to assess statistical significance using the statistical software package SPSS 16.0. Results were considered statistically significant at the probability (*P*) values < 0.05 level.

Results

Angelica Sinensis Polysaccharide (ASP) Treatment Affects K562 cell line and AML $CD34^+CD38^-$ cells Proliferation but limited to normal $CD34^+CD38^-$ cells

Initial studies were performed to identify the effects of ASP on human myeloid leukemia K562 cell line using CCK-8 assay. The cell viabilities were determined after 48h co culture. As shown in Figure 1a, Exposure of

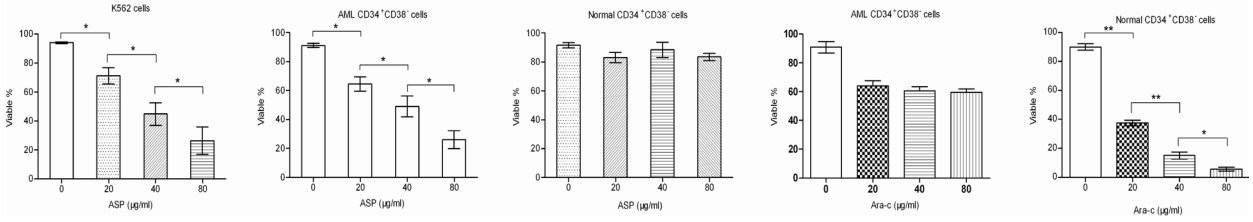

Figure 1. ASP Inhibits K562 Cells And $CD34^+CD38^-$ Cells Proliferation But Limited Effects to Normal AML $CD34^+CD38^-$ Cells. Effects of ASP treatment on (a) K562 cells (b) AML $CD34^+CD38^-$ cells (c) normal AML $CD34^+CD38^-$ cells (n=3). Effects of Ara-c treatment on (d) AML $CD34^+CD38^-$ cells (e) normal AML $CD34^+CD38^-$ cells (n=3). Each error bar represents the SEM. $*P < 0.05$, $**P < 0.01$

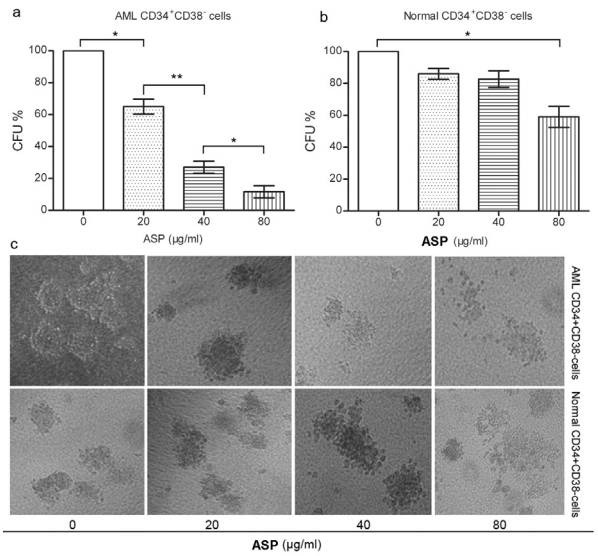

Figure 2. ASP Ablates The Colony-forming Ability of Primary AML Stem/Progenitor Cells But not Normal Counterparts. (a) The colony-forming ability of AML $CD34^+CD38^-$ cells and (b) normal $CD34^+CD38^-$ cells was examined in the absence or presence of ASP in vitro. All assays were performed in triplicate. Each error bar represents the SEM. $*P < 0.05$, $**P < 0.01$. (c) The representative CFU microscopy images are shown as indicated

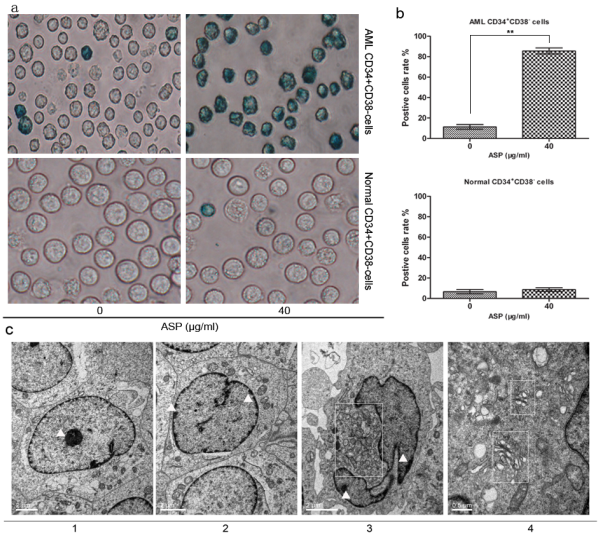

Figure 3. ASP Treatment Leads AML $CD34^+CD38^-$ Cells to Senescence Profiles. (a) AML $CD34^+CD38^-$ cells and normal$CD34^+CD38^-$ cells were treated with 40μg/ml ASP for 48h stained by SA-β-Gal staining kit (b) The relative value of positive staining cells (n=3). Each error bar represents the SEM. (c) 1: triangular symbol represent the integrated nucleolus of AML$CD34^+CD38^-$ cells absence of ASP. 2: triangular symbol represent chromatin converge into cell membrane boundary, senescence associated heterochromatin formation (SAHF). 3: Square areas represent lots of mitochondria aggregation and swell. 4: Square area represents edema of Golgi complex. 2, 3, 4: These changes of AML$CD34^+CD38^-$ cells presence of 40 μg/ml ASP for 48 h

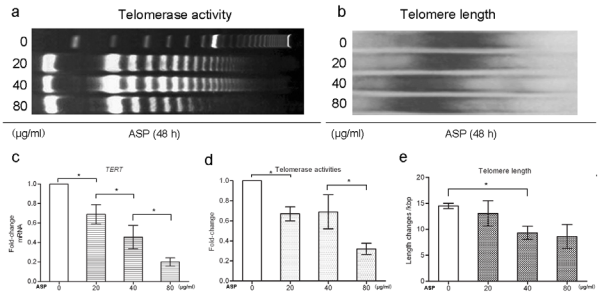

Figure 4. Telomerase Activities Inhibition and Telomere Length Shorten Effects of ASP on AML $CD34^+CD38^-$ Cells. (a) Telomerase activities changes of AML $CD34^+CD38^-$ cells exposed to (0, 20, 40, 80 μg/ml) ASP for 48h performed by TRAP-PCR assay. (b)Telomere length changes of AML $CD34^+CD38^-$ cells exposed to (0, 20, 40, 80 μg/ml) ASP for 48h performed by southern blotting assay. (c) The changes of mRNA value of TERT gene upon ASP treatment. The relative expression levels were normalized to GAPDH expression (n=3). $*P < 0.05$; $**P < 0.01$. (d) The relative value of telomerase activities changes normalized to control group cells. (n=3). $*P < 0.05$; $**P < 0.01$. (e) The definite value of telomere length (kb) changes upon ASP treatment. Each error bar represents the SEM.$*P < 0.05$; $**P < 0.01$

K562 cells to ASP (0-80 μg/ml) exerted a relative dose dependent decrease in cell viability. Because LSC are responsible for leukemia initiation and progression as well as relapse. So we further observe the ASP effect on LSC-enriched $CD34^+CD38^-$ subpopulation cells. The AML $CD34^+CD38^-$ cells are sensitive to (0-80 μg/ml) ASP for 48h treatment with a dose dependent manner (Figure 1b). To avoid the potentially probable side effects of ASP on normal tissue, the normal bone marrow derived $CD34^+CD38^-$ cells were subjected to ASP with the same concentration. As described in Figure 1c, a limited effect on normal $CD34^+CD38^-$ cells was observed after ASP treatment with various concentrations for 48h. As a means to further assess the relative efficacy of ASP, we also performed side-by-side comparison studies with the standard chemotherapy drug Ara-C. Analysis of $CD34^+CD38^-$ cells showed Ara-C had a moderate effect on cell viability in AML $CD34^+CD38^-$ cells (Figure 1d), but a remarkable cytotoxic effect on normal $CD34^+CD38^-$ cells (Figure 1e).

This is consistent with previous studies (Guzman et al. 2005), Ara-C effects on LSC viability appear to plateau at levels above 7.5 μM, even at concentrations as high as 200 μM (approximately 56 μg/ml). These experiments indicate that APS has greater toxicity to AML cells than Ara-C and has less nonspecific toxicity to normal cells.

Angelica Sinensis Polysaccharide Suppresses Colony Formation of AML $CD34^+CD38^-$ cells

The numbers of Colony-forming units (CFUs) represent self-renewal and multi-directional differentiation potentials of stem cells. To determine whether ASP would functionally affect cells capable of forming leukemic cell colonies. Colony-forming units culture assay were performed and data showed that the CFUs number of AML $CD34^+CD38^-$ cells is dramatically reduced by ASP treatment (mean CFU inhibition, 57.3%, 24.7%, 14.7% for 20, 40, 80 μg/ml, respectively). In contrast, the colony forming ability of normal $CD34^+$ $CD38^-$ hematopoietic progenitor cells was not substantially affected by 40μg/ml ASP, but a moderate impairment by 80μg/ml (Figure

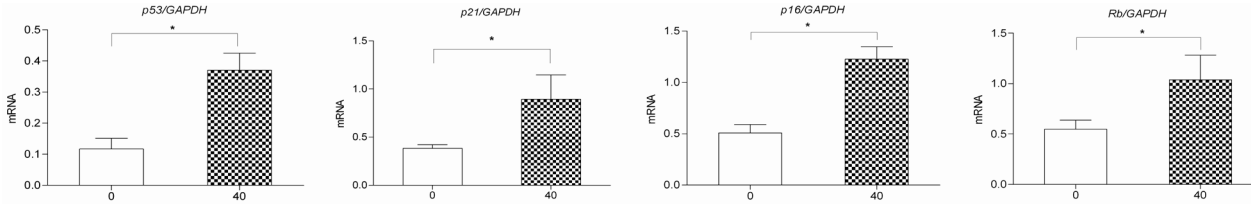

Figure 5. ASP Enhanced Expression of Senescence Associated Regulators Involved in p53/p21 and p16^{INK4a}/pRb Pathways. The changes of mRNA value of p16, p21, p19 and Rb genes upon 40 μg/ml ASP treatment for 48 h. The relative expression levels were normalized to GAPDH expression (n=3). *$P < 0.05$; **$P < 0.01$

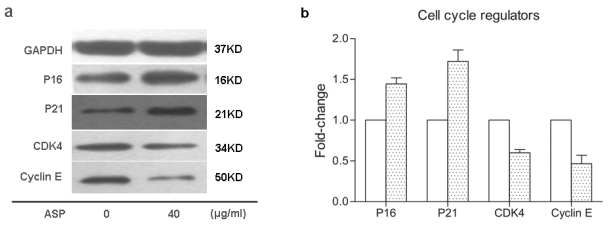

Figure 6. The Cell Cycle Regulators Are Involved in Mediating ASP-induced AML CD34$^+$CD38$^-$ Cells Senescence. (a) Expression of P16, P21, CDK4 and Cyclin E proteins of AML CD34$^+$CD38$^-$ cells exposed to 40 μg/ml ASP treatment for 48 h performed by western blot assay. (b) The relative gray value of P16, P21, CDK4 and Cyclin E proteins normalized to GAPDH was calculated (n=3). *$P < 0.05$; **$P < 0.01$

2). Therefore, the concentration of 40μg/ml was used in the subsequent experiments. Data together, ASP have a remarkable effect on leukemia cells and relative stem and progenitor stage AML CD34$^+$CD38$^-$ cells but limited onto normal hematopoietic progenitor cells.

Angelica Sinensis Polysaccharide (ASP) Treatment Leads AML CD34$^+$CD38$^-$ cells to Senescence Profiles

Cellular senescence, a state of irreversible growth arrest, can be triggered by multiple mechanisms such as telomere shortening and DNA damage. Based on proliferation arrest effects of ASP on AML CD34$^+$CD38$^-$ cells, senescence changes and underlying mechanisms have been intriguing. Senescence associated beta-galactosidase (SA-β-Gal) staining is a classical method for cell senescence evaluation (Severino et al., 2000). To fully take advantage of available resource, the single ASP concentration was performed for next assay. After 40 μg/ml ASP treatment for 48h, the staining positive cells are much more than CD34$^+$CD38$^-$ cells absence of ASP performed by SA-β-Gal staining assay. ASP had a minor effect on positive rate in normal CD34$^+$CD38$^-$ cells (Figure 3a, b). In addition to losing the ability to divide, cells in senescent state exhibit dramatic alterations in morphology and dynamics of their subcellular organelles, such as enlarged and flat cellular morphology, mitochondria aggregation and swell, edema of Golgi complex, formation of nuclear marker senescence-associated heterochromatin foci (SAHF) (Narita ., 2003; Hwang et al., 2009). These ultra structure changes on AML CD34$^+$CD38$^-$ cells after ASP treatment have been observed under transmission electron microscope in current work (Figure 3c). Data together, analysis of senescence associated tests showed that ASP has a relative senescence-inducible effect on AML CD34$^+$CD38$^-$ cells.

Telomerase Activities Inhibition and telomere length shortening are Associated With the ASP Senescence-inducible Mechanisms in AML CD34$^+$CD38$^-$ cells

The telomerase complex consists of a telomerase RNA component (TERC) and the reverse transcriptase catalytic subunit (TERT), which has fundamental roles in the protection of telomeres at chromosome ends (Martínez, 2011). Mammalian telomeres are composed of TTAGGG repeats at the ends of chromosomes that protect chromosome integrity. Telomere shortening occurs accompanying with each division of cell cycle, which accumulation contributing to cellular senescence (Blasco et al, 2007). The majority of human cancer cells possesses high telomerase activity and strong proliferation capacity, but can also be destined to a state of senescence when exposed to certain DNA damaging assaults (Hoffmeyer et al., 2012). In current study, changes on telomerase activities and telomere length associated with ASP-induced AML CD34$^+$CD38$^-$ cells senescence were investigated. We detected the transcriptional level of TERT expression in AML CD34$^+$CD38$^-$ cells after ASP treatment by qRT-PCR. As described in figure3c, ASP treatment (0-80μg/ml) of AML CD34$^+$CD38$^-$ cells for 48h reduced TERT mRNA expression in a dose-dependent manner. We also performed TRAP-PCR assays for observation of telomerase activities expression level. The telomerase activities of AML CD34$^+$CD38$^-$ cells have been decreased after (0, 20, 40, 80μg/ml) ASP treatment (Figure 4a).

The extent of telomerase activities is sensitive to ASP concentration with a dose-dependent manner (Figure 4d). Telomeres are composed of TTAGGG repeats at the ends of chromosomes that protect chromosome integrity, which gradually shortens in the process of cellular ageing. As is demonstrated in figure 4b, the telomere lengths of AML CD34$^+$CD38$^-$ cells chromosome ends have been sharply shortened after ASP treatment presented by Southern Blotting (mean lengths 14.5±1.3, 11.1±4.2, 10.3±2.8, 9.3±3.4 kb for 0, 20, 40, 80 μg/ml, respectively) (Figure 4e).

The p53/p21 and p16^{INK4a}/Rb pathways are responsible for ASP-induced AML CD34$^+$CD38$^-$ cells senescence

Telomere uncapping induces senescence mainly through two important pathways, p53/p21 and p16INK4a/pRb. Hence, we investigated some major factors of senescence related cell cycle regulators for further understanding the main mechanisms of ASP-induced senescence on AML CD34$^+$CD38$^-$ cells. We tested the mRNA fold changes on p53, p16, p21 and pRb genes of CD34$^+$CD38$^-$ cells after 40 μg/ml ASP treatment.

As is shown in figure 5, the expressions of these genes have remarkably been enhanced due to ASP treatment, indicating that p53/p21 and p16INK4a/pRb pathways play an important role in the process of ASP-induced AML $CD34^+CD38^-$ cells senescence.

The cell cycle is driven by the waves of cyclin formation that begin when growth factors activate the transcription of cyclin D. Cyclin D then combines with cyclindependent kinase 4 (CDK4) to form an active complex that phosphorylates the retinoblastoma protein (Rb), which normally acts to repress the transcriptional activator E2F. When Rb is phosphorylated, this repression is removed, and E2F can begin to activate its various gene targets that include E2F itself, cyclin E and cyclin A (Rhee et al., 2006). Limited activity response to mitogens for cell cycle progress and arrested at G1 phase modulated by cyclin, cyclin dependant kinase (CDK) and cyclin dependant kinase inhibitor (CKI) is an outstanding hallmark of ageing cells. To confirm the roles of these pathways during the process of AML $CD34^+CD38^-$ cells senescence induced by ASP, we further explored the proteins expressions of cell cycle regulator on AML $CD34^+CD38^-$ cells. As a result, increased level expression of P16INK4a and P21 but decreased level of CDK4 and CyclinE were observed after 40 μg/ml ASP treatment for 48 h (Figure 6).

Discussion

LSCs are crucial in leukemia initiation, progression, metastasis, and relapse. Their residual levels are strongly correlated with the prognosis of individual AML patients. Recently, much attention has been paid to the development of strategies that selectively eliminate LSC, which may prevent the recurrence of leukemia and eventually cure the disease (Zhang et al., 2013). Generally, this study demonstrates that Angelica sinensis Polysaccharide (ASP) is able to induce senescence of human acute myeloid leukemia $CD34^+CD38^-$ cells and the underlying mechanism is associated with telomerase activities inhibition and telomere shortening. It shows that proliferation and the colony forming ability of AML $CD34^+CD38^-$ cells population were effectively ablated by (0 -80μg/ml) ASP in a dose-dependent manner. Subsequently, analysis of senescence associated makers show that the number of staining positive AML $CD34^+CD38^-$ cells has been significantly increased after ASP treatment performed by SA-β-Gal staining assay and ultra structure changes including mitochondria aggregation and swell, edema of Golgi complex, chromatin boundary converge, formation of nuclear marker senescence associated heterochromatin foci (SAHF) are observed under electronic transmission microscope. These phenotypic changes are frequently taken as indications for cellular senescence. Investigations of the detailed underlying mechanisms showed that telomere shortening can be achieved and the level of telomerase activities has been decreased upon ASP treatment for 48h. Taken together, these data suggest that ASP has intriguing potential as a senescence-inducible agent, particularly with regard to primitive AML stem and progenitor cells. These data also indicate that ASP have an important role in activation of p53/p21 and p16INK4a/pRb, which are effectively responsible for LSC-enriched $CD34^+CD38^-$ subpopulation cells senescence.

Traditional chemotherapies have limited efficacy for acute myelogenous leukemia patients, and new therapeutic agents are therefore needed. Unlike chemotherapeutic agents, it elicit systemic effects on healthy tissue, LSC-specific targeted drugs has the promise to eradicate AML sparing normal cells. Others reports that a natural product has the potential to improve tshe survival of patients by eliminating cancer cells (Gunn et al., 2011; Liu et al., 2012), demonstrating that Chinese herbal or active gradient may be a good candidate for LSC-specific targeted drug. Angelica sinensis, a Chinese herbal medicine, was therefore identified as a candidate for further study. In addition, Angelica sinensis polysaccharide, which was isolated from the acetone extract polysaccharose of Angelica sinensis. Previous studies have described several characteristics of ASP in other systems. Perhaps most infatuatedly, ASP is known to be potent anti-tumor drug (Shang et al., 2003; Zong et al., 2012). In the present study, we investigated the molecular mechanisms underlying the anticancer effects of ASP on leukemia stem cells. It exhibits an effective inhibition on leukemia stem cell proliferation, highlighting the promise of being as an LSC-specific targeted drug.

Although stem cells are defined by their ability to self-renew, several lines of evidence suggest that adult stem cells do age (Signer et al., 2013). Telomere shortening is one of the mechanisms that can limit the self-renewal of adult stem cell (Günes et al, 2013). SA-β-Gal, taken as indications for cellular senescence (Severino et al., 2000), showed an increase on staining positive cells after ASP administration. In addition to losing the ability to divide, cells in the senescent state exhibit dramatic alterations in morphology, mass, and dynamics of their sub-cellular organelles, and thereby display structural and functional differences compared to proliferating cells. These differences include an enlarged and flat cellular morphology, mitochondria aggregation and swell , lipofuscins and granular particles, altered mass and functionality of mitochondria and lysosomes, and certain cytosolic and nuclear markers such as senescence-associated heterochromatin foci (SAHF) (Hwang et al., 2009), in common with results of ultra structure analysis in current work.

It has been an accepted belief that telomere shortening is one of the mechanisms contributing cell senescence (Herbig et al., 2004). Telomeres are the termini of eukaryotic chromosomes that protect chromosomes from end to end fusions and the loss of genetic material. Telomerase is the enzyme responsible for maintaining telomere length and stability of the chromosome structure in eukaryotic cells (Martínez et al., 2011; Günes et al., 2013). Telomerase activity in stem cells is important to maintain the self-renewal of stem cells. However, telomeres shorten in human hematopoietic stem cell during ageing suggesting that the level of telomerase activity is not sufficient to maintain telomere length in ageing stem cells (Song et al., 2012; Sperka et al., 2012). It implied that telomerase activities dependent therapy is

potential candidate for cancer stem cell aging, contributing to limit the self renewal capacity of stem cells. Based on our results presented here, we found that the promising effects of ASP on telomerase activities inhibition and telomere length shortening.

Senescence depends on a number of signaling pathways that together lead to a permanent and irreversible cell cycle arrest. Telomere shortening induces senescence mainly through the DNA damage pathway, activating the ATM/ATR pathway and Chk1/Chk2. p53 activation is achieved by phosphorylation, performed by the ATM/ATR and Chk1/Chk2 proteins, and by the p19ARF product of the INK4a locus, which sequesters Mdm2 in the nucleolus. p53 can activate senescence by activating Rb through p21 activation. Rb activates senescence by shutting down the transcription of E2F target genes, Since E2F increases both E2F and cyclin E and it sets up a positive-feedback loop, which serves to increase the phosphorylation of Rb to provide further transcription of the components necessary to drive the cycle on (Ben-Porath et al., 2005). In human cells p16 is activated in certain settings in response to telomere uncapping. P16 is encoded by Ink4a/Arf gene, which is located on human chromosome 9p21 coordinate a signaling network that depends on the activities of the retinoblastoma (RB) protein and the p53 transcription factor, capable of inducing cell arrest (Quelle et al., 1997; Sherr etal., 2012; Provinciali et al., 2013). Tumor suppressor p16 acts through the retinoblastoma pathway to inhibit cyclin dependant kinases, leading to G1 cell cycle arrest and senescence and is unregulated in various tissues during mammalian ageing. p21 is best known as a broad-specificity inhibitor of cyclin/cyclin-dependent kinase complexes, which is essential for the onset of cell cycle arrest in damage response and cell senescence (Masgras et al., 2013). In this study, we found that ASP treatment effectively abrogate the level of p53, p16, p21 and pRb genes expressions of AML $CD34^+CD38^-$ subpopulation cells. It implied that p53/p21 and p16INK4a/pRb have an important role being responsible for ASP induced LSC-enriched $CD34^+CD38^-$ subpopulation cells senescence. There is experimental evidence that p16 can activate p53-independent checkpoints in response to telomere dysfunction induced by inhibition of TRF2, a telomere binding protein, which is necessary for telomere capping function (Phalke et al., 2012; Carnero et al., 2013). Taken together, telomere dysfunction can induce cell intrinsic DNA damage checkpoints that limit function and maintenance of adult stem cell during ageing. A further characterization of these checkpoints could ultimately lead to the development of new molecular therapies aiming to improve stem cell function and organ maintenance during ageing.

In summary, this study demonstrates that selective targeting of $CD34^+CD38^-$ subpopulation cells is possible by exploiting unique molecular characteristics of ASP on LSC-enriched $CD34^+CD38^-$ cells. ASP as a single agent induces cooperating molecular events that are sufficient to cause primarily stem and progenitor cells senescence *in vitro*. Going forward, the main challenge will be to translate these findings to a clinically relevant setting and demonstrate that LSC can be targeted *in vivo*.

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