Ginsenoside Rg1 induces senescence of leukemic stem cells by upregulating $p16^{INK4a}$ and downregulating hTERT expression

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Abstract

Background. Leukemic stem cells (LSCs) play an important role in the pathogenesis of leukemia. This research attempted to clarify effects of the telomere system on ginsenoside Rg1-induced senescence of LSCs.

Objectives. This research attempted to clarify effects of the telomere system on ginsenoside Rg1-induced senescence of LSCs.

Materials and methods. $CD34^+CD38^-$ LSCs were isolated, sorted, and divided into a control group and a Rg1 group (treated with 40 μmol/L Rg1). Cell Counting Kit-8 (CCK-8) was used to evaluate cell proliferation, and flow cytometry was used to assess the cell cycle of $CD34^+CD38^-$ LSCs. The senescence-associated β-galactosidase (SA-β-Gal) staining and CFU-Mix assay were conducted to measure senescence of $CD34^+CD38^-$ LSCs. The mRNA transcription and protein expression of $p16^{INK4a}$ and human telomerase reverse transcriptase (hTERT) were determined using a real-time polymerase chain reaction (RT-PCR) and western blot assay, respectively.

Results. The Rg1 treatment significantly attenuated proliferative activity and decreased the proliferative index (PI) of $CD34^+CD38^-$ LSCs compared to those of the control group ($p < 0.05$). It remarkably increased positive SA-β-Gal staining rate, and suppressed formation of the CFU-Mix of $CD34^+CD38^-$ LSCs compared with those of the control group ($p < 0.05$). The Rg1 treatment markedly boosted telomere effector, $p16^{INK4a}$, in $CD34^+CD38^-$ LSCs compared with that of control group ($p < 0.05$). Such treatment obviously reduced telomere regulator, hTERT, in $CD34^+CD38^-$ LSCs compared with the control group ($p < 0.05$).

Conclusions. Ginsenoside Rg1-induced senescence of $CD34^+CD38^-$ LSCs through upregulating $p16^{INK4a}$ and downregulating hTERT expression, both of which are associated with telomere systems. The present study would be beneficial for the treatment of leukemia by providing a promising strategy to induce senescence of $CD34^+CD38^-$ LSCs.

Key words: senescence, telomere, ginsenoside Rg1, leukemic stem cells

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Background

Leukemic stem cells (LSCs), important pathogenic factors of leukemia, play an important role in the initiation of leukemia.[1,2] The clinical recurrence or relapse of leukemia is correlated with decreased therapeutic response for LSCs.[3] Strong resistance of LSCs to traditional, cell cycle-dependent drugs, it leads to a poor therapeutic effect, easy recurrence and drug resistance.[4] Drug resistance has become a difficult problem in the treatment of leukemia.[5] Therefore, it will be a breakthrough in the treatment of leukemia to find drugs that can specifically target LSCs and effectively inhibit the proliferation of LSCs without damaging normal tissue cells.

Cell aging or senescence is closely related to a tumor and is considered to be one of the mechanisms of tumor self-inhibition.[6] Therefore, inducing tumor cell senescence is considered to be an effective way to treat cancer. The $CD34^+CD38^-$ LSCs are the first identified LSCs; therefore, their inhibition might be beneficial to the cell senescence of tumor cells in leukemia patients.[7,8] Ginsenoside Rg1 (shorter: Rg1) is an important pharmacological active component of *Panax ginseng* and functions as an anti-tumor agent by promoting the proliferation of blood cells.[9] It can effectively promote the proliferation of normal hematopoietic stem/progenitor cells in the blood system and delay their aging.[10] Moreover, Rg1 can inhibit the proliferation and induce the senescence of leukemia K562 cells within an abnormal blood system.[11]

The human telomerase reverse transcriptase (hTERT) is an important catalytic component for regulating telomerase activity and, therefore, could be effective in inhibiting telomerase activity, thereby preventing the progression of the cell cycle, and suppressing tumor growth.[12,13] The $p16^{INK4A}$ is considered to be a cyclin-dependent kinase inhibitor illustrating many biological functions, such as inhibition of the cell cycle.[14,15] Therefore, we speculated that hTERT and $p16^{INK4A}$ might be involved in the aging or senescence of the LSC, and furthermore, participate in tumor growth.

Objectives

In this study, Rg1 was applied to the $CD34^+CD38^-$ LSCs, and the Rg1-induced targeted regulation and mechanism in $CD34^+CD38^-$ LSCs were discussed. This study provides an experimental basis for the application of aging, promotes novel research methods in cancer treatment and provides new ideas for the research of leukemia balanced utilizing effective components of natural drugs.

Materials and methods

Experiment grouping

According to the previous study by our team,[1] the $CD34^+CD38^-$ LSCs were successfully sorted and identified by staining with an allophycocyanine (APC)-labeled anti-CD38 antibody and a FITC-labeled anti-CD34 antibody. Therefore, the formerly sorted $CD34^+CD38^-$ LSCs were used in this study.

In our present research, the $CD34^+CD38^-$ LSCs were grouped into a control group and a Rg1 group (subdivided into 20 μmol/L, 40 μmol/L and 80 μmol/L groups). In the control group, the $CD34^+CD38^-$ LSCs were cultured in the Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 5% CO_2 at 37°C for 48 h. In the Rg1 group, the $CD34^+CD38^-$ LSCs were cultured as those in the control group, but also treated with Rg1 (dissolved in dimethyl sulfoxide (DMSO)) for 48 h at final dosages of 20 μmol/L, 40 μmol/L and 80 μmol/L. Moreover, an equal volume of DMSO was also added to the cell medium of the control group. The ginsenoside Rg1, with purity of more than 95% (Cat. No. 060427), was purchased from Jinlin Hongjiu Biotech (Changchun, China).

Measurement for proliferative activity with CCK-8 assay

The proliferative activity for the $CD34^+CD38^-$ LSCs was measured with the Cell Counting Kit-8 (CCK-8) method using a commercial CCK-8 Kit (Cat. No. C0037; Beyotime, Shanghai, China), and introduced protocol of the manufacturer. In short, the density of cells was adjusted to 1×10^8 cells per well (96-well plates) and cultured for 48 h at conditions of 5% CO_2 and 37°C. Then, the above cells were incubated with the CCK-8 reagent at a final dosage of 20 μL/well for 2 h. Optical density (OD) values for the cells in 96-well plates were determined with a professional enzyme-linked immunosorbent assay (ELISA) reader (ELx808; Bio-Tek, Winooski, USA) at 450 nm. The cell proliferation inhibitive rate (%) calculation was described in our previous study.[1]

Evaluation for cell cycle with flow cytometry assay

The $CD34^+CD38^-$ LSCs were cultured, harvested, washed using phosphate-buffered saline (PBS), fixed with 70% ethanol (cold), and incubated with bovine pancreatic ribonuclease (1 mg/mL medium; Sigma-Aldrich, St. Louis, USA), as described in our previous study.[16] The $CD34^+CD38^-$ LSCs were stained using propidium-iodide (with a dosage of 50 μg/mL) for 30 min in the dark, and then analyzed with a flow cytometer (FACS Aria IIU; Becton Dickinson Biosciences, Oxford, UK). Finally, the cell cycle was analyzed with the Multi-Cycle software (Phoenix, Tokyo, Japan).

Colony formation assay

In the present study, the colony formation assay was carried out as described in previous studies, with some modifications.[1,17] Briefly, the $CD34^+CD38^-$ LSCs were seeded onto 96-well plates, cultured and incubated with methylcellulose (final concentration of 0.8%; Sigma-Aldrich) with 5% CO_2 at 37°C for 2 weeks. A total of 50 or more colony formation units (CFU) of cells were defined as 1 mixed CFU (CFU-Mix). The formed CFU-Mix was counted with a light microscope (BX51; Olympus Corp., Tokyo, Japan).

Determination for senescence with SA-β-Gal staining

The senescence-associated β-galactosidase (SA-β-Gal) staining was conducted as reported in our previous study.[12] The SA-β-Gal staining was carried out according to the protocol of the SA-β-Gal Staining Kit (Cell Signaling Technology, Beverly, USA). In short, the cells were stained with the SA-β-Gal reagent, seeded on slices, and sealed using 70% glycerol (Sigma-Aldrich). Eventually, slices carrying 400 or more LSCs were selected, and the positive-staining LSCs were counted under the inverted microscope.

Evaluation for $p16^{INK4a}$ and hTERT expression using western blot assay

Total protein in $CD34^+CD38^-$ LSCs was extracted with the protein lysate buffer (Applygen Tech. Inc., Beijing, China). Concentrations for the total proteins were evaluated using the BCA Protein Detection Kit (Cat. No. P0010S; Beyotime) according to the protocol of the manufacturer. The same dosage of proteins was separated with the SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Beyotime), and then incubated using 5% skimmed milk. The PVDF membrane was incubated with rabbit anti-human $p16^{INK4a}$ antibody (Cat. No. ab108349), rabbit anti-human hTERT antibody (Cat. No. ab32020) and rabbit anti-human GAPDH antibody (Cat. No. ab8245) at 4°C overnight. Subsequently, the PVDF membranes were washed using phosphate-buffered saline with Tween (PBST) buffer and incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Cat. No. ab6721) at room temperature for 1.5 h. All of the above antibodies were purchased form Abcam (Cambridge, USA). The bands in the PVDF membranes were visualized using the enhanced ECL Western Blotting Substrate (Cat. No. 32106; Thermo Fisher Scientific, Rockford, USA) and analyzed with the Gel Imaging System GelDoc It TS2L (Bio-Rad, Hercules, USA).

Evaluation for $p16^{INK4a}$ and hTERT mRNA transcription with RT-PCR assay

The cells were lysed using TRIzol solution, and total RNAs were extracted and reversely transcribed to the complementary DNA (cDNAs) under the following conditions: 42°C for 30 min, 99°C for 5 min and 5°C for 5 min. The mRNA transcriptions of $p16^{INK4a}$, hTERT and *GAPDH* were amplified using a real-time polymerase chain reaction (RT-PCR) assay, under the following conditions: 95°C for 2 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and termination at 72°C for 10 min. Here, the *GAPDH* was defined as the internal control for amplifying hTERT mRNA, and actin was defined as the internal control for amplifying $p16^{INK4a}$. The primers for amplifying the above targeting genes are illustrated in Table 1. The RT-PCR data were analyzed with the previous reported $2^{-\Delta Ct}$ method.[18]

Statistical analyses

Data are presented as mean ± standard deviation (SD) and analyzed using IBM SPSS v. 19.0 (IBM Corp., Armonk, USA). Data were analyzed using a one-way analysis of variance (ANOVA) test followed by Tukey's post hoc test for comparing the differences between the groups. A p-value less than 0.05 was defined as a statistically significant.

Results

Rg1 treatment attenuated proliferative activity of $CD34^+CD38^-$ LSCs

The CCK-8 assay findings showed that the Rg1 treatment (all dosages of 20 μmol/L, 40 μmol/L and 80 μmol/L) significantly attenuated the proliferative activity compared with the control group (Table 2, all $p < 0.05$). The inhibitive rates of $CD34^+CD38^-$ LSCs were increased with increases in Rg1 treatment concentrations, with an obvious Rg1 concentration-dependent manner. The condition

Table 1. The specific primers for the PCR assay

Primers	Sense (5′-3′)	Anti-sense (5′-3′)	Length [bp]
$p16^{INK4a}$	AAGACATCGTGCGATATTTGCG	TGAGCTGAAGCTATGCCCGTC	121
β-actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG	205
hTERT	TTGGAATCAGACAGCACTTG	GTAGTCCATGTTCACAATCG	155
GADPH	AGATCCCTCCAAAATCAAGTGG	GGCAGAGATGATGACCCTTTT	130

Table 2. Inhibiting rate of Rg1 on proliferation of $CD34^+CD38^-$ LSCs in vitro (±SD, n = 8)

Groups	Dosage [μmol/L]	Inhibitive rate [%]		
		24 h	48 h	72 h
Control	—	1.57 ±0.24	5.68 ±0.36	12.47 ±1.21
Rg1	20	13.63 ±1.02*	24.16 ±1.21*	31.44 ±2.02*
Rg1	40	28.13 ±1.44*,#	52.01 ±2.14*,#	62.47 ±2.03*,#
Rg1	80	38.76 ±2.01*,Δ	64.46 ±3.12*,Δ	76.04 ±0.92*,Δ

*p < 0.05 compared to the control group; #p < 0.05 compared to the 20 μmol/L group; Δp < 0.05 compared to the 40 μmol/L group.

Table 3. Effect of Rg1 on distribution of cell cycle to $CD34^+CD38^-$ LSCs (±SD, n = 8)

Groups	Dosage [μmol/L]	G_0/G_1 phase [%]	G_2/M phase [%]	S phase [%]	PI
Control	—	70.35 ±5.02	17.24 ±2.12	12.41 ±1.35	29.65 ±1.06
Rg1	40	86.89 ±4.32*	6.12 ±1.03*	6.99 ±1.06*	13.11 ±1.17*

*p < 0.05 compared to the control group.

of the 40 μmol/L Rg1 treatment for 48 h (with an inhibitive rate of 52.01%), demonstrated a half-proliferation inhibition. Therefore, the regimen of 40 μmol/L Rg1 for 48 h in treating $CD34^+CD38^-$ LSCs was used for the following experiments and tests (in the Rg1 group).

Rg1 treatment decreased proliferative index (PI) of $CD34^+CD38^-$ LSCs

The cell cycle findings indicated that $CD34^+CD38^-$ LSCs in the G0/G1 phase were remarkably increased, and in the G2/M phase and S phase were significantly decreased in the Rg1 group compared with the control group (Table 3, all p < 0.05). Through calculating the cell amounts in G0/G1, G2/M and S phases, we found that the Rg1 treatment markedly decreased the proliferative index (PI) compared with the control group (Table 3, p < 0.05).

Rg1 treatment raised positive SA-β-Gal staining rate of $CD34^+CD38^-$ LSCs

According to the SA-β-Gal staining images, there were obviously SA-β-Gal-stained $CD34^+CD38^-$ LSCs in the Rg1 group, with no obvious staining in the control group (Fig. 1A). The statistical analysis illustrated that the SA-β-Gal staining rate in the Rg1 group (44.74%) was significantly higher compared with the control group (12.03%) (Fig. 1B, p < 0.05).

Rg1 treatment suppressed formation of CFU-Mix of $CD34^+CD38^-$ LSCs

As a marker for cell aging, CFU-Mix, is also observed in the $CD34^+CD38^-$ LSCs (Fig. 2A).[19] The results exhibited that the Rg1 treatment markedly suppressed the formation of the CFU-Mix of $CD34^+CD38^-$ LSCs compared with the control group (Fig. 2B, p < 0.05).

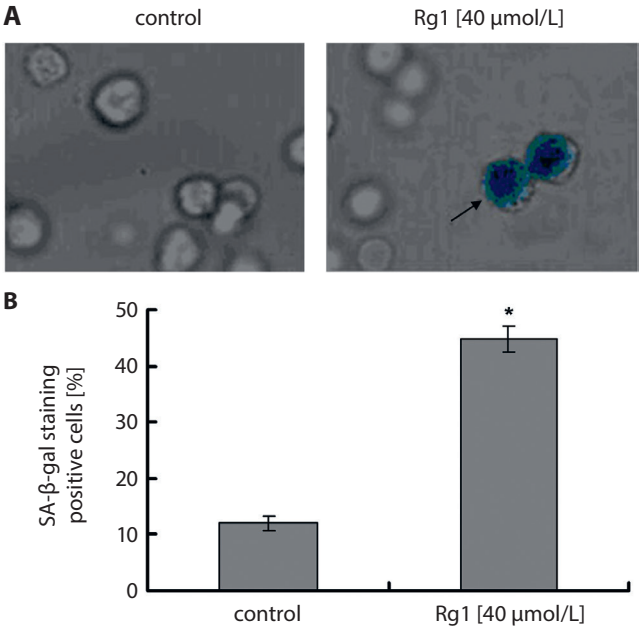

Fig. 1. SA-β-Gal staining for the $CD34^+CD38^-$ LSCs (± SD, n = 8). A. Negative SA-β-Gal staining of cells in control group; B. Positive SA-β-Gal staining of cells in Rg1 group. Black arrow represents the positive SA-β-Gal staining cells; ×400 magnification; *p < 0.05 compared to the control group

Rg1 treatment boosted telomere effector, $p16^{INK4a}$, in $CD34^+CD38^-$ LSCs

The telomere damage system associated effector, $p16^{INK4a}$ was also determined using both a RT-PCR assay (Fig. 3) and a western blot assay (Fig. 4A).[20] The findings showed that the Rg1 treatment obviously boosted both $p16^{INK4a}$ mRNA transcription (Fig. 3) and $p16^{INK4a}$ protein expression (Fig. 4B) in $CD34^+CD38^-$ LSCs compared with the control group (p < 0.05). These results suggest that $CD34^+CD38^-$ LSCs undergoing Rg1 treatment demonstrate obvious aging characteristics of stem cells.

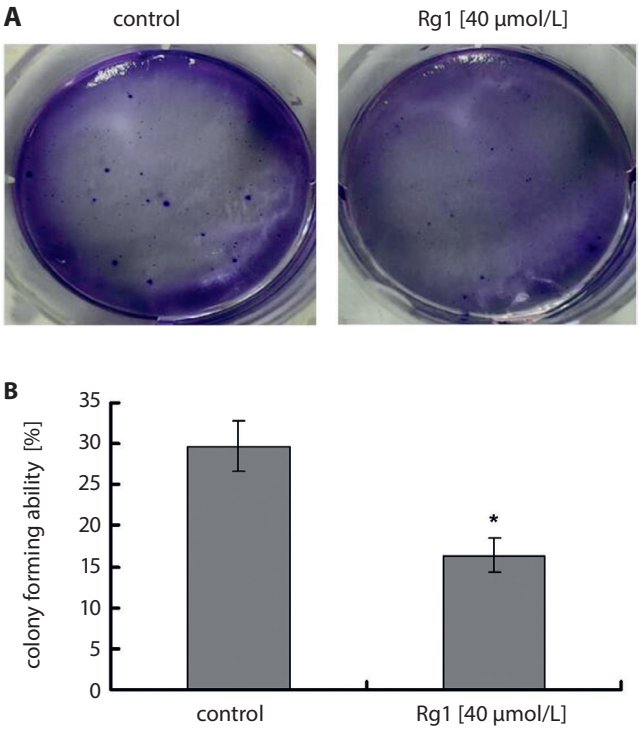

Fig. 2. Effects of Rg1 on the colony formation of CFU-Mix of $CD34^+CD38^-$ LSCs (± SD, n = 8). A. CFU-Mix formation in control group; B. CFU-Mix formation in Rg1 group; *p < 0.05 compared to the control group

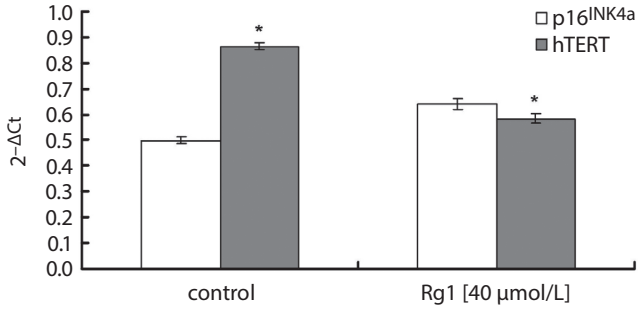

Fig. 3. Effects of the Rg1 on the $p16^{INK4a}$ mRNA transcription and hTERT mRNA transcription in $CD34^+CD38^-$ LSCs (± SD, n = 8). The mRNA transcriptions were determined using the RT-PCR assay; *p < 0.05 compared to the control group

Rg1 treatment reduced telomere regulatory biomarker, hTERT, in $CD34^+CD38^-$ LSCs

The telomere regulatory biomarker, hTERT,[21] was evaluated using a RT-PCR (Fig. 3) and a western blot assay (Fig. 5A). As the results demonstrated, Rg1 treatment remarkably reduced the hTERT mRNA transcription (Fig. 3) and hTERT protein expression (Fig. 5B) when compared with the control group ($p < 0.05$). These results imply that $CD34^+CD38^-$ LSCs undergoing Rg1 treatment indirectly attenuate telomere activity by reducing hTERT expression.

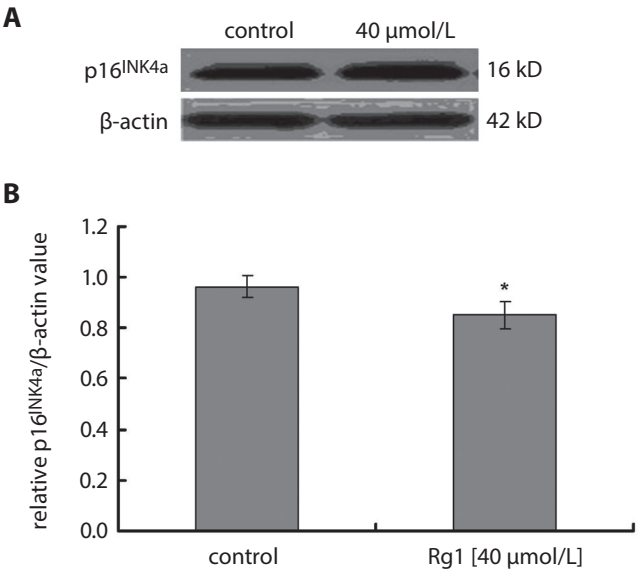

Fig. 4. Effects of Rg1 on the $p16^{INK4a}$ protein expression in $CD34^+CD38^-$ LSCs (± SD, n = 8). A. Western blot image of the $p16^{INK4a}$ protein expression; B. Rg1 treatment increased $p16^{INK4a}$ protein expression; *p < 0.05 compared to the control group

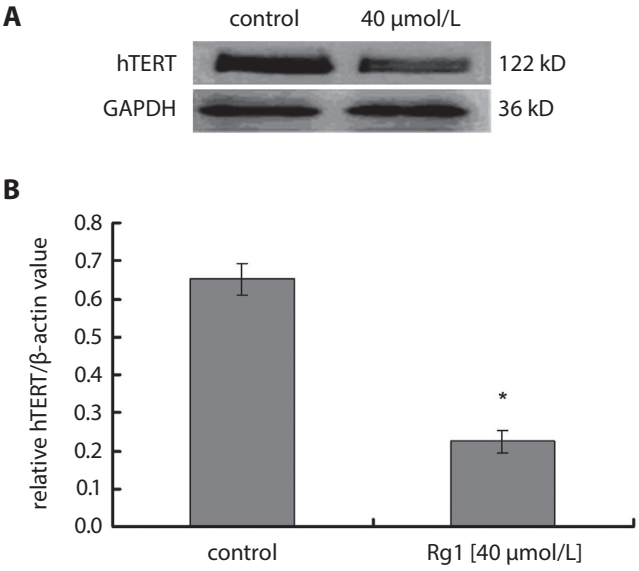

Fig. 5. Effects of Rg1 on the hTERT protein expression in $CD34^+CD38^-$ LSCs (± SD, n = 8). A. Western blot image of the hTERT protein expression; B. Rg1 treatment decreased hTERT protein expression; *p < 0.05 compared to the control group

Discussion

The LSCs have been proven as a risk factor for acute myeloid leukemia (AML) and are associated with chemotherapy resistance and relapse of disease.[22,23] Therefore, discovering a novel reagent or drug targeting LSCs might hold promise for the clinical treatment of AML. According to former studies,[24,25] Traditional Chinese medicine (TCM) normally demonstrates drug acceptance when applied in the disease therapy. Therefore, we speculated

that TCM might play a feasible role in treating the senescence of $CD34^+CD38^-$ LSCs in certain signaling pathways.

Ginsenoside Rg1, in TCM, could benefit the Qi and nourish the blood, as described by Chinese medicine theory,[26] as well as protect against injury, aging and oxidants, and promote immunity, as described by modern medicine theory.[27] Many reports have documented and proved the anti-senescence function of Rg1 through different pathways, including activating the SIRT1/TSC2 signaling pathway,[1] the SIRT3/SOD2 signaling pathway[16] and the SIRT6/NF-kB signaling pathway,[15] all of which target $CD34^+CD38^-$ LSCs. In this study, we clarified a novel signaling pathway that promotes the effects of Rg1 on senescence of $CD34^+CD38^-$ LSCs.

We found that Rg1 treatment remarkably attenuated $CD34^+CD38^-$ LSCs proliferation and obviously decreased proliferative index (PI) by modulating cell cycle, which is consistent with the findings of previous studies.[1,28] Therefore, the Rg1 significantly inhibits the proliferation and blocks the cell cycle of $CD34^+CD38^-$ LSCs. The previous study[29] reported that SA-β-Gal staining could reflect the senescence of $CD34^+CD38^-$ LSCs. Our results indicated that Rg1 treatment increased the positive SA-β-Gal staining rate of $CD34^+CD38^-$ LSCs and suppressed the formation of the CFU-Mix of $CD34^+CD38^-$ LSCs. These results suggest that Rg1 inhibits the CFU-Mix formation and senescence of $CD34^+CD38^-$ LSCs, which is consistent with the previous study.[1]

The activation of the telomerase could protect against telomere damage by delaying the senescence of cells and mediating the apoptosis.[30] Therefore, the status of telomere might be correlated with the senescence of cells. The p16 plays a critical role in the telomere damage-associated senescence by limiting the apoptosis.[31] Meanwhile, the telomerase reverse transcriptase (TERT) modulates the telomere-associated senescence by triggering the DNA-damage response of cells.[32] Previous research[33,34] also documents that ginsenoside Rg1 could ameliorate proliferation of hematopoietic progenitor cells/hematopoietic stem cells (HPCs/HSCs) through reducing the expression of $p16^{INK4a}$. Therefore, in the present study, expressions of both telomere damage system-associated effector ($p16^{INK4a}$)[20] and telomere regulatory biomarker (hTERT)[21] in $CD34^+CD38^-$ LSCs administrated with Rg1 were determined. Our findings illustrated that Rg1 treatment boosted $p16^{INK4a}$ expression and reduced hTERT expression in the $CD34^+CD38^-$ LSCs. These results suggest that Rg1 triggers the senescence of $CD34^+CD38^-$ LSCs via upregulating $p16^{INK4a}$ expression and downregulating hTERT expression.

Limitations

In this study, whether ginsenoside Rg1 plays role in Rg1-triggered enhancive effects on $CD34^+CD38^-$ LSCs growth have not been clarified, which is a limitation of this study.

Conclusions

Our findings indicate that Rg1 could suppress proliferation and decrease the proliferative index of $CD34^+CD38^-$ LSCs. Ginsenoside Rg1 demonstrated positive SA-β-Gal staining and inhibited formation of the CFU-Mix, both of which are indicators for senescence of cells. Also, Rg1 boosted $p16^{INK4a}$ expression and reduced hTERT expression in $CD34^+CD38^-$ LSCs. In summary, ginsenoside Rg1 induces the senescence of $CD34^+CD38^-$ LSCs through upregulating $p16^{INK4a}$ expression and downregulating hTERT expression, both of which are associated with the telomere system. The present study would be beneficial in the treatment of AML by providing a promising strategy to induce senescence of $CD34^+CD38^-$ LSCs.

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