

RESEARCH ARTICLE

Senescence as A Consequence of Ginsenoside Rg₁ Response on K562 Human Leukemia Cell Line

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Abstract

Aims and Background: Traditional chemotherapy strategies for human leukemia commonly use drugs based on cytotoxicity to eradicate cancer cells. One predicament is that substantial damage to normal tissues is likely to occur in the course of standard treatments. Obviously, it is urgent to explore therapies that can effectively eliminate malignant cells without affecting normal cells. Our previous studies indicated that ginsenoside Rg₁ (Rg₁), a major active pharmacological ingredient of ginseng, could delay normal hematopoietic stem cell senescence. However, whether Rg₁ can induce cancer cell senescence is still unclear. **Methods:** In the current study, human leukemia K562 cells were subjected to Rg₁ exposure. The optimal drug concentration and duration with K562 cells was obtained by MTT colorimetric test. Effects of Rg₁ on cell cycle were analyzed using flow cytometry and by SA-β-Gal staining. Colony-forming ability was measured by colony-assay. Telomere lengths were assessed by Southern blotting and expression of senescence-associated proteins P21, P16 and RB by Western blotting. Ultrastructural morphology changes were observed by transmission electron microscopy. **Results:** K562 cells demonstrated a maximum proliferation inhibition rate with an Rg₁ concentration of 20 μmol·L⁻¹ for 48h, the cells exhibiting dramatic morphological alterations including an enlarged and flat cellular morphology, larger mitochondria and increased number of lysosomes. Senescence associated-β-galactosidase (SA-β-Gal) activity was increased. K562 cells also had decreased ability for colony formation, and shortened telomere length as well as reduction of proliferating potential and arrestin G₂/M phase after Rg₁ interaction. The senescence associated proteins P21, P16 and RB were significantly up-regulated. **Conclusion:** Ginsenoside Rg₁ can induce a state of senescence in human leukemia K562 cells, which is associated with p21-Rb and p16-Rb pathways.

Keywords: Ginsenoside Rg₁ - K562 cells - senescence - mechanisms

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Introduction

Human leukemia is a malignant disease characterized by an aberrant accumulation of immature hematopoietic cells. Primary strategies for leukemia therapeutics were focused on complete removal of cancer cells with chemical drugs. Although remission can be achieved in most patients, relapse is common and long-term survival is poor for most cases, as well as the unavoidable toxicities to local normal tissues. Based on this awkward situation, an alternative strategy of induction of cellular senescence has been widely considered, which is taken as a tumor suppressor that protects against cancer progression (Collado et al., 2007; Ewald et al., 2010). The K562 leukemia cell line, originally established from a patient with chronic myelogenous leukemia (Lozzio et al., 1975), has been characterized as an early precursor of the granulocytic series with a block for differentiation and widely used for in vitro studies of human leukemia (Lozzio

et al., 1981; Park et al., 2000). Accumulating studies have provided convincing evidences that several tumor cells can be induced senescence with some traditional Chinese drugs or main ingredients (Lin et al., 2011; Tan et al., 2011).

The process of cellular senescence was first described in a seminal study by Hayflick and Moorhead in which they observed that normal human fibroblasts were able to enter a state of irreversible growth arrest after serial cultivation in vitro (Hayflick et al., 1961). Cellular senescence is a response to nonlethal stress that results in persistent cytostasis with a distinct morphological and biochemical phenotype. Cells in the senescent state typically exhibit phenotypic features, such as an increased senescence associated-β-galactosidase activities (SA-β-Gal), irreversible growth arrest, senescence-associated heterochromatinization foci (SAHF), which are frequently taken as biomarkers for cellular senescence (Dimri et al., 1995; Shi et al., 2012).

To be a Traditional Chinese herb ginseng has traditionally been administered to treat “deficiency” conditions associated with symptoms such as fatigue, irritability, thirst and dryness of the mouth or respiratory tract in Traditional Chinese Medicine (Cho et al., 2011), another role has been recently demonstrated as a potential supplementary therapy to enhance host immune response to anticancer in cancer treatment. Some researches show that the ginseng was used not only to decrease proliferation of human cancer cells in vitro (Oh et al., 1999; Popovich et al., 2002), but also as an adjuvant to enhance efficacy and reduce side-effects of active cancer therapies such as chemotherapy and radiotherapy (Jia et al., 2009). How does the ginseng act its function to anti-cancer cells and what are the underling mechanisms with the anticancer effects interaction by ginseng? These problems remain unclear.

On the basis of ginseng’s reputation as an adaptogenic agent, our previous work performed that ginsenoside Rg₁, a major active pharmacological ingredient of ginseng, could delay normal hematopoietic stem cell senescence (Zhou et al., 2011). Therefore, to seek a better agent for human leukemia treatment, the ginsenoside Rg₁ (Rg₁) was taken into account to investigate the effect on human leukemia K562 cells in current study. For the purpose of better understanding the effects of ginsenoside Rg₁ on K562 cells, the efforts of identifying the underling mechanism and the morphological changes has been made, which will greatly contribute to the development of effective cancer treatment strategies.

Materials and Methods

Reagents and Drugs

Ginsenoside Rg₁ was purchased from Jinlin Hongjiu Co, LTD (Jilin, China), and the purity was more than 98.6%; Human leukemia K562 cell line was given as a gift from the college of laboratory medicine of Chongqing medical university; Roswell Park Memorial Institute (RPMI-1640) medium and fetal bovine serum were purchased from Gibco (Gaithersburg, USA); 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Beijing Zhongshan Goldbridge Biotechnology Co. (Beijing, China). Anti-P16, Anti-P21/CDKN1A, anti-Rb/P105 RB, Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG were from Bioss (Beijing, China). Western blotting kit was from Beyotime (Shanghai, China).

Cell culture and drug treatment

The K562 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and passaged every 2-3 days. Cells in their logarithmic growth phase were cultured in 96-well plates with 1×10⁶ cells in each well and divided into the different groups with varying Rg₁ concentration 0, 5, 10, 20, 40 and 80 μ mol·L⁻¹. At the different time of 24h, 48h, 72h with cells exposed to drugs, all the groups were detected by MTT Colorimetric Assay. The optimal drug concentration and duration of proliferating inhibition was acquired from the analysis of MTT Colorimetric Assay results and was taken as

the optimal strategy for inhibition of cell proliferation. Thereafter, K562 cells were subjected to the optimal strategy for following assays.

Cell Cycle Analysis

To observe cell-cycle distribution, K562 cells were treated with optimal drug concentration with different time 24h, 48h, 72h in Rg₁ treatment groups and treated with RPMI-1640 supplemented with 10% fetal bovine serum in control group. All the group cells were harvested after release at the indicated times, then fixed with 70% cold ethanol and stained with PI for 30 min at room temperature. The fluorescence intensity of PI was analyzed with a FACS vantage SE flow cytometry (Becton Dickinson, USA) equipped with Cell Quest software (Becton Dickinson, USA).

SA-β-Gal activity Assay

Briefly, the group cells were fixed with 0.5% glutaraldehyde for 15min. After fixation, cells were incubated with SA-β-Gal staining solution 1ml at 37°C separated from CO₂ through over night. The results were documented using a charge-coupled device camera attached to a phase-contrast microscope.

Colony formation assay

Cells of two group were respectively harvested and plated with 3×10³ per well in 96-wells plate with each well containing 1.1ml 0.8% methylcellulose during the environment of 37°C, 5%CO₂ for 2w prior to fixation with 4% paraformaldehyde and stained with 0.1% crystal violet. The colony number were counted under the optical microscope, cell sphere with more than 50 cells was taken as one colony.

Southern Blot Analyses

To perform Southern Blot assay, the DNA was separated by 0.7% agarose gel electrophoresis after cells were digested by Hinf/RsaI enzyme for 2h. After neutralization with Tris-HCl, pH 7.5, DNA was transferred onto a porous membrane by capillary action using absorbent paper to soak solution through the gel and the membrane and hybridized with a gene-specific probe. After hybridization and washing, signals were visualized by exposure to X-Imager Screen. The telomere length was measured by VIDAS of Alpha Innotech Corporation.

Western Blotting Analyses

For the Western blotting assay, cellular proteins were obtained from total cell lysates resuspended in Lysis Buffer. Cellular proteins were electrophoresed by SDS-poly-acrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk in phosphate-buffered saline for 2h and then reacted with specific antibodies Anti-P16, Anti-P21/CDKN1A, Anti-RB (Bioss, Beijing) with the ratio of 1:200 dilution. Horseradish peroxidase-conjugated goat anti-rabbit antibodies were used as secondary antibody with the ratio of 1:5000 dilutions for 2h. Specific proteins were Detected using enhanced chemiluminescent reagents.

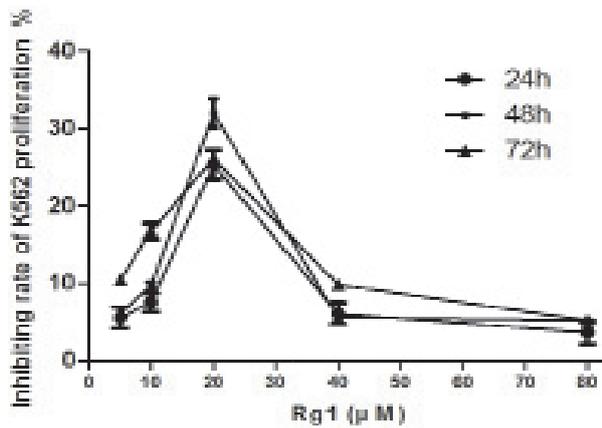


Figure 1. Inhibiting Rate of K562 Cells Proliferation Induced by Rg₁. The proliferation of K562 cells was significantly inhibited by Rg₁ within limits (0~20 µmol·L⁻¹) in a dose-dependent manner. The cells have a top proliferating inhibition rate with Rg₁ concentration 20 µmol·L⁻¹ for 48h

Ultra structure analyses

Cells of each group were harvested and fixed at 1×10^5 with 2.5% glutaraldehyde for 6h at 4°C and then with 1% osmium tetroxide for 2h prior to 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.

Statistics analyses

Results were presented as mean \pm standard error of the mean for a given number of observations. Group differences of the positive percentage were introduced into Statistics Package for Social Sciences (SPSS) software vision 14.0 to Student's t test. Statistical significance was assigned to $P < 0.05$.

Results

Rg₁ inhibited the proliferation of K562 cell line

To identify the effects of Rg₁ on K562 cells, we utilized the above described MTT Colorimetric Assay to obtain the optimal drug concentration and duration with cells. The results demonstrated that the proliferation of K562 cells was significantly inhibited by Rg₁ within limits (0~20 µmol·L⁻¹) in a dose-dependent manner. The cells have a top proliferating inhibition rate with Rg₁ concentration 20 µmol·L⁻¹ for 48h (Figure 1). Thereafter, K562 cells were subjected to the optimal Rg₁ concentration 20 µmol·L⁻¹ prior to harvest to analyze cell cycles. The findings show that the distributions of cell cycles in Rg₁ treatment group cells were remarkably changed compared to that of the control group cells. The cells significantly arrested at G₂/M phase with interaction of Rg₁ concentration 20 µmol/L at the point of 24 h compared to control treatment group cells.

Rg₁ has decreased the ability of colony-formation in K562 cells

Senescent tumor cells generally have limit ability of colony-formation, K562 cells were submitted to colony-formation assay after induction with Rg₁ in this study.

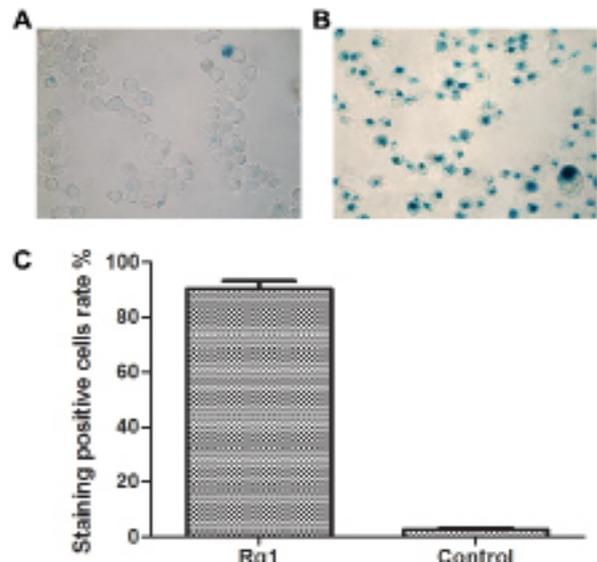


Figure 2. Effects of Rg₁ on SA-β-Gal Staining of K562 Cells. A: K562 cells in control group (cultured in RPMI-1640 supplemented with 10% fetal bovine serum for 48h); B: K562 cells in interference group (exposed to Rg₁ with concentration 20 µmol·L⁻¹ for 48h), showing a higher positive rate compared to control group cells presented by the SA-β-Gal staining assay

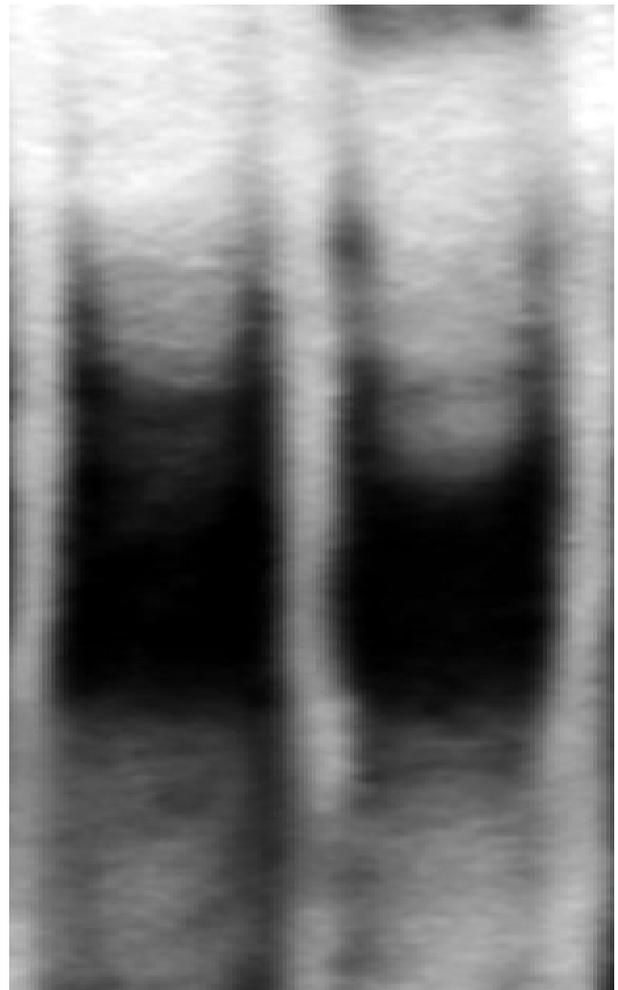


Figure 3. Effects of Rg₁ on Telomere Length of K562 Cells. A: control group (K562 cells cultured in RPMI-1640 supplemented with 10% fetal bovine serum for 48h); B: interference group (K562 cells exposed to Rg₁ with concentration 20 µmol·L⁻¹ for 48h); both group cells were presented by Southern Blotting Assay. * $P < 0.05$, compared with control group

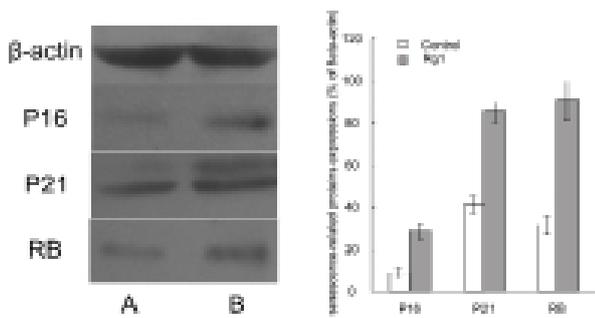


Figure 4. Effects of Rg₁ on Expressions of Senescence Related Proteins in K562 Cells. A: control group (K562 cells cultured in RPMI-1640 supplemented with 10% fetal bovine serum for 48h); B: interference group (K562 cells exposed to Rg₁ with concentration 20 μ mol·L⁻¹ for 48h); The expressions of senescence-associated proteins P16, P21 and RB have been dramatically increased after induction with Rg₁ compared to that in control group

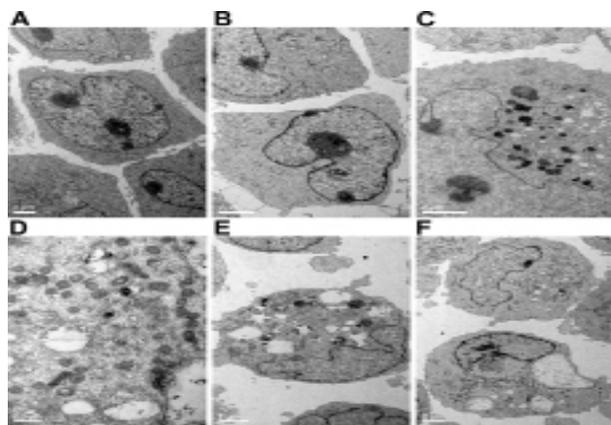


Figure 5. Ultra-structural Characteristics of Rg₁ on Senescence of K562 Cells. A, B: control group (K562 cells cultured in RPMI-1640 supplemented with 10% fetal bovine serum for 48h); C, D, E, F: Rg₁ treatment group (K562 cells exposed to Rg₁ with concentration 20 μ mol·L⁻¹ for 48h); C: Increased volume and number of lysosomes; D: Increased volume of mitochondria; E: Nuclear membrane retraction; F: Senescence-associated heterochromatin foci (SAHF). The K562 cells exhibit some ultra-structural changes associated with senescence after Rg₁ induction under H-600 transmission electron microscopy

The number of colony-formation is 111.3±12.0 in cells with Rg₁ induction which is less than 358.3±15.0 in cells without Rg₁.

K562 cells emerged senescence features with induction of Rg₁

To further investigate the effects of Rg₁ on K562 cells, a serial of assays was applied to identify the senescence-associated biological markers. The positive staining rate of K562 cells is (90.4±3.0)% in interference group cells, which is significantly higher than (2.6±0.9)% in control group cells presented by the SA-β-Gal staining assay (Figure 2). Occasionally cell steps into senescence accompany telomere length shortens, so the telomere length was also tested by southern blotting assay in this study. From the figure below, we can find that the telomere length is (6.7±0.1) Kb in Rg₁ treatment group cells, which is shorter than (7.5±0.2) Kb in control treatment group cells (Figure 3). Senescent cells generally exhibit some

molecular biological traits, such as the high level of P21, P16 and RB proteins expression. Thus, these proteins were tested in Rg₁ treatment groups and had a high level expression compared to that in control groups displayed by the below figure with the western blotting assay (Figure 4).

Rg₁ has changed the Ultra-structure characteristics of K562 cells

The K562 cells exhibit dramatic morphological alterations with induction of Rg₁ compared to cells in control group. These alterations include an enlarged and flat cellular morphology, Increased volume of lysosome and mitochondrial, Nuclear membrane retraction,, an occurrence of certain cytosolic and nuclear marker senescence-associated heterochromatin foci (SAHF) (Figure 5).

Discussion

In current study, we have studied the effects of ginsenoside Rg₁ on human leukemia K562 cell line with a serial of assays. And the encouraging evidence demonstrated that Rg₁ remarkably inhibited proliferation of K562 cells and arrested the cells in the G₂/M phase (Figure 1). Western blotting assay showed that Rg₁ significantly increased expression of P21, P16, RB proteins (Figure 4). Rg₁-induced senescent cells exhibit ultra-structural changes of an enlarged and flat morphology, larger mitochondria and increased number of lysosome by transmission electron microscopy (Figure 5). According to the result of south blotting and SA-β-Gal staining assay, the biomarkers of cellular senescence, such as telomere length shortening and high activity of SA-β-Gal, have been obviously emerged (Figure 2, 3).

Cancer therapy has traditionally relied on cytotoxic treatment strategies on the assumption that complete cellular destruction of tumors optimizes the potential for patient survival. These approaches may produce complete cell death and can cause severe side effects in patients. More recently, it is of the clinical interest that some traditional Chinese medicine could provide equivalent or prolonged survival with fewer and less severe side effects related to cytotoxicity and may provide a more realistic goal for the chronic management of some cancers including human leukemia. Ginseng, an ancient drug used as adaptogens in traditional Chinese medicine, has attracted worldwide interest because it shows substantial anticancer activity in patients. Its role in cancer treatment previously has been demonstrated as a supplementary therapy to enhance host immune response to cancer. Recently some researches suggest that ginsenoside, pharmacological active ingredients derived from ginseng, have a function of inhibiting cancer development (Jia et al., 2011). Our previous work shows that ginsenoside Rg₁ could delay normal hematopoietic stem cell senescence (Zhou et al., 2011). These studies made us confidence to deem that Rg₁ can increase viability of normal cells and decreased that of cancer cells to perform the function of adaptogenic activity. Thus, the aim of this study was to observe the effects of ginsenoside Rg₁ on human leukemia

K562 cells and to explore the underlying molecular mechanisms.

Recent reports show that ginsenoside Rg₁ have anticancer effect in osteosarcoma MG-63 cells (Li et al., 2008). In current study, the MTT assay results showed that human leukemia K562 cells had a reduced proliferation inhibited by Rg₁, which suggest that Rg₁ could abrogate the developmental progression of cancers with the inhibition of cancer cells proliferation. The senescence-associated β -galactosidase (SA- β -Gal) activity can be detected at pH 6.0 in many cultured cells undergoing replicative and induced senescence, but is absent from actively proliferating cells (Dimri et al., 1995; Itahana et al., 2007). To further investigate effects of Rg₁ on K562 cells, the widely accepted SA- β -Gal staining assay was performed and the results showed that Rg₁ increased the positive rate of SA- β -Gal staining. One of the most pronounced morphological changes occurring in senescent cells is an increase in cell surface area. This enlargement underlying cellular senescence occurs in various cells types including fibroblasts and cancer cells (Greenberg et al., 1977; Shin et al., 2011). In this study, the ultra structure changes showed an enlarged and flat morphology and increased volume and number of mitochondria and lysosomes occurred in K562 cells after induction by Rg₁. Indeed, these evidences indicated that the cells stepped into the process of senescence.

In addition, it is accepted that the biological clock limiting division potential is telomere length. Telomeres are the termini of eukaryotic chromosomes that protect chromosomes from end to end fusions and the loss of genetic material. Human cell telomeres consist of tandem repeats of TTAGGG sequence (Bodnar et al., 1998). In human cells, progressive telomere shortening appears to be the primary cause of cellular senescence (Harley et al., 1990; Serrano et al., 2010). The telomere length of K562 cells has been shortened after interaction with Rg₁ from the above study indicated that Rg₁ can be used to induce cancer cell senescence. Growth arrest is achieved and maintained in either the G1 or G₂/M stage of the cell cycle, in part, by the increased expression of specific cyclin-dependent kinase inhibitors (CDKIs), including p16Ink4a, p21Waf1, and p27Kip1 (Larsson et al., 2011), that is consistent with the result that Rg₁ arrest the proliferation of K562 cells.

Phosphorylated Rb should be altered with senescence (Burd et al., 2010). Rb, a major tumor suppress genes, is crucial in regulating cell cycles, that is involved in preventing cancers (Campisi et al., 2011). It has been clearly shown that telomere shortening or dysfunction induces a DNA damage response mediated by p53 (Moiseeva et al., 2011). P21 induction by P53 inhibits CDK2/Cyclin E activity. Activity of CDK4/Cyclin Ds can also be inhibited by P21. Inhibition of activity of CDKs by P21 results in phosphorylation of RB, which very likely mediates cell cycle arrest during senescence (Collado et al., 2010). Cells with functional Rb appear more sensitive to stress and oncogene activities that stimulate senescence (Campisi et al., 2007). Studies have demonstrated in prostate epithelial and urothelial cells that p21Waf1/Cip1 appears more important in the cell cycle arrest

associated with early senescence, whereas p16Ink4a is more important to maintaining this phenotype (Schwarze et al., 2001). Accumulating data show that the p21-pRB and p16-pRB pathways are most critical for genesis and maintenance of the senescent phenotype in various types of cells. Western Blotting Assay results showed in this study that the senescence-associated proteins P16, P21 and RB have significantly been increased after induction by Ginsenoside Rg₁, indicated that Ginsenoside Rg₁ could break the integrity of DNAs in K562 cells and lead to DNA damage responses developing into state of senescence. This promising finding give us an enlightenment that the senescence-induced strategy of cancer cells could be achieved as an alternative option for cancer treatment and ginsenoside Rg₁ play an important role in the development and maintenance of senescence on cancer cells like human leukemia K562 cells. Moreover, our future work will focus on the field of the in vivo study to investigate the anti-aging effects of ginsenoside Rg₁, and more kinds of cell lines will be applied to these studies.

In conclusion, a series of assays was presented to identify the ability of colony-formation, the telomere length and the expression of senescence associated proteins and ultra structure changes of K562 cells treatment with Rg₁ and the underlying molecular mechanisms over those changes. Those findings gave us full evidences of illustrating the senescence on human leukemia K562 cells induced by Rg₁. Moreover, p21-Rb and p16-Rb pathways have an important role in modulating these changes during this senescence process.

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