

Sun Ginseng Protects Endothelial Progenitor Cells From Senescence Associated Apoptosis

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Endothelial progenitor cells (EPC) are a population of cells that circulate in the blood stream. They play a role in angiogenesis and, therefore, can be prognostic markers of vascular repair. Ginsenoside Rg₃ prevents endothelial cell apoptosis through the inhibition of the mitochondrial caspase pathway. It also affects estrogen activity, which reduces EPC senescence. Sun ginseng (SG), which is heat-processed ginseng, has a high content of ginsenosides. The purpose of this study was to investigate the protective effects of SG on senescence-associated apoptosis in EPCs. In order to isolate EPCs, mononuclear cells of human blood buffy coats were cultured and characterized by their uptake of acetylated low-density lipoprotein (acLDL) and their binding of Ulex europaeus agglutinin I (ulex-lectin). Flow cytometry with annexin-V staining was performed in order to assess early and late apoptosis. Senescence was determined by β-galactosidase (β-gal) staining. Staining with 4'-6-Diamidino-2-phenylindole verified that most adherent cells (93±2.7%) were acLDL-positive and ulex-lectin-positive. The percentage of β-gal-positive EPCs was decreased from 93.8±2.0% to 62.5±3.6% by SG treatment. A fluorescence-activated cell sorter (FACS) analysis showed that 4.9% of EPCs were late apoptotic in controls. Sun ginseng decreased the apoptotic cell population by 39% in the late stage of apoptosis from control baseline levels. In conclusion, these results show antisenescent and antiapoptotic effects of SG in human-derived EPCs, indicating that SG can enhance EPC-mediated repair mechanisms.

Key words: *Panax ginseng*, Apoptosis, Endothelial progenitor cells (EPCs), Aging, Sun ginseng

INTRODUCTION

Bone-marrow-derived endothelial progenitor cells (EPCs) play an integral role in the regulation and protection of the endothelium. EPCs from peripheral blood can also enhance angiogenesis when they are infused into host animals [1,2]. The number of EPCs can be used as a surrogate biologic marker of vascular function, and the number of EPCs is inversely correlated with the cumulative cardiovascular risk [3-7]. In addition, reduced numbers of EPCs in patients with cerebrovascular dysfunctions have been noted [8,9], suggesting that the number of EPCs can be a biological marker in patients with migraine, ischemic strokes, cerebral large artery atheroscle-

rosis, and even in Alzheimer's disease (AD) [8].

Sun ginseng (SG), which is processed at 120°C, results in an increased production of ginsenoside Rg₃, Rg₅, and Rk₁. This processed ginseng includes ingredients, such as ginsenoside Rk₂, Rk₃, Rs₄, Rs₅, Rs₆, and Rs₇ [10-13]. These ginsenosides protect endothelial cells (ECs) from apoptosis through the inhibition of mitochondrial caspase. ECs undergo apoptosis and exhibit increased levels of caspase-9 and caspase-3 activities and DNA fragmentation after 24 h of serum deprivation. However, caspase-9 and caspase-3 levels were suppressed by the addition of (20S)Rg₃. (20S)Rg₃ prevents EC apoptosis

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Received 25 May. 2011, Revised 03 Aug. 2011, Accepted 03 Aug. 2011

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through the Akt-dependent inhibition of the mitochondrial apoptotic signaling pathway [14]. A dysfunction of ECs or apoptosis has been proposed to be an underlying mechanism in neurodegenerative disorders. For example, in AD dementia, the decreased clearing of β -amyloid deposits can contribute to the pathogenesis of this disorder [15,16].

In this study, we investigated the antisenescent and antiapoptotic activities of SG on EPCs, which may be potentially related to EC function in degenerative disorders, as well as in vascular repair.

MATERIALS AND METHODS

Materials

SG (Ginseng Science Inc., Seoul, Korea), endothelial basal medium (EBM-2, Clonetics Corporation, San Diego, CA, USA), EGM-2-MV-SingleQuots (Clonetics Corporation), fetal bovine serum (FBS, GIBCO, Invitrogen Corporation, Burlingame, CA, USA), FITC-labeled Ulex europaeus agglutinin I (ulex-lectin, Sigma-Aldrich Co., St. Louis, MO, USA), DiI-labeled acetylated low-density lipoprotein (acLDL; Invitrogen Corporation) [17], Annexin V-FITC (BD Biosciences, San Jose, CA, USA), and Propidium iodide (PI, Sigma-Aldrich Co.) were used in this study.

Isolation and Characterization of EPCs

Human EPCs were cultured as previously described [18,19]. Briefly, EPCs were obtained by isolating mononuclear cells using a Ficoll density-gradient centrifugation of human blood buffy coats. Cells were resuspended in EBM-2 that was supplemented with EGM-2-MV-SingleQuots containing vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor-1, epidermal growth factor, and 5% FBS. A total of 1×10^6 mononuclear cells/cm² was plated onto fibronectin-coated tissue culture flasks. After 4 d of culture, nonadherent cells were discarded by washing with PBS. In order to confirm the EPC phenotypes, adherent cells were incubated with DiI-labeled acLDL for 1 h. After fixation, they were incubated with FITC-labeled ulex-lectin for 1 h. Cells were visualized with an inverted fluorescent microscope, and adherent cells that stained positive for both FITC-ulex-lectin and DiI-acLDL were determined to be EPCs. Blood samples were acquired with donors' consent, and the experimental procedures were approved by the institutional review board of Seoul National University Hospital.

Experimental protocol and treatment with SG

In order to determine the apoptotic and antiapoptotic effects of SG, a flow cytometric analysis was performed. Flow cytometry was performed on EPCs treated with vehicle or SG. In brief, EPCs were seeded onto a 6-well plate at a concentration of 1×10^5 , cultured for 4 days, and then treated with SG (200 μ g/mL) [20,21] after removal of the supernatant since there was the most effective at a dose of 200 μ g/mL SG (data not shown). In 24 h, EPCs were stained with Annexin-V and PI after the detachment of cells with 0.25% trypsin-EDTA (Invitrogen Corporation).

A β -galactosidase (β -gal) assay was done in order to test for antiaging effects after the isolation of EPCs [22]. EPCs were plated on coverslips coated with poly-L-lysine and cultured for 4 days. Cells were resuspended in media supplemented with SG and cultured for 24 h. The number of β -gal-stained cells (blue structures) was counted in 4 fields. The area of a field was magnified at 100X by an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

Flow cytometric analysis

To analyze the patterns of apoptosis in EPCs after treatment with SG, a flow cytometric analysis was performed with cells after labeling with Annexin-V-FITC and PI. Adherent cells were collected, washed, and resuspended in cold binding buffer (PBS [pH 7.4], 140 mM NaCl, and 2.5 mM CaCl₂) and diluted to a final concentration of 5×10^5 cells/mL. Aliquots of 1×10^5 cells were incubated with 0.5 μ L of Annexin-V-FITC and 10 μ L of PI per tube. After 15 min at room temperature, 400 μ L of binding buffer was added before the flow cytometric analysis. For each sample, 1×10^4 cells were analyzed on a FACS II flow cytometer (BD Biosciences). CellQuest™ Pro software (BD Biosciences) was used to perform the flow cytometric analysis.

Flow Cytometric Analysis of Annexin-V in EPCs

Annexin-V staining indicates the stages of cell death resulting from either apoptotic or necrotic processes. Cells in early apoptosis are Annexin-V-positive and PI-negative, and cells that are in late apoptosis or are already dead are both Annexin-V- and PI-positive.

Cytochemical detection of β -gal staining

The monolayers of cells were washed 2 times with PBS and then fixed with 2% formaldehyde and 0.2% glutaraldehyde for 5 min. Staining solution was added [1 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactosidase

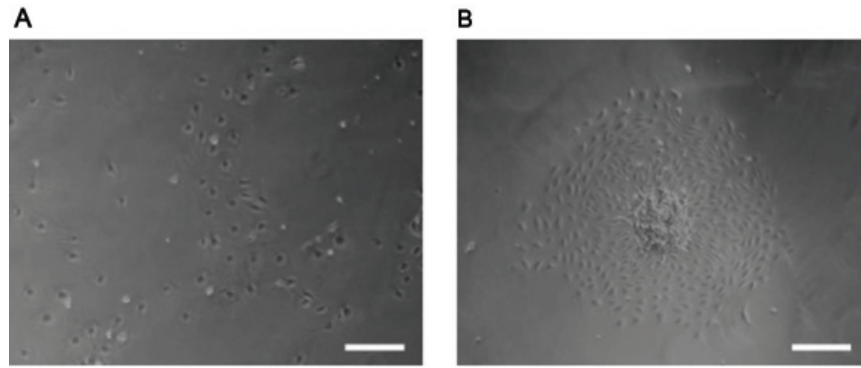


Fig. 1. Endothelial progenitor cell culture. Peripheral Blood Mononuclear Cells at day 2 (A). A central core of rounded cells that are surrounded by elongated and spindle-shaped cells appears after 7 days in culture conditions. They are defined and counted as Colony-Forming Units (B). Bar=100 μ m.

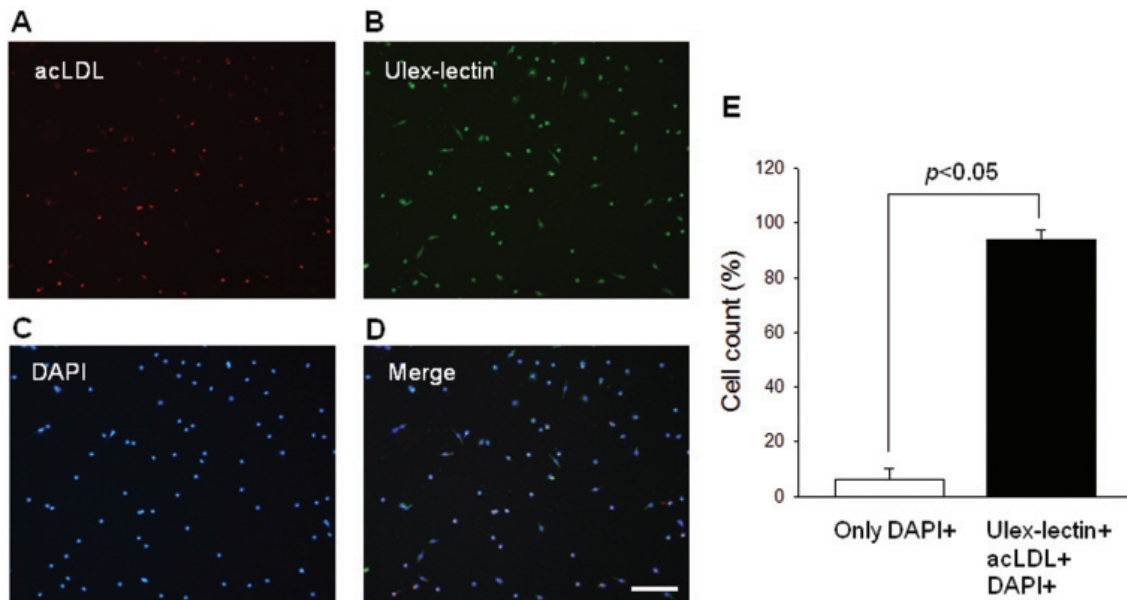


Fig. 2. Endothelial progenitor cell characterization (EPCs). Adherent cells are positive for the uptake of acetylated low-density lipoprotein (acLDL, A) and the binding of FITC-Ulex europaeus agglutinin I (ulex-lectin, B). EPCs stained with the nuclear stain DAPI (C) show that all nuclei are found in cells that are acLDL-positive and bind ulex-lectin (D). There is 93 \pm 2.7% of ulex-lectin and acLDL-positive cells in total cells (E) ($p < 0.05$). Bar=100 μ m.

(X-gal) in dimethylformamide, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂], and the cells were incubated at 37°C for 18 h. After incubation, the cells were washed 2 times with PBS.

Data analysis

All values are expressed as mean \pm standard deviation. The analysis was conducted using repeated measures of analysis of variance and an unpaired Student's *t*-test. A 2-tailed *p*-value less than 0.05 was considered to be significant.

RESULTS

Culture and characterization of endothelial progenitor cells

On day 2, cultured peripheral blood mononuclear cells (PBMCs) were scattered on the culture plate, and on day 7, the cells showed the colony-forming units (CFU) or outgrowth cells (Fig. 1). In previous study, the EPC-CFU numbers were developed to 72.4 \pm 24.5 when 1 \times 10⁴ EPCs derived from normal person were cultured in 12 well-plate [21,23]. The EPCs exhibited a common endothelial phenotype that was defined by the uptake of acLDL and

the binding of ulex-lectin [4,6,24]. In order to confirm the phenotypes of PBMCs cultured with EGM2MV, we subcultured CFU cells and stained them with acLDL and ulex-lectin. Fig. 2 shows that the subcultured CFU cells were positive for acLDL and showed ulex-lectin binding. The staining of nuclei with DAPI verified that most adherent cells ($93\pm 2.7\%$) were acLDL-positive and ulex-lectin-positive.

Senescence-associated β -gal staining in endothelial progenitor cells and effect of sun ginseng

In order to investigate the anti-senescent effect of SG, we compared the differences in β -gal-positive cell number between EPCs treated with SG (200 $\mu\text{g}/\text{mL}$) or vehicle for 24 h. The mean number of senescence-associated (SA)- β -gal-stained cells treated with SG was $62.5\pm 3.6\%$, whereas that in controls was $93.8\pm 2\%$. Compared with vehicle, SG resulted in a significant diminution of the proportion of SA-stained cells ($p < 0.001$, Fig. 3B).

The antiapoptotic effects of sun ginseng on endothelial progenitor cells

A flow cytometric analysis of Annexin-V-positive cells was performed in order to investigate the antiapoptotic effects of SG in EPCs. In control conditions, 4.9% of cells were undergoing late apoptotic cell death. EPCs treated with SG for 24 h had lower percentages of late apoptotic cells, with 3.0% in the late stage of apoptosis. Therefore, the population of apoptotic cells was reduced by SG compared to controls, which had 39% in late apoptosis ($p < 0.05$) (Fig. 4).

DISCUSSION

In this study, we attempted to test the antisenescent and antiapoptotic effects of SG on EPCs. The number of SA- β -gal staining-positive EPCs was lower after SG treatment than that with vehicle. Moreover, apoptotic cells were decreased in SG-treated EPCs compared to controls. Based on our data, SG appears to have effects on EPCs that involve both antisenescent and antiapoptotic mechanisms.

Recent studies have shown that degenerating neuronal cells display characteristics of apoptosis [25,26]. Intra-hippocampal injections of amyloid β peptide ($A\beta$) induced a spatial memory deficit, apoptosis, and caspase-9 activation in hippocampal neurons [27]. Interference with or alterations of the Akt signaling pathway can be a feature in several neurodegenerative diseases that are characterized by neuronal attrition [28-30]. Therefore, reagents that suppress neuronal apoptosis have been developed as therapeutic agents in several neurodegenerative disorders [31,32]. *Panax ginseng* has been reported to have an antiapoptotic effect in both *in vivo* and *in vitro* experiments [33]. In this experiment, SG lowered the number of Annexin-V-positive EPCs in a flow cytometric analysis [34,35], supporting the previous findings that Rb_3 inhibited apoptosis [10-12].

SG, which removes peroxide from the human body, has been reported to have strong antioxidant activities compared to the current ginsengs. SG also has effects on fatigue relief, antiaging, and improvements of circulation [21,36]. Rg_3 in SG affects estrogen activity that reduces

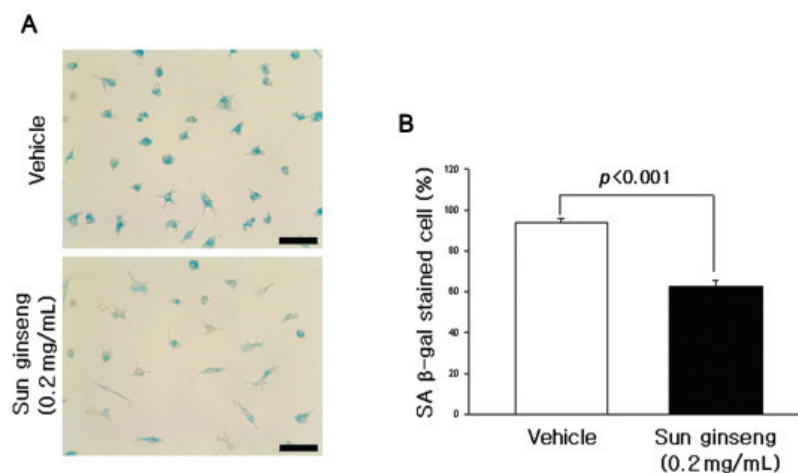


Fig. 3. Senescence-associated (SA) β -galactosidase (β -gal) staining in endothelial progenitor cell (EPCs) after treatment with Sun ginseng. Cytochemically detectable β -gal staining at pH 6.0 was conducted as a marker of cellular senescence. Sun ginseng-treated EPCs exhibit a lower level of SA β -gal staining than that of vehicle-treated controls ($p < 0.05$).

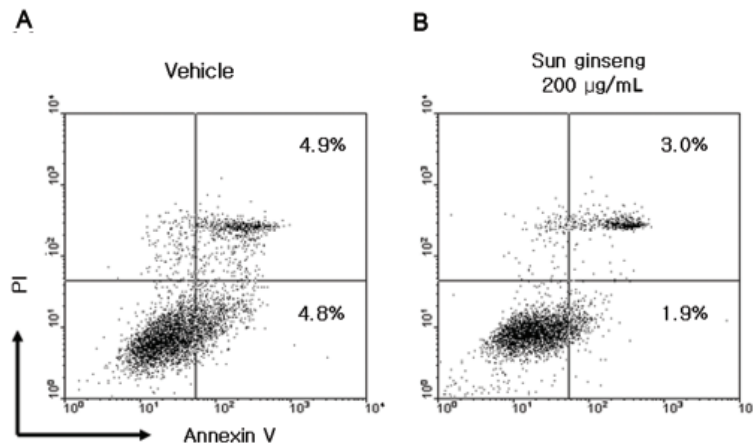


Fig. 4. Flow Cytometric Analysis of Annexin-V in EPCs. The lower and right box in the figure shows the early apoptotic population (PI-negative, Annexin-V-positive), whereas the upper and right box shows the late apoptotic population (PI-positive, Annexin-V-positive). The percentage of late apoptotic cells is 4.9% in A and 3.0% in B.

EPC senescence through the augmentation of telomerase activity [37]. Therefore, it can be plausible that SG has effects on slowing senescence in EPCs, as shown with SA- β -gal staining [38-40]. In this study, we confirmed that SA β -gal staining in EPCs was decreased when compared to that of vehicle controls, indicating that SG has antisenescent effects on EPCs.

Dementia is one of the most common human disorders associated with aging. AD, which is the most frequent type of dementia, exhibits degenerating neuronal loss in the brain [41,42]. Amyloid plaques in the extracellular space are known to be the main mediator of the pathogenesis of AD [43,44]. Circulating angiogenic cells (CACs), such as EPCs, have been shown to have functions in the clearance of amyloid β peptide (A β) [45,46]. Therefore, endothelial senescence in AD is attributable to the faulty clearance of A β , which accelerates the progression of disease [47,48]. CACs participate in the maintenance of the endothelium by replacing dysfunctional ECs or by releasing angiogenic growth factors [49,50]. Recently, dysfunctions of EPCs have been proposed to be a cause of AD [9]. In addition, efficacies of treatment with Korean red or *Panax ginseng* in patients with AD have been reported [51], warranting a correlation study of ginsenosides, EPCs, and AD.

In conclusion, SG reduced the senescence and apoptosis in EPCs. Given that EPCs constitute a circulating pool of cells and augment neovascularization by integrating into newly developing capillaries [3,52,53], we suggest that SG can be an effective treatment for vascular repair and for disorders associated with the function and number of EPCs [3-7,23,54].

ACKNOWLEDGEMENTS

This work was supported by Ginseng science inc., the Korea Health 21 R&D Project(A092058) and WCU Neurocytomics.

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