BMB
Reports

BMB Rep. 2017; 50(11): 572-577
www.bmbreports.org

Antioxidant effects of selenocysteine on replicative senescence in human adipose-derived mesenchymal stem cells

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In most clinical applications, human mesenchymal stem cells (hMSCs) are expanded in large scale before their administration. Prolonged culture *in vitro* results in cellular senescence-associated phenotypes, including accumulation of reactive oxygen species (ROS) and decreased cell viabilities. Profiling of stem cell-related genes during *in vitro* expansion revealed that numerous canonical pathways were significantly changed. To determine the effect of selenocysteine (Sec), a rare amino acid found in several antioxidant enzymes, on the replicative senescence in hMSCs, we treated senescent hMSCs with Sec. Supplementation of Sec in the culture medium in late-passage hMSCs reduced ROS levels and improved the survival of hMSCs. In addition, a subset of key antioxidant genes and Sec-containing selenoproteins showed increased mRNA levels after Sec treatment. Furthermore, ROS metabolism and inflammation pathways were predicted to be downregulated. Taken together, our results suggest that Sec has antioxidant effects on the replicative senescence of hMSCs. [BMB Reports 2017; 50(11): 572-577]

INTRODUCTION

Mesenchymal stem cells (MSCs) are promising sources for development of novel therapeutics due to their abilities for tissue repair and regeneration (1). These cells can be isolated from the stroma of virtually all tissues, including bone marrow, adipose, and umbilical cord (2-4). Numerous animal model experiments have demonstrated that transplantation of *ex vivo* expanded MSCs have therapeutic effects in various disease settings. Currently, thousands of clinical trials with human MSCs (hMSCs) have been reported for testing their clinical efficacies worldwide (5). To achieve sufficient cell dosage numbers for cell therapy, a reproducible and efficient *ex vivo* expansion process is required. However, hMSCs undergo a limited number of cell divisions under standard culture conditions, as do all primary human cells (6). This process is known as cellular senescence and is characterized by loss of proliferative capacity, morphological transformation, induction of tumor suppressor networks, increased senescence-associated β-galactosidase (SA-β-gal) activity, and accumulation of reactive oxygen species (ROS) (7). Therefore, it is critical to understand the molecular nature of cellular senescence in hMSCs and to identify intrinsic and/or extrinsic factors affecting this process for clinical applications.

Selenium is an essential nutrient for many organisms, including humans (8, 9). At the organismal level, selenium deficiency was associated with various diseases in humans, including Keshan disease, a congestive cardiomyopathy, and Kashin-Beck disease, a chronic, endemic type of osteochondropathy (10). In addition, a selenium concentration decrease in the blood was associated with mortality in elderly human populations (11-13) indicating its role in longevity. At the cellular level, the beneficial effect of selenium on replicative senescence of human fibroblasts has been confirmed (14).

Selenium is thought to function biologically largely through selenoproteins (15). To date, 25 selenoproteins have been identified in humans, and approximately only half of them are known for their functions in redox homeostasis and antioxidant defenses (16). The best-studied selenoproteins are: glutathione peroxidases (Gpx1-Gpx6), which catalyze the glutathione-dependent reduction of organic hydroperoxides and hydrogen peroxide (H_2O_2); thioredoxin reductases (Txnrd1-Txnrd3), which reduce thioredoxins, as well as other substrates; and iodothyronine deiodinase (Dio1-Dio3), which deiodinate thyroid hormones, thereby regulating their activities (17).

All selenoproteins contain selenium in the form of selenocysteine (Sec), a rare amino acid found in both prokaryotes and eukaryotes, in their active sites (18). In contrast to selenium, the beneficial effect of Sec on human health is less clear. Mutations in selenocysteine synthase, which catalyze Sec synthesis, cause neurological disorders in humans (19). However, in Drosophila, selenocysteine synthesis was not essential for oxidative stress and longevity (20). Interestingly, a recent study demonstrated that dietary supplementation of Sec could modulate resistance to environmental stress, with

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https://doi.org/10.5483/BMBRep.2017.50.11.174

Received 1 September 2017, Revised 14 September 2017, Accepted 18 September 2017

Keywords: Cellular senescence, Human mesenchymal stem cell (hMSC), Reactive oxygen species (ROS), Selenocysteine (Sec)

ISSN: 1976-670X (electronic edition)

anti-aging effects in *Caenorhabditis elegans* (*C. elegans*) (21). Despite these observations at the organismal level, a potential role for Sec in mammalian cells during cellular senescence has not been demonstrated. To test this hypothesis, we demonstrated that supplementation of Sec in the culture medium of late-passage hMSCs significantly decreased ROS levels and improved hMSC survival. Furthermore, when all the selenoproteins were examined, *Gpx2*, Selenoprotein O (*SelO*), and *SelX* showed altered mRNA expression levels after Sec treatment. In addition, genes involved in ROS metabolism − including superoxide dismutase 2 (*Sod2*), NADPH oxidase 1 (*Nox1*), and *Nox4* − also significantly changed their expression with Sec exposure. Extensive bioinformatics analyses revealed that the Sec treatment of senescent hMSCs was predicted to downregulate ROS metabolism and inflammation pathways. Taken together, our results suggest beneficial antioxidant effects of Sec on replicative senescence of hMSCs.

RESULTS AND DISCUSSION

The hMSC cell lines were established from three donors and expanded *in vitro* to determine the senescence-associated phenotypes. A long-term growth curve revealed that early-passage cells maintained high proliferation rates until they reached senescence, with 35 ± 3.69 cumulative population doublings (CPDs) (Fig. 1A). Compared to early-passage hMSCs, late-passage cells showed characteristic flattened-cell morphologies (Fig. 1B). The number of viable cells was then determined by a MTS [3-(4,5-dimethylthiazol-2-ul)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Consistent with results from long-term growth experiments, the MTS assay showed more than a 60% reduction of proliferation capacities of late-passage cells (Fig. 1C). It has been well known that oxidative stress and ROS production are major causes of cellular senescence and organismal aging (22). Using Amplex® Red reagents, which reacts with H_2O_2 to produce a fluorescent oxidation derivative in the presence of peroxidase, we observed a 2.2-fold increase in H_2O_2 in senescent cells (Fig. 1D). Based on the observed senescence phenotypes, we categorized hMSCs into two groups (early [E, passage 3-6] and late [L, passage 20-26]) and performed further analyses.

To determine the molecular regulation of cellular aging in hMSCs, we performed a PCR array with early- and late-passage hMSCs. The expression levels of 84 genes related to identification, growth, and differentiation of stem cells were examined. Consistent with previous reports on the mRNA expression profiles of bone marrow-derived MSCs (23), early- and late-passage hMSCs showed extensive expression profile changes in stem cell-related genes. Compared with early-passage hMSCs, 7 genes were upregulated, and 20 genes were down-regulated more than 2-fold in late-passage hMSCs (Fig. 2A, black and gray dots respectively) (Fig. 2A). Interestingly, stem cell specific markers, such as cell cycle regulators (*Ccna2*,

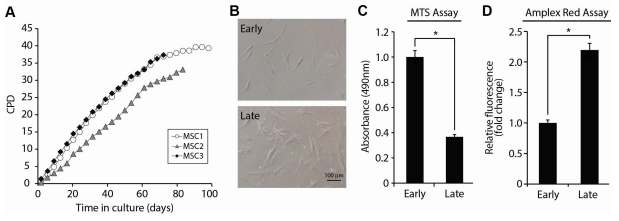

Fig. 1. Characterization of senescence-associated changes in *ex vivo* expanded-hMSCs. (A) hMSCs were isolated from the adipose tissue of three donors and evaluated for the cumulative population doublings (CPD). (B) Representative images of hMSCs at early and late passage. Scale bar: 100 μm. (C) Viable cells were examined by MTS assay in young (passage 3-6) and senescent (passage 20-26) hMSCs. Error bars denote the standard error of the mean of 3 experiments (*P < 0.0001). (D) H_2O_2 levels produced in young and senescent cells were quantified by the Amplex® Red assay. Error bars denote the standard error of the mean of 3 experiments (*P < 0.0001).

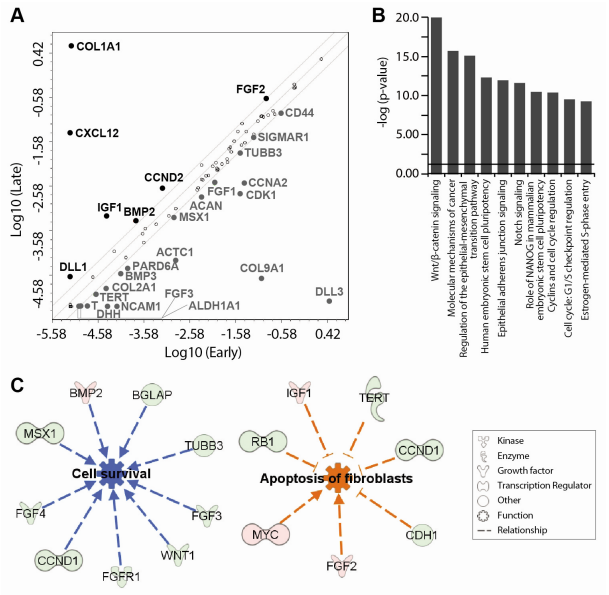

Fig. 2. Gene expression profiles of hMSCs during *in vitro* aging. (A) The scatter plot of young and senescent hADSCs using RT^2 Profiler™ PCR Array Human Stem Cell, which contains 84 genes related to identification, growth, and differentiation of stem cells. Genes up-regulated or down-regulated in late-passage hMSCs by at least 2-fold are indicated with black and gray dots, respectively. (B) Regulated top 10 canonical pathways significantly altered by *ex vivo* amplification of hMSCs were shown by using Ingenuity Pathways Analysis (IPA) (Benjamini-Hochberg multiple test-corrected P-values < 0.05). (C) Analysis of the expression profile data with the cellular functions by using IPA software. The molecule types and relationships were indicated in a boxed legend. The colors in the molecular types reflect the expression levels in the dataset. The relationships were presented in blue and orange dashed lines representing the inhibition and activation of the cellular functions, respectively.

Cdk1, *Fgf1*, *Fgf3*, *Pard6a*), genes regulating symmetric/asymmetric cell division (*Dhh*, *Pard6a*) and cell adhesion molecules (*CD44*, *Col9a1*, *Ncam1*), were significantly down-regulated. In addition, stem cell differentiation markers, including embryonic cell lineage markers (*Actc1*, *Msx1*), mesenchymal cell lineage markers (*Acan*, *Col2a1*, *Col9a1*), and neural cell lineage markers (*CD44*, *Ncam1*, *Sigmar1*, *Tubb3*), were also greatly downregulated in senescent cells. However, cytokines and growth factors were both upregulated (*Bmp2*, *Cxcl12*, *Fgf2*, *Igf1*) and downregulated (*Bmp3*, *Fgf1*, *Fgf3*), implying a dramatic change in secreted molecules.

We next evaluated canonical pathways associated with *in vitro* aging of hMSCs using Ingenuity Pathway Analysis (IPA) software. Function and network analysis revealed predominant enrichment of 118 canonical pathways (Benjamini-Hochberg multiple test-corrected P-values < 0.05) including those of Wnt/β-catenin, Notch, and cancer signaling (Fig. 2B). To better understand the underlying mechanisms for senescence phenotypes, we focused on the changes in cellular functions, which were predicted based on the expression profile data. Notably, in late-passage hMSCs, significant changes were observed in several important cellular functions such as the inhibition of cell survival and the activation of apoptosis in fibroblasts (Fig. 2C). Taken together and consistent with previous reports, senescent hMSCs exhibited systematic deregulation of stem cell properties, including proliferation/differentiation capabilities, secretion of trophic factors, and associated signaling pathways.

It has been reported that serum levels of micronutrient selenium are closely related to human mortality (12, 13). At the cellular level, selenium levels can extend the replicative life span of human fibroblasts when added during early-stage expansion (14). In addition, dietary supplementation of Sec, a selenium-containing cysteine, leads to lifespan extension in *C. elegans* (21). However, the relationship between Sec and replicative senescence in hMSCs is not known. To determine the effect of Sec during replicative senescence, we treated senescent hMSCs with two different concentrations of Sec. The proliferation rate of hMSCs was measured by MTS assay. Sec supplementation increased the proliferation capability to 28.7% (50 nM Sec) and 36.9% (100 nM Sec) compared to the untreated senescent hMSCs (Fig. 3A). Next, we monitored the H_2O_2 status after Sec treatment. Compared with untreated late-passage hMSCs, H_2O_2 levels were significantly reduced to 14.6% and 22.4% with 50 nM and 100 nM Sec, respectively (Fig. 3B). Moreover, late-passage hMSCs grown in 100 nM Sec for 24 hours showed more discrete cell morphologies compared to the flattened morphologies seen in senescent cells (Fig. 3C). In conclusion, Sec supplementation exerts positive effects on proliferation capability of senescent hMSCs in part by reducing H_2O_2 accumulation.

To gain further insight into the underlying mechanisms of Sec treatment on senescent hMSCs, we analyzed the expression levels of all 25 human selenoproteins using

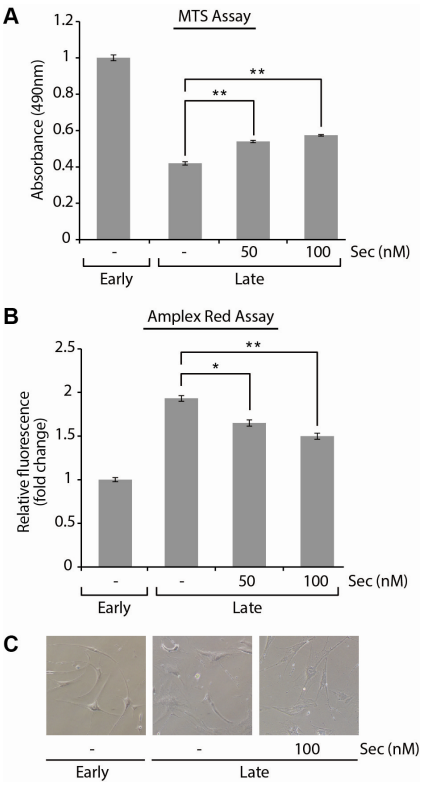

Fig. 3. Effects of Sec on survival and H_2O_2 accumulation in senescent hMSCs. (A) Senescent hMSCs were cultured with 0, 50, and 100 nM of Sec for 24 hours and then cell growth rate was measured using MTS assay. Error bars denote standard error of the mean of 6 experiments (**P $<$ 0.0001). (B) H_2O_2 production was quantified by Amplex® Red on early, late, and late-passage hMSCs treated with 50 nM and 100 nM Sec for 24 hours. Error bars denote the standard error of the mean of 6 experiments (*P = 0.001; **P $<$ 0.0001) (C) Representative photographs of hMSCs on young and senescent cells with or without 100 nM selenocysteine treatment.

qRT-PCR analysis (Fig. 4A). The other 24 selenoproteins were all expressed in hMSCs, except for Dio1 (which was not detectable in this condition). We observed that in response to Sec treatment, *Gpx2* was dramatically up-regulated (32.9X relative to the untreated late-passage hMSCs, P $<$ 0.001), whereas *SelO* (0.76X, P $<$ 0.05) and *SelX* (0.77X, P $<$ 0.05) were statistically down-regulated. Since selenium supplementation can modulate selenoproteins at both mRNA and protein levels (14), we cannot rule out the possibility of translational control after Sec treatment. We next monitored the expression of genes involved in ROS metabolism, including superoxide dismutases (*Sod* 1-3) and NADPH oxidases (*Nox1* and *4*), after Sec treatment (Fig. 4B). A mitochondrial protein, *Sod2* (2.1X compared to the untreated late-passage hMSCs, P $<$ 0.01) and *Nox1* (22.3X, P $<$ 0.001) were significantly up-regulated and *Nox4* (0.35X, P $<$ 0.05) was down-regulated.

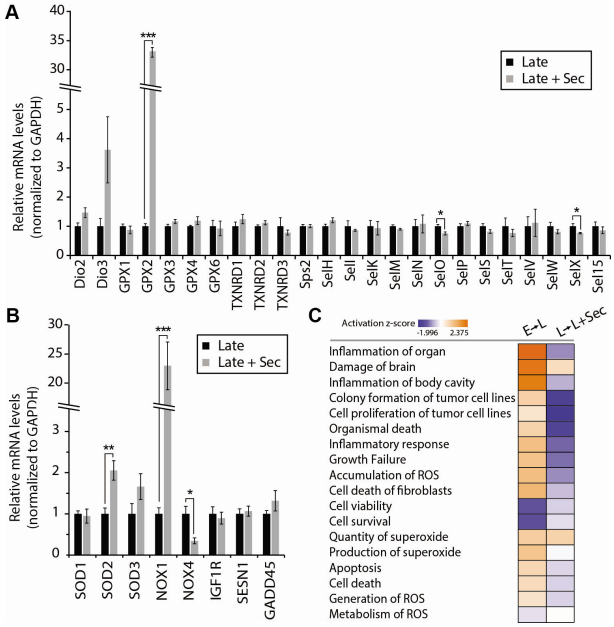

Fig. 4. Regulation of selenoproteins and genes involved in ROS metabolism in response to Sec supplementation in senescent hMSCs. (A) mRNA levels of selenoproteins were evaluated by qRT-PCR analysis. Error bars denote the standard error of the mean of four experiments (*P < 0.05; ***P < 0.001). (B) Expression levels of genes associated with ROS metabolism were measured by qRT-PCR analysis upon Sec treatment. Error bars denote standard error of the mean of four experiments (*P < 0.05; ** P < 0.01; ***P < 0.001). (C) The heat map of a comparison analysis on disease and function was generated using IPA software. Pathways were sorted by activation z-scores, which were calculated by total −log(P-value) from Fisher's Exact test across compared observations (observation 1, early to late transition; observation 2, late to late with Sec treatment transition).

Using mRNA expression data on both selenoproteins and other genes involved in ROS metabolism, we next performed a comprehensive bioinformatic analysis using IPA software. Specifically, we compared the diseases and functions of early to late-passage and late to late-passage cells with Sec treatment (Fig. 4C). As expected, during *ex vivo* expansion of hMSCs, inflammation, ROS metabolism, cell death, and apoptotic pathways were all increased (Fig. 4C, left column, red) in senescent cells. In contrast, cell viability and survival functions were greatly decreased in senescent cells (Fig. 4C, left column, blue). However, there were striking changes in those pathways when cells were exposed to the Sec (Fig. 4C, right column). Indeed, Sec supplementation on senescent hMSCs was predicted to decrease ROS metabolism pathways, such as ROS accumulation, superoxide quantity, superoxide production, and generation and metabolism of ROS (Fig. 4C, right column, activation z-score from −0.8825 to 0.038964). Furthermore, cell viability and survival pathways were predicted to increase upon Sec treatment (activation z-score: −0.40095 and −0.30488). Interestingly, inflammation-related biofunctions, including inflammation of organ and inflammatory response, were anticipated to decrease with Sec supplementation (activation z-score: −0.8849 and −1.13137). Considering the anti-inflammatory properties of selenium in human health (24), this was not a surprising finding.

In conclusion, our findings show the beneficial effects of Sec during hMSC senescence for the first time. We observed a significant increase in cell viability and a selective regulation of selenoproteins and genes involved in ROS metabolism upon Sec treatment on senescent hMSCs. Comparison analysis on diseases and functions further provide evidence for the antioxidant response of Sec on cellular senescence in hMSCs.

MATERIALS AND METHODS

Isolation and culture conditions of hMSCs

This study was carried out according to the guidelines of the Institutional Review Board of Soon Chun Hyang University. hMSCs were isolated from human adipose tissue of three donors (age range of 42-51 years) and cultured as previously described (25-28). The hMSCs obtained were cultured in DMEM with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco, Gaithersburg, MD, USA) in a humidified atmosphere containing 5% CO_2 at 37°C. Medium was replaced every 3 days and the cells were used at passage 3 to 6 for early passages and passage 20 to 26 for late passages. For Sec treatment, late-passage hMSCs were incubated with different concentrations (0, 50, or 100 nM) of Sec (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours.

Cell proliferation assay

Cell proliferation was measured using a colorimetric assay with 3-(4,5-dimethylthiazol-2-ul)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI, USA) (29). Briefly, cells were plated 3×10^3 cells per well in 96-well plate on day 1 and then treated with different concentrations (0, 50, or 100 nM) of Sec. on day 2. After 24 hours of incubation, 20 μl of MTS solution was added to each well and incubated for 4 hours at 37°C. The absorbance was then recorded at 490 nm using a BioTek Epoch2 microplate reader (BioTek, Winooski, VT, USA). Each assay was performed in three replicate wells for each condition.

Hydrogen peroxide (H_2O_2) measurement

For the quantitative measurement of H_2O_2 levels, an Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) was used in accordance with the manufacturer's instructions. Briefly, hMSCs were plated 6×10^4 cells per well in a 6-well plate, and senescent hMSCs were incubated with different concentration (0, 50, or 100 nM) of Sec. After 24 hours, cells were detached with 0.25% trypsin/EDTA and suspended in Krebs-Ringer phosphate (KRPG) buffer (145 mM NaCl, 5.7 mM sodium phosphate,

4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, pH 7.35). The reaction mixture (50 μM Amplex® Red reagent and 0.1 U/ml HRP in KRPG buffer) was pre-warmed at 37°C for 10 minutes and then incubated with 1×10^4 cells in 96-well plate. After incubation for 90 minutes, fluorescence was measured at 540 nm excitation and 590 nm emission using BioTek Epoch2 microplate reader.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured cells using Trizol (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. For quantitative real-time PCR (qRT-PCR) analysis of mRNA, 300 ng of total RNAs were transcribed with random hexamers and SuperScript IV reverse transcriptase (ThermoFisher Scientific, Waltham, MA, USA). The qRT-PCR assay was then performed using a Power SYBR Green PCR master mix on a QuantStudio 6 Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA, USA). The primers used in the qRT-PCR can be seen in Supplementary Table 1. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous normalization control.

PCR array and network analysis

The RT^2 Profiler™ PCR Array Human Stem Cell (PAHS-405Z) (Qiagen, Hilden, Germany) was used as previously described (25, 30). Briefly, 400 ng of total RNAs were treated with DNase I and then reverse transcribed using the RT^2 First Strand Kit (Qiagen, Hilden, Germany). PCR quantification was done on a 7900 Real-Time PCR system using RT^2 SYBR Green qPCR Master Mixes. Data analysis was performed using online analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) (Qiagen, Hilden, Germany). The canonical pathways, functional network, and comparison analysis were performed using Ingenuity Pathway Analysis (IPA) software (Qiagen, Hilden, Germany).

ACKNOWLEDGEMENTS

This work was supported by the Soon Chun Hyang University Research Fund (No. 20160225), and the Basic Science Research Program through the National Research Foundation of Korea (NRF) from the Ministry of Education, Science, and Technology (2016R1D1A1B03935929).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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