Biochemical and Biophysical Research Communications 496 (2018) 1162–1168

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Senescence-messaging secretome factors trigger premature senescence in human endometrium-derived stem cells

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ARTICLE INFO

Article history:
Received 16 January 2018
Accepted 26 January 2018
Available online 31 January 2018

Keywords:
Mesenchymal stem cells
SASP
Stress-induced senescence
Signaling pathway
DNA damage response
Senescence propagation

ABSTRACT

Accumulating evidence suggests that the senescence-messaging secretome (SMS) factors released by senescent cells play a key role in cellular senescence and physiological aging. Phenomenon of the senescence induction in human endometrium-derived mesenchymal stem cells (MESCs) in response to SMS factors has not yet been described. In present study, we examine a hypothesis whether the conditioned medium from senescent cells (CM-old) may promote premature senescence of young MESCs. In this case, we assume that SMS factors, containing in CM-old are capable to trigger senescence mechanism in a paracrine manner. A long-term cultivation MESCs in the presence of CM-old caused deceleration of cell proliferation along with emerging senescence phenotype, including increase in both the cell size and SA-β-Gal activity. The phosphorylation of p53 and MAPKAPK-2, a direct target of p38MAPK, as well as the expression of p21Cip1 and p16Ink4a were increased in CM-old treated cells with senescence developing whereas the Rb phosphorylation was diminished. The senescence progression was accompanied by both enhanced ROS generation and persistent activation of DNA damage response, comprising protein kinase ATM, histone H2A.X, and adapter protein 53BP1. Thus, we suggest that a senescence inducing signal is transmitted through p16/MAPKAPK-2/Rb and DDR-mediated p53/p21/Rb signaling pathways. This study is the first to demonstrate that the SMS factors secreted in conditioned medium of senescent MESCs trigger a paracrine mechanism of premature senescence in young cells.

1. Introduction

Mesenchymal stem cells (MSCs) in response to different stimuli secrete a broad array of bioactive factors such as proteins, lipids, nucleic acids and vesicles which shape the secretome. The secreted factors may affect the function of surrounding tissues, including the promotion of regenerative processes in the damaged tissue [1,2]. However, the regenerative capacity of MSCs decreases dramatically in process of stress-induced senescence, in particular under oxidative stress which accompanies the multiple age-related pathologies [3]. An important consequence of senescence is the profound alterations in the secretome of senescent cells, secreting a complex mixture of factors referred to as the senescence-associated secretory phenotype (SASP) [4,5] or senescence-messaging secretome (SMS) [6]. These include increased secretion of growth factors and proteins binding to them, pro-inflammatory and anti-inflammatory cytokines, extracellular matrix remodeling factors, proteases etc. The SMS can exert the opposite effects. So, the initial studies focused on the pro-tumorigenic properties of SMS [4,7] but it also may mediate tumor suppressive effects [5,6]. Moreover, the SMS contributes to surveillance and elimination of pre-malignant senescent cells by the immune system [8,9].

Depending on the physiological context, the SMS factors can either reinforce senescence by inducing a stable growth arrest in an autocrine manner or relay the senescence phenotype to surrounding cells in a paracrine manner, greatly accelerating the senescence spread in the cell population [10,11]. Thus, a loss of proliferative (regenerative) potential during stress-induced senescence, as well as the senescence expansion due to SMS can significantly reduce the beneficial effects of MSCs transplantation in cell-based therapy and limit their use in regenerative medicine. In this

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https://doi.org/10.1016/j.bbrc.2018.01.163
0006-291X/

regard, a role of SMS factors in regulation of cellular senescence is a current topic of modern investigations.

Earlier we have shown that human endometrium-derived mesenchymal stem cells (MESCs) in response to H_2O_2-induced oxidative stress enter the premature senescence [12]. Although the intracellular molecular mechanism of stress-induced senescence of these cells was studied in details [13–16], the paracrine mechanism of SMS-mediated signal transduction from senescent MESCs to neighboring cells in the population remained so far unexplored. In the current work, we provide evidence that the SMS of MESCs implements a senescence response in young cells, suggesting that the secreted factors are capable to trigger senescence mechanisms through paracrine signaling cascades.

2. Materials and methods

The manuscript was written in accordance with Uniform Requirements for manuscript submitted to Biomedical journals.

2.1. Reagents

All reagents except indicated otherwise were from Sigma-Aldrich (St. Louis, MO, USA). Plastic dishes and plates were from Nalge Nunc International (Neerijse, Belgium) and Eppendorf AG (Hamburg, Germany). Cell culture reagents from Gibco Life Technologies Corporation (Gibco BRL, USA) and serum (HyClone, USA) were used. Nitrocellulose and PVDF membranes and electrophoretic equipment were from Bio-Rad Laboratories (Hercules, CA, USA). Primary antibodies were rabbit polyclonal from Cell Signaling Technology (Danvers, MA, USA); the exceptions are indicated below. Antibodies against the following epitopes were used: p16Ink4a (monoclonal, BD Transduction Laboratories), p21Cip1/Waf1, phospho-ATM (Ser1981) (Invitrogen, Rockford, IL, USA), phospho-Histone-H2A.X (Ser139), phospho-53BP1 (Ser1778), phospho-Rb (Ser807/811), phospho-p53 (Ser15), phospho-MAPKAPK-2 (Thr334), GAPDH. FITC-Ki67 conjugate for FACS was from Dako. Secondary antibodies for immunoblotting – GAR-HRP and GAM-HRP were from Cell Signaling Technology, secondary antibodies for immunofluorescence - Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse were from Invitrogen. ECL substrates SuperSignal West Femto were from Thermo Scientific (Rockford, IL, USA). Hyperfilm (CEA) was from Amersham (Sweden).

2.2. Cell culture

Human mesenchymal stem cells (MESCs, line 2304) were isolated from desquamated endometrium in menstrual blood from healthy donors as described previously [17]. MESCs had a positive expression of CD13, CD29, CD44, CD73, CD90 and CD105 markers; expression of the hematopoietic cell surface antigens CD19, CD34, CD45, CD117, CD130, and HLA-DR was absent. Besides, MESCs partially (over 50%) expressed the pluripotency marker SSEA-4 but not Oct-4. Multipotency of isolated MESCs was confirmed by their ability to differentiate into other mesodermal cell types, such as osteocytes and adipocytes. Cells were characterized by high rate of cell proliferation (doubling time 22–23 h) and high cloning efficiency (about 60%). Cells were cultured in DMEM/F12 supplemented with 10% fetal calf serum, 1% penicillin-streptomycin and 1% GlutaMax at 37 °C, 5% CO_2. Cells were harvested by trypsinization and plated at a density of $15*10^3$ or $7{,}5*10^3$ cells per cm^2 where it was necessary. For microscopy experiments, cells were grown on glass coverslips. To avoid complications of replicative senescence, cells at early passages (between 5 and 8 passages) were used in all experiments.

2.3. Cell treatment and preparation of conditioned medium

H_2O_2-treated cells and intact (H_2O_2-untreated) cells were referred to as senescent and young MESCs, respectively. To induce the premature senescence in young MESCs, the cell treatment with H_2O_2 was performed as previously reported [13]. Briefly, cells were treated with 200 μM H_2O_2 for 1 h, then washed twice with serum-free medium to remove H_2O_2, and re-cultured in fresh complete medium for 7 days. The preparation of conditioned media (CM) was performed according to references [18]. So, the senescent cells were washed twice with PBS and re-cultured in serum-free medium for 24 h. The CM collected from senescent cells is referred to as CM-old. In the parallel experiments, young MESCs after seeding were cultured for 2 days, then washed twice with PBS and re-cultured in serum-free medium for 24 h. The CM collected from young cells is referred to as CM-young. Importantly, MESCs were seeded at similar density to prepare both CM-old and CM-young. The collected CM-old and CM-young were used at 50% in complete growth medium for in vitro cultivation of young MESCsduring various time period as specified in individual experiments. In both cases, three independent culture preparations were prepared and pooled to avoid biological variation.

2.4. SA-β-Gal activity assay

Cells expressing senescent-associated β-galactosidase (SA-β-Gal) were detected with senescence β-galactosidase staining kit (Cell Signaling Technology) according to manufacturer's instructions and quantified microscopically by counting X-gal-positive cells among not less 500 cells in random fields of view. The kit detects β -galactosidase activity at pH 6,0 in cultured cells which is present only in senescent cells and is not found in pre-senescent, quiescent, or immortal cells.

2.5. FACS analysis of cell viability and cell size

The cell viability of CM-treated MESCs was determined by FACS as reported earlier [12]. Cells were detected by size and granularity using FSC/SSC and cell debris was gated out. The cell size was evaluated by cytometric light scattering of PI-negative stained cells. To discriminate the live and dead cells, two-parameter histogram was used (PILOG vs. FSLOG). Analysis at least 3000 events of each sample was performed by flow cytometry using the CytoFLEX (Beckman Coulter, CA, USA), and the obtained data were analyzed using CytExpert software version 1.2.

2.6. Ki67/DAPI staining

The samples were prepared using Nuclear Factor Fixation and Permeabilization Buffer Set (BioLegend, USA). Briefly, after fixation and permeabilization cells were stained with FITC-Ki67 conjugate (10 μl/10^6 cells) and DAPI (1 μg/ml) and then analyzed by FACS, using the CytoFLEX; the obtained data were analyzed using CytExpert software version 1.2.

2.7. Measurements of intracellular ROS

Measurements of intracellular ROS levels with applying redox-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Invitrogen) were performed as described previously [13]. Mean fluorescence intensity from 10,000 cells was acquired. The DCF fluorescence was measured using a flow cytometer CytoFLEX, and the obtained data were analyzed using CytExpert software version 1.2.