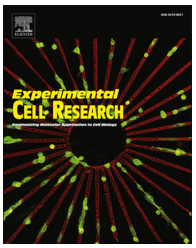
EXPERIMENTAL CELL RESEARCH 319 (2013) 2977–2988

Available online at www.sciencedirect.com

ScienceDirect

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



Research Article

Simvastatin rises reactive oxygen species levels and induces senescence in human melanoma cells by activation of p53/p21 pathway

*Fernanda Augusta de Lima Barbosa Guterres, Glaucia Regina Martinez, Maria Eliane Merlin Rocha, Sheila Maria Brochado Winnischofer**

Department of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba, PR, Brazil

ARTICLE INFORMATION

Article Chronology:
Received 2 April 2013
Received in revised form
24 July 2013
Accepted 26 July 2013
Available online 8 August 2013

Keywords:
Senescence
Simvastatin
ROS
Melanoma cells

ABSTRACT

Recent studies demonstrated that simvastatin has antitumor properties in several types of cancer cells, mainly by inducing apoptosis and inhibiting growth. The arrest of proliferation is a feature of cellular senescence; however, the occurrence of senescence in melanoma cells upon simvastatin treatment has not been investigated until now. Our results demonstrated that exposure of human metastatic melanoma cells (WM9) to simvastatin induces a senescent phenotype, characterized by G1 arrest, positive staining for senescence-associated β-galactosidase assay, and morphological changes. Also, the main pathways leading to cell senescence were examined in simvastatin-treated human melanoma cells, and the expression levels of phospho-p53 and p21 were upregulated by simvastatin, suggesting that cell cycle regulators and DNA damage pathways are involved in the onset of senescence. Since simvastatin can act as a pro-oxidant agent, and oxidative stress may be related to senescence, we measured the intracellular ROS levels in WM9 cells upon simvastatin treatment. Interestingly, we found an increased amount of intracellular ROS in these cells, which was accompanied by elevated expression of catalase and peroxiredoxin-1. Collectively, our results demonstrated that simvastatin can induce senescence in human melanoma cells by activation of p53/p21 pathway, and that oxidative stress may be related to this process.

Introduction

Cellular senescence was first described by Hayflick and Moorhead and characterized as the finite capacity for replication of cells in culture [1]. The main mechanism involved in replicative senescence is telomere shortening, which functions as an internal clock, preventing chromosomal instability after division [2]. Therefore, short telomeres are associated with age-related human diseases, as well as several premature-aging syndromes [3]. Salient features of senescent cells are the cell-cycle arrest, altered responsiveness to apoptotic stimuli, altered gene expression of various cell growth-regulatory proteins, morphological changes (increase in cell size, flattened appearance, and frequent multinucleation), and senescence-associated β-galactosidase activity [4]. It was also reported that activation of cellular oncogenes may promote senescence, as a defense mechanism against

*Corresponding author. Fax: +55 41 3266 2042.
E-mail addresses: sheilambw@ufpr.br, shewinnisch@gmail.com (S.M.B. Winnischofer).

0014-4827/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.yexcr.2013.07.026

malignant transformation [5]. Agents that change chromatin structure, damage non-telomeric DNA, or induce oxidative stress also could lead to cellular senescence [6].

Recently, the induction of senescence in tumor cells through the use of anticancer agents has been described. Therapy-induced senescence (TIS) can be induced by drugs such as doxorubicin, cisplatin and methotrexate [7–9]. The clinical application of TIS could be based on some advantages: (1) the persistent induction of cell-growth arrest, (2) an increased susceptibility to cytotoxic immune response, and (3) possible reduction of side effects caused by drug toxicity, due to the observation that TIS can be induced by the use of lower concentrations of chemotherapeutic agents, while high concentrations induce apoptosis [10].

Among the agents with anticancer properties are the statins. Their properties are characterized by apoptosis induction and cell-proliferation arrest in several types of tumor cells [11–14]. Simvastatin was first described as an antilipemic drug derived from fungi, and its anticancer effect began to be investigated after clinical studies indicated that patients who used simvastatin were less prone to develop some types of tumors, such as melanoma [15,16]. In vitro studies showed that simvastatin is able to induce cell death and cell-cycle arrest in various melanoma cell lines, together with decreased cell viability, morphological changes, and increased levels of p21 and p27 [17]. In addition, simvastatin also decreases cell invasion [18]. Together, these lines of evidence indicate the possibility of using simvastatin as an adjuvant in chemotherapy.

This study investigated the induction of TIS mediated by simvastatin in the human metastatic melanoma cell line WM9, and its possible relationship to the stress response caused by disruption of the intracellular ROS balance. We demonstrated that treatment of WM9 cells with simvastatin resulted in a senescent phenotype, with activation of p53/p21 pathway and oxidative stress mediated by an increase in ROS levels and changes in the cellular antioxidant status.

Materials and methods

Cell culture and simvastatin treatment

The WM9 and WM35 human melanoma cell lines were kindly provided by Dr. Meenhard Herlyn of the Wistar Institute, Philadelphia, PA, USA. These cells were cultured in Tu medium (80% MCDB medium and 20% L-15 medium, Sigma-Aldrich) supplemented with 2% fetal bovine serum (FBS, Gibco), 2 mM $CaCl_2$, 5 μg/mL insulin and 50 μg/mL gentamicin (Sigma-Aldrich). Melanoma cells were maintained at 37 °C in a 5% CO_2 atmosphere. Simvastatin was obtained as courtesy of Merck Sharp and Dohme Laboratories, and was diluted in ethanol. The cells were treated with simvastatin at 0.05, 0.1, 0.25, 0.5 and 1 μmol/L. Cultures were incubated for periods of time from 72 h to 168 h, and were replenished with simvastatin every 72 h. Control conditions consisted of melanoma cells maintained in culture medium and melanoma cells maintained in culture medium plus 0.025% ethanol (equivalent to 0.25 μmol/L simvastatin treatment).

Cell viability analysis

Cell viability was evaluated by crystal violet staining. Cells were seeded in 24-well culture plates at a density of 1×10^3 cells/well and incubated for adhesion during 24 h. Then, the normal medium was replaced by medium containing the above-mentioned concentrations of simvastatin, and incubated for the mentioned times. After treatment, the medium was removed, and the cells were washed with phosphate saline buffer (PBS) solution and fixed with 100% methanol during 10 min. The cells were immersed in a solution of 0.2% crystal violet in 2% ethanol for 3 min, and then washed with PBS until the excess crystal violet was cleared. Then, an elution step was performed with 0.05 mol L^{-1} sodium citrate diluted in 50% ethanol, during 10 min. The absorbance was determined in a microplate reader (Infinite 200, Tecan Group) at 540 nm.

Annexin V-FITC/PI staining

The occurrence of cell death was assessed by staining WM9 cells with Annexin V-FITC/PI. Briefly, cells were plated in 60-mm culture plates (1×10^5 cells/plate) and treated with simvastatin. After 72 h, the supernatant was collected, and the adherent cells were washed, harvested and added to the collection tubes. The cell suspension was centrifuged (2000 rpm, 5 min), the supernatant was discarded, and the cell pellet was resuspended in 0.5 mL of ligation buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, pH 7.4). Then, the cell suspension (0.1 mL) was incubated with 5 μL of Annexin V-FITC (BD Biosciences) for 15 min. After this time, 0.4 mL of ligation buffer was added to each sample and the fluorescence was measured in a FACSCalibur flow cytometer (BD Biosciences), using a FL1-H filter. In addition, cell suspensions were incubated with 50 μg/mL of propidium iodide (PI), and fluorescence was measured using FL2-H filter. Graphical analyses were performed by the Cyflogic software 1.2.1 (developed by Cyflo Ltd., available at http://www.cyflogic.com).

Cell cycle analysis

To evaluate the cell-cycle distribution of WM9 cells after simvastatin treatment, a flow cytometry analysis was performed. For this, cells were plated in 60-mm culture plates at a density of 1×10^5 cells/plate and incubated for 24 h to allow them to adhere. Next, they were treated with simvastatin for 72 h. The cells were collected, and the pellet was washed twice by centrifugation in PBSA. After the wash procedure, the cell pellet was incubated with a cold staining solution of propidium iodide (PI) 50 μg/mL, 0.1% Triton X-100, 0.1% sodium citrate and 0.2 mg/mL RNAse A for 30 min in the dark. Subsequently, the samples were run in a FACSCalibur System and analyzed using Cyflogic software.

Senescence-associated β-galactosidase staining and morphological analysis

Senescence-associated β-galactosidase activity was evaluated using the Senescence β-galactosidase Staining Kit (Cell Signaling), following the manufacturer's instructions. Briefly, 1.5×10^4 cells per well were plated on 6-well culture plates and incubated for 24 h to allow them to adhere. The medium was removed, and the simvastatin treatment was applied. After this, the medium was withdrawn, and cells were washed with a PBS solution. The cells were fixed with 1 mL of fixative solution during 15 min, and then washed twice with PBS. Subsequently, the cells were incubated