Kinetics of the Cell Biological Changes Occurring in the Progression of DNA Damage-Induced Senescence

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Cellular senescence is characterized by cell-cycle arrest accompanied by various cell biological changes. Although these changes have been heavily relied on as senescence markers in numerous studies on senescence and its intervention, their underlying mechanisms and relationship to each other are poorly understood. Furthermore, the depth and the reversibility of those changes have not been addressed previously. Using flow cytometry coupled with confocal microscopy and Western blotting, we quantified various senescence-associated cellular changes and determined their time course profiles in MCF-7 cells undergoing DNA damage-induced senescence. The examined properties changed with several different kinetics patterns. Autofluorescence, side scattering, and the mitochondria content increased progressively and linearly. Cell volume, lysosome content, and reactive oxygen species (ROS) level increased abruptly at an early stage. Meanwhile, senescence-associated β-galactosidase activity increased after a lag of a few days. In addition, during the senescence progression, lysosomes exhibited a loss of integrity, which may have been associated with the accumulation of ROS. The finding that various senescence phenotypes matured at different rates with different lag times suggests multiple independent mechanisms controlling the expression of senescence phenotypes. This type of kinetics study would promote the understanding of how cells become fully senescent and facilitate the screening of methods that intervene in cellular senescence.

INTRODUCTION

Normal cells enter a state of replicative senescence after a prolonged division. Short and unprotected telomeres resulting from prolonged DNA replication trigger a continuous DNA damage response, which leads to permanent arrest of the cell cycle (Campisi et al., 2001). Senescence results in specific cell biological changes, which include enlargement and flattening of the cytoplasm, increased production of reactive oxygen species (ROS), accumulation of lipofuscin, increased mitochondrial and lysosomal mass and their cellular contents, and loss of mitochondrial membrane potential (MMP) (Hwang et al., 2009). Senescent cells also express cytosolic and nuclear markers such as senescence-associated β-galactosidase (SA β-Gal) activity (Dimri et al., 1995) and senescence-associated heterochromatin foci (SAHF) (Nakata et al., 2006). These have been referred to as senescence phenotypes, but their underlying mechanisms and relevance to aging physiology have only been discussed recently (Adams et al., 2008; Campisi and d’Adda di Fagagna, 2007; Krizhanovsky et al., 2008). Cells can be driven to enter a state of senescence through ways that do not involve continuous cell division. Normal cells can be acutely induced to enter senescence by the overexpression of oncogenic Ras or Raf genes (Serrano et al., 1997; Zhu et al., 1998). This ‘oncogene-induced premature senescence’ has led the hypothesis that senescence might have developed as a cellular device to suppress tumor development (Campisi et al., 2007). In addition, cells can undergo senescence after exposure to a DNA-damaging insult (Toussaint et al., 2000). This ‘stress-induced senescence’ can be induced in normal as well as cancer cells. Both premature senescence and stress-induced senescence are generally assumed to express the same panel of phenotypes that are expressed in replicative senescence. The only difference may be that, in the induced cases, the phenotypes are acutely expressed within several days of the oncogene expression or stress imposition. Interest in induced senescence has recently increased because of its potential physiological role. First, for oncogene-induced senescence, the hypothesis regarding its tumor-suppressive role has been well supported by the finding that cells express senescence phenotypes in tumor masses or nevi in model animals (Mooi and Peeper, 2006). Second, the DNA damage-induced senescence of cancer cells suggests that, in addition to apoptosis, senescence may play a role in the cancer treatment effects of chemotherapeutic drugs or radiation. While apoptosis is a dominant mode of tumor cell death during the treatment of certain cancers such as leukemia and lymphoma, it is becoming more apparent that senescence is the predominant fate of cells in the treatment of solid-type tumors (Elmore et al., 2005; Gewirtz et al., 2008). Importantly, these findings suggest a possibility that accelerating the onset or the process of senescence may be beneficial for protecting against cancer development as well as for cancer therapy.

For effective senescence modulation, a better understanding of the properties of senescent cells is required. However, so far, senescence phenotypes have rarely been studied for their ex-

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pression kinetics or analyzed in quantitative terms. Therefore, it is not known whether all senescence phenotypes are fully expressed once cells are growth arrested or after a certain incubation period and whether their pattern of expression is abrupt or progressive. Quantitative measures of the expression levels of the phenotypes may help grading the depth or matureress of the senescence of a population of cells. This information may be utilized in determining the effectiveness of an intervening treatment, improving the methods used to detect senescent cells in tissues, and facilitating identification of the pathological and physiological roles of senescence in vivo. In addition, such studies are practically important. Most studies on cellular senescence have examined only one or two phenotypes at a single time point without quantitative measures. Such approach may easily lead to under- or over-estimations of the properties related to the senescence of the cells in the study. For example, an assumption that a population of cells is senescent based solely on SA-β-Gal activity, which is positive in only a fraction of the cells, can provide misleading results.

MCF-7 cells, a human breast cancer line, undergo senescence after a pulse of a moderate dose of adriamycin (doxorubicin) (Elmore et al., 2002; Song and Hwang, 2005). In the present study, a time-course study was carried out on the levels of the senescence phenotypes expressed in the adriamycin-treated MCF-7 cells. In response to the DNA damage, MCF-7 cells were immediately arrested, and various senescence phenotypes were subsequently expressed during the chase period. The quantitative changes of the cell volume, SA-β-Gal activity, and the cellular content and morphology of mitochondria and lysosomes were determined through flow cytometry. This study reveals that senescence indeed quantitatively matures with time. Interestingly, the various cell biological changes did not follow a single pattern of kinetics, but rather were grouped into several different patterns. This study, although restricted to a case of DNA damage-induced senescence, can serve as a model for future studies aiming to understand the cell biology of senescence and its manipulation.

MATERIALS AND METHODS

Cell culture

MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Lonza, USA). To induce senescence, cells were pulsed with 0.25 μM adriamycin (doxorubicin hydrochloride; Sigma-Aldrich Co., USA) for 4 h and chased in fresh medium which was replaced every two days.

Analysis of cell cycle distribution

At each time point, 1 × 10^6 cells were collected and stored in 70% ethanol. Cells were then stained with 10 μg/ml of propidium iodide in phosphate-buffered saline (PBS) containing 1 mM EDTA and 0.2 mg/ml RNaseA. The raw data from flow cytometry were analyzed by CellQuest 3.2 software (BD Biosciences, USA).

In situ staining of SA-β-Gal activity and β-galactosidase assay in solution

For the SA-β-Gal assay, the protocol reported by Dimri et al. (Dimri et al., 1995) was used. Briefly, cells that had been fixed with 3% formaldehyde were washed in PBS (pH 6.0) containing 2 mM of MgCl₂. After incubation overnight at 37°C in β-galactosidase-staining solution [1 mg/ml of X-gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 2 mM MgCl₂], cells were observed with bright-field microscopy. To determine the β-galactosidase activity per cell, cells were lysed in PBS by freeze-thawing. The supernatant was incubated at 37°C for 6 h in reaction buffer [5 mM MgCl₂, 0.55 mg/ml chloromethyl-β-D-galactopyranoside (CMPPR) in 0.1 M phosphate buffer (pH 6.0)]. The reaction was stopped by adding 1 M Na₂CO₃, and the transmittance of light at 570 nm was measured.

Measurement of autofluorescence and contents of lysosomes and mitochondria

For measurement of autofluorescence, cells that were collected in PBS containing 1 mM EDTA were applied to flow cytometry (488 nm excitation and 530 nm emission; BD FACS Canto II, BD Biosciences, USA). For the measurement of lysosome and mitochondria content, the washed cells were incubated with 50 nM of LysoTracker Red or 30 nM MitoTracker Green (Invitrogen/Molecular Probes, USA) for 30 min and then applied to flow cytometry with 488 nm excitation/S585 nm emission or 488 nm excitation/S530 nm emission, respectively.

Assessment of lysosomal integrity

An acridine orange (AO) uptake and relocation assay (Erdal et al., 2005; Kokkonen et al., 2004) was used to determine lysosomal integrity. Briefly, cells cultured on a cover slip were treated with 20 μg/ml AO for 15 min. Confocal microscopy was carried out with excitation at 488 nm and emission through the filter for either Texas red (for red emission) or FITC (for green emission).

Measurement of ROS and mitochondrial membrane potential (MMP; ΔΨm)

For quantification of the superoxide anions produced in the mitochondria, cells were incubated with 0.1 μM MitoSox (Invitrogen/Molecular Probes) for 30 min and applied to flow cytometry. For the measurement of MMP, cells were incubated with 0.3 μg/ml JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide) for 30 min and applied to flow cytometric analysis with excitation at 488 nm radiation. The emissions at 530 nm (FL-1; for fluorescence from monomeric JC-1) and at 585 nm (FL-2; for that from JC-1 aggregates) were monitored, and the FL2/FL1 ratio of the individual cells was calculated by using WEASEL software (http://www.wehi.edu.au/cytometry/WEASELv2.html). The mean values of the FL2/FL1 ratio of the samples were plotted by using SigmaPlot 9.01 software (Systat Software, Inc.).

Western blotting

Cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with NaF, NaVO₃, and a protease-inhibitor mixture (Sigma). Typically 30-40 μg of proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Hybond ECL; Amersham, USA), and blotted with one of the following primary antibodies: human ERK 1/2 (C-16) (Santa Cruz Biotechnology, Inc.), p53 (DO-1) (Santa Cruz Biotechnology, Inc.), p35 (DO-1) (Santa Cruz Biotechnology, Inc.), p21WAF1/CIP1 (C-19) (Santa Cruz Biotechnology, Inc.), E2F1 (C-20) (Santa Cruz Biotechnology, Inc.), 4E-BP1 (Cell Signaling Technology, Inc.), phospho-4E-BP1 (Cell Signaling Technology, Inc.), p70 S6Kinase (Cell Signaling Technology, Inc.), and phospho-p70 S6Kinase (Thr421/Ser424) (Cell Signaling Technology, Inc.).

RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer’s protocol. A total of 5 μg of RNA was