Mechanisms of Ageing and Development 146–148 (2015) 65–71

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

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

Relief of delayed oxidative stress by ascorbic acid can suppress radiation-induced cellular senescence in mammalian fibroblast cells

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ARTICLE INFO

Article history:
Received 24 December 2014
Received in revised form 6 April 2015
Accepted 4 May 2015
Available online 6 May 2015

Keywords:
Ionizing radiation
Oxidative stress
Radiation-induced cellular senescence
Ascorbic acid

ABSTRACT

Ionizing radiation-induced cellular senescence is thought to be caused by nuclear DNA damage that cannot be repaired. However, here we found that radiation induces delayed increase of intracellular oxidative stress after irradiation. We investigated whether the relief of delayed oxidative stress by ascorbic acid would suppress the radiation-induced cellular senescence in Syrian golden hamster embryo (SHE) cells. We observed that the level of oxidative stress was drastically increased soon after irradiation, then declined to the level in non-irradiated cells, and increased again with a peak on day 3 after irradiation. We found that the inductions of cellular senescence after X-irradiation were reduced along with suppression of the delayed induction of oxidative stress by treatment with ascorbic acid, but not when oxidative stress occurred immediately after irradiation. Moreover, treatment of ascorbic acid inhibited p53 accumulation at 3 days after irradiation. Our data suggested a delayed increase of intracellular oxidative stress levels plays an important role in the process of radiation-induced cellular senescence by p53 accumulation.

1. Introduction

It is well known that normal human fibroblasts have limited division potential and become cellular senescence (Kashino et al., 2003; Yang et al., 1998). Cellular senescence is also known to be induced by ionizing radiation (Suzuki et al., 2001), hydrogen peroxide (H_2O_2) (Chen et al., 1998), and oncogenes such as Rat sarcoma protein (Ras) (Serrano et al., 1997). Senescent cells are still metabolically active, but they irreversibly stop their growth and change their morphological characteristics (Blander et al., 2003; Hagen et al., 1997). These changes are caused via the activation of tumor protein p53 (p53), p16 and other senescence associate proteins, which are well-known tumor suppressor genes. Therefore, it is thought that senescence suppresses cellular immortalization and protects against carcinogenesis. However, the mechanism of the induction of cellular senescence is still unclear, but there are a number of reports suggesting the involvement of reactive oxygen species (ROS) (Chen et al., 1998; Dimri et al., 1995; Hayflick and Moorhead, 1961; Kashino et al., 2003; Linford et al., 2006; Orr and Sohal, 1994; Packer and Fuehr, 1977; Schriner et al., 2005). One of these reports showed that treatment with hydrogen peroxide or cell culturing under hyperoxic conditions may lead to premature cellular senescence (Chen et al., 1998; Packer and Fuehr, 1977; von Zglinicki et al., 1995), and the relief of oxidative stress could retard this cellular senescence (Kashino et al., 2003; Linford et al., 2006; Orr and Sohal, 1994; Packer and Fuehr, 1977; Schriner et al., 2005). These findings support the free radical theory of aging.

Radiation-induced senescent cells have morphologies similar to spontaneously senescent cells, with enlarged and flattened shapes, and are stained positive by senescence-associated beta-galactosidase (SA-β-gal) staining, a marker of cellular senescence (Suzuki et al., 2006). However, the involvement of oxidative stress in radiation-induced cellular senescence has been less studied. Because radiation-induced cellular senescence is thought to be caused by p53 activation related to DNA double-strand breaks (DSB) induced by radiation exposure (Suzuki et al., 2001). Ataxia-telangiectasia mutated (ATM) protein is well known key protein for DNA damage response. ATM changes its formation dimer to monomer with autophosphorylation it's Ser1981 in response to DSB (Bakkenist and Kastan, 2003). Activated ATM phosphorylates many proteins such as p53 at Ser15. Phosphorylated p53 promotes transcription of p21 then activates G1/S cell cycle arrest (Mirzayans et al., 2013). Recently we and other groups reported there is delayed increase of oxidative stress from mitochondria after radiation

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http://dx.doi.org/10.1016/j.mad.2015.05.002
0047-6374/

exposure (Hong et al., 2010; Kobashigawa et al., 2015, 2011; Passos et al., 2010; Yamamori et al., 2012). Several studies reported that radiation induced delayed oxidative stress mediate genomic instability such as increase chromosome aberrations, mutations and reduce clonogenic survival after irradiation (Dahle and Kvam, 2004; Kim et al., 2006; Limoli et al., 2003; Samper et al., 2003). However, it would be interesting to determine why a delayed increase of oxidative stress may be induced after irradiation. Recent years it is reported that oxidative stress in mitochondria oxidize ATM and activates (Guo et al., 2010). Hence, high intracellular oxidative stress possibly cause ATM and p53 activation. We hypothesized the delayed increase of oxidative stress is involved in radiation-induced cellular senescence via activation of p53, and thereby avoiding damaged cells to proliferate.

In the present study, we found that there was a remarkable increase of oxidative stress 3 days after irradiation, and this increase was a trigger of radiation-induced cellular senescence in Syrian golden hamster embryo (SHE) cells. Ascorbic acid suppresses the delayed induction of oxidative stress and thereby reduces total amount of p53, phosphorylated p53 at Ser15, phosphorylated p38, and then the radiation-induced cellular senescence.

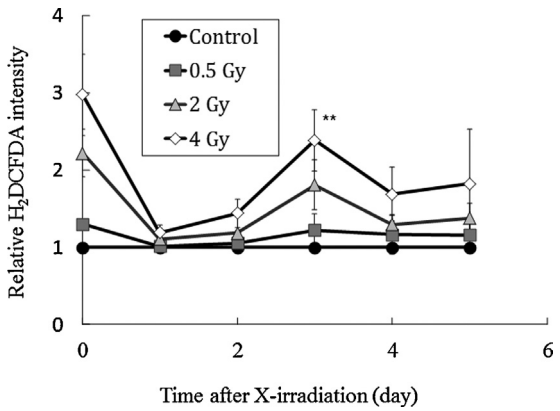

Fig. 1. Generation of reactive oxygen species following X-irradiation of SHE cells. Intracellular ROS levels were detected by the intensity of H_2DCFDA-formed fluorescence determined by flow cytometry analysis. ROS generation was detected each day after irradiation. Error bars indicate the standard error of the mean (SEM) for $N = 3$ independent experiments. The data were significantly different among each dose (ANOVA, $p < 0.005$). ** represents $p < 0.01$ (t test).

2. Materials and methods

2.1. Cell culture and X-irradiation

Syrian golden hamster embryo (SHE) cells were cultured in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT, USA) in a 5% CO_2 incubator at 37 °C (Watanabe et al., 1992). Briefly, 3×10^5 cells were incubated into a 25-cm^2 flask (Becton Dickinson, and Co., Lincoln, NJ, USA) and subcultured every 3 days. Cells were irradiated with X-rays from an X-ray generator (Softex Co., Ebina, Kanagawa, Japan) at 150 kVp and 5 mA with a 0.1-mm copper filter. X-irradiation was administered at a rate of 0.492 Gy/min.

2.2. Measurement of intracellular oxidative stress level

Cells were washed with phosphate buffered saline containing Mg^{2+} and Ca^{2+} (PBS $^+$) twice and then treated with 5 mM 2′,7′-dichlorofluorescein (H_2DCFDA; Invitrogen, Molecular Probes, Carlsbad, CA, USA) in PBS$^+$ solution for 20 min in a 37 °C 5% CO_2 incubator. To measure the oxidative stress level immediately after irradiation, cells were treated with H_2DCFDA solution for 20 min before irradiation, and this treatment was continued during irradiation. After the treatment, cells were trypsinized and suspended in phosphate buffered saline without Mg^{2+} and Ca^{2+} (PBS$^-$) solution, and green fluorescent intensities were measured using a BD FACScan™ (Becton Dickinson, and Co.). Ten thousand cells were measured for each sample, and the data were analyzed using FlowJo software (Tomy Digital Biology Co., Ltd., Nerima, Tokyo, Japan). The experiment was performed more than three times for each time point.

2.3. Detection of radiation-induced senescent cells (SA-β-gal staining)

Cells were washed once with PBS$^-$ and fixed with 2% paraformaldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 0.2% glutaraldehyde (Wako Pure Chemical Industries, Ltd.) for 5 min at room temperature. After fixation, the cells were washed 3–4 times with PBS$^-$ and then incubated with staining solution (40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal; Wako Pure Chemical Industries, Ltd.) overnight. The stained cells were photographed and counted, and the percentage of stained cells to the total number of cells was determined for each sample. More than three independent experiments were performed.

2.4. Ascorbic acid treatment

L-Ascorbic acid (Sigma–Aldrich Co., Tokyo, Japan; Tokyo Chemical Industry, Tokyo, Japan) was dissolved in water to a concentration of 1 M. Then, the solution was added to the culture medium at a final concentration of 1 mM.

2.5. Measurement of cell viability

Cells plated onto T25 flask was irradiated with 4 Gy of X-ray. Twenty minute after irradiation, cells were treated with fresh medium containing 1 mM ascorbic acid, then 72 h after irradiation, cells were trypsinized and suspended in medium containing 0.2%

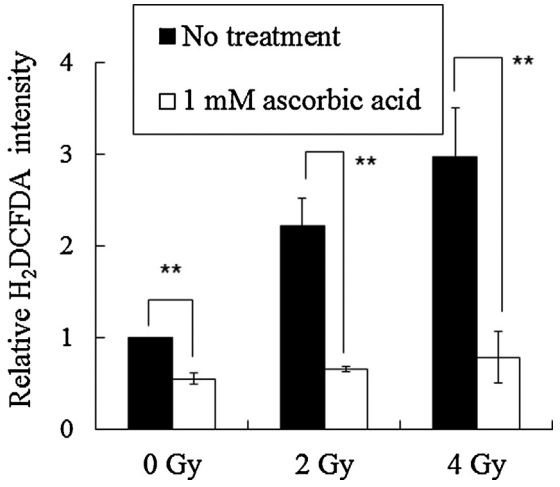

Fig. 2. ROS generation detected immediately after X-irradiation. Intracellular ROS levels were detected by the intensity of H_2DCFDA-formed fluorescence determined by flow cytometry analysis. SHE cells were treated with 5 μM H_2DCFDA from 20 min before and during irradiation. White bars indicate the cells treated with 1 mM ascorbic acid for 1 h. Error bars indicate the standard error of the mean (SEM) for $N = 3$ independent experiments. ** represents $p < 0.01$ (t test).