Biochemical and Biophysical Research Communications 407 (2011) 406–411

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Loss of p21^{Sdi1} expression in senescent cells after DNA damage accompanied with increase of miR-93 expression and reduced p53 interaction with p21^{Sdi1} gene promoter

Ok Ran Choi, In Kyoung Lim *

Department of Biochemistry and Molecular Biology, Ajou University School of Medicine, Suwon 443-721, Republic of Korea

ARTICLE INFO

Article history:
Received 4 March 2011
Available online 12 March 2011

Keywords:
p21$^{sdi1/cip1/waf1}$
p53
miR-93
Doxorubicin
HDF
Senescence

ABSTRACT

To answer what is a critical event for higher incidence of tumor development in old than young individuals, primary culture of human diploid fibroblasts were employed and DNA damage was induced by doxorubicin or X-ray irradiation. Response to the damage was different between young and old cells; loss of p21^{sdi1} expression in spite of p53^{S15} activation in old cells along with [^{3}H]thymidine and BrdU incorporation, but not in young cells. The phenomenon was confirmed by other tissue fibroblasts obtained from different donor ages. Induction of miR-93 expression and reduced p53 binding to p21 gene promoter account for loss of p21^{sdi1} expression in senescent cells after DNA damage, suggesting a mechanism of *in vivo* carcinogenesis in aged tissue without repair arrest.

1. Introduction

A growing body of evidence suggests that cellular senescence is an important and evolutionarily conserved tumor-suppression mechanism, acting as a natural barrier to cell immortalization and transformation [1,2]. However, a striking link between advanced age and increased incidence of cancer is also evident in human and laboratory animals [3]. Inactivation of p53 or pRb tumor suppressor gene is essential to bypass replicative senescence; disruption of only p53 or pRb results in a slight increase of life span, but inactivation of both genes causes somewhat greater increase of life span, however, the cells then enter into crisis and result in cell death [4–6].

The p21$^{waf1/cip1/sdi1}$ (p21), a cyclin E/Cdk2 inhibitor, is a direct transcriptional target of p53 and links the p53 and pRb pathways in human cells [7–9]. p21 is involved in the Ras^{G12V}-induced senescence in different human cells; inhibition of p21 expression results in Ras^{G12V}-resistant growth in BJ foreskin fibroblasts [10] and LF1 human lung fibroblasts [11]. Treatment of various human cells with low dose doxorubicin (200 ng/ml) also induces growth arrest with high expressions of p53 and p21 [12]. Increased p53 in response to genotoxic damage temporally regulates selective binding to p21 and MDM2 promoters; p21 induces growth arrest with DNA damage repair, whereas MDM2 degrades p53 in human cells. In contrast to the genotoxic damage, replicatively senescent cells reveal failure of p53 activation by phosphorylation on serine residues and by acetylation on lysine residues, therefore, p53 binding to p21 and GADD45 is specifically higher than the target genes regulating apoptosis [13]. On the other hand, critically short human telomeres induce senescence either by activating p53 or p16^{INK4a}/pRB pathway, and suppression of both pathways is required to suppress senescence of aged human cells. Loss of p53 function was sufficient to completely abrogate senescent growth arrest in human cells, indicating that the p16^{INK4a}/pRB response to telomere dysfunction is not active in mouse cells [14]. These reports provide us how cells are directed to senescence by tumor suppressor p53 and why senescent cells are resistant to apoptosis, instead the cells reveal deleterious effects including ageing phenotypes and tumor promotion in neighboring cells.

By using primary cultures of human diploid fibroblasts (HDF), we have previously reported that expression of p21 in senescent HDF is dependent of Sp1 phosphorylation on serine^{59} by PKCα and its downstream kinase Erk1/2, activated by reactive oxygen species [15]. siRNA constructs against the kinases make senescent HDF to be proliferative. In this study, we addressed what is a molecular mechanism of higher development of cancer in aged person, in which most cells are already senescent.

It has been reported that DNA double strand breaks (DSBs) are repaired by nonhomologous end joining (NHEJ) and homologous recombinational repair (HRR), and NHEJ is faster and more efficient than HRR in human cells [16]. The activity of NHEJ varies depending on cell cycle phase (G1 < S < G2/M) in which the damage is inflicted [17] and Ku protein is significantly reduced in senescent

* Corresponding author. Fax: +82 31 219 5059.
E-mail address: iklim@ajou.ac.kr (I.K. Lim).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.03.038

cells [18], therefore, efficiency of end joining is reduced up to 4.5-fold in senescence and old cells contain more mutations, indicating that NHEJ might be activated more in senescent cells, because HRR is active only in S phase [19]. In this report, we examined damage response to DSB inducing agent, doxorubicin and X-ray, and found differential response of p21 expression in young and senescent cells: young cells highly induced p21 expression after DSB, whereas senescent cells rather reduced p21 expression along with increased miR-93 expression and decreased p53 binding to p21 promoter in spite of $p53^{S15}$ activation in both cells. Since, p21 is important for DNA damage-induced senescence as well as growth arrest for DSB repair, we proposed a model whereby the inability of presenescent cells to induce p21 expression in response to DSB (and perhaps other cellular stresses) underlies an increased tendency toward tumor development among aged individuals with greater number of replicatively aged cells.

2. Materials and methods

2.1. Material

Antibodies against PARP, procaspase 3 and $p53^{S15}$ were purchased from Cell Signaling. Anti-p53, -p21 and -GAPDH were from Santa Cruz Biotechnology. BrdU and anti-actin were from Sigma. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and trypsin were from GIBCO-BRL. [^{3}H]thymidine was from Amersham (Buckinghamshire, England).

2.2. Preparation of human diploid fibroblasts from child foreskin and adult buttock

Primary culture of HDF was prepared in our laboratory from foreskin of 4 year-old boy [15,20–22] and maintained in DMEM-high glucose supplemented with 10% heat inactivated fetal bovine serum under 5% CO_2 in air. To evaluate characteristics of *in vivo* cellular senescence, skin fibroblasts were freshly isolated from buttock of 24–75 year-old men and cultivated until use. Doubling times (DT) of HDF used in this study were calculated based on the equation [15]; mean 26 h in young cells, 2–10 days in presenescent cells and over 14 days in senescent cells.

2.3. Immunoblot analyses

Cells were washed 3 times with ice-cold PBS and lysed with modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid) plus protease and phosphatase inhibitors (1.0 μg/ml leupeptin, 100 μg/ml PMSF, 1 mM Na_3VO_4, 1 mM NaF). Lysates were clarified by centrifugation at 12,000*g* for 10 min. Cell lysates (50 μg) were resolved by 8–13% SDS–PAGE and hybridized with antibody, then developed with ECL.

2.4. Reverse transcription-polymerase chain reaction

Total cellular RNAs (0.5 μg) isolated with RNAiso Plus were used for cDNA preparation and then amplified by PCR kit (Takara Inc., Japan); denaturation at 94 °C for 30 s, annealing at 52 °C for 40 s, and elongation at 72 °C for 30 s for GAPDH in 28 cycles and 30 cycles for p21. Oligonucleotide primer pairs used for amplification of p21 and GAPDH were 5′-CGACTGTGATGCGCTAATGG-3′ and 5′-CCGTTTTCGACCCTGAGAG-3′, and 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′, respectively.

2.5. [^{3}H]Thymidine incorporation analysis

Young and senescent HDF cells were plated in 30% confluent and the monolayers were stabilized for 48 h before doxorubicin treatment for 24 h. Before harvesting the cells, 2 μCi/ml [^{3}H]thymidine was added for 4 h, and the monolayers were then thoroughly rinsed 3 times with ice-cold PBS before lysis with 200 μl of RIPA buffer. Radioactivity incorporated was measured by liquid scintillation counter (Tri-Carb^R Liquid Scintillation Analyzer 2100 TR, Packard) in 2 ml of EcoLite(+)™ cocktail solution (MPbio, CA).

2.6. BrdU incorporation assay

It was followed by the method [23]: Cells were prelabelled with 10 μM BrdU for 2 h before trypsinization, fixed in 70% ethanol, and

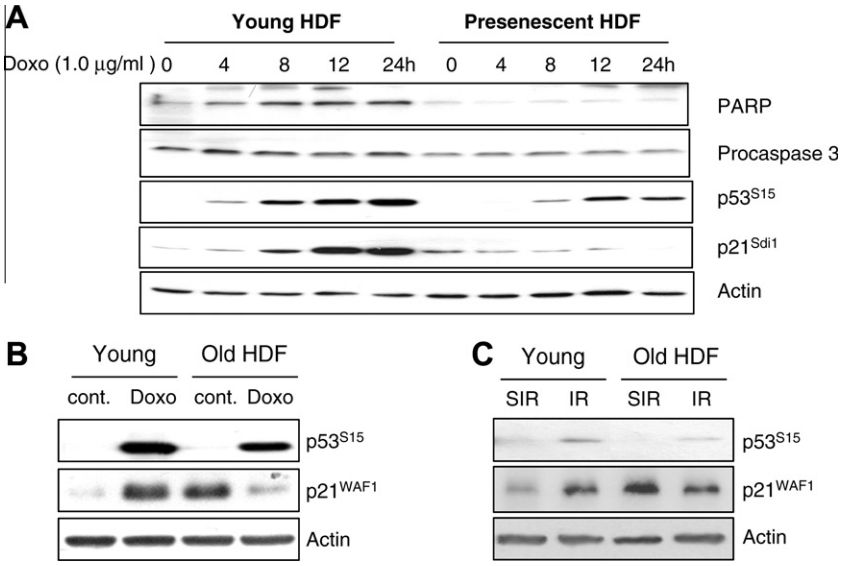

Fig. 1. Loss of $p21^{Sdi1}$ expression in senescent cells in response to DNA strand breaks. Young and senescent HDF were treated with doxorubicin for 24 h, and damage response was monitored; (A) the treatment induced PARP expression in young cells, but not in presenescent cells. Young and senescent HDF normally responded to DNA damage by $p53^{S15}$ phosphorylation, however, presenescent cells failed to induce p21 expression until 24 h. Note no change of procaspase 3 cleavage in young and presenescent cells. Loss of p21 induction in the fully senescent HDF cells (DT14d and DT18d) either by doxorubicin treatment for 24 h (B) or X-ray irradiation (C). Note marked induction of p21 expression in young HDF, but the loss in old cells. IR; X-ray irradiation (6 Gy, 2 h incubation), SIR; sham irradiation.