Journal of Life Science 2009 Vol. 19. No. 4. 436~441 ©JLS / ISSN 1225-9918

The Stability of p53 in Ras-mediated Senescent Cells in Response to Nucleolar Stress

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Received January 14, 2009 / Accepted January 30, 2009

B23/nucleophosmin, a nucleolar protein, translocates into the nucleus from the nucleolus when cells are damaged by extracellular stresses. Recently, it was shown that such translocation of B23/nucleophosmin in normal fibroblasts under stress conditions increases both the stability and activation of the p53 protein by disrupting its interaction with MDM2. Senescent cells have a single large nucleolus and a diminished capacity to induce p53 stability upon exposure to various DNA damaging agents. To investigate the role of B23/nucleophosmin in p53 stability in senescent cells, we established a senescence model system by expressing the ras oncogene in IMR90 cells. The stability of p53 was reduced in these cells in response to nucleolar stress, although the level of B23/nucleophosmin protein was not changed. In addition, p53 did not accumulate in the nucleus and B23/nucleophosmin did not translocate into the nucleoplasm. The binding affinity of B23/nucleophosmin with p53 was reduced in senescent cells, whereas the interaction between MDM2 and p53 was stable. Taken together, the stability of p53 in ras-induced senescent cells may be influenced by the ability of B23/nucleophosmin to interact with p53 in response to nucleolar stress.

Key words : B23/nucleophosmin, senescence, p53, IMR90, nucleolar stress

Introduction

According to the Hayflick limit, normal diploid fibroblast cells have a finite proliferative potential [6]. After reaching the passage limit the cells enter replicative senescence, undergoing irreversible growth arrest. The cellular morphologies become altered and are easily detected by staining for senescence-associated beta-galactosidase (SA-β-gal) activity and imaging of senescence-associated heterochromatic foci (SAHF) [11]. Replicative senescence is caused by progressive telomere shortening, leading to genomic instability.

The tumor suppressor p53 acts as a cellular stress sensor. In response to different types of stress including DNA damage, p53 becomes stabilized and is activated to limit cellular proliferation by inducing either cell cycle arrest or apoptosis [5]. However, in senescent cells the ability of p53 to become stabilized is impaired, and so the cells are unable to undergo p53-dependent apoptosis in response to DNA damage [15]. The absence of p53-dependent apoptosis is caused by senescence-specific post-translational modification of p53 [1,14,19]. However, it is not fully understand at the molecular level why p53 fails to become stabilized and activated in senescent cells in response to stress on DNA.

A number of recent studies of the nucleolus suggest that, in addition to its role in ribosomal biogenesis, the nucleolus may act as a stress sensor linked to p53 activation [10,13,23]. In response to cellular stresses, the nucleolus undergoes fragmentation and releases proteins to the nucleoplasm to induce p53 activation. On such nucleolar factor is the phosphoprotein B23/nucleophosmin [22]. In normal cells in response to DNA damage, B23/nucleophosmin binds to MDM2 as a negative regulator of the p53-MDM2 complex, leading to induced p53 stabilization and transcriptional activation [2,7]. However, little is known about p53 stability associated with B23/nuclophosmin in senescent cells under genotoxic stress. Therefore, in the present study, we investigated the association of B23/nucleophosmin with p53 in ras-induced senescent cells in response to nucleolar stress.

Materials and Methods

Cell culture and transfection

Human lung primary fibroblasts (IMR-90) were cultured in Dulbecco's modified medium supplemented with 10% fe-

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tal bovine serum, and incubated in a humidified incubator at 37°C with 5% CO_2. IMR90 cells were infected by a retrovirus system as described below. Cells were grown to 70~80% confluence on coverslips in 35-mm dishes and transfected with GFP-B23 or HP1β-DsRed vector by using ExGen500 reagent (MBI Fermentas, Hanover, USA) following the manufacturer's instructions. At 24 hr post-transfection, cells were treated with UVC (ULTRA-LUM; 254 nm, 40 J/m^2) or actinomycin D (50 ng/ml). After 6 hr, cells were harvested and stored at -80°C until they were processed for western blot analysis or immunofluorescence assay.

Retrovirus infection

Retroviral stocks were produced by using the pBabe retroviral vector expressing a human H-RasV12 cDNA [9]. Retroviral infection was performed using high-infectivity retroviral stocks generated by transient transfection of the HD29 retrovirus packaging cell line as described [16]. Infected IMR90 cells were selected by puromycin (1.5 μg /ml) for 2 days to eliminate uninfected cells. At post-infection day 6, cells were analyzed.

Senescence-associated-β-galactosidase staining

Cells were washed twice with PBS (pH 7.2), and fixed with 0.5% glutaraldehyde/2% formaldehyde in PBS (pH 7.2) for 5 min. After washing, cells were stained with X-gal solution [1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$] overnight at 37°C and observed using microscope.

Immunoprecipitation

Prepared cells were lysed in RIPA buffer [50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% NP-40, 50 mM NaF, 200 μM Na_3VO_4, 1 mM EDTA, 0.5% sodium deoxyhcolate, 0.1% SDS and protease inhibitor cocktail tablets (Roche, Meylan, France)] on ice for 30 min. After centrifugation, lysates were reacted with specific antibodies (1 μg) for 2 hr and protein G resins were added for an additional 1 hr in a 4°C cold chamber. After immunoprecipitation, samples were boiled in SDS loading buffer for 5 min and analyzed by 12.5% SDS-PAGE. The samples were transferred to nitrocellulose membrane for western blotting.

Western blot analysis

Ras-infected cells were washed with cold PBS and lysed in NP-40 lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 50 mM NaF, 200 μM Na_3VO_4, protease inhibitor cocktail tablets (Roche, Meylan, France) and 0.1% SDS]. After 30 min on ice, lysates were cleared by centrifugation. Cell lysates were resolved by 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose. Western blot analyses were done according to standard procedures using ECL detection (Amersham). The primary antibodies used were as follow: p53 (1:1,000 dilution, Santa Cruz SC-126), MDM2 (1:250 dilution, Abcam ab-16895) and B23 (1:1,000 dilution, Zymed 32-5200).

Immunofluorescence assay

IMR90 cells expressing the H-RasV12 gene from a retroviral vector were cultured on coverslips for 24 hr and then treated with actinomycin D (50 ng/ml). Six hours later, cells were fixed with 4% paraformaldehyde for 15 min, and washed with PBS. After blocking with 10% horse serum, coverslips were incubated with p53 antibody at a 1:200 dilution in a humidified chamber for 1 hr. After washing with PBS containing 0.1% NP-40, samples were incubated with secondary antibodies conjugated to TRITC (Sigma Aldrich, Saint Louis, MO, USA) at a dilution of 1:100 in PBS (0.1% NP-40) for 45 min. Cells were stained with DAPI (0.1 μg/ml) for 5 min to detect nuclei. To stain SAHF, cells were fixed with 4% paraformaldehyde for 15 min and stained with DAPI (0.1 μg/ml). Samples were mounted and visualized using an LSM 510 microscope (Carl Zeiss, Oberkochen, Germany).

Results

Establishment of ras-mediated senescent IMR90 cells

To study whether B23/nucleophosmin regulates the stability and activity of p53 protein in aging cells, we used a well-characterized oncogenic ras-mediated cellular senescence system to induce senescence in normal fibroblasts that is known to be indistinguishable from replicative senescence [9,16]. Oncogenic ras was introduced into normal human diploid IMR90 fibroblasts using a retroviral method. Most of the ras-infected cells became flat and enlarged at 6 day after puromycin selection. To confirm the senescence phenotype, we examined SA-β-gal activity in the cells. A significant proportion of the ras-transduced cells showed staining typical of senescent cells (Fig. 1A). In addition, these cells displayed another senescence marker, SAHF, which was

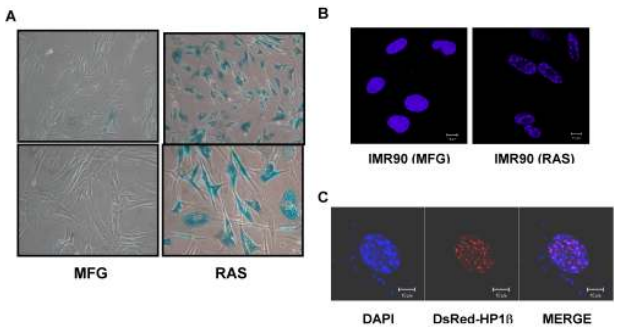

Fig. 1. Establishment of ras-mediated senescent IMR90 fibroblast cells by using a retroviral system. (A) SA-β-gal activity assay was measured in IMR90 cells transduced with an empty vector (MFG) or oncogenic ras (RAS) at 6~8 days after puromycin selection. The cells were visualized by light microscopy. Magnification: ×100 (top), ×200 (bottom). (B) DAPI staining to detect SAHF (senescence associated heterochromatic foci) formation. The retrovirus-infected cells were observed by fluorescence microscopy. Magnification: ×400. (C) IMR90 cells were transduced with an oncogenic ras gene construct for 6~8 days and transfected with HP1b-DsRed plasmid to identify SAHF.

demonstrated by the co-localization of HP1b-DsRed and DAPI foci (Fig. 1B, C). These results indicated that our oncogenic ras-mediated senescence system is appropriate for this study.

Destabilization of p53 in ras-mediated senescent cells under genotoxic stresses

Various stresses, including UV radiation, actinomycin D, and doxorubicin, can lead to nucleolar fragmentation, resulting in activation of p53. It is known that old human fibroblasts are unable to stabilize p53 in response to genotoxic damage [15]. In this study, we determined whether ras-mediated senescent cells could accumulate p53 in response to nucleolar stress caused by UVC radiation (40 J/m^2) or a low dose of actinomycin D (50 ng/ml), which only inhibits RNA polymerase I. As shown in Fig. 2A, p53 expression in normal IMR90 cells was strongly induced by UV treatment; in contrast, no such induction of p53 was observed in senescent IMR90 cells exposed to UV. Treatment with actinomycin D induced an attenuated p53-response in senescent IMR90 cells compared to normal IMR90 cells (Fig. 2B). Taken together, these results show that p53 protein failed to undergo stabilization in senescent cells in response to nucleolar stress.

To examine whether the instability of p53 in senescent cells undergoing nucleolar stress depends on the proteasome

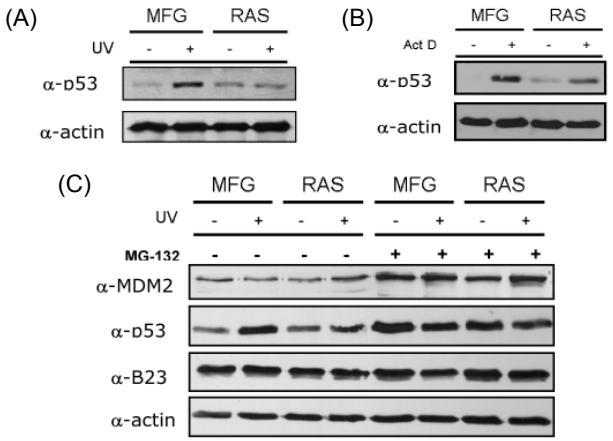

Fig. 2. Protein expression in retroviral ras-infected senescent cells exposed to nucleolar stress. (A) Control vector and oncogenic ras-induced IMR90 cells were irradiated with UV light (40 J/m^2). After 6 hr, cells were harvested for immunoblot analysis. Total cell lysates were analyzed by SDS-PAGE and were immunoblotted by using anti-p53, p21 and pRb antibodies. Actin is shown as a loading control. (B) Cells were treated with MG-132 (+) or not (-). These samples were resolved by SDS-PAGE for immunoblotting with anti-p53, MDM2, B23/nucleophosmin and p21 antibodies. Actin is used as a loading control.

pathway, cells were treated with the proteasome inhibitor MG-132. Interestingly, p53 protein gave a highly intense signal in senescent cells treated with MG-132 (Fig. 2C), in contrast to the results above. In addition, the expression level of B23/nucleophomin was constant, even showing no change in the UV-irradiated cells (Fig. 2C). These results indicate that p53 protein was unable to accumulate in senescent cells subjected to nucleolar stress even though the MDM2-dependent p53-degradation pathway was normal.

Localization of B23/nucleophosmin protein in senescent cells under nucleolar stress

B23/nucleophosmin's interaction with p53 plays a critical role in p53 stability and transcriptional activation in response to genotoxic stresses [2]. Furthermore, B23/nucleophosmin translocates to the nucleoplasm from the nucleolus upon various stresses [13,21]. Therefore, we studied senescent cells to determine whether B23/nucleophosmin could distribute to the nucleoplasm under nucleolar stress. Again we used a low concentration of actinomycin D (50 ng/ml) as a nucleolar stressor. As shown in Fig. 3, GFP-tagged B23/nucleophosmin was distributed to the nucleoplasm in proliferating IMR90 cells exposed to actinomycin D, whereas

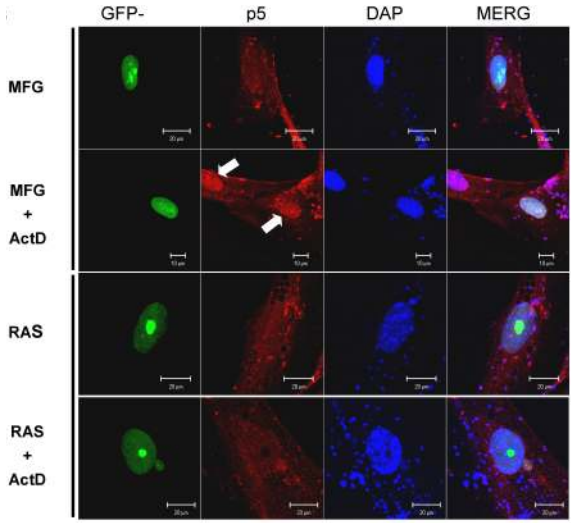

Fig. 3. Distribution of B23/nucleophosmin and p53 in ras-mediated senescent cells under actinomycin D treatment. After transduction with empty vector (MFG) or oncogenic ras (RAS), IMR90 cells were transfected with GFP-B23/nucleophosmin vector to allow its transient expression. After 24 hr, cells were treated with actinomycin D (50 ng/ml). Six hour later, cells were fixed and stained with TRITC-conjugated p53 antibody. DAPI staining indicates the chromosomes. Arrows indicate accumulated p53 in the nucleus.

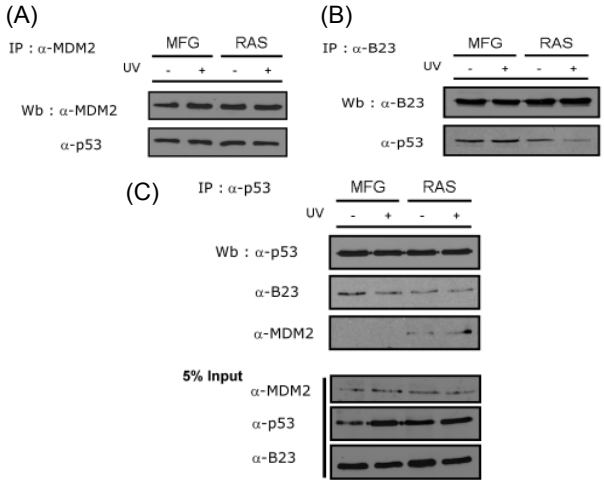

Fig. 4. Formation of complexes between MDM2 and p53 or between B23/nucleophosmin and p53. (A). Virus-infected cells were irradiated with UV light (40 J/m^2) as described in Materials and Methods. Cell lysates were immunoprecipitated with anti-MDM2 and immunoblotted with the indicated antibodies. (B) B23/nucleophosmin antibody was used to immunoprecipitate, and the samples were analyzed by immunoblotting. (C) Samples were immunoprecipitated with anti-p53 antibody and analyzed by immunoblotting. 'Input' indicates whole-cell lysate loaded onto the SDS-polyacrylamide gel.

in senescent cells exposed to the drug, B23/nucleophosmin remained in the nucleolus. In addition, p53 accumulated in proliferating cells but not in senescent cells in response to actinomycin D-induced nucleolar stress. Therefore, in senescent cells, B23/nucleophosmin may be sequestered, preventing its association with p53 in response to nucleolar damage.

Dissociation of B23/nucleophosmin from p53 in senescent cells in response to nucleolar stress

The stability of p53 is regulated by MDM2 and B23/nucleophosmin. In this study, we observed that the interaction between p53 and MDM2 was strong in senescent cells (Fig. 4A), suggesting that the MDM2-mediated p53 degradation pathway is functionally normal. Based on the observed B23/nucleophosmin localization pattern in senescent cells, we estimated that the interaction between p53 and B23/nucleophosmin would be weak in such cells subjected to nucleolar stress. Indeed, as shown in Fig. 4B, the binding affinity between p53 and B23/nucleophosmin in senescent cells was diminished by UV damage. Moreover, when lysates of virus-infected senescent cells were subjected to immunoprecipitation by p53 antibody, MDM2 protein showed a slightly improved binding affinity with p53 (Fig. 4C). However, the interaction of B23/nucleophosmin with p53 was decreased in senescent cells. These data indicate that B23/nucleophosmin has a reduced capacity to form complexes with p53 in senescent cells, leading to reduced p53 stability in response to stress.

Discussion

In old fibroblasts undergoing senescence, p53 protein is unstable in response to many types of stresses, leading to cell necrosis instead of apoptosis [15]. The reduction in p53 stability in aged cells undergoing genotoxic stress may be caused by impaired function of the upstream signaling pathway such as a senescence-specific phosphorylation pattern [1,14,19]. In this study, we used the ras-mediated cellular senescence model system to mimic aging cells.

The expression of p53 protein is tightly regulated to induce apoptosis and cell-cycle arrest. p53 stability is mediated through the MDM2-dependent proteasome pathway [3,20]. In the absence of DNA damage, p53-MDM2 complex is stable for exporting p53 protein into the cytoplasm to be degraded. Upon cellular DNA damage, the association of

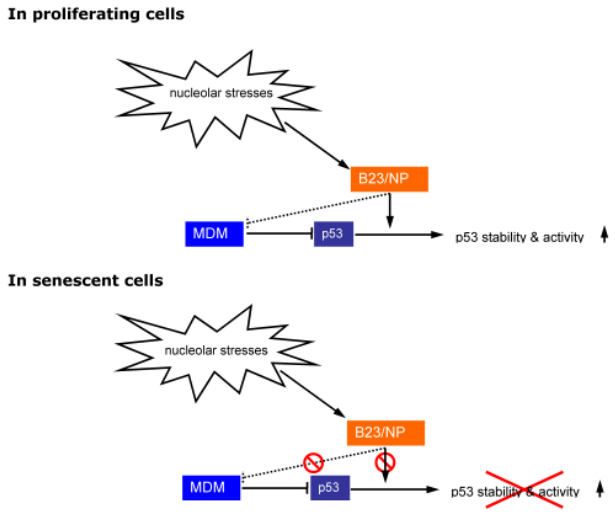

Fig. 5. The signaling pathway of p53 stabilization and activation by B23/nucleophosmin (NPM) in proliferating and senescent cells. In proliferating cells, the formation of complexes of p53 and MDM2 is constant, leading to the degradation of p53 by the ubiquitination system. Under damage conditions from a variety of sources, p53 protein is stablized and activated through its interaction with B23/nucleophosmin or because the MDM2-mediated p53-degradation pathway is blocked by B23/nucleophosmin (dotted line). However, in senescent cells, B23/nucleophosmin does not accomplish its function in p53 stabilization and activation.

p53 with MDM2 is broken by post-translational modification of p53, such as phosphorylation, sumoylation, and acetylation, resulting in greater p53 stability and activity [4,8]. Besides these post-translational modifications of p53 upon DNA damage, B23/nucleophosmin also enhances the stability and activity of p53 by inhibiting proteasome-dependent p53 degradation under damage [7]. B23/nucleophosmin is able to bind p53 directly to enhance both its stability and transcriptional activity [2]. Here, we observed that the level of p53 protein was not induced in ras-mediated senescent cells in response to nucleolar stress, consistent with the results of a previous study of aging cells [15]. Moreover, we proved that the destabilization of p53 protein was dependent on the proteasome pathway and the interaction of B23/nucleophosmin with p53. In our senescent cells, B23/nucleophosmin protein was retained in the nucleolus upon nucleolar damage by treatment with actinomycin D, resulting in sequestration of B23/nucleophosmin away from p53 and destabilization of the latter. In addition, we could observe that p53 protein had reduced binding affinity with B23/nucleophosmin in senescent cells. In a previous study [7], B23/nucleophosmin was reported to bind MDM2 directly to compete with p53 in response to damage. However, we could not observe such an effect of B23/nucleophosmin on p53-MDM2 complex formation by competing with MDM2. This discrepancy may be a consequence of the different cell systems used. In addition, B23/nucleophosmin has chaperone activity to prevent protein aggregation and ensure proper protein folding [12,17,18]; this function of B23/nucleophosmin may also be impaired in senescent cells, resulting in reduced p53 activity. As depicted in Fig. 5, B23/nucleophosmin protein may have multiple pathways to regulate p53 stability in response to cellular damage.

In conclusion, we demonstrated that nucleoplasmic redistribution of B23/nucleophosmin and its interaction with p53 are blocked in ras-mediated senescent cells, leading to destabilization of p53 in response to nucleolar stress.

Acknowledgement

This work was supported by a grant from BAERI of National Nuclear R& D program, Korean Ministry of Science and Technology.

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초록 : Ras에 의해 유도된 노화세포에서 핵인 스트레스에 의한 p53 안정화 연구

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B23/nucleophosmin은 핵인 단백질로서 외부 스트레스에 의해 핵인에서 핵으로 이동하게 된다. 이러한 세포 내 위치변화는 MDM2에 의한 p53단백질의 안정화에 영향을 미친다. 노화세포는 거대한 단일 핵인을 가지고 있으며, 외부 스트레스에 의해 p53 안정성이 감소한다. 그렇지만, 노화세포에서 어떠한 기전에 의해 p53의 불안정성이 증가하는 지는 아직 밝혀진 바가 없다. 따라서 본 연구에서는 노화세포에서 B23/nucleophosmin과 p53간의 상호 관련성을 조사하여 p53 안정성에 미치는 영향을 규명하고자 하였다. 본 연구에서는 IMR90세포주에 ras oncogene을 과발현시켜 노화세포를 유도하였다. 핵인 스트레스에 의해 노화세포 내 p53 단백질 발현은 감소하였으나, B23/nucleophosmin 단백질의 발현은 정상세포와 큰 차이가 없었다. 그렇지만, 두 단백질의 세포 내 위치는 노화세포에서 변화가 있었다. 즉, 정상세포와 달리, 노화세포에서는 스트레스에 의해 핵 내 p53발현이 증가하지 않았으며, B23/nucleophosmin은 핵 내로 이동하지 않고, 핵인에 그대로 머물러 있었다. 노화세포에서 MDM2와 p53간 상호결합이 안정적으로 유지된대 비하여, p53과 B23/nucleophosmin간의 상호결합은 감소하였다. 이러한 결과는 노화세포에서 핵인 스트레스에 의한 p53단백질의 안정성은 B23/nucleophosmin 결합이 감소하여 일어나는 것으로 해석된다.