

Vitamin C inhibits p53-induced replicative senescence through suppression of ROS production and p38 MAPK activity

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Abstract. We previously reported that tumor cells expressing p53 increase intracellular levels of reactive oxygen species (ROS). In this study, we described an inhibitory effect of vitamin C on replicative senescence. Vitamin C was found to inhibit p53-induced senescence in human bladder cancer EJ cells. The senescence-like phenotype (SLP) induced by p53, which showed a morphological change and an irreversible cell cycle arrest, was not observed in vitamin C-treated EJ cells. In addition, vitamin C did not significantly affect normal cell proliferation. We investigated the molecular mechanisms of the inhibitory effect of vitamin C on the development of replicative senescence in EJ cells. We found that vitamin C inhibited this p53-induced ROS generation. Moreover, p38 kinase which was activated during p53-induced senescence was not observed in vitamin C-treated EJ cells. SB203580, a chemical inhibitor of p38 kinase, was found to consistently inhibit p53-induced senescence. Therefore, it is suggested that vitamin C inhibits p53-induced senescence by preventing ROS generation, which in turn leads to the activation of p38 MAPK. These results reveal the inhibitory mechanism of vitamin C on cellular senescence.

Introduction

Vitamin C acts as an antioxidant in mammalian cells. The majority of previous studies have reported that vitamin C protects cells from oxidative stress, which can induce cancer

and aging via DNA damage (1-3). However, the molecular mechanisms of vitamin C cellular senescence have not been fully elucidated.

Senescent cells display characteristic phenotypic markers, such as an enlarged and flattened morphology, irreversible growth arrest and the expression of senescence-associated β -galactosidase (SA- β -Gal) activity at pH 6.0 (4). It has been demonstrated that the expression of *ras* and *raf* oncogenes can induce a rapid onset of replicative senescence in normal fibroblast cells and that this is associated with the induction of p53, p21, p16 and p19, and the hypophosphorylation of Rb protein (5-7). This implies that replicative senescence, such as apoptosis, is a programmed response of an organism to a potentially oncogenic impact.

Several studies have shown that oxidants are important factors for the induction of cellular senescence. Studies with human diploid fibroblasts have demonstrated that cells treated with hydrogen peroxide (H₂O₂) reveal a shortened telomere length per population doubling (8) and induce cellular senescence (9-11). The role of oxidants in cellular senescence has been further understood by observations demonstrating that tumor cells expressing tumor suppressor genes, such as p53 and p21, increase the intracellular ROS levels (6,12). Antioxidants consistently inhibit the induction of senescence phenotypes (12). Thus, ROS is essential for the induction of senescence phenotypes in cells expressing p53 or p21.

In this study, we investigated the effects of vitamin C on p53-induced replicative senescence. ROS levels are increased by overexpression of p53 in the p53-negative cancer cell line EJ and subsequent activation of p38 mitogen activated protein kinase (MAPK). Thus, we used the ROS-mediated senescence system to examine whether vitamin C inhibits replicative senescence through the blockage of ROS induction or other responses.

Materials and methods

Cell cultures, recombinant adenovirus and materials. Human bladder cancer EJ cells, were maintained in DMEM supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY) and penicillin-streptomycin (50 U/ml). Recombinant adenoviruses encoding wild-type p53 were purchased from

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Vector BioLabs (Philadelphia, PA). Vitamin C and *tert*-butylhydrogen peroxide (t-BTH) were purchased from Sigma Chemical Company (Sigma, St. Louis, MO).

Senescence-associated β -galactosidase (SA- β -Gal) analysis. Cells were washed in PBS and fixed in a solution of 0.25% glutaraldehyde in PBS, and 2 mM MgCl₂ was added at room temperature for 20 min. SA- β -Gal activity at pH 6.0 was detected as previously described (12).

Measurement of ROS levels. Cells were incubated with 10 μ M H₂DCF-DA (Molecular Probes, Carlsbad, CA) for 30 min, washed with PBS, trypsinized and collected in 1 ml of PBS. Fluorescence-stained cells were transferred to polystyrene tubes with cell-strainer caps (BD Biosciences, San Jose, CA) and sorted using a fluorescence-activated cell sorter (FACScalibur™, BD Biosciences). Cell Quest 3.2 software (Becton Dickinson, NJ, USA) was used for analysis (12).

Immunoblot analysis. Cell lysates were prepared by lysis in RIPA buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 μ M EGTA, 1% Triton X-100, 50 mM NaF, 5 mM Na₃VO₄, 10 mM Na₄P₂O₇, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 1 μ g/ml leupeptin and 1 mM DTT). Extracts normalized for protein concentrations using the Bradford assay, and 20 μ g of total cell protein per sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PolyScreen membrane (Nen, Boston, MA). Membranes were incubated in a solution of 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) and probed with one of the following antibodies: p-p38 (Cell Signaling Technology, Beverly, MA) p38, p53, Hdm2/Mdm2 and tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies were detected with either goat anti-mouse or goat anti-rabbit, horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Results

Vitamin C inhibits p53-induced replicative senescence. Since vitamin C has been shown to affect various cell functions such as cell growth and apoptosis, we first examined the effects of vitamin C on cell proliferation before analyzing its effects on senescence (Fig. 1A). Dead cells were not found in EJ cells treated with 0.15 or 0.3 mM vitamin C, but they were found in cells treated with 0.5 or 1 mM. Therefore, low doses (0.15 and 0.3 mM) of vitamin C did not affect normal cell proliferation, but high doses (0.5 and 1 mM) induced cell death regardless of the growth stage (Fig. 1A).

To determine whether a low dose (0.15 mM) of vitamin C inhibits senescence phenotypes, we first examined the effect of vitamin C on the proliferation of cells infected with p53-adenovirus. The cells were treated with 0.15 mM vitamin C after infection with p53-adenovirus, and the total number of cells was counted (Fig. 1B). Growth of the cells treated with vitamin C was not affected by p53-adenovirus and was similar to that of normally proliferating cells (Fig. 1B). We then examined SA- β -Gal activity and morphological characteristics

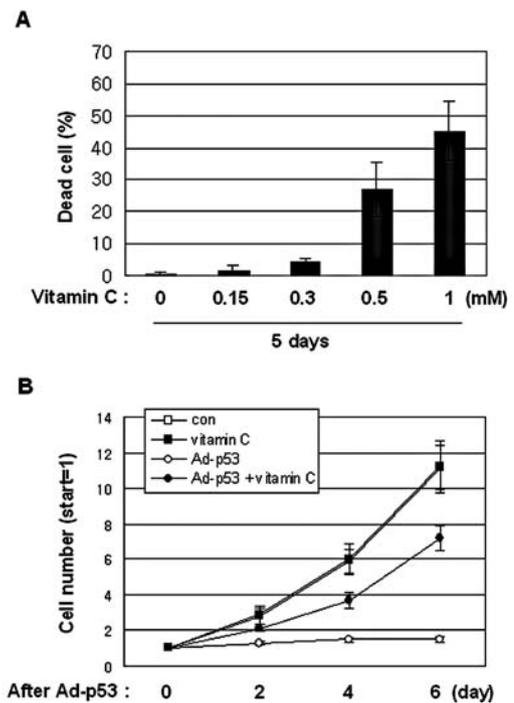


Figure 1. The effects of vitamin C on cell proliferation. (A) Normally growing EJ cells were treated with 0.15, 0.3, 0.5 and 1 mM vitamin C, and the number of cells was counted 5 days later. Cell death was determined by counting trypan blue-stained cells. (B) Normally growing EJ cells and p53-expressing EJ cells were treated with 0.15 mM vitamin C (■, ●) or were left untreated (□, ○), and the number of cells was counted at the indicated time points. Each point represents the average of 3 independent experiments.

of the cells treated with vitamin C in comparison with controls and cells infected with p53-adenovirus (Fig. 2A and B). Consistent with SA- β -Gal activity, the morphological characteristics of senescent cells were significantly reduced in the cells infected with p53-adenovirus following the treatment of vitamin C without induction of cell death. Notably, vitamin C decreased the protein level of p53 which was expressed by infection with p53-adenovirus of p53-negative EJ cells (Fig. 2C). SA- β -Gal activity was also decreased by 0.3 mM vitamin C in the cells infected with p53-adenovirus. However, 0.15 mM vitamin C produced a greater decrease than 0.3 mM. Unlike the effects of 0.3 mM vitamin C alone on cell proliferation, dead cells were observed in the cells infected with p53-adenovirus following treatment of 0.3 mM vitamin C (data not shown). These results indicate that vitamin C inhibits replicative senescence induced by p53 in the p53-deficient cell line, EJ.

Vitamin C inhibits an increase in ROS levels by p53. It has been reported that tumor suppressor genes, such as p53 and p21, increase intracellular reactive oxygen species (ROS) levels, which play a key role in senescence (12-14) and *in vivo* aging (15-17). Thus, an increased ROS level by p53 is important for the induction of senescence. We, therefore, investigated whether vitamin C affected ROS production by p53 (Fig. 3). ROS levels in EJ cells expressing p53 were found to significantly decrease one day after vitamin C treatment (Fig. 3B and C). In addition, ROS levels in control EJ cells were also found to decrease after vitamin C treatment

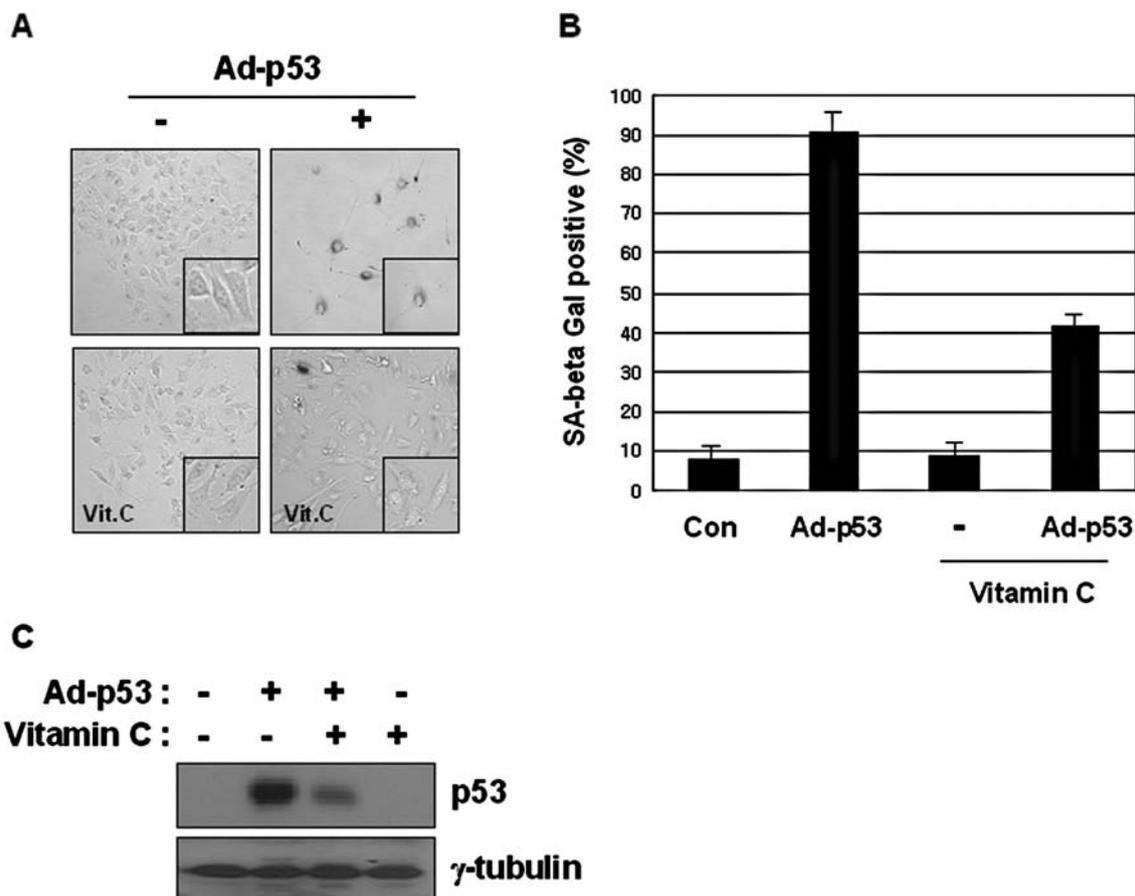


Figure 2. Vitamin C inhibits senescence phenotypes induced by p53. (A) Cells were treated with 0.15 mM vitamin C 2 h after infection with p53-adenovirus, and SA- β -Gal activity was analyzed. The results represent the mean \pm SD of 3 independent experiments. (B) SA- β -Gal staining 5 days after the infection with p53-adenovirus following vitamin C treatment. (C) Expression of p53 protein in EJ cells was analyzed by immunoblotting.

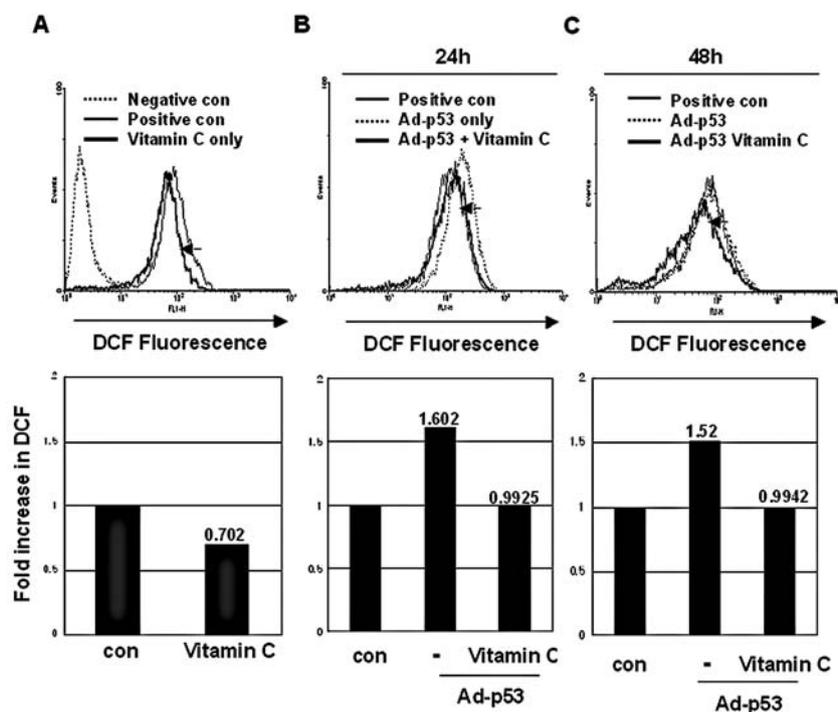


Figure 3. Vitamin C inhibits ROS generation by p53. (A) Normally growing EJ cells and p53-infected EJ cells were treated with 0.15 mM vitamin C or were left untreated, and were cultured for 24 h (B), 48 h (C) and 3×10^5 cells were subjected to FACS analysis after staining with DCF, a fluorescent probe. Each point represents the average of 3 independent experiments.

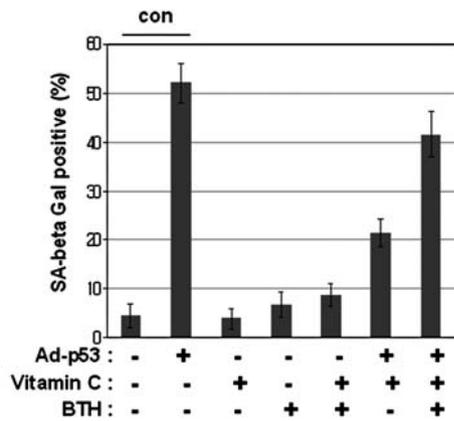


Figure 4. Senescence phenotypes inhibited by vitamin C were overridden by pro-oxidant, t-BTH. p53-infected EJ cells treated with vitamin C were cultured with 10µM t-BTH for 5 days, and SA-β-Gal activity was analyzed. The data represent the mean ±SD of 3 independent experiments.

(Fig. 3A), showing that vitamin C prevents ROS generation by p53 in EJ cells.

To support the aforementioned results, we aimed to confirm whether senescence phenotypes inhibited by vitamin C are recovered by pro-oxidants such as *tert*-butyl-H₂O₂ (t-BTH). We post-treated p53-expressing EJ cells with t-BTH after vitamin C treatment. SA-β-gal activity was significantly increased after t-BTH addition in p53-expressing cells treated with vitamin C (Fig. 4). These results suggest that the inhibitory effect of vitamin C on p53-induced ROS production was offset after t-BTH treatment in EJ cells.

Vitamin C inhibits the activity of p38 kinase induced by p53. MAPK can promote growth arrest, replicative senescence and apoptosis when cells are exposed to different apoptotic stimuli. A previous study reported that p38 kinase is activated during p53-induced senescence (12). Based on this study, we examined whether vitamin C inhibits the activity of p38 kinase induced by p53 in EJ cells. The phosphorylation of p38 kinase induced by p53 was dramatically suppressed by vitamin C (Fig. 5A). In addition, the inhibitory effect of vitamin C on senescence phenotypes was almost the same as that of SB203580, a specific chemical inhibitor of p38 kinase (Fig. 5B). These results suggest that vitamin C inhibits senescence phenotype through suppression of p38 kinase activity induced by p53 in EJ cells.

Discussion

In this study, we investigated the effects of vitamin C on cellular senescence. Although it has been suggested that vitamin C has an anti-aging effect (18,19), its molecular mechanism is unclear. Based on the results of our previous study suggesting that Bcl-xL and E1B-19K prevent ROS production by p53 and subsequently inhibit cellular senescence (12), we focused on the inhibitory mechanisms of vitamin C on cellular senescence by using this experimental method. We found that vitamin C inhibited p53-induced senescence through blockage of the ROS-p38 kinase pathway.

Treatment with a low dose of vitamin C, which did not affect cell death, inhibited senescence phenotypes induced by p53 in EJ cells (Figs. 1 and 2). In addition, the expression of exogenous p53 protein was decreased after vitamin C treatment (Fig. 2C). We, therefore, aimed to confirm whether

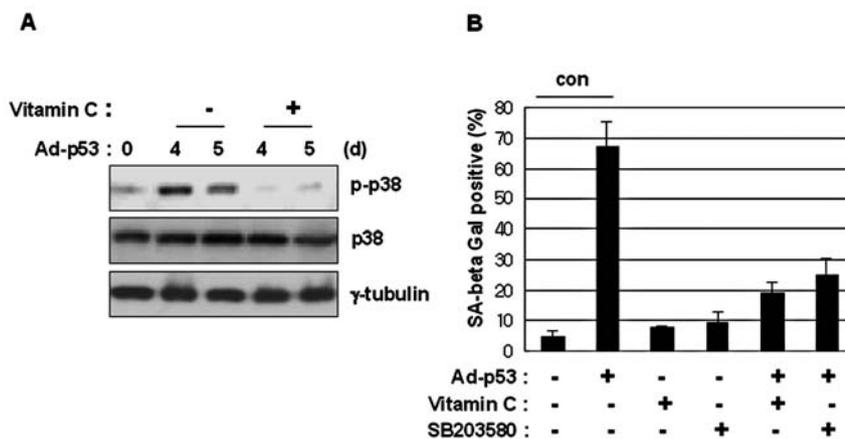


Figure 5. Vitamin C inhibits the activity of p38 kinase in p53-induced senescence. (A) Immunoblot analysis of phosphorylated p38 kinase at the indicated time points after EJ cells infected with adenovirus p53 were treated with 0.15 mM vitamin C. (B) A comparison of vitamin C and t-BTH in terms of the inhibitory effects of senescence phenotypes induced by p53. EJ cells infected with p53-adenovirus were treated with either vitamin C (0.15 mM) or SB203580 (10 µM). The number of SA-β-Gal-positive cells was counted 5 days later. The results represent the mean ± SD of 3 independent experiments.

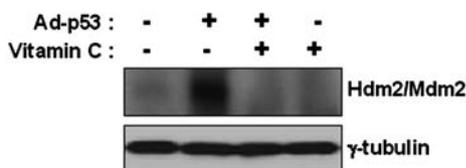


Figure 6. The effect of vitamin C on Hdm2 expression. p53-infected EJ cells were treated with 0.15 mM vitamin C or were left untreated, and immunoblotting was performed as shown in Fig. 2C.



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 decreased p53 was related to the expression of Hdm2, which directly binds to and degrades it (Fig. 6).

However, our data showed that the expression of exogenous p53 protein was regulated independently of Hdm2/mdm2, indicating that vitamin C can down-regulate the expression of p53 via other E3 ligases. Thus, these results demonstrate that vitamin C suppresses p53 expression and subsequently inhibits senescence phenotypes. Further studies are required to confirm the inhibitory mechanism of p53 by vitamin C in p53-induced senescence.

Numerous studies have reported that vitamin C functions as an antioxidant (21). We previously reported that ROS induction by p53 is essential for senescence induction (12), and thus we aimed to confirm whether vitamin C regulates ROS generation. ROS induced by p53 was significantly reduced by vitamin C (Fig. 3). This was further supported by the finding that the inhibition of senescence phenotypes by vitamin C was overcome by exogenous ROS (Fig. 4). To elucidate the molecular mechanisms by which vitamin C inhibits p53-induced senescence, we then focused on the effect of vitamin C on the activity of p38 kinase. We reported that p38 kinase is activated during p53-induced senescence and its phosphorylation is paralleled by ROS induction (12). Collectively, our results confirm that vitamin C blocks the phosphorylation of p38 kinase in p53-induced senescence (Fig. 5). The activation of p38 kinase, after infection with adenovirus p53, was blocked by vitamin C. The results of this study suggest that vitamin C may have a novel function in its involvement with the inhibition of p53-induced senescence.

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