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The Molecular Mechanisms of Vitamin C on Cell Cycle Regulation in B16F10 Murine Melanoma

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Abstract Vitamin C has inconsistent effects on malignant tumor cells, which vary from growth stimulation to apoptosis induction. It is well known that melanoma cells are more susceptible to vitamin C than any other tumor cells, but the precise mechanism remains to be elucidated. In the present study, the proliferation of B16F10 melanoma cells was suppressed by vitamin C, which induced growth arrest in a dose-dependent manner without cytotoxic effects. Therefore, we investigated the changes in cell cycle distribution of B16F10 melanoma cells by staining DNAs with propidium iodide (PI). The growth inhibition of B16F10 melanoma by vitamin C was associated with an arrest of cell cycle distribution at G1 stage. In addition, the levels of p53-p21$^{Waf1/Cip1}$ increased during G1 arrest, which were essential for vitamin C-induced cell cycle arrest. The increased p21$^{Waf1/Cip1}$ inhibited CDK2. Moreover, the activity of p53-p21$^{Waf1/Cip1}$ pathway was closely related with the activation of checkpoint kinase 2 (Chk2). Inhibitor of the PI3K-family, LY294002 and the ATM/ATR inhibitor, caffeine, blocked vitamin C-induced growth arrest in B16F10 melanoma cells. These results suggest that vitamin C might be a potent agent to inhibit proliferative activity of melanoma cells via the regulation of Chk2-p53-p21$^{Waf1/Cip1}$ pathway. J. Cell. Biochem. 102: 1002–1010, 2007.

Key words: G1 arrest; vitamin C; melanoma; p53; p21$^{Waf1/Cip1}$; Chk2; CDK2

The incidence of melanoma is rapidly increasing in the U.S. population. The overall survival of patients with metastatic melanoma ranges from 4.7 to 11 months, with a median survival of 8.5 months. The standard treatment of patients with metastatic melanoma has been undetermined [Sun and Schuchter, 2001]. Chemotherapy and immunotherapy were introduced, but these are associated with significant toxicity [Buzaid and Legha, 1994; Legha et al., 1996]. At present, there is no effective treatment for invasive melanoma.

It has been shown that vitamin C (L-ascorbic acid) is an important antioxidant and enzyme co-factor [Levine, 1986, 1995; Sies and Stahl, 1995]. However, Schwartz [1996] demonstrated that vitamin C may sometimes help to destroy

Abbreviations used: PI, propidium iodide; Chk2, checkpoint kinase 2; CDK2, cyclin-dependent kinase 2; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering ribonucleotides acid; ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia Rad3-related.

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tumors by acting as a pro-oxidant. In addition, vitamin C works by stimulating the immune system and protecting against infections [Hemila, 1992; Jeng et al., 1996]. It is well known that melanoma cells are more susceptible to vitamin C than any other tumor cells [Bram et al., 1980]. We previously reported the anti-tumor activities of vitamin C in B16F10 murine melanoma cells [Cho et al., 2003]. Vitamin C is found to induce the apoptosis of B16F10 murine melanoma cells in a time- and dose-dependent manners [Kang et al., 2003]. In addition, it has been reported that vitamin C induces apoptosis of melanoma cells via the inhibition of iron uptake [Kang et al., 2005]. In the present study, we have investigated vitamin C-induced growth arrest in B16F10 murine melanoma cells.

Cell growth (an increase in cell mass and size through macromolecular biosynthesis) and cell cycle progression are tightly coupled, allowing cells to proliferate continuously while maintaining their size [Fingar and Blenis, 2004]. The progression from G1 phase to S phase is controlled by G1 cyclins that bind to cyclin-dependent kinases (CDKs) to form holoenzymes which phosphorylate substrates faciliating the progression [Sherr, 1994]. In normal cells, the transition from G1 phase to S phase requires the activity of two classes of CDKs, namely CDK4/6 and CDK2. Recently, two families of mammalian G1 CDK inhibitors, the CIP/KIP (p21, p27, and p57) and the INK-4 (p15, p16, p18, and p19), have been described [Sherr, 1996]. These CDK inhibitors bind to cyclin-CDK complexes, particularly to G1-cyclin-CDK complexes, thus inducing cell cycle arrest [Xaus et al., 1999].

The recent studies have focused on antiproliferative effects of vitamin C on tumor cells. Furthermore, our results indicate that vitamin C mediates these effects by increasing p53, $p21^{Waf1/Cip1}$ expression and inhibiting CDK2 expression. In the present study, the effect of vitamin C on the cell growth and cell cycle progression of B16F10 murine melanoma cells was investigated.

MATERIALS AND METHODS

Cell Line

The murine melanoma cell line B16F10 was obtained from the American type culture collection and used as a target. The target cell line was maintained in continuous log phase growth and cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS).

Cell Proliferation Assay

Cell proliferation was assessed using Alamar Blue assay. As a redox indicator, Alamar Blue (BUF012A, Serotec Ltd, Oxford, United Kingdom) is reduced by reactions innate to cellular metabolism and thus provides an indirect measure of the number of viable cells. B16F10 melanoma cells were seeded onto a 96-well plate and treated with various doses of vitamin C (L-ascorbic acid sodium salt, Sigma–Aldrich, St. Louis, MI) for 24 h. Then Alamar Blue (10 v/v% in medium) was added to the cells, and 6 h later, fluorescence was determined in a spectrofluorometer at 530 nm excitation and 590 nm emission wavelength in a spectrofluorometer (Fluoroskan Asent FL, Labsystems, Helsinki, Finland).

Dye Exclusion Assay

Exponentially grown cells (1×10^6) were seeded in 150-mm tissue culture dishes for 15–18 h to undergo attachment and treated with various doses of vitamin C. After 24 h, cells were harvested with trypsin-EDTA and washed once with phosphate-buffered saline (PBS). Cell number and viability were determined by the trypan blue dye exclusion test. Quadruplicate wells were run for each group.

Assay of Apoptosis

The cells were prepared and cultured under the same conditions as dye exclusion assay. After 24-h incubation, trypsinized cells were washed and stained with fluorescein isothiocyanate (FITC)-labeled annexin V (AV) and 7-amino-actinomycin D (7-AAD). Five thousand cells were analyzed using flow cytometry (EPICS profile, Coulter).

DNA Cell Cycle Analysis

The cells were treated with vitamin C (0.05, 0.1, 0.15, and 0.2 mM) in complete media for 24 h. The cells were thereafter trypsinized, washed twice with cold PBS, and centrifuged. The pellet was resuspended in 1 ml of cold PBS and 4 ml of cold ethanol for 30 min at 4°C. The cells were centrifuged at 1,500 rpm for 5 min; the pellet was washed twice with cold PBS, suspended in

500 μl of propidium iodide (PI)-staining solution (0.1% Triton X-100, 0.1% sodium citrate in PBS), and incubated with RNase (final concentration, 25 μg/ml) at 37°C for 30 min. The cells were stained with PI (final concentration, 50 μg/ml) for 15 min and analyzed by flow cytometry.

Western Blot Analysis

Logarithmically growing cells were treated with vitamin C for 24 h. At various time points after the beginning of exposure to vitamin C, the cells were lysed and proteins extracted in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail. The protein concentration was measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (30–100 μg) were resolved over 12% (for p53, CDK2, p-CDK2, CDK4, cyclin D1), and 15% (for $p21^{Waf1/Cip1}$) SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk and 0.1% Tween 20-PBS for 1 h, washed with 0.1% Tween 20-PBS, and then exposed to primary antibody for 1 h at RT. Primary antibodies were diluted 1:200 (anti-p21 [sc-6246], anti-CDK2 [sc-6248], anti-CDK4 [sc-260], and anti-cyclin D1 [sc-717] Ab, Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1% Tween 20-PBS. After washing, blots were exposed to biotin-conjugated secondary antibodies for 1 h at RT. The membrane was then washed, incubated with a 1:5,000 dilution of streptavidin-HRP, and the immunoreactive proteins were visualized with the ECL detection system (Amersham Biosciences Corp., Piscataway, NJ).

RNA Interference

B16F10 melanoma cells were transfected for 24 h with control small interfering ribonucleotides acid (siRNA) or p53- p21 siRNA (20 pmol/well, Santa Cruz Biotechnology) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) in six-well plates. After transfection, the cells were treated with 0.2 mM vitamin C for 24 h. Cell proliferation was analyzed by BrdU incorporation assay.

BrdU Incorporation Assay

Proliferation assay was performed using a 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit II (Roche Molecular Biochemicals, Mannheim, Germany). B16F10 melanoma cells were seeded on cover glasses, grown for 24 h before vitamin C treatment (0.2 mM). The cells were pretreated with each 25 μM LY294002 (inhibitor of the phosphatidylinositol 3-kinase (PI3K)-family, Calbiochem, 495620) or 20 μM olomoucine (chemical inhibitor of CDK2, Calbiochem, 440202) or 4 mM caffeine (inhibitor of ataxia-telangiectasia mutated/ataxia-telangiectasia Rad3-related (ATM/ATR), Calbiochem, 205548) for 1 h. The cells on each cover glass were labeled for 1 h with 10 μM BrdU at 24 h after treatment with 0.2 mM vitamin C and fixed with acetic acid/ethanol. BrdU-incorporated cells were counted.

Chk2 Immunoprecipitation (IP) Kinase Assay

For IP kinase assay, after vitamin C treatment or not, checkpoint kinase 2 (Chk2) was immunoprecipitated from B16F10 melanoma cells with active Chk2 kinase immunoprecipitation beads, and kinase assay was performed according to the manufacturer's instructions (DuoSet® IC, R&D System, Minneapolis, MN). The presence of Chk2 protein in the immunoprecipitates was determined by immunoblot using an anti-Chk2 antibody (R&D System).

Statistical Analysis

The statistical significance was determined by using ANOVA at $P < 0.05$. All results are expressed as a two-tail P-value.

RESULTS

It has been established that human tumor cells induce cell cycle arrest by vitamin C treatment [Jamison et al., 2004]. We determined whether vitamin C inhibits the growth of B16F10 melanoma cells. Cell proliferation was measured by Alamar blue assay and trypan blue dye exclusion assay in vitamin C-treated B16F10 melanoma cells. Proliferation of exponentially growing B16F10 melanoma cells was dose-dependently inhibited by vitamin C as shown in Figure 1a,b. Especially, treatment with vitamin C (0.2 mM) for 24 h significantly inhibited the growth of melanoma cells by approximately 50%, compared with the untreated control. As shown in Figure 1a,b, treatment with vitamin C resulted in the decrease of cell growth in a dose-dependent manner. In addition, the number of cells treated with the indicated dose of vitamin C was also counted at 12 and 24 h (Fig. 1c). The result was

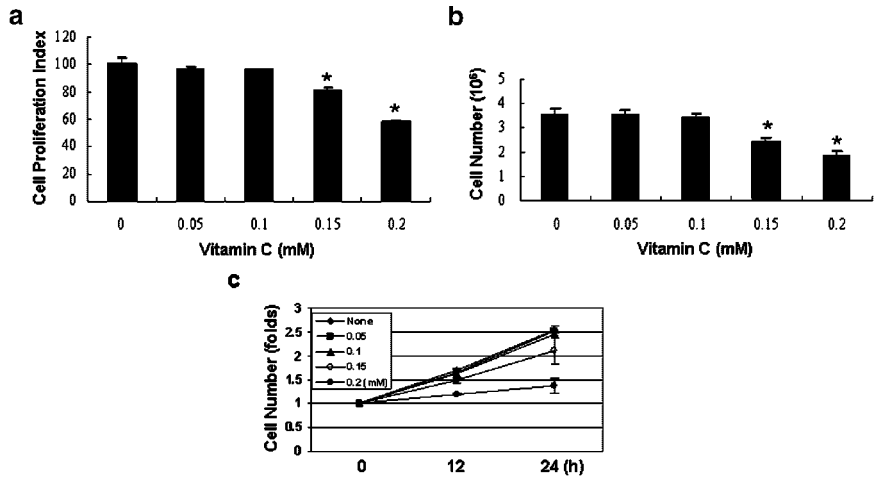


Fig. 1. Vitamin C inhibits proliferation of B16F10 murine melanoma cells. **a**: B16F10 melanoma cells were exposed to vitamin C (0.05, 0.1, 0.15, and 0.2 mM) and then incubated for 24 h. Cell proliferation was measured by Alamar blue assay. **b**: After vitamin-C treatment for 24 h, the cells were trypsinized and then cell number was counted by the trypan blue dye exclusion assay. **c**: Proliferation of B16F10 murine melanoma cells was examined after treatment with or without the indicated dose of vitamin C at the time points indicated, and the number of cells was counted by the trypan blue dye exclusion assay at the indicated times. Data represent the mean and SD (error bar) of three independent experiments. (*) Significant differences in comparison to the medium control.

also same as Figure 1a,b. To evaluate the toxicity of vitamin C in melanoma cells, the cells were cultured for 24 h in the absence or presence of vitamin C, stained with annexin V, and 7-AAD, and then measured by flow cytometric analysis (Fig. 2). Apoptosis was negligible in the culture, in both the presence and the absence of vitamin C. Taken together, vitamin C inhibited the growth of B16F10 melanoma cells in a dose-dependent manner without induction of apoptosis.

To further understand the nature of cell growth arrest, melanoma cells were cultured for 24 h in the presence of various concentrations of

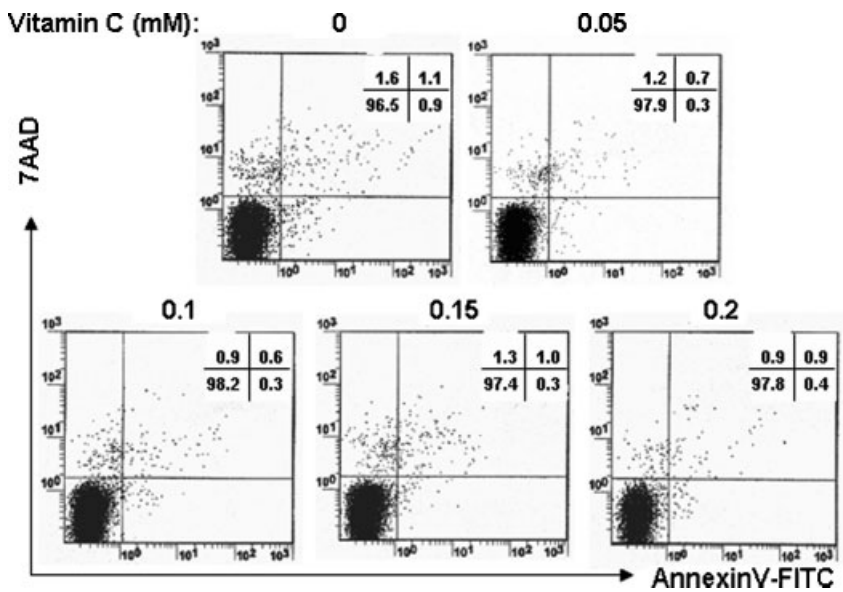
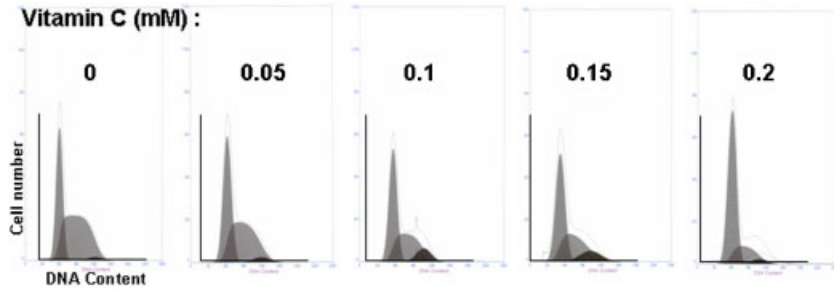


Fig. 2. Vitamin C has no effect on cell death of B16F10 murine melanoma cells. B16F10 melanoma cells were exposed to vitamin C (0.05, 0.1, 0.15, and 0.2 mM) and then incubated for 24 h. The cytotoxic effect of vitamin C on B16F10 melanoma cells was assessed by Annexin V-FITC/7-AAD staining. Cell death analysis was performed with flow cytometry. The numbers indicate the percentages of the viable (annexin V−; 7-AAD-) cell population (**lower left** quadrant), the apoptotic (annexin V+) cell population (**lower right** quadrant), and the necrotic cell populations in the **upper right** (annexin V+; 7-AAD+) and the **upper left** quadrants (annexin V−; 7-AAD+).



Vitamin C (mM)	Cell Cycle Distribution (%)		
	G0/G1	S	G2/M
0.00	48.0	44.1	7.9
0.05	48.0	48.7	3.4
0.10	46.0	45.8	8.2
0.15	54.9	28.5	16.6
0.20	74.6	20.0	5.4

Fig. 3. Induction of cell cycle arrest in B16F10 murine melanoma cells by vitamin C. B16F10 melanoma cells were exposed to vitamin C (0.05, 0.1, 0.15, and 0.2 mM) and then incubated for 24 h. Cell cycle analysis was performed with flow cytometry as described in Materials and Methods. Percentages of G0/G1-, S-, and G2/M-phase cells are shown in this figure.

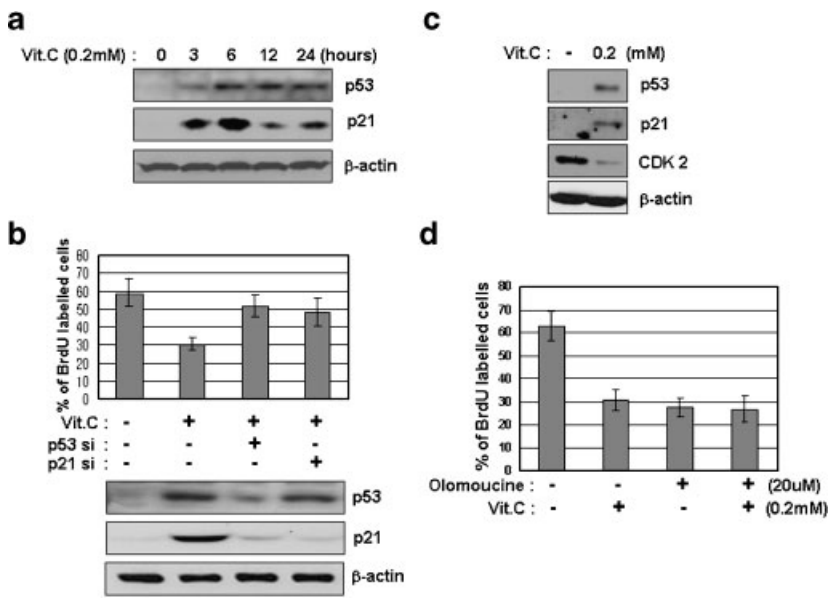


Fig. 4. Vitamin C is indispensable for p53-p21$^{Waf1/Cip1}$ dependent growth arrest. **a**: B16F10 melanoma cells were harvested at the indicated times after incubation with vitamin C (0.2 mM). The cells were then lysed. The supernatants were subjected to western blot analysis and immunoblotted with anti-p53, and anti-p21$^{Waf1/Cip1}$ antibodies. **b**: The cells were transfected for 24 h with control siRNA or p53-p21$^{Waf1/Cip1}$ siRNA (20 pmol/well, Santa Cruz Biotechnology) using Lipofect-AMINE 2000 (Invitrogen) in six-well plates. After transfection, the cells were treated with 0.2 mM vitamin C for 24 h. Cell proliferation was analyzed by BrdU incorporation assay. Immunoblots shows RNAi efficacy. Rectangles and error bars represent mean values and standard deviations, respectively, of the three independent experiments. **c**: Same as the methods described in (a), together with detection using anti-CDK2 antibody. **d**: The cells were treated with 20 μM olomoucine 1 h before vitamin-C treatment. BrdU incorporation of the cells was counted 24 h after treatment with or without olomoucine or vitamin C at the indicated times. Data represent the mean and SD (error bar) of three independent experiments for each olomoucine or vitamin C.

vitamin C and subjected to cell cycle analysis (Fig. 3). A significant increase in the cell population at G1 phase of cell cycle (48.0% vs. 74.6%, $P < 0.05$) and a significant decrease in the cell population at S phase of cell cycle (44.1% vs. 20.0%, $P < 0.05$) were observed after addition of 0.2 mM vitamin C to growing-phase cells.

Several cyclin-dependent kinase inhibitors (CDKIs) are known to regulate the transition from G1 phase to S phase. Since vitamin C induced G1 arrest in melanoma cells, we determined whether the p53-p21$^{Waf1/Cip1}$ is involved in vitamin C-induced reduction of proliferation. As shown in Figure 4a, western blot analysis revealed that the levels of p53-p21$^{Waf1/Cip1}$ protein were significantly increased in vitamin C-treated cells. The time course of the increased expression of p53-p21$^{Waf1/Cip1}$ in 0.2 mM vitamin C showed that p53-p21$^{Waf1/Cip1}$ expression increased by 3 h, reaching a peak in 6 h. The siRNA technique was applied to knockdown the expression of endogenous p53 and p21$^{Waf1/Cip1}$. As shown in Figure 4b, vitamin C had no effect on p53- and p21$^{Waf1/Cip1}$-knockdown cells, implying that vitamin C can induce p53-p21$^{Waf1/Cip1}$ mediated cell cycle arrest. The CDK2 levels were suppressed by increased p21$^{Waf1/Cip1}$ expression (Fig. 4c). In the cells treated with 20 μM olomoucine, a chemical inhibitor of CDK2, vitamin C had the same significant effect on cell cycle (Fig. 4d).

Recent studies have reported that Chk2 is essential for p53-dependent G1 cell cycle arrest [Jack et al., 2002]. It has also been reported that p53 is phosphorylated at Ser-15, a site targeted by ATM and ATR [Banin et al., 1998; Tibbetts et al., 1999]. Based on these studies, we determined whether Chk2 can play a role in p53-dependent G1 growth arrest. Chk2 kinase activity was analyzed after vitamin-C treatment. The cells incubated with 0.2 mM vitamin C for 6 h were immunoprecipitated by IP-Kinase assay, as previously described in Materials and Methods, and Chk2 activity was induced by vitamin C, but not untreated cells (Fig. 5a). Co-treatment of vitamin C with LY294002, specific inhibitor of the PI3K family, significantly decreased phosphorylation of p53 at Ser-15 and the expression levels of p53 protein, but increased CDK2 levels. However, there were no changes by the treatment of LY294002 only

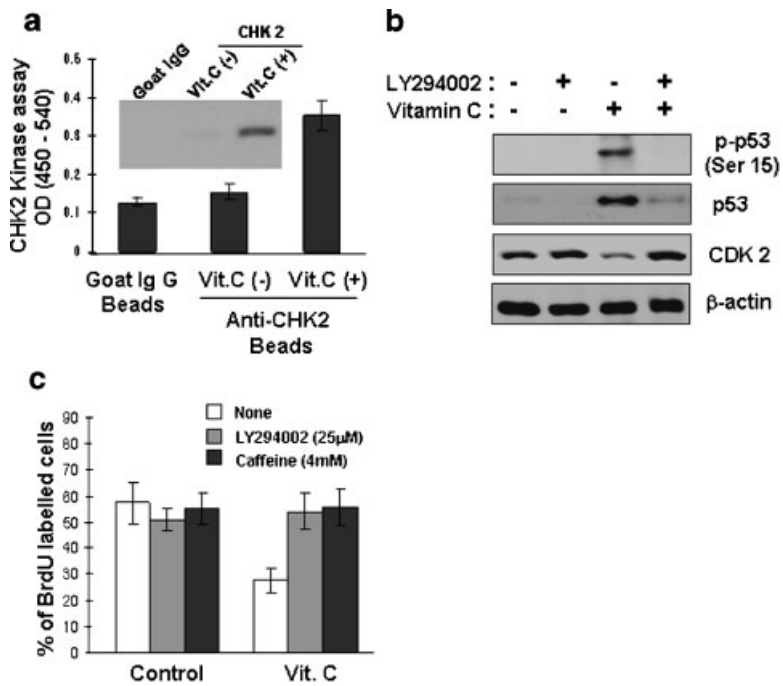


Fig. 5. Chk2 is essential for p53-depednet cell cycle arrest by vitamin C. **a**: After vitamin C treatment or not, chk2 was immunoprecipitated from B16F10 melanoma cells with Active Chk2 kinase Immunoprecipitation Beads, as described in Materials and Methods. **b**: The cells were harvested 12 h after treatment with 0.2 mM vitamin C and immunoblotted with anti-p53, phospho-p53, and anti-CDK2 antibodies. **c**: The cells were treated with 25 μM LY294002 or 4 mM caffeine 1 h before vitamin-C treatment. BrdU incorporation was same as described in Materials and Methods. Data represent the mean and SD (error bar) of three independent experiments.

(Fig. 5b). Cell cycle arrest in B16F10 melanoma cells by vitamin C was not observed by inhibitor of the PI3K-family, LY294002, and the ATM/ATR inhibitor, caffeine (Fig. 5c).

DISCUSSION

The present study was conducted to elucidate the roles of vitamin C in the regulation of cell growth in murine melanoma cells. It is well known that melanoma cells are be more susceptible to vitamin C than any other tumor cells [Bram et al., 1980]. We previously reported that vitamin C down-regulates the production of IL-18, which is known to be involved in the pathogenesis of melanoma [Cho et al., 2000]. Moreover, a high dose of vitamin C may induce the apoptosis of B16F10 murine melanoma cells via the induction of cytochrome *c* release from the mitochondria and inhibition of iron uptake [Kang et al., 2003, 2005]. Based on these studies, we hypothesized that a low dose of vitamin C might inhibit growth of murine melanoma cells. We assessed cell proliferation after vitamin-C treatment. As expected, the proliferation was inhibited by vitamin-C treatment in a dose-dependent manner (Fig. 1), which indicated that vitamin C could modulate tumor cell growth. Vitamin C influenced tumor cells by inhibiting growth at a low dose of $\leq$0.2 mM and by inducing apoptosis at a high dose of $>$1 mM. To the best of our knowledge, the present study is the first one regarding inhibition of melanoma cell growth by a low dose of vitamin C.

Many studies have shown an association between cell cycle and cancer. In recent years, inhibition of cell cycle progression has been considered as a target for the management of cancer. Recent studies have demonstrated that cell cycle aberrations occurring at the G1/S checkpoint often lead to uncontrolled cell proliferation and tumor growth [Cordon-Cardo, 1995; Sherr, 1996; Obana et al., 2003]. The precise mechanisms of growth inhibition in melanoma cells are unknown. To investigate the effect of vitamin C on cell cycle progression, we analyzed DNA cell cycle. Consistent with inhibition of cell proliferation, vitamin C increased the G0/G1 fraction of cell cycle and concomitantly decreased cell population in S phase, suggesting G1 arrest (Fig. 2). Therefore, we have focused on changes in cell cycle regulators involved in G1 phase of cell cycle.

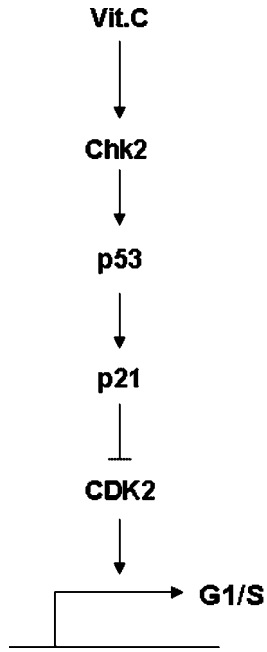


Fig. 6. Schematic drawing of vitamin-C network in B16F10 murine melanoma cells. Vitamin-C signal is indirectly generated by activity of Chk2, which may occur as a consequence of phosphorylation of p53 at Ser-15 and subsequent accumulation of p53 released from Mdm-2, and in turn activate the transcription of $p21^{Waf1/Cip1}$, a cell cycle inhibitor, ultimately leading to G1 cell cycle arrest by blocking CDK2 activity.

Here, we report that vitamin C arrests B16F10 melanoma cells in the G1 phase of the cell cycle by inducing p53 accumulation and expression of the CDKI $p21^{Waf1/Cip1}$.

Several reports have shown that p53 mediates growth arrest especially at G1 stage or apoptosis in response to many types of stress [Ryan et al., 2001]. We observed that the expression of p53 is induced by treatment of vitamin C, but G1 arrest is not induced in p53-deficient cells (Fig. 4). It suggests that vitamin C-induced cell cycle arrest in B16F10 murine melanoma is totally is dependent of p53 expression. The intrinsic CDK inhibitor, such as $p21^{Waf1/Cip1}$, binds to the CDK-cyclin complex and inhibits its kinase activity [Peter and Herskowitz, 1994]. Other studies on growth inhibition have been undertaken in several types of tumor cells through cell cycle arrest that is mediated by $p21^{WAF1/Cip1}$ [Wu et al., 1997; Agarwal et al., 2003; Wesley et al., 2004]. In the present study, within 3 h after addition of vitamin C to the cell culture, there was a marked increase in cellular levels of the cell

cycle inhibitor protein $p21^{Waf1/Cip1}$. After 6 h, the expression of $p21^{Waf1/Cip1}$ reached a peak. Although the reason why the band disappeared after 12 h is not clear, it seems that $p21^{Waf1/Cip1}$ was rapidly induced, degraded, and played a role as an early regulator. Taken together, these data suggest that p53 exerts cell-cycle arrest through its target, $p21^{Waf1/Cip1}$.

In the cell cycle of mammalian cells, cyclins and CDKs play a central role and promote G1/S transition. Sherr and Roberts [1999] have demonstrated that $p21^{Waf1/Cip1}$ induces G1 arrest via the suppression of CDK2 activity by binding to the cyclin E-CDK2 complex. We evaluated the effects of vitamin C on the expression of cyclins and CDKs involved in G1–S phase transition. Western blot analysis for CDK2, CDK4, cyclin D1, and cyclin E were performed. However, there were no changes in the expression of these molecules except CDK2 (data not shown). By vitamin-C treatment, the expression of CDK2 was also modulated at early time points (3–12 h) by $p21^{Waf1/Cip1}$. The subsequent decrease in CDK2 may be associated with early induction of p53 and $p21^{Waf1/Cip1}$ to arrest cells in G0/G1 and thereby further contribute to vitamin C-induced growth inhibition.

Chk2 plays an important role in phosphorylation of p53 and induction of G1 arrest in response to DNA damage [Chehab et al., 2000]. In the present study, vitamin C markedly induced p53 expression. Consistent with induction of p53, Chk2 kinase activity increased after vitamin-C treatment. There are several reports that Chk2-dependent p53 phosphorylation causes its stabilization and activation, which in turn results in transcriptional regulation of various genes involved in cell cycle regulation and apoptosis, including $p21^{Waf1/Cip1}$ [Fei et al., 2003; Fridman and Lowe, 2003; Kastan and Bartek, 2004]. Therefore, our data suggest that vitamin C increases accumulation of p53 and $p21^{Waf1/Cip1}$ through the activation of Chk2 (Fig. 6).

The results of the present study suggest that a low dose of vitamin C may inhibit melanoma cell proliferation and this antiproliferative effect of vitamin C may be linked to its ability to regulate the expression and activity of cell cycle-related proteins. Treatment of melanoma cells with vitamin C induces accumulation of p53 and expression of $p21^{Waf1/Cip1}$, followed by inactivation of CDK2, resulting in growth arrest in G1 phase of cell cycle. Chk2 kinase activity is necessary for vitamin C-induced, p53-dependent growth arrest in B16F10 melanoma cells. It is concluded that vitamin C can be used as one of new therapeutic agents for the treatment of melanoma by its antiproliferative effect and tumor cell-specific cytotoxic effect.

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