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Vanadate-induced cell growth arrest is p53-dependent through activation of p21 in C141 cells

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Abstract

Vanadium is widely used in industry. It is a potent toxic agent and carcinogen. The mechanisms involved in its toxicity and carcinogenesis are still unclear. Improper cell growth is believed to be involved in cancer development. The present study investigated the regulation of p53 on vanadate-induced cell growth arrest using both p53 wild type C141 cells and p53 deficient embryo fibroblasts (p53 −/−). On vanadate stimulation, C141 cells exhibited a dose- and time-dependent S phase arrest as determined by DNA content analysis. In contrast, vanadate was unable to increase the percentage of S phase in p53 −/− cells. Luciferase assay showed that vanadate induced p53 activation in a dose- and time-dependent manner in p53 wild type C141 cells. Addition of pifithrin-α (PFT), a specific inhibitor of p53, reduced the activation of p53 with a concomitant decrease in growth arrest at S phase. Western blotting analysis demonstrated that vanadate caused a dose- and time-dependent increase of p21 level in C141 cells. Pretreatment of C141 cells with PFT decreased p21 expression induced by vanadate while the p21 expression did not vary in vanadate stimulated p53 −/− cells. The results obtained from the present study suggest that vanadate is able to induce S phase arrest through p53- and p21-dependent pathway.

Keywords: Vanadate; Cell cycle; p53; p21

1. Introduction

Vanadium exists in water, rocks, and soils in low concentrations, and in coal and oil deposits in relatively high concentrations. Vanadium is widely used in mining, steel and steel-alloy manufacture, and in the chemical industry. Epidemiological studies have shown a correlation between vanadate exposure and the incidence of lung cancer [1,2]. Workers occupationally exposed to vanadium are at risk as respirable particulates may penetrate deep into the tracheobronchial tree. Vanadium mimics the effects of insulin, and stimulates or inhibits several enzymes in vivo and in vitro. Many studies have focused on the mitogenic effects of vanadate, which is mediated by inhibiting the activity of tyrosine phosphatase and triggering the autophosphorylation of tyrosine kinase on cell membrane receptors [3]. However, other mechanisms are plausible. For example, as a pro-oxidant, vanadate causes DNA damage, DNA strand breaks, and cell transformation [4,5].

The ability of cells to maintain genomic integrity is vital for cell survival and proliferation. Lack of fidelity in DNA replication and maintenance can result in deleterious mutations leading to cell death or, in multicellular organisms, cancer [6]. Minor cell damage is repaired by a temporary pause of cell cycle. If the damage is severe, cells will undergo apoptosis and enter into a dormant G_0 state. Signal transduction pathways play a key role in the regulation of cell cycle progression and stabilization of DNA under genotoxic stress. It is known that signal transduction pathways control the activation of transcription factors and the regulation of gene expression as well as a temporary pause of cell progression to allow the damaged DNA to be repaired. The mechanisms involved in the regulations of vanadate on cell cycle control remain unknown.

The p53 tumor suppressor is a multifunctional protein that exerts a variety of different effects and plays a central

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role in the regulation of the normal cell cycle. A number of different stimuli are known to activate p53 [7]. Overexpression of the p53 protein was found to induce cell growth arrest associated with G_0/G_1 checkpoint [8–10] and to induce apoptosis that occurs either through the G_0/G_1 checkpoint or the S phase [11,12], or cell differentiation. Overexpression of the p53 protein has also been suggested to be associated with G_2/M checkpoint regulation [13,14]. Furthermore, p21 (WAF-1/CIP1) is known to be a transactivation target of p53, and is believed to mediate p53-induced growth arrest triggered by DNA damage [15,16]. Induction of p21 expression could serve as an indicator for transactivation by p53, although p21 was also shown to be transactivated by p53-independent mechanisms [17].

The purpose of the present study was to identify the possible mechanisms of vanadate-induced cell growth arrest. The specific questions to be addressed were: (a) Does vanadate induce cell growth arrest? (b) If yes, does p53 play an important role? (b) What is the mechanism involved in p53 regulated cell growth arrest?

2. Materials and methods

2.1. Reagents

Sodium metavanadate was from Aldrich (Milwaukee, WI). RNase A, DMEM and EMEM medium were from Sigma (St. Louis, MO). Propidium iodide (PI) was from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS) was purchased from Gibco BRL (Life Technologies, Gaithersburg, MD). Pifithrin-α (PFT) was purchased from Alexis (San Diego, LA). Antibody to p21 was from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary AP linked anti-rabbit IgG was from Cell Signaling (Beverly, MA).

2.2. Cell culture

The JB6 P^+ mouse epidermal cell line, C141 and its stable p53 luciferase reporter plasmid transfectant, C141 p53 cells, were cultured in MEM medium containing 5% FBS, 2 mM L-glutamine and 25 μg/ml gentamicin in an incubator at 5% CO_2 and 37 °C. p53-deficient embryo fibroblasts (p53 −/−) derived from p53 gene knockout mice were incubated in DMEM medium with 10% FBS, 2 mM L-glutamine and 25 μg/ml gentamicin at 5% CO_2 and 37 °C.

2.3. Treatments

For the time-course study, the cells were treated with 50 μM vanadate for 6, 12, 24, and 48 h. For the dose–response study, the cells were treated with 10, 25, 50, and 100 μM vanadate for 24 h. For the inhibitory studies of p53, the cells were pre-incubated with PFT for 0.5 h prior to the vanadate treatment.

2.4. Measurement of cell cycle/DNA content

DNA content in S phase was analyzed using flow cytometry according to the methods described previously [18,19]. Cells were first fixed and permeabilized with 70% ice-cold ethanol for more than 2 h, followed by incubation with the freshly prepared staining buffer (0.1% Triton X-100 in PBS, 200 μg/ml RNase A, and 20 μg/ml PI) for 30 min at room temperature. Cell cycle analysis was performed by flow cytometry with at least 10,000 cells for each sample. The DNA content histogram was abstracted and the percentage of cells in S phase was then calculated using ModFit LT software.

2.5. Luciferase assay for p53 activity

After C141 p53 cells were 80–90% confluent, 1×10^4 cells were added into 96-well plate with 100 μl medium in each well. The cells were subjected to different treatments according to the experimental design. At various time points, the cells were extracted with lysis buffer. The p53 luciferase activity was measured using a luminometer (moonlight 3010). The results were expressed as p53 activity relative to the control.

2.6. Western blot analysis

Whole cell extracts were mixed with Tris-Glycine SDS sample buffer and then subjected to Tris-Glycine gel electrophoresis. The resolved proteins were transferred to a PVDF membrane. A Western blot assay was performed using antibodies against p21 and secondary anti-rabbit IgG. After reaction with ECF substrate, the signal was detected using a Storm Scanner (Molecular Dynamics, Sunnyvale, CA).

2.7. Statistical analysis

All data were based on at least three independent experiments. Cell growth arrest and relative p53 activity data were presented as means±S.D. and analyzed using one-way ANOVA with Scheffe's test. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. The effects of vanadate in cell cycle in C141 cells and p53 −/− cells

To study vanadate-induced cell growth arrest, DNA content was used to measure the percentages of different

phases. Fig. 1a shows that the percentage of S phase increased with the increase in vanadate concentration up to 100 μM in C141 cells. The percentage of S phase increased from 8 to 30% when vanadate concentration increased from 0 to 100 μM. Fig. 1b shows the results from time-dependent study of vanadate-induced cell growth arrest in C141 cells. Treatment of C141 cells with 50 μM vanadate for 6 h did not significantly increase the S phase percentage compared to the control. However, the difference of S phase percentage increased between the treatment and the control with time in C141 cells (Fig. 1b), being 12, 19 and 20% when the cells were treated with 50 μM vanadate for 12, 24 and 48 h, respectively. In p53 −/− cells, treatment with different concentrations of vanadate for 24 h did not cause any significant increase in S phase (Fig. 2a). The S phase percentage was 20 and 16%

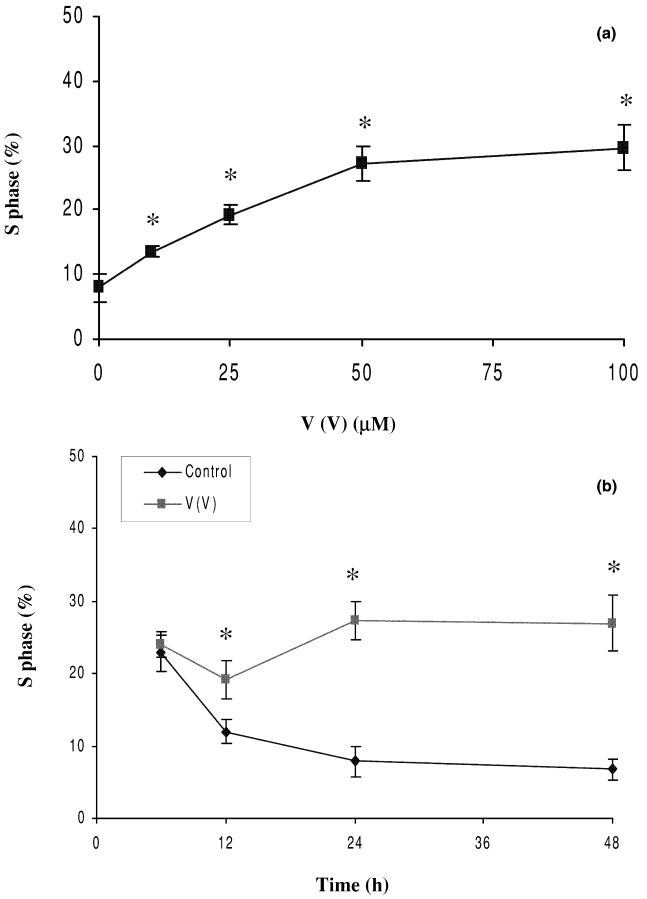

Fig. 1. Vanadate-induced cell growth arrest in C141 cells. C141 cells were suspended in 5% fetal bovine serum (FBS) MEM in a 100-mm dish. After 80–90% confluence, cells were washed with PBS three times, and treated with 10, 25, 50, and 100 μM vanadate for 24 h (a) or 100 μM vanadate for 6, 12, 24 and 48 h (b). Cells were harvested and DNA content was measured by flow cytometry. Each point represents ±S.D. of the percentage of S phase of three independent experiments. *$P<0.05$ compared to control (one-way ANOVA with Scheffe's test).

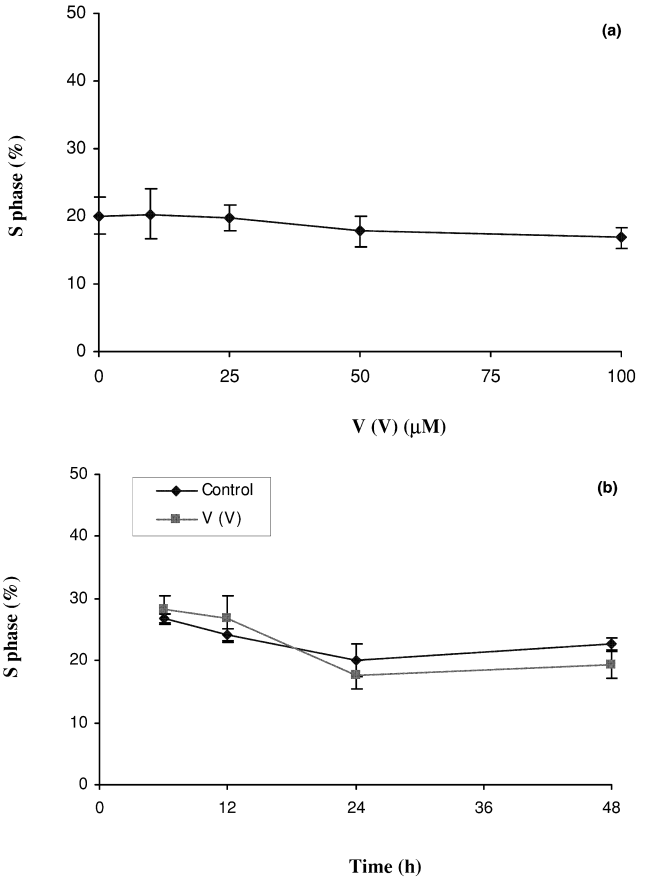

Fig. 2. The effect of vanadate on cell growth in p53-deficient embryo fibroblasts (p53 −/−). p53 −/− cells were suspended in 10% fetal bovine serum (FBS) DMEM in a 100-mm dish. After 80–90% confluence, cells were washed with PBS three times, and treated with 10, 25, 50, and 100 μM vanadate for 24 h (a) or 50 μM vanadate for 6, 12, 24 and 48 h (b). Cells were harvested and DNA content was measured by flow cytometry. Each point represents ±S.D. of the percentage of S phase of three independent experiments. *$p<0.05$ compared to control (one-way ANOVA with Scheffe's test).

in control cells and 100 μM vanadate stimulated cells, respectively (Fig. 2a). There was no significant difference between the treatment of vanadate and the control in p53 −/− cells for incubation time up to 48 h (Fig. 2b).

3.2. Vanadate-induced p53 activation

Luciferase assay was used to measure the p53 activity in C141 cells. Vanadate treatment caused a dose-dependent increase in p53 activity (Fig. 3a). Upon treatment with 10 μM vanadate for 24 h, the p53 activity increased 1.8-fold compared to the control. It was ~6-fold when cells were treated with 100 μM vanadate. Vanadate also caused a time-dependent activation of p53 as shown in Fig. 3b. The relative p53 activity increased from 1.8- to 6-fold when vanadate treatment time was increased from 6 to 48 h.

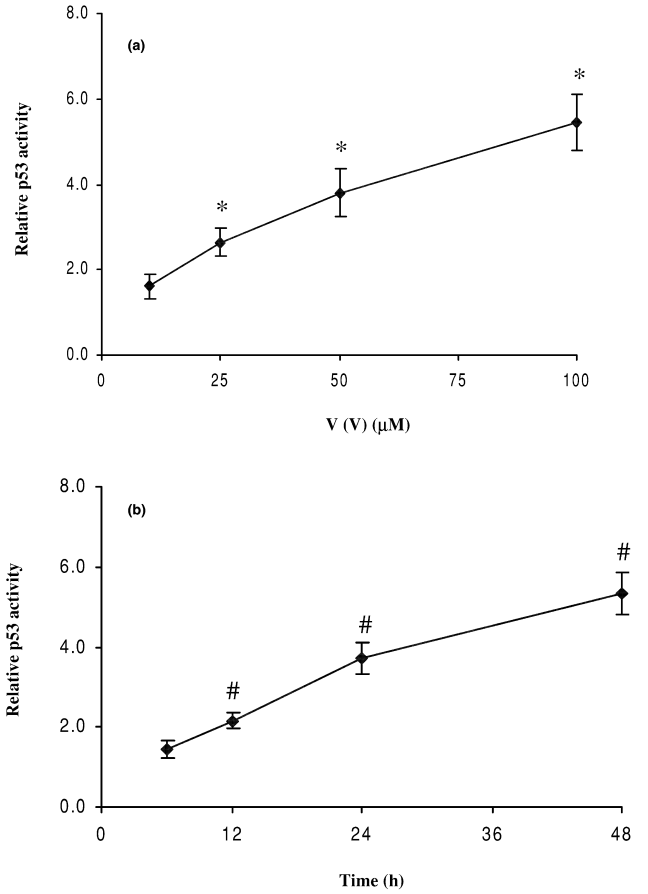

Fig. 3. Vanadate-induced p53 activation in C141 cells. A total of 1×10^4 cells were seeded into 96-well plate with 100 μl medium each well. The cells were treated with 10, 25, 50, and 100 μM vanadate for 24 h (a) or 50 μM vanadate for 6, 12, 24 and 48 h (b). At time points, the cells were extracted with lysis buffer. The p53 luciferase activity was measured using a luminometer (moonlight 3010). The results were expressed as p53 activity relative to the control. Each point represents ±S.D. of the percentage of S phase of three independent experiments. $^*p<0.05$ compared to 10-μM vanadate treatment. $^{\#}p<0.05$ compared to 6-h vanadate treatment (one-way ANOVA with Scheffe's test).

3.3. The inhibition of vanadate-induced cell growth arrest

PFT, a specific inhibitor of p53, was used to investigate the effect of p53 in vanadate-induced cell growth arrest in C141 cells. As shown in Fig. 4, 50 μM vanadate significantly increased the percentage of S phase. Pretreatment with 10 or 20 μM PFT for 0.5 h decreased the percentage of S phase to the control level.

3.4. The inhibition of vanadate-induced p53 activation

To examine whether PFT is able to inhibit vanadate-induced p53 activation, the effect of PFT on p53 activity was measured. Fig. 5 shows the results of inhibitory effect of PFT on vanadate-activated p53 in C141 cells using luciferase assay. Addition of PFT (10 μM) to the stimulated cells significantly decreased the relative p53 activity induced by 50 μM vanadate. An increase in PFT concentration (20 μM) further attenuated the relative p53 activity.

3.5. The effects of vanadate on p21 activation and the role of p53

Western blotting was used to detect p21 protein level. Fig. 6 shows that C141 cells treated with 50 μM vanadate for 6–48 h displayed a time-dependent increase in p21 level (panel A). In contrast, p21 decreased when p53 deficient cells were treated with 50 μM vanadate for 6 and 12 h (panel B). In addition, vanadate activated p21 in a dose-dependent manner when the cells were treated with different concentrations of vanadate for 24 h (Fig. 7, panel A). In p53 deficient cells, p21 remained the same level as the control when the cells were stimulated with 10, 25 and 50 μM vanadate for 24 h (Fig. 7, panel B). Expression of p21 decreased at 100 μM vanadate treatment. Addition of PFT in stimulated C141 cells decreased the p21 level (Fig. 8). These results indicate that p53 is required for vanadate-induced p21 activation.

4. Discussion

The present study investigated vanadate-induced cell growth arrest and the role of p53. The results obtained from this study demonstrate that: (a) vanadate induced S phase arrest in a dose- and time-dependent manner in p53 wild type C141 cells, while no significant change was observed in p53 −/− cells; (b) vanadate caused a dose- and time-dependent increase in p53 activity in C141 cells; (c) PFT, a specific inhibitor of p53, blocked the p53 activity with a concomitant decrease in the percentage of S phase induced by vanadate; (d) vanadate activated p21 in a dose- and time-dependent manner in C141 cells while no change was observed in p53 −/− cells; (e) PFT inhibited p21 expression in vanadate stimulated C141 cells.

The ability of cells to maintain genomic integrity is vital for cell survival and proliferation. The important part in cell growth regulation is the ability of cells to pause transiently during the cell cycle in response to agents that cause damage, particularly to DNA. Surveillance controls the mechanisms that check and ensure proper completion of early events and cellular integrity before initiation of subsequent events in cell cycle progression. This process can cause a transient delay that has been suggested to allow the cells more time to repair damage before progressing to the next phase [20,21]. Alternatively, if the damage is too severe to be adequately repaired, the cells may undergo apoptosis or enter an irreversible senescence

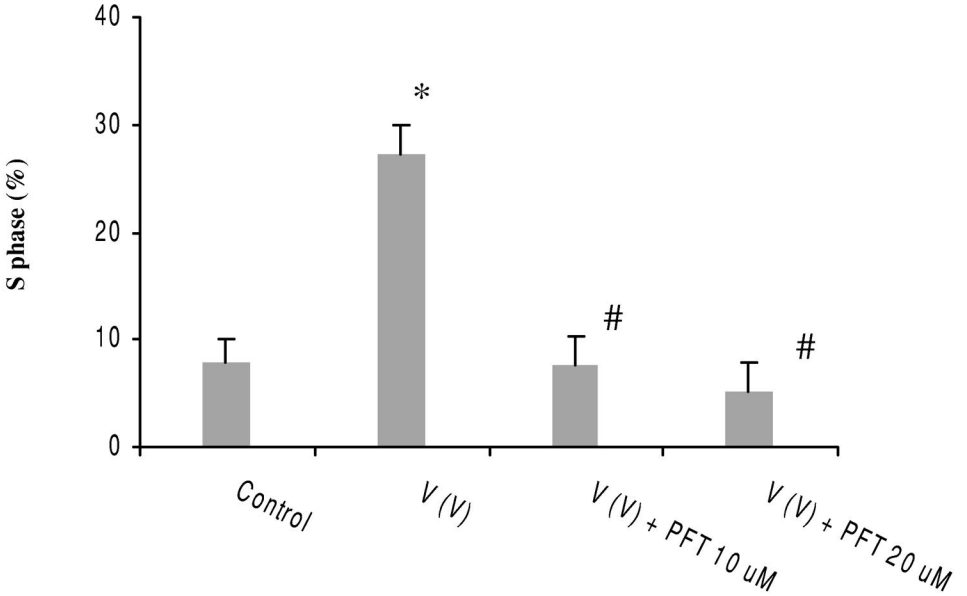

Fig. 4. Inhibitory effects of PFT on vanadate-induced cell growth arrest in C141 cells. The cells were incubated in a 100-mm dish and pretreated with 10 and 20 μM PFT for 0.5 h before vanadate treatment (50 μM). After 24 h, cells were harvested, and DNA content was measured by flow cytometry. Each point represents ±S.D. of the percentage of S phase of three independent experiments. $*p<0.05$ compared to control. $^{\#}p<0.05$ compared to vanadate treatment (one-way ANOVA with Scheffe's test).

like state [21]. In general, the toxic effects of vanadium are due to its inhibitory effects on certain enzymatic systems, mimicing and regulating growth factor activity and gene expression [22,23]. It has been reported that sodium vanadate causes G_2/M arrest and Rb hypophosphorylation in T98 glioma cells [24], demonstrating that the G_2/M arrest induced by a peroxovanadium compound was related to the reduced activity of $p34^{cdc2}$ and inhibition of Cdc25C [25]. A 2-h exposure of the melanoma cells to sodium vanadate led to a decrease in the activity of cyclin D [26]. Other studies have indicated that the mitogenesis induced by vanadate in CSV3-1 cells was associated with the induction of certain protooncogenes, c-*jun* and *jun B*, two major components of the AP-1 transcription factor [27].

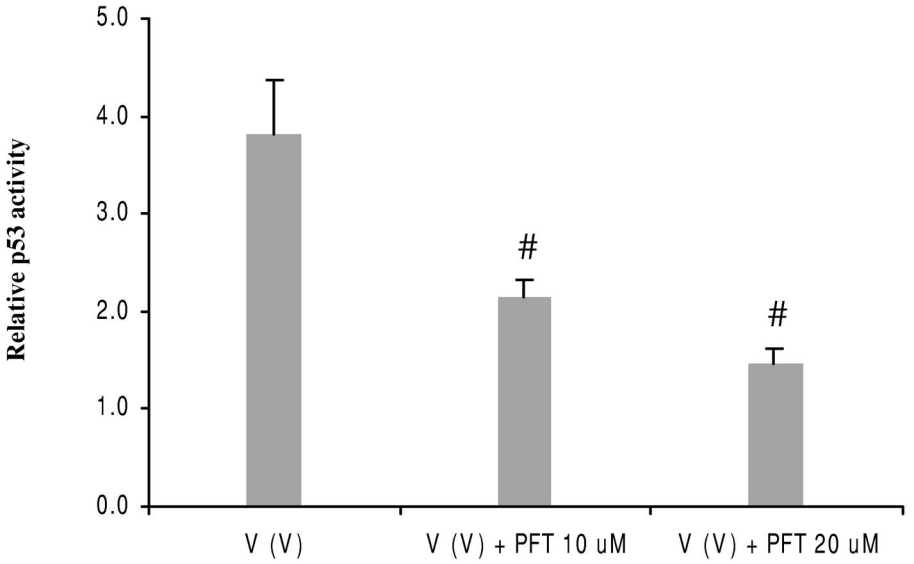

Fig. 5. Inhibitory effects of PFT on vanadate-induced p53 activation in C141 cells. A total of 1×10^4 cells were incubated in 96-well plate. The cells were pretreated with 10 and 20 μM PFT for 0.5 h prior to vanadate stimulation (50 μM). After 24 h, the cells were extracted with lysis buffer. The p53 luciferase activity was measured using a luminometer (moonlight 3010). The results were expressed as p53 activity relative to the control. Each point represents ±S.D. of the percentage of S phase of three independent experiments. $*p<0.05$ compared to control. $^{\#}p<0.05$ compared to 6-h vanadate treatment (one-way ANOVA with Scheffe's test).

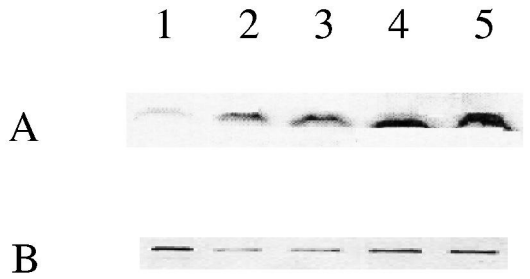

Fig. 6. Time dependence of vanadate on p21 level in C141 cells and p53 −/− cells. Both types of cells were incubated in 6-well plate. The cells were treated with 50 μM vanadate for different times. The whole cell lysates were collected for Western blotting using specific antibodies to p21. Panel A represents C141 cells and panel B represents p53 −/− cells. Lane 1, control; lane 2, 6 h; lane 3, 12 h; lane 4, 24 h; lane 5, 48 h. The results are representative of three separate experiments.

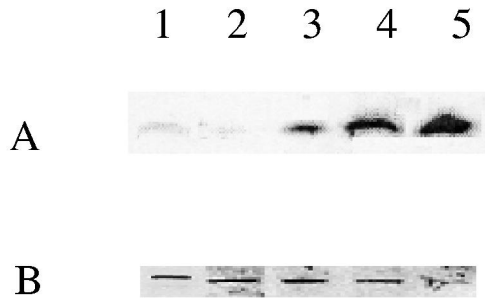

Fig. 7. Dose–response study of vanadate on p21 level in C141 cells and p53 −/− cells. The method used is the same as in Fig. 6. The cells were treated with different concentrations of vanadate for 24 h. Panel A represents C141 cells and panel B represents p53 −/− cells. Lane 1, control; lane 2, 10 μM vanadate; lane 3, 25 μM vanadate; lane 4 50 μM vanadate; lane 5, 100 μM vanadate. The results are representative of three separate experiments.

Our earlier study demonstrated that vanadate induced G_2/M phase arrest in a dose- and time-dependent manner in human epithelial cell line, A549 [28]. In the present study, the results showed that vanadate caused S phase arrest in mouse epidermal C141 cells. The difference in phase arrest induced by vanadate may be cell line specific.

The p53 tumor suppressor is a multifunctional protein that exerts a variety of different effects and plays a central role in the regulation of the normal cell cycle [14,29]. Overexpression of this protein was found to induce growth arrest associated with the G_0/G_1 checkpoint [8–10]. At

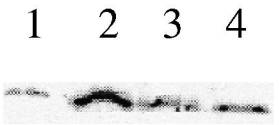

Fig. 8. The inhibitory effects of PFT on p21 expression in C141 cells. The cells were pretreated with 10 and 20 μM PFT for 0.5 h prior to vanadate treatment (50 μM, 24 h). Western blotting was used to examine the p21 level. Lane 1, control; lane 2, 50 μM vanadate for 24 h; lane 3, 50 μM vanadate+10 μM PFT for 24 h; lane 4, 50 μM vanadate+20 μM PFT for 24 h. The results are representative of three separate experiments.

G_0/G_1 checkpoint, wild type p53 was associated with DNA repair activity, and this association prevented the entry into S phase [30,31]. At G_2 checkpoint, p53 functions prior to mitosis and takes part in the spindle checkpoint associated with mitosis [32]. p53 is important for securing the stability and integrity of the genome of normal cells and was suggested to be the 'guardian of the genome' [33]. A variety of DNA insults were shown to stabilize the protein, which in turn can either cause growth arrest, permit the induction of the DNA repair process, or alternatively, direct cells to undergo apoptosis [34]. p53 was suggested to be a sensor of damaged DNA, and may be involved in the repair process [35]. It has been shown that cells containing wild type p53 alleles undergo G_1 arrest in response to γ-radiation, whereas cells lacking functional p53 alleles enter S phase regardless of dose of γ-radiation [36]. It has been demonstrated that vanadate-mediated generation of reactive oxygen species (ROS) plays an important role in various adverse biological effects induced by this metal [37,38]. Moreover, ROS are believed to be capable of damaging DNA [39]. Our earlier study showed that vanadate-induced apoptosis was p53 dependent which was mediated by H_2O_2 [40]. Our previous study also suggested that ROS are mediators of vanadate-induced cell growth arrest [28]. The results from the present study show that vanadate not only induces S phase arrest in C141 cells, but also causes an increase in p53 activity. Our study also showed that vanadate caused S phase arrest in p53 +/+ cells derived from mouse embryo fibroblasts similarly to that observed in C141 cells (unpublished observations). Due to lack of p53 in p53 −/− cells, vanadate failed to induce growth arrest at S phase. Although the mechanism involved in the vanadate-induced cell growth arrest remains to be investigated, it is known that ROS generated by vanadate-mediated reactions cause DNA damage. This DNA damage may activate signal transduction pathways, such as mitogen-activated protein kinase family, leading to an increase in p53 protein level and its phosphorylation. PFT, an inhibitor of p53, mainly inhibits p53 function [41]. The results obtained from the present study show that addition of 10 μM PFT decreased the p53 activity by 42% compared to the stimulated C141 cells (treated with 50 μM vanadate for 24 h). The inhibition was stronger when the concentration of PFT was increased to 20 μM, being ~60%. More importantly, pretreatment with PFT significantly decreased the percentage of S phase induced by vanadate. The percentage of S phase in stimulated cells was 27%. It was 8 or 5% after addition of PFT 10 or 20 μM, respectively. There was no significant difference in S phase between the PFT pretreated, vanadate-stimulated cells and the control cells (S phase, 8%). p21 (WAF-1/CIP1) is known to be a transactivation target of p53, and has been suggested to mediate p53-induced growth arrest triggered by DNA damage [42]. Induction of p21 expression could serve as an indicator for transactivation by p53, although p53-independent mecha-

nisms were also observed [14,29]. Induction of p21 may inhibit cell progression in two ways: (a) by inhibiting a variety of cyclin/cdk complexes and (b) by inhibiting DNA synthesis through proliferation cell nuclear antigen (PCNA) binding [43]. It is known that cyclin A accumulates at the G_1/S phase transition and persists through S phase. Cyclin A initially associates with cdk2 and then, in late S phase, associates with cdk1. Cyclin A-associated kinase activity is required for entry into S phase, completion of S phase, and entry into M phase [44–46]. The p21 protein inhibits the activity of cyclin A/cdk2, which in turn phosphorylates the E2F heterodimerization DP1, resulting in an inhibition of E2F DNA-binding activity [43]. The present study shows that vanadate indeed is able to cause a dose- and time-dependent increase in p21 protein level in C141 cells. In contrast, it was unable to activate p21 in p53 $-/-$ cells regardless of dose or time of vanadate treatment. Furthermore, addition of PFT significantly decreased p21 protein level activated by vanadate, indicating that vanadate-induced activation of p21 is through p53-dependent pathway. Our preliminary data also showed that during p21 activation in p53 wild type cells vanadate reduced both cyclin A and cdk2 expression. This observation suggests that both activated p21 binding to and inhibition of cdk2 are involved in vanadate-induced S phase arrest.

In conclusion, the results obtained from the present study demonstrate that vanadate is capable of inducing S phase arrest in C141 cells that contain wild type p53 alleles, and this interruption of cell cycle is p53-dependent. Activation of p53 leads to an increase in p21 expression, resulting in a pause in growth progression at S phase. Thus, vanadate-induced S phase arrest is mediated by p53 through activation of p21.

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