Vitamin B_6 activates p53 and elevates p21 gene expression in cancer cells and the mouse colon

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Abstract. Increasing evidence indicates vitamin B_6 acts as a protective factor against colon cancer. However, the mechanisms of the effect of vitamin B_6 are poorly understood. The present preliminary study using DNA microarray and real-time PCR indicates p21 mRNA is upregulated in human colon carcinoma (HT29) cells exposed to pyridoxal (PL, 500 μM). A similar effect was observed in human epithelial colorectal adenocarcinoma (Caco2) cells, human colon adenocarcinoma (LoVo) cells, human embryonic kidney (HEK293T) cells, and human hepatoma (HepG2) cells. Adding other B_6-vitamers such as pyridoxal 5'-phosphate (PLP), pyridoxine (PN), and pyridoxamine (PM) caused no such effect. In order to understand the mechanism of higher mRNA expression of p21 by PL, effect of PL on the p53 activation was examined (the upstream factor for p21 mRNA transcription) in HT29 cells, LoVo cells, and HepG2 cells. PL increased the phosphorylated p53 protein levels (active form) in whole-cell lysates and the nuclei of the cells. Noteworthy, the consumption of a vitamin B_6-deficient diet for 5 weeks significantly reduced p21 mRNA levels and tended to reduce phosphorylated p53 protein levels (P=0.053) in the colons of mice compared to a diet with adequate vitamin B_6. Thus, these results suggest vitamin B_6 plays a role in increasing p21 gene expression via p53 activation in several cancer cells and the mouse colon.

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Abbreviations: AOM, azoxymethane; IGFBP1, insulin-like growth factor-binding protein 1; IGFBP3, insulin-like growth factor-binding protein 3; PL, pyridoxal; PN, pyridoxine; PM, pyridoxamine; PLP, pyridoxal 5'-phosphate; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride

Key words: vitamin B_6, pyridoxal, p53, p21, colon cancer cells, mouse

Introduction

We previously reported that vitamin B_6 supplemented diet markedly reduces colon tumorigenesis in mice exposed to azoxymethane (AOM) (1,2). Consistently, increasing epidemiological evidence indicates vitamin B_6 acts as a protective factor against colon cancer (3-6). We also reported that vitamin B_6 decreases oxidative stress, inflammation, cell proliferation, epithelial cell damage, and angiogenesis, which may lead to lower tumorigenesis (1,2,7-10). Moreover, we recently found that high concentrations of pyridoxal (PL) increase the expression of insulin-like growth factor-binding protein 1 (IGFBP1), a putative tumor suppressor, in human hepatoma (HepG2) cells via the upregulation of the ERK/c-Jun pathway (11). However, the molecular mechanisms involved in the antitumor effect of vitamin B_6 remain unclear.

Our preliminary experiment involving DNA microarray analysis shows several genes are upregulated by 500 μM PL in human colon carcinoma (HT29) cells. Among these upregulated genes, higher p21 expression was confirmed by real-time PCR. It is well known that p21 negatively regulates cell cycle progression and is an antitumor factor (12). The gene expression of p21 is tightly controlled by the tumor-suppressor protein p53 (13). A recent study shows that a high dose of pyridoxine (PN, 10 mM) induces insulin-like growth factor-binding protein 3 (IGFBP3) mRNA expression in MCF-7 cells in a p53-dependent manner (14). Therefore, the present study examined the effect of vitamin B_6 on p21 gene expression and p53 activation in cancer cells and the colon of mice.

Materials and methods

Materials. PL hydrochloride, PN hydrochloride, and pyridoxal 5'-phosphate (PLP) were obtained from Nacalai Tesque (Kyoto, Japan), and pyridoxamine (PM) dihydrochloride was obtained from Calbiochem (La Jolla, CA, USA). Human colorectal cancer (HT29) cells, human epithelial colorectal adenocarcinoma (Caco2) cells, human colon adenocarcinoma (LoVo) cells, human embryonic kidney (HEK293T) cells, and human hepatoma (HepG2) cells were purchased from the Health Science Research Resources Bank (Japan) and the Japan Health Science Foundation (Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma (St. Louis, MO, USA). Anti-p-p53 antibody was obtained from Cell Signaling Technology (USA). Anti-p53 antibody

was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-tubulin antibody was obtained from Harlan Sera-Lab (UK).

Cell cultures and treatment. HT29, Caco2, LoVo, HEK293T, and HepG2 cells were maintained in a DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2. PL, PN, PM, or PLP was dissolved directly into the culture medium and filtered through a Millex-HV (0.45 μm; Millipore, Billerica, MA, USA).

Animals and diets. Four-week-old male ICR mice (Charles River, Japan) were housed in groups of 3 in metal cages in a room with controlled temperature (24±1˚C) and a 12:12-h light/dark cycle (lights on from 800-2000 h) according to the Guide for the Care and Use of Laboratory Animals established by Hiroshima University Animal Research Committee. After one week of acclimation on commercial stock diet (MF, Oriental Yeast, Tokyo, Japan), the mice were divided into two groups (n=9 each) fed diets with different vitamin B_6 concentrations. The basal diet consisted of the following components (g/kg diet): α-cornstarch, 302; casein, 200; sucrose, 200; corn oil, 200; cellulose, 50; AIN-93G mineral mixture, 35; AIN-93 vitamin mixture (PN-free), 10; and l-cysteine, 3. PN HCl (Nacalai Tesque) was added to the basal diet at 0 or 7 mg/kg diet (15). The level of PN HCl/kg diet recommended in the AIN-93 diet is 7 mg (16). Animals had free access to food and water *ad libitum* for 5 weeks.

mRNA analysis. Total RNA from HT29, Caco2, LoVo, HEK293T and HepG2 cells was isolated using TRIzol™ (Invitrogen, Carlsbad, CA, USA). The Qiagen Midi kit was used to isolate total RNA from mouse colons, which were subsequently prepared according to the standard protocol. Total RNA (1 μg) was reverse-transcribed using the First Strand cDNA Synthesis kit (Toyobo, Japan) according to the manufacturer's instructions. Real-time PCR was performed with a StepOne™ Real-Time PCR System (Applied Biosystems, Japan) using Thunderbird SYBR qPCR Mix (Toyobo, Japan). The human primer sets for p21, p53, and GAPDH were purchased from Greiner Bio-One (Japan) (p21: 5'-TGGA GACTCTCAGGGTCGAAA-3' and 5'-CGGCGTTTGGAG TGGTAGA-3'; p53: 5'-ATCTACTGGGACGGAACAGC-3' and 5'-GTGAGGCTCCCCTTTCTTG-3'; GAPDH: 5'-CAAT GACCCCTTCATTGACC-3' and 5'-TGGAAGATGGTGATG GGATT-3'). The mouse primer sets for p21 and GAPDH were also purchased from Greiner Bio-One (p21, 5'-AGTGTG CCGTTGTCTCTTCG-3' and 5'-ACACCAGAGTGCAAG ACAGC-3'; GAPDH: 5'-CATGGCCTTCCGTGTTCCTA-3' and 5'-CCTGCTTCACCACCTTCTTGAT-3'). The cycling parameters were as follows: initial step at 90˚C for 1 min, followed by 40 cycles of 90˚C for 15 sec and 60˚C for 1 min. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method normalized to GAPDH expression levels, and fold differences in expression were calculated relative to those of control samples.

Western blot analysis. Western blot analyses for p53 and p-p53 detection were performed using HT29, LoVo, and HepG2 cell lysates. The cells were grown to 70% confluence in 6-well plates. After PL treatment, the cells were washed twice with PBS and subsequently lysed in RIPA buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$] with 1% Triton X-100. Cell lysates were then centrifuged at 12,000 x g for 10 min to pellet debris. For mouse colons, ~0.1 g tissue homogenized with buffer [50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, 1 mM EDTA, protease inhibitor cocktail (1 mM phenylmethysulfonyl fluoride, 5 μg/ml aprotinin and 5 μg/ml leupeptin)] were added before homogenization. After homogenization, samples were centrifuged at 12,000 x g for 10 min to pellet debris. The total protein of cells or colons was assayed using a Bio-Rad Protein Assay kit (Bio-Rad, Bath, UK).

To extract nuclear fractions, HT29, LoVo and HepG2 cells were collected and washed twice with ice-cold PBS. The harvested cells were lysed in nuclear protein extraction buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA and 0.1 mM EGTA with 50 μl 10% NP-40, 20 μl 0.1 M DTT, 5 μl 2 μg/ml aprotinin, and 5 μl 2 μg/ml leupeptin in 1 ml buffer added before use], and pipetted up and down to disrupt cell clumps. The cell lysates were subsequently centrifuged at 12,000 x g for 3 min at 4˚C, and the supernatant was removed. Then, 500 μl buffer A was added to the tube, which was subsequently vortexed for 10 sec and centrifuged at 12,000 x g for 3 min at 4˚C. The supernatant was subsequently discarded. The pellet was lysed with 60 μl nuclear protein extraction buffer B [20 mM HEPES (pH7.9), 0.4 mM NaCl, 1 mM EDTA, and 1 mM EGTA with 10 μl 0.1 M DTT, 5 μl 2 μg/ml aprotinin, and 5 μl 2 μg/ml leupeptin in 1 ml buffer added before use] and incubated on ice for 15 min with intermittent vortexing. The lysate was subsequently centrifuged at 12,000 x g for 10 min at 4˚C, and the supernatant was collected as the nuclear fraction. Protein concentration was measured with the Bio-Rad Protein Assay kit.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the protein pellets, and the samples were heated for 3 min at 95˚C. Samples were loaded (10 μg of total protein for whole-cell lysate or nuclear fraction), and electrophoretically separated on 10% polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Western blotting was performed according to standard protocols, and proteins were visualized using primary antibodies against p53 (mouse monoclonal antibody, diluted 1:1,000), p-p53 (rabbit polyclonal antibody, diluted 1:1,000), and tubulin (rat monoclonal antibody, diluted 1:1,000).

Statistical analysis. The SSRI Excel Statistics 2006 software (Japan) was used in the statistical analysis. Data are presented as means ± SE. Differences among the means of treatment groups were analyzed by one-way ANOVA; significance was determined by Scheffe's multiple-range test. Student's t-test was used for comparisons between 2 groups. The level of significance for all tests was set at $P<0.05$.

Results

Effect of vitamin B_6 on p21 mRNA expression in cancer cells. DNA microarray analysis revealed p21 mRNA expression was upregulated in HT29 cells in response to treatment with

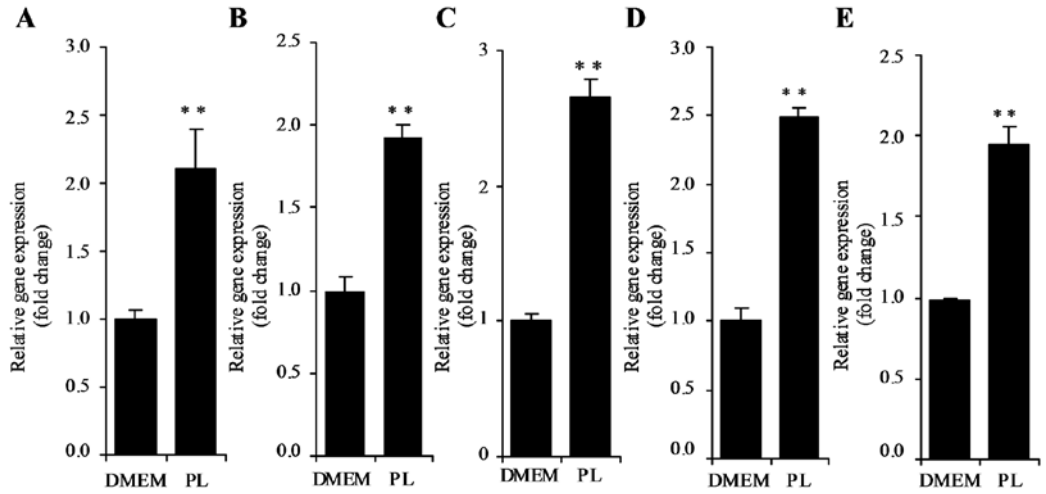

Figure 1. Stimulation of p21 mRNA expression by PL in HT29, Caco2, LoVo, HEK293T, and HepG2 cells. HT29 (A), Caco2 (B), LoVo (C), HEK293T (D) and HepG2 (E) cells were incubated in presence or absence of PL at 500 μM over 24 h. p21 mRNA level was determined by real-time PCR. Cells cultured with medium (DMEM with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin) were used as control (DMEM). Values represent means ± SE (n=4). **P<0.01 compared with relative control.

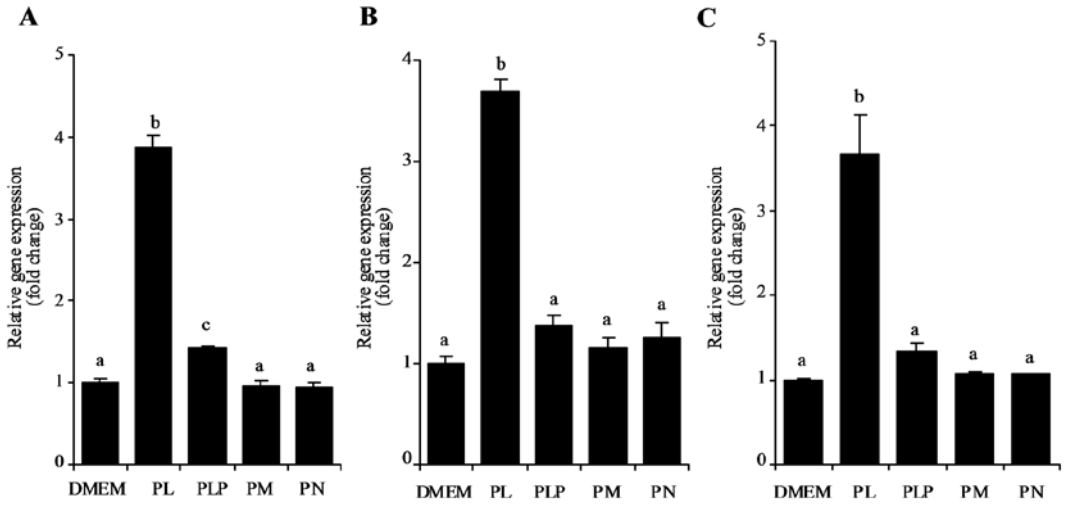

Figure 2. Effect of B_6-vitamers on p21 gene expression in HT29, LoVo and HepG2 cells. HT29 (A), LoVo (B) and HepG2 (C) cells were incubated with different B_6-vitamers (PL, PLP, PM or PN) at the concentration of 500 μM over 24 h. p21 mRNA level was determined by real-time PCR. Values represent means ± SE (n=4). Groups with different letters are significantly different from each other (P<0.05).

500 μM PL for 24 h. Stimulation of p21 mRNA expression by PL in HT29 cells was confirmed by real-time PCR (Fig. 1A). Caco2, LoVo, HEK293T and HepG2 cells were incubated in the presence or absence of 500 μM PL for 24 h, and p21 mRNA levels were examined by real-time PCR. As shown in Fig. 1B-E, p21 mRNA levels were significantly increased by PL in Caco2, LoVo, HEK293T, and HepG2 cells (P<0.01).

The effect of adding B_6-vitamers including PL, PM, PN, and PLP at 500 μM on p21 mRNA expression in HT29, LoVo, and HepG2 cells were analyzed. The results indicate PL significantly stimulated p21 mRNA expression (P<0.05) whereas other B_6-vitamers had no such effect in HT29 (Fig. 2A), LoVo (Fig. 2B), or HepG2 cells (Fig. 2C).

Time- and dose-dependent effect of PL on p21 mRNA expression. We investigated the effect of different concentrations of PL on p21 mRNA expression. After incubating HT29, LoVo or HepG2 cells with 100, 250 or 500 μM PL for 24 h, p21 mRNA levels increased dose-dependently from 100 to 500 μM (Fig. 3A-C). At 500 μM, PL significantly stimulated p21 mRNA expression in HT29, LoVo and HepG2 cells (P<0.05). To examine the time-dependent effect of PL, HT29, LoVo or HepG2 cells were cultured with or without 500 μM PL for 6, 12 or 24 h. PL treatment increased p21 mRNA expression from 6 to 24 h in these cells (P<0.01) (Fig. 4A-C).

Effect of PL on p53 activation. As the transcription of p21 gene is tightly controlled by p53, p53 gene expression was examined in HT29, LoVo, and HepG2 cells in response to treatment with 500 μM PL for 24 h. There was no significant difference in p53 mRNA expression between the control and PL-treated cells (data not shown). In order to understand the activation of the p53 pathway, p-p53 protein was analyzed in HT29 (Fig. 5A), LoVo (Fig. 5B), and HepG2 cells (Fig. 5C). PL increased p-p53

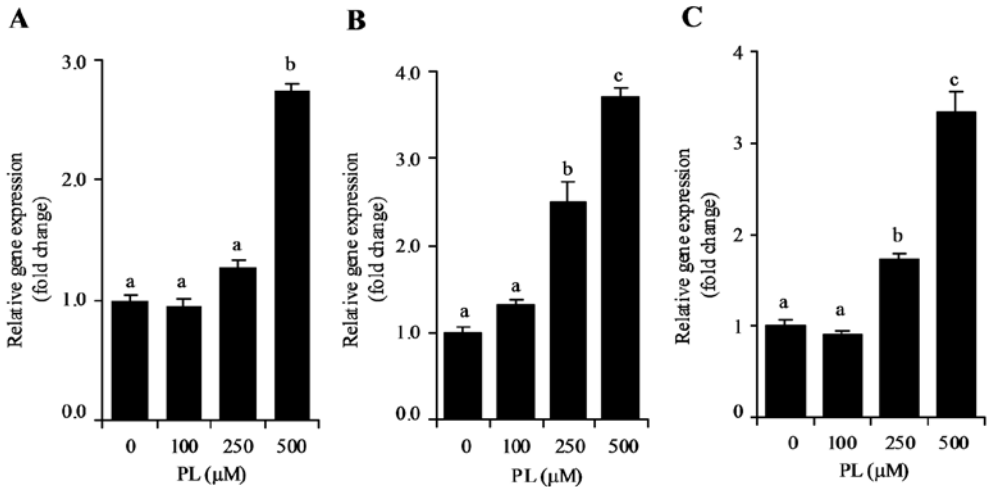

Figure 3. The mRNA expression of p21 in response to PL incubation at different doses. HT29 (A), LoVo (B) and HepG2 (C) cells were incubated with PL at 0, 100, 250 or 500 μM over 24 h. p21 mRNA level was determined by real-time PCR. Results are means ± SE (n=4). Groups with different letters are significantly different from each other (P<0.05).

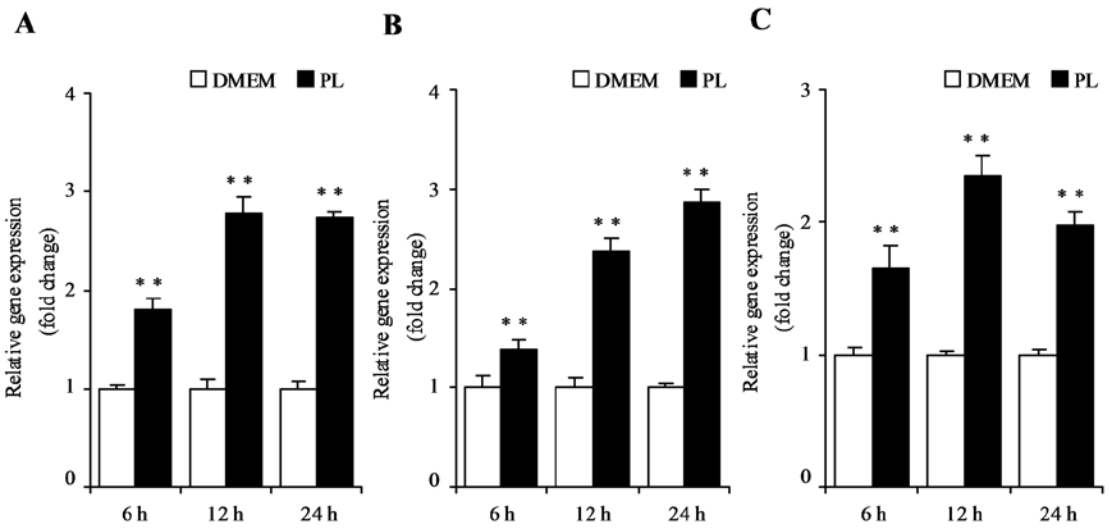

Figure 4. The p21 mRNA expression in response to incubation with PL for different time periods. HT29 (A), LoVo (B) and HepG2 (C) cells were incubated with PL at the concentration of 500 μM for 6, 12 or 24 h. p21 mRNA level was determined by real-time PCR. Results are means ± SE (n=4). **P<0.01 compared with relative control.

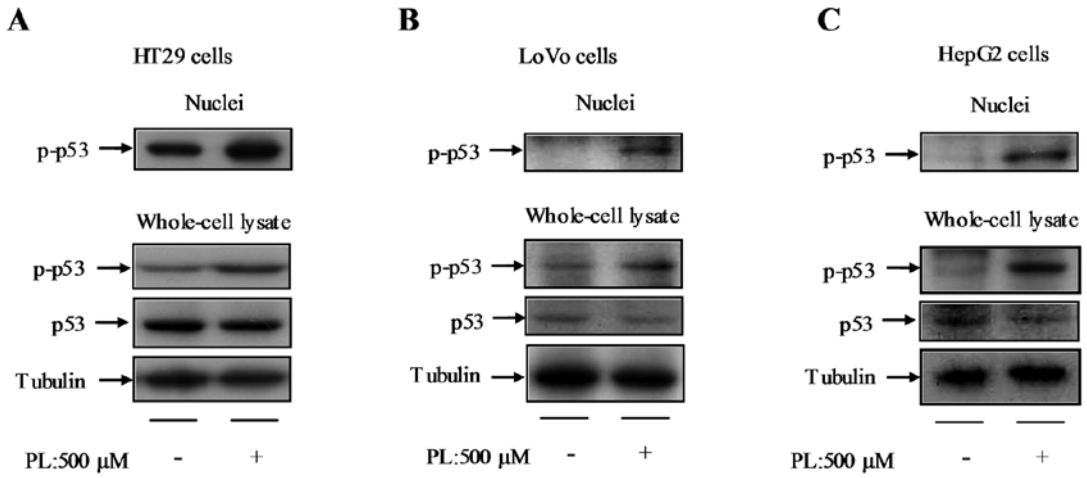

Figure 5. Activation of p53 pathway in HT29, LoVo and HepG2 cells by PL. HT29 (A), LoVo (B) and HepG2 (C) cells were incubated with PL at the concentration of 500 μM for 24 h. The p53 of whole-cell lysate, p-p53 of whole-cell lysate and nuclei in HT29 (A), LoVo (B) and HepG2 (C) cells were analyzed by western blotting.

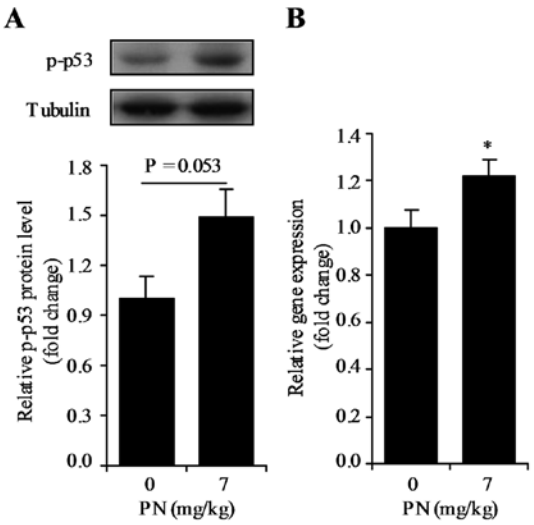

Figure 6. Elevation of p21 mRNA level in colon of mice by dietary vitamin B_6. The p-p53 in colon of mice (A) was analyzed by western blotting (n=6). The p21 mRNA in colon of mice (B) was determined by real-time PCR (n=9). Results are means ± SE. *P<0.05 compared with 0 mg PN HCl/kg diet group.

protein expression in whole-cell lysates and the nuclei of these cell lines, but did not increase the total protein level of p53 (Fig. 5A-C).

Effect of vitamin B_6-deficient diet on p-p53 protein level and p21 mRNA expression in the mouse colon. To understand the role of vitamin B_6 in the upregulation of p21 mRNA expression *in vivo*, 2 groups of mice fed diets with different vitamin B_6 contents (i.e., 0 and 7 mg PN HCl/kg) were used. There was a small, but significant difference in final body weight between the mice fed the 0 and 7 mg PN HCl/kg diets (41.1±1.0 g and 46.2±1.4 g, respectively) (P<0.05). There was no significant difference in total food intake between the 2 groups (data not shown). Mice fed the PN-deficient diet tended to exhibit reduced p-p53 protein expression (P=0.053) (Fig. 6A) and significantly reduced p21 mRNA expression (P<0.05) (Fig. 6B) in the colon. There was no association between final body weight and p-p53 protein expression or p21 mRNA expression (P>0.05). This implies the effect of dietary vitamin B_6 may not be related to changes in body weight.

Discussion

The results of the present study demonstrate PL increases p21 mRNA expression in HT29, Caco2, LoVo, HEK293T and HepG2 cells. This implies the upregulating effect of PL on p21 mRNA expression might occur in a wide variety of cancer cells. On the other hand, other B_6-vitamers including PLP, PN and PM had no such effect on p21 mRNA expression. We recently found that PL remarkably increases IGFBP1 mRNA whereas no other B_6-vitamers have a similar effect (11). PL can freely pass through the cell membrane, and only PL is reported to interact with the cell surface of RAW264.7 cells cultured in culture medium treated with B_6-vitamers (i.e., PL, PM, PN and PLP) (17,18). Therefore, the stimulation of p21 mRNA expression might be related to the cell surface interaction and penetration of PL.

The results further indicate PL increases p-p53 protein levels in both the whole-cell lysate and nuclei of HT29, LoVo and HepG2 cells. This implies PL activates p53. Concordant with these cell culture experiments, mice fed the vitamin B_6-deficient diet exhibited decreased p21 mRNA expression and tended to exhibit decreased phosphorylated p53 protein levels in the colon than mice fed the diet containing adequate vitamin B_6. Therefore, higher p21 expression by vitamin B_6 may be at least be partially mediated by the activation of p53. Nakari *et al* show that a high dose of PN (10 mM) inhibits the growth of MCF-7 cells and induces IGFBP3 expression in a p53-dependent manner (14). However, in the present study, 500 μM PN did not increase p21 gene expression. Therefore, these findings suggest PL rather than PN affects the p53/p21 pathway.

We previously suggested dietary vitamin B_6 supplementation suppresses colon tumorigenesis by decreasing colon cell proliferation, inflammation and oxidative stress in mice treated with AOM (1). Accumulating evidence from *in vitro* and *in vivo* studies suggests p21 and p53 suppress cell proliferation (2,11,19-25). In addition, p21 protects against oxidative stress (26). Importantly, p53 and p21 are reported to be anti-inflammation factors (27-29). Furthermore, p21 is a negative regulator of macrophage activation; in particular, it inhibits the lipopolysaccharide-dependent stimulation of TNF-α and IL-1β (29,30). Moreover, the inhibition caused by p21 inhibits the NF-κB activity (29,30). Yanaka *et al* (7) reported the inhibitory effect of PL on the lipopolysaccharide-dependent activation of NF-κB in macrophages. In addition, activation of p53 is reported to lead to cell cycle arrest, DNA repair, and genomic stability (31). Taken together, these findings raise the question of whether increased p21 expression and p53 activation by PL are associated with decreased cell proliferation, oxidative stress and inflammation.

In conclusion, this study provides evidence of a role of PL in the upregulation of p21 gene expression in HT29, Caco2, LoVo, HEK293T and HepG2 cells. Furthermore, the p53 pathway, which is responsible for controlling p21 mRNA transcription, is activated by PL in cancer cells. Importantly, p21 mRNA levels were higher in the colon of mice fed a diet with adequate vitamin B_6 than those fed a vitamin B_6-deficient diet. Thus, these findings may help us understand the antitumor effect of vitamin B_6 via the activation of p53 and elevation of p21 mRNA.

Acknowledgements

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