**Abstract.** Increasing evidence indicates vitamin B\textsubscript{6} acts as a protective factor against colon cancer. However, the mechanisms of the effect of vitamin B\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{6}. Thus, these results suggest vitamin B\textsubscript{6} plays a role in increasing p21 gene expression via p53 activation in several cancer cells and the mouse colon.

**Introduction**

We previously reported that vitamin B\textsubscript{6} supplemented diet markedly reduces colon tumorigenesis in mice exposed to azoxymethane (AOM) (1,2). Consistently, increasing epidemiological evidence indicates vitamin B\textsubscript{6} acts as a protective factor against colon cancer (3-6). We also reported that vitamin B\textsubscript{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\textsubscript{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\textsubscript{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% CO₂. 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 B₆ concentrations. The basal diet consisted of the following components (g/kg diet): α-corn starch, 302; casein, 200; sucrose, 200; corn oil, 200; cellulose, 50; AIN-93G mineral mixture, 35; AIN-93 vitamin mixture (PN-free), 10; and 1-cysteine, 3. PHCl (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) using Thunderbird SYBR qPCR Mix (Toyobo, Japan). The mouse primer sets for p21 and GAPDH were purchased from Greiner Bio-One (Japan) (p21: 5'-TGGA GACTCTCAGGGTGCAAA-3' and 5'-CGCGGTTTGGAG TGTTAGA-3'; p53: 5'-ATCTACTGGGACGGAACAGC-3' and 5'-GTGAGGCCTCCTTCTTGG-3'; GAPDH: 5'-CATT GACCCTCTTGGACC-3' and 5'-TGGAAGATGGTGATG GGATT-3'). The mouse primer sets for p21 and GAPDH were also purchased from Greiner Bio-One (p21, 5'-AGTGTG CCCTTTGGCTCTTTC-3' and 5'-ACACCCAGGCTCGAA ACAG-3'; GAPDH: 5'-CATTGGGCTTCTGTGCTCTA-3' and 5'-CCTGCTTCACCACCTTCTGAT-3'). The human primer sets for p21, p53, and GAPDH were purchased from Greiner Bio-One (p21: 5'-TGGA GACTCTCAGGGTGCAAA-3' and 5'-CGCGGTTTGGAG TGTTAGA-3'; p53: 5'-ATCTACTGGGACGGAACAGC-3' and 5'-GTGAGGCCTCCTTCTTGG-3'; GAPDH: 5'-CATT GACCCTCTTGGACC-3' and 5'-TGGAAGATGGTGATG GGATT-3'). The primer sets for p21 and GAPDH were purchased from Greiner Bio-One (Japan) using Thunderbird SYBR qPCR Mix (Toyobo, Japan). The human primer sets for p21, p53, and GAPDH were purchased from Greiner Bio-One (Japan) using Thunderbird SYBR qPCR Mix (Toyobo, Japan). The human primer sets for p21, p53, and GAPDH were purchased from Greiner Bio-One (Japan) using Thunderbird SYBR qPCR Mix (Toyobo, Japan).

Protein concentration was measured with the Bio-Rad Protein Assay kit (Toyobo, Japan). The human primer sets for p21, p53, and GAPDH were purchased from Greiner Bio-One (Japan) using Thunderbird SYBR qPCR Mix (Toyobo, Japan).

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 B₆ on p21 mRNA expression in cancer cells. DNA microarray analysis revealed p21 mRNA expression was upregulated in HT29 cells in response to treatment with...
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 B6-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 B6-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
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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.
show that a high dose of PN (10 mM) inhibits penetration of PL. Therefore, the stimulation of p21 mRNA in culture medium treated with B6 may be at least 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 B6 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-inflammatory 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 B6 than those fed a vitamin B6-deficient diet. Thus, these findings may help us understand the antitumor effect of vitamin B6 via the activation of p53 and elevation of p21 mRNA.

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References


