Note

Dietary Vitamin B₆ Suppresses Colon Tumorigenesis, 8-Hydroxyguanosine, 4-Hydroxynonenal, and Inducible Nitric Oxide Synthase Protein in Azoxymethane-Treated Mice

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Summary Recently we reported that the supplementation of vitamin B₆ to low vitamin B₆ diet caused suppression in colon tumorigenesis and cell proliferation of azoxymethane-treated mice in a dose-dependent manner among 1, 7, and 14 mg pyridoxine HCl/kg diet (*J Nutr* 131: 2204–2207, 2001). To examine the mechanism of the anticolon tumor effect of vitamin B₆, male ICR mice were fed the diet containing 1, 7, 14, and 35 mg pyridoxine HCl/kg diet for 22 wk and simultaneously given a weekly injection of azoxymethane for an initial 10 wk. The supplementation of vitamin B₆ to a low vitamin B₆ diet (1 mg pyridoxine HCl/kg) suppressed the levels of colonic 8-hydroxyguanosine and 4-hydroxynonenal and inducible nitric oxide synthase protein. The results suggest that the preventive effect of vitamin B₆ against colon tumorigenesis is at least in part mediated by reducing oxidative stress and nitric oxide production.

Key Words vitamin B₆, colon tumorigenesis, cell proliferation, oxidative stress, nitric oxide

A population-based case-control study in the United States has indicated an inverse association between the intake of vitamin B₆ and colon cancer (1). We have recently examined the effect of the dietary level of vitamin B₆ (pyridoxine HCl, 1, 7, 14, and 35 mg/kg) on colon tumorigenesis in mice that have received azoxymethane (2). The supplementation of vitamin B₆ (pyridoxine HCl) to the 1 mg pyridoxine HCl/kg diet caused a marked suppression in colon tumorigenesis in a dose-dependent manner among 1–14 mg/kg pyridoxine HCl (2). This antitumor effect of vitamin B₆ was associated with a reduction in the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into the colonic cells (BrdU-labeling index) and the colonic expression of *c-myc* and *c-fos* proteins (oncogene products relating to cell proliferation), implying the involvement of reduced colonic cell proliferation in the mechanism of the effect of vitamin B₆ (2). It has been reported that *c-myc* and *c-fos* expression can be induced by oxidative stress (3, 4). Since vitamin B₆ has been found to have a strong antioxidative effect (5), we postulated that the suppression effect of vitamin B₆ on the cell proliferation might be mediated

through reduced oxidative stress.

Nitric oxide (NO), one of the oxygen free radicals, has been considered to play an important role in colon carcinogenesis (6, 7). Moreover, the expression and activity of inducible NO synthase (iNOS) is higher in human adenomas (8). The production of NO and the expression of iNOS mRNA is elevated by oxidative stress, which is induced by superoxide (9). Therefore in this study we examined the effect of dietary vitamin B₆ on colonic tumorigenesis, oxidative stress, and iNOS expression in azoxymethane (AOM)-treated mice.

Materials and Methods

Animals and diets. Three to four male CD-1 (ICR): Crj mice (4 wk old, Charles River Japan Inc., Hino, Japan) were kept in a metal cage at a controlled temperature (24±1°C) in a 12 h light:dark cycle (light on, 0800–2000 h). They had free access to diet and deionized water. The mice were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. After being fed commercial stock diet (MF, Oriental Yeast, Tokyo, Japan) for 1 wk, the mice (average 26 g) were divided into four groups of 34 mice each. The basal diet included the following components (g/kg diet): α -corn starch, 402; casein, 200; sucrose, 200; corn oil, 100; cellulose, 50; AIN-93G mineral mixture, 35; AIN-93 vitamin mix-

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Abbreviations: AOM, azoxymethane; 8-OHdG, 8-hydroxydeoxyguanosine; 4-HNE, 4-hydroxynonenal; NO, nitric oxide.

ture (pyridoxine free), 10; and L-cystine, 3 (10). Vitamin B₆ (pyridoxine HCl, Nacalai Tesque Inc., Kyoto, Japan) was supplemented along with basal diet at a concentration of 1, 7, 14, and 35 mg/kg diet. A 7 mg pyridoxine HCl/kg diet is the level recommended as the AIN-93 diet (10), and a 1 mg pyridoxine HCl/kg diet has been reported to be the minimum level required for preventing growth depression caused by vitamin B₆ deficiency (11). The feeding continued for 22 wk. The mice were given azoxymethane (AOM, 5 mg/kg body weight, Sigma Chemical, St. Louis, MO, USA), diluted by saline by subcutaneous injection once a week for the initial 10 wk of the experiment. The food intake and body weight were measured every day.

At the termination of the experimental treatment, the colon was removed, slit open longitudinally from cecum to anus, placed on a paper towel, and fixed in neutral formalin for 24 h. The tumor-bearing areas and volumes were observed under a microscope and embedded in paraffin.

Oxidative stress markers. The level of lipid peroxide in serum was measured as thiobarbituric-acid-reacting substances (TBARS) by the use of kit (Kasankashishitsu-Test Wako, Wako Pure Chemical Industries, Osaka, Japan). An immunohistochemical analysis of 8-hydroxydeoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) labeling was performed for colonic mucosa. The 8-OHdG and 4-HNE staining methods were as follows. After deparaffinization, a monoclonal anti-8-OHdG antibody (Nippon Yushi, Tokyo, Japan) and a monoclonal anti-4-HNE antibody (Nippon Yushi) were added to the specimens, and they were stained by "Vectastain Universal Quick Kit" (Vector Laboratories, Burlingame, CA). The 8-OHdG and 4-HNE positive cells in the colonic mucosal epithelium were counted under the microscope at a magnification of 200 $\times$.

Expression of iNOS protein. An immunohistochemical analysis of iNOS protein was performed for the normal colonic mucosa. The iNOS staining was as follows. After deparaffinization, the rabbit polyclonal anti-iNOS

antibody (Santa Cruz Biochemistry, Santa Cruz, CA) was added to the specimens, and they were stained by "Vectastain Elite ABC Kit" (Vector Laboratories). The iNOS-positive cells in the colonic mucosal epithelium were counted under the microscope at a magnification of 200 $\times$.

Statistical analysis. Values are presented as means $\pm$ SE. The statistical significance of mean values was estimated at $p<0.05$ according to one-way ANOVA and Duncan's multiple-range tests (12). Some data were analyzed by regression analysis, and the correlation coefficient was calculated.

Results

Body weight and food intake

The food intake and final body weight did not differ between the experimental and control groups.

Oxidative stress markers

The labeling index of 8-OHdG in the colonic crypt was significantly reduced by higher dietary vitamin B₆ (ANOVA analysis, $p<0.05$, Table 1). Seven, 14, and 35 mg pyridoxine HCl/kg diets significantly reduced this parameter as compared to the 1 mg pyridoxine HCl/kg diet group in all areas of the colon. The labeling index of 4-HNE in the colonic crypt was also significantly reduced by the higher dietary vitamin B₆ (ANOVA analysis, $p<0.05$, Table 1). A similar reduction was observed in the 7, 14, and 35 mg pyridoxine HCl/kg diet groups as compared to the 1 mg pyridoxine HCl/kg diet group in the distal colon and proximal colon ($p<0.05$). In the rectum, the 35 mg pyridoxine HCl/kg diet group significantly reduced this parameter compared to the 7 mg pyridoxine HCl/kg diet group ($p<0.05$). The labeling index of 8-OHdG was correlated with the expression of *c-myc* ($r=0.57$, $p<0.01$) and *c-fos* protein ($r=0.83$, $p<0.01$) in all the colon epithelium, as previously reported (2). The labeling index of 4-HNE was also correlated with the expression of *c-myc* ($r=0.72$, $p<0.01$) and *c-fos* protein ($r=0.76$, $p<0.01$), as previously reported (2). The concentration of serum

Table 1. Effect of dietary vitamin B₆ on oxidative stress markers in azoxymethane-treated mice.

	Pyridoxine HCl (mg/kg diet)			
	1	7	14	35
Labeling index of 8-OHdG expression cells (%)				
Rectum	7.28 $\pm$ 0.22 ^a	5.81 $\pm$ 0.16 ^b	5.26 $\pm$ 0.21 ^b	5.36 $\pm$ 0.29 ^b
Distal colon	6.67 $\pm$ 0.22 ^a	5.73 $\pm$ 0.18 ^b	5.20 $\pm$ 0.23 ^b	5.12 $\pm$ 0.27 ^b
Proximal colon	6.10 $\pm$ 0.25 ^a	4.71 $\pm$ 0.18 ^b	4.54 $\pm$ 0.26 ^b	4.51 $\pm$ 0.36 ^b
All colon	6.68 $\pm$ 0.14 ^a	5.42 $\pm$ 0.10 ^b	5.00 $\pm$ 0.14 ^c	4.99 $\pm$ 0.18 ^c
Labeling index of 4-HNE expression cells (%)				
Rectum	11.8 $\pm$ 0.5 ^a	9.1 $\pm$ 0.3 ^b	8.0 $\pm$ 0.5 ^{bc}	7.4 $\pm$ 0.4 ^c
Distal colon	10.5 $\pm$ 0.4 ^a	8.7 $\pm$ 0.4 ^b	7.9 $\pm$ 0.5 ^b	7.8 $\pm$ 0.5 ^b
Proximal colon	9.3 $\pm$ 0.5 ^a	7.4 $\pm$ 0.3 ^b	7.1 $\pm$ 0.5 ^b	7.2 $\pm$ 0.7 ^b
All colon	10.5 $\pm$ 0.3 ^a	8.4 $\pm$ 0.2 ^b	7.7 $\pm$ 0.3 ^b	7.5 $\pm$ 0.3 ^b
Serum TBARS (μ mol/L)	7.69 $\pm$ 0.68 ^a	5.72 $\pm$ 0.61 ^b	5.64 $\pm$ 0.53 ^b	5.68 $\pm$ 0.54 ^b

Values are means $\pm$ SE ($n=17$). Means in a row not sharing a superscript letter are significantly different by Duncan's multiple-range test ($p<0.05$).

Table 2. Effect of dietary vitamin B₆ on colonic expression of iNOS protein in azoxymethane-treated mice.

	Pyridoxine HCl (mg/kg diet)			
	1	7	14	35
Labeling index of iNOS expression cells (%)				
Rectum	12.5 ± 0.3 ^a	11.6 ± 0.4 ^{ab}	10.8 ± 0.5 ^{bc}	10.0 ± 0.6 ^c
Distal colon	12.5 ± 0.4 ^a	11.7 ± 0.3 ^{ab}	10.6 ± 0.4 ^{bc}	9.6 ± 0.5 ^b
Proximal colon	9.7 ± 0.4 ^a	9.2 ± 0.3 ^{ab}	8.4 ± 0.5 ^{ab}	8.0 ± 0.7 ^b
All colon	11.5 ± 0.2 ^a	10.8 ± 0.2 ^b	9.9 ± 0.3 ^c	9.2 ± 0.3 ^c

Values are means ± SE (n=17). Means in a row not sharing a superscript letter are significantly different by Duncan's multiple-range test ($p < 0.05$).

TBARS was significantly higher in the 1 mg pyridoxine HCl/kg diet group than in the other groups. However, it did not differ significantly among 7, 14, and 35 mg pyridoxine HCl/kg diet groups ($p < 0.05$).

Expression of iNOS protein

The labeling index of iNOS protein in the colonic crypt was significantly reduced by the supplementation of vitamin B₆ (ANOVA analysis, $p < 0.05$, Table 2). Fourteen and 35 mg pyridoxine HCl/kg diets significantly reduced this parameter as compared to the 1 mg pyridoxine HCl/kg diet in the rectum and distal colon. However, in the proximal colon only the 35 mg pyridoxine HCl/kg diet group significantly lowered the expression of iNOS protein as compared to the 1 mg pyridoxine HCl/kg diet group. All the colon-labeling index were significantly lower in the 7, 14, and 35 mg pyridoxine HCl/kg diet groups than in the 1 mg pyridoxine HCl/kg diet group. The labeling index of iNOS in all the colon epithelium was correlated with that of 8-OHdG ($r = 0.70$, $p < 0.01$) and 4-HNE ($r = 0.54$, $p < 0.01$).

Discussion

The present study has demonstrated that a dietary supplementation of vitamin B₆ reduced the level of colonic 8-OHdG and 4-HNE as well as colonic tumorigenesis. The serum level of TBARS was also higher in the 1 mg pyridoxine HCl/kg diet group as compared to other groups. These findings suggest that the antitumor effect of vitamin B₆ is at least in part mediated through a reduction of oxidative stress. The free radical dependent lipid peroxidation has been suggested to be involved in the tumor promotion and progression of carcinogenesis (13). The production of 8-OHdG by oxygen radicals induces mutagenesis and carcinogenesis by causing a misreading of DNA bases (14). 4-HNE is produced as a major product of the peroxidation of ω -6 polyunsaturated fatty acids, and it possesses cytotoxic, hepatotoxic, mutagenic, and genotoxic properties (15). Recently an in vitro study demonstrated a strong antioxidative effect of vitamin B₆ (5). Thus the supplementation of vitamin B₆ appears to directly inhibit the oxidative stress in the colorectal mucosa treated with AOM, and this leads to lower tumorigenesis.

In our study, the labeling index of 8-OHdG and 4-HNE in the colon epithelium was significantly associated with the expression of *c-myc* and *c-fos* proteins.

The oxidative stress has been reported to cause an increase in the expression of *c-myc* and *c-fos* (3, 4), implying a proliferative response to oxidative stress. Thus it is likely that vitamin B₆ suppresses tumorigenesis through a reduction in oxidative stress and hypercell proliferation in the colorectal mucosa treated with AOM.

The present study has further demonstrated that the supplemental vitamin B₆ suppressed the expression of iNOS protein in colon mucosa. NO has been considered to play a role in the colon carcinogenesis by elevating cyclooxygenase-2 and angiogenesis (6, 7). The higher expression and activity of iNOS have been found in human colon adenomas (9). Some studies have also demonstrated that AOM-induced colon tumors have increased an expression and/or activity of iNOS when compared to levels in adjacent colonic tissue (16). It has been reported that the expression of iNOS protein and mRNA and NO synthesis are increased by oxidative stress induced by superoxide (8). Our data also indicated an association between the levels of oxidative stress markers and iNOS expression in the colon. Taken together, the lower expression of iNOS protein by the supplementation of vitamin B₆ may also be partially involved in the mechanism of its preventive effect against the tumorigenesis. Although the present study indicated the reductions in the markers of oxidative stress and iNOS protein expression in the colon by a dietary supplementation of vitamin B₆, these alterations appeared to be not enough to give a satisfactory explanation of the suppression in the tumorigenesis previously reported (2). Therefore the possibilities of other mechanisms seem to remain.

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