Effect of High Dose of Pyridoxine on Mammary Tumorigenesis

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Abstract: The effect of high-dose pyridoxine (PN) on mammary tumorigenesis was examined in female Sprague-Dawley rats. The first mammary tumors appeared between 84 and 90 days after 7,12-dimethylbenzanthracene treatment. There was no effect of PN level on tumor incidence at 90 days but at 98, 104, and 111 days. Tumor incidence was lower in the high-dose group (35 mg PN/kg diet) compared with the controls (7 mg PN/kg diet). All tumors were identified as adenocarcinoma and most as papillary type. The number of microcarcinomas in mammary glands of the 35-mg PN group tended to be reduce than that of the 7-mg group. The number of proliferating Ki67-positive cells was significantly reduced by supplementation with PN.

Introduction

Vitamin B₆ is an essential water-soluble vitamin required for normal growth and development (1). Its physiologically active form, pyridoxal (PL) 5'-phosphate (PLP), is derived from inactive precursors and functions as a cofactor in numerous enzymatic reactions of amino acid metabolism (2). PLP plays a key role as coenzyme for serine hydroxymethyltransferase, which is involved in one-carbon metabolism, and enzymes involved in the trans-sulfuration pathway of homocysteine catabolism. Vitamin B₆ deficiency in animals and humans has been correlated with atrophy of lymphoid organs and impairment of both humoral and cell-mediated immunity (3). In addition, a deficiency of vitamin B₆ during brain development has been shown to result in neurochemical and morphologic changes expressed behaviorally as tremors, irritability, abnormalities in motor function, and spontaneous seizures (4–6). On the other hand, some studies have reported that pyridoxine (PN) deficiency can inhibit the development of tumors in experimental animals (7,8), whereas others have suggested that alterations in vitamin B₆ nutrition are responsible for clinical manifestations of certain cancers (9–12). Gridley et al. reported that high dietary intake of vitamin B₆ might suppress tumor development by either immune enhancement or PLP growth regulation of H238-induced tumors (13). On the other hand, Hartman et al. found significantly lower risk of lung cancer among men who had higher serum vitamin B₆ levels in the nested case-control study (14).

Our recent studies have shown that vitamin B₆ modulates expression of the albumin gene by inactivating tissue-specific DNA-binding protein in rat liver; that is, binding PLP with tissue-specific transcription factors such as NHF-1 and C/EBP reduces the capacity of these factors to interact with the regulatory region of the albumin gene, resulting in the inhibition of gene expression (15,16). These reports suggest the possibility that vitamin B₆ suppresses the expression of oncogenes. Moreover, Molina et al. revealed that the growth of HepG2 cells, a human hepatoma cell line, is severely inhibited by the addition of high levels of PN in the culture medium and is accompanied by marked inhibition of albumin gene expression (17). In addition, more recent studies have shown that dietary supplementation of vitamin B₆ markedly suppresses azoxymethane-induced colon carcinogenesis in mice by reducing cell proliferation and expressions of oncogenes (18).

Breast cancer is the most common cancer affecting women and the second most common cause of death from cancer in women after lung cancer. Many cases of breast cancer in women revealed no obvious risk factors; however, age, obesity, lack of exercise, and alcohol use are thought to be relevant. A recent study by Zhang et al. reported that higher plasma levels of folate and possibly vitamin B₆ might reduce the risk of developing breast cancer (19). In this study, the effect of high-dose PN on mammary tumorigenesis induced by 7,12-dimethylbenzanthracene (DMBA) was examined in the Sprague-Dawley rat. The recommended AIN-93 dietary level of 7 mg PN HCl/kg (20) was used as a control, and the level reportedly required for preventing colon tumorigenesis (35 mg PN HCl/kg) (18) was used for the high-level group.
Table 1. Composition of the Experimental Diets (g/kg)

<table>
<thead>
<tr>
<th></th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; (7 mg)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; (35 mg)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>t-Cysteine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Corn oil</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Salt mix&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>50</td>
</tr>
<tr>
<td>Sucrose</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Corn starch</td>
<td>402</td>
<td>402</td>
</tr>
</tbody>
</table>

a: Pyridoxine hydrochloride.
b: AIN-93 pyridoxine free.
c: AIN-93.

Materials and Methods

Animals

Twenty female Sprague-Dawley rats were housed under controlled temperature and in a 12-h light–dark cycle and given free access to food and water. After feeding with a commercial stock diet (CE-2, CLEA, Inc., Tokyo, Japan) for 1 wk, animals (average weight = 64.9 g) were divided into two groups (n = 10 each); one (the control group) was fed 7 mg/kg vitamin B<sub>6</sub> in their diet (PN HCl, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the other (the high-dose group) was fed 35 mg/kg. The diet compositions are shown in Table 1.

At 50 days, all rats were given 10 mg DMBA (Wako Pure Chemical, Osaka, Japan) dissolved in 1 ml sesame oil by gastric infusion. Following this, animals were palpated for a mammary mass once a week. All animals with masses were sacrificed at occurrence, whereas those with no palpated masses were sacrificed at age 187 days. Blood was collected from the abdominal aorta under anesthesia with diethyl ether, and then major organs, including mammary glands, were immediately excised and weighed. The present study was approved using the IACUC guidebook, and the rats were maintained in accordance with Kagoshima University guidelines for the care and use of laboratory animals.

Histochemical Examination

The monoclonal antibody NCL-Ki67-MM1 was used for the assay of cell proliferation because Ki67 is expressed in all proliferating cells during late G1, S, M, and G2 phases of the cell cycle (21). Mammary glands and masses were fixed in 10% phosphate-buffered formalin for 24 h at room temperature and embedded in paraffin. Sections were cut from paraffin-embedded tissue blocks, deparaffinized, and rehydrated and then subjected to standard avidin–biotin complex immunoperoxidase assays using the Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). Briefly, deparaffinized and rehydrated sections were treated with 0.3% hydrogen peroxidase in methanol solution to block endogenous pero-

Statistical Analysis

Mean differences were evaluated by Student’s t-test. Tumor incidences and the total number of mammary tumors in each group were tested using the fourfold contingency table (22) and analyzed by χ² test. Survival rates of rats were estimated using the Kaplan-Meier method and analyzed using the log-rank test (Stata 8.0, Stata Corp., College Station, TX).

Results

Mammary Tumors

There were no differences in the body weight gain between dietary groups (Fig. 1). The first mammary tumors appeared between Days 84 and 90. At this time ~12% of the rats had tumors, and there were no differences between dietary groups. At 98, 104, and 111 days, tumor incidence increased progressively, and there were significant differences between the control and high-dose PN diets. High-dose PN decreased tumor incidence by 30%, 30%, and 50% at these three time points. Tumor incidence of the high-dose PN group at 111 days was significantly decreased compared with the control group (n = 10; χ² test P < 0.05) (Fig. 2A). Furthermore, the estimated survival rates of the high-dose group were significantly increased compared with the control (n = 10; χ² test P < 0.05) (Fig. 2B). Thus, it seems that the administration of high-dose PN suppressed mammary tumorigenesis.

Subtypes of Mammary Tumors

All tumors in this study were identified as mammary adenocarcinomas, with papillary (70–80%), solid (6–20%), and secretory type (8–9%) observed (Table 2) and no significant differences between the experimental groups. In addition to these macroscopically detectable tumor-forming lesions, microscopic lesions were also observed in the mammary glands. Microcarcinomas are induced as early lesions of mammary dysplasia by DMBA (23). In this study, the number of
Microcarcinomas was examined in three areas: the abdominal, breast, and neck areas of the mammary gland. The numbers of microcarcinomas in any area of the high-dose group tended to be lower than in the control, although the differences were not statistically significant (35-mg group, 3.1 ± 2.4; 7-mg group, 9.2 ± 6.1 in abdominal areas) (Fig. 3).

Effect of Vitamin B6 on Cell Proliferation

To examine the effect of vitamin B6 on proliferating cells, an immunohistochemical study was undertaken using Ki67, which is expressed in all proliferating cells during late G1, S, M, and G2 phases of the cell cycle (24). The numbers of

Figure 1. Body weight gain in the two experimental groups. Open circle, 7 mg pyridoxine (PN) HCl/kg; closed circle, 35 mg PN HCl/kg. The mean ± SD is shown.

Figure 2. Effect of vitamin B6 on the appearance of (A) mammary tumors and (B) survival rate. (A) Values represent the percentage of rats bearing mammary tumors. Open bar, 7 mg pyridoxine (PN) HCl/kg; closed bar, 35 mg PN HCl/kg. Rats were divided in two groups and fed two levels of vitamin B6, 7 and 35 mg/kg, respectively. At 50 days, all rats were given 10 mg 7,12-dimethylbenzanthracene dissolved in 1 ml sesame oil by gastric infusion, and then the tumor incidence and total number of mammary tumors in each group were tested using the fourfold contingency table (22) and analyzed using the $\chi^2$ test; * $P < 0.05$. (B) Survival rates were estimated using the Kaplan-Meier method and analyzed using the log-rank test. Solid line, 35 mg PN HCl/kg; interrupted line, 7 mg PN HCl/kg ($\chi^2$ test $P < 0.05$).
Ki67-positive cells obtained from 10 tissues were statistically analyzed, revealing a significant reduction in mammary tumors with supplementation of high-dose PN \((n = 10; \text{Student’s } t\text{-test } P < 0.05)\) (Fig. 4B). Representative photos of tumor tissues from each group are shown in Fig. 4A.

**Discussion**

These present findings suggest that a high level of supplemental PN suppressed the development of mammary tumorigenesis. Further, Ki67-positive proliferative cells in the high-dose group tended to decrease compared with the control group, suggesting that supplemental vitamin B6 suppressed mammary tumorigenesis by suppressing cell proliferation. In addition to macroscopically detectable tumor-forming lesions, microscopic lesions were also observed in mammary glands. The numbers of microcarcinomas in the high-dose group also tended to decrease compared with the control group; however, no morphological differences in the microcarcinomas were observed between groups.

Previous studies have shown that culturing a variety of rodent and human tumor cell lines in a PN-supplemented medium for several days resulted in an inhibition of cell growth (24–26). Molina et al., for example, demonstrated that growth of the human hepatoma cell line HepG2 was severely inhibited in PN-supplemented medium (17). They also reported that vitamin B6 inhibits albumin gene expression in HepG2, resulting in an interaction with the transcription factors. In this study, the effect of vitamin B6 on cell proliferation of mammary carcinomas seems to have been mediated through an alteration of gene expression (15,16). Komatsu et al. previously reported that dietary supplementation of vitamin B6 markedly suppressed azoxymethane-induced colon carcinogenesis in mice by reducing cell proliferation and expressions of the oncogenes c-fos and c-myc (18). Further, they suggested that the suppression of colon tumors was mediated through reduced nitric oxide production (27). Many reports have documented the antitumor effect of vitamin B6 in vivo and in vitro; however, the antitumor effect in vitro seems to be more effective than in vivo. These differing effects are possibly due to the effective concentration of vitamin B6 in cells. The effect of vitamin B6 on colon carcinogenesis is more effective than other in vivo models because of direct interaction between vitamin B6 and colon tumors.

The involvement of vitamin B6 in the regulation of steroid hormone action is a potential mechanism of the antitumor effect in breast cancer (28,29). However, Davis and Cowing reported that PL supplementation reduced cell proliferation and DNA synthesis in both estrogen receptor–positive (MCF-7 and T-47D) and estrogen receptor–negative (BT-20) breast cancer cell lines (30). Thus, PL supplementation regulates breast cancer cell growth in a steroid-independent manner.

In summary, this study revealed that supplementation with high-dose PN suppresses the development of mammary tumors though suppression of the proliferation of mammary tumor cells.

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**Table 2. Subtypes of DMBA-Induced Mammary Tumors**

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>No. of Tumors</th>
<th>Papillary</th>
<th>Solid</th>
<th>Secretory</th>
</tr>
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<tbody>
<tr>
<td>7 mg</td>
<td>26</td>
<td>19 (73.0%)</td>
<td>5 (19.2%)</td>
<td>2 (7.7%)</td>
</tr>
<tr>
<td>35 mg</td>
<td>35</td>
<td>30 (85.7%)</td>
<td>2 (5.7%)</td>
<td>3 (8.6%)</td>
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</table>

\(a\): Abbreviation is as follows: DMBA, 7,12-dimethylbenzanthracene.
Acknowledgments and Notes

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Figure 4. Immunostaining of Ki67. Ki67 is expressed in all proliferating cells during late G1, S, M, and G2 phases of the cell cycle (24). Immunostaining of Ki67 was conducted as noted in Materials and Methods, and the numbers of positive cells were counted in one view field of the microscopy. (A) Representative photos of tumor tissues from each group. (B) Numbers of positive cells obtained from 10 tissues were statistically analyzed using the Student’s t-test; *P < 0.05.

References


