Doctorial Thesis

Studies on the anti-disease function of vitamin B6

(Summary)

Peipei Zhang

Graduate School of Biosphere Science
Hiroshima University

September 2013

Vitamin B6 is a water-soluble vitamin and is part of the vitamin B complex group. Among several forms of B6-vitamers, pyridoxal 5'-phosphate (PLP) is the active form and is a cofactor in amino acid metabolism, gluconeogenesis and lipid metabolism. There are seven forms of this vitamin: pyridoxine (PN), pyridoxine 5'-phosphate (PNP), pyridoxal (PL), PLP, pyridoxamine (PM), pyridoxamine 5'-phosphate (PMP) and 4-pyridoxic acid (PA).

There is growing epidemiological evidence for vitamin B6 acting as a protective factor against colon cancer. Our animal studies have suggested that the anti-colon tumor effect of dietary vitamin B6 is, at least, partially ascribed to lowering colon cell proliferation, oxidative stress, inflammation, and epithelium cell damage. However, the molecular mechanisms involved in the anti-tumor effect of vitamin B6 are not yet clearly understood.

According to my preliminary experiment in this study using DNA microarray analysis, several genes were upregulated by PL at 500 μM in human colon cancer cells (HT29). Insulin-like growth factor-binding protein 1 (IGFBP1) was one of these upregulated genes in the HT29 cells, and confirmed by real-time PCR. IGFBP1 is mainly produced in the liver and secreted from the liver. It binds to insulin growth factors (IGFs), modulating their actions. A line of studies has suggested that IGFBP1 may be a tumor suppressor. Low serum IGFBP1 levels are associated with some chronic diseases, including colon cancer, cardiovascular disease and diabetes. A recent study has shown that increased circulating IGFBP1 levels can improve insulin sensitivity, lower blood pressure, and protect against atherosclerosis. IGFBP1 is rapidly induced during liver regeneration, and is implicated in the maintenance of hepatocyte differentiation and metabolism. The liver is a central organ involved in regulating vitamin B6 metabolism. Additionally, Nakari et al. have recently demonstrated that a high dose of pyridoxine (PN: 10 mM) induced the expression of the insulin-like growth factor-binding protein 3 (IGFBP3) in human breast adenocarcinoma MCF-7 cells. Therefore, the objective of this study was to explore the effect of vitamin B6 on the expression of IGFBP1 and other IGFBPs in human hepatoma HepG2 cells.

In the first study, I mainly focused on the effect of vitamin B6 on gene and protein expression of IGFBP1. IGFBP1 is mainly produced in the liver and secreted from the liver. IGFBP1 has been reported to be a tumor suppressor via binding to insulin growth factors (IGFs), modulating their actions. IGFBP1 is also known to be a hepatoprotective factor. Expression of IGFBP1 is elevated by fasting and exercise, and suppressed by insulin. Recent study further suggested that IGFBP1 has a protective role in the development of cardiovascular disease. Thus, experiments were performed to examine the effect of vitamin B6 on the expression of IGFBP1 in hepatocarcinoma HepG2 cells. Adding PL (500 μM) markedly elevated the mRNA expression of IGFBP1 in HepG2 cells at 6 h, 12 h and 24 h, whereas other vitamers (500 μM), including pyridoxal 5'-phosphate (PLP), pyridoxine (PN) and pyridoxamine (PM), caused no such effect. PL caused a dose-dependent increase in mRNA expression of IGFBP1 in HepG2 cells. The protein expression of IGFBP1 in the cell lysate and culture medium was also markedly elevated in the presence of PL in a time-dependent manner. PL elevated the expression of p-ERK1 (active form of ERK1) and the p-c-Jun proteins (a down stream factor of ERK). Furthermore, the elevation in mRNA and protein of IGFBP1 by PL was suppressed by PD98059, an ERK inhibitor. Treatment of cycloheximide, a protein synthesis inhibitor, completely inhibited the elevation in protein expression of IGFBP1 by PL, suggesting that the higher protein expression of IGFBP1 induced by PL is dependent on the protein synthesis. Thus, the possibility of the suppressed degradation of IGFBP1 protein by PL treatment appears to be negated. Furthermore, PL stimulated the mRNA expression of IGFBP3 and IGFBP4 in a time- and dose-dependent manner in HepG2 cells. Adding of the ERK inhibitor suppressed the stimulation of the gene expression of IGFBP3 and IGFBP4 by PL. These results suggest that PL can induce the expressions of IGFBP1, IGFBP3 and IGFBP4 in hepatoma cells via a mechanism involving the ERK pathway. Higher expression of these IGFBPs by vitamin B6, together with their inhibitory effects on cell proliferation, suggests the anti-tumor effect of vitamin B6 via elevating IGFBPs.

In addition to the anti-colon tumor effect of vitamin B6, our group reported that

vitamin B6 inhibited lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in mouse macrophage RAW264.7 cells via suppressing nuclear factor-κB (NF-κB) activation. In addition, dietary vitamin B6 inhibited iNOS activity in the liver of rats exposed to LPS. Importantly, our group has previously suggested that high dietary vitamin B6 suppressed colon cell proliferation. However, the molecular mechanisms involved in the suppression effect of vitamin B6 on colon cell proliferation are not yet clearly elucidated.

According to the preliminary experiment in this study, PL significantly suppressed the growth of human colon cancer cells (HT29) at 500 μM for 24h. The results of the DNA microarray analysis showed that several genes were upregulated by PL at 500 μM in HT29 cells. In the upregulated genes, p21 was confirmed by real-time PCR. It is well known that p21 functions as a negative regulator of cell cycle progression. The gene expression of p21 is tightly controlled by the tumor suppressor protein p53. Recently, a study has shown that a high dose of pyridoxine (PN: 10 mM) induced the mRNA expression of the insulin-like growth factor-binding protein 3 (IGFBP3) in human breast adenocarcinoma MCF-7 cells in a p53 dependent manner. Therefore, the objective of the second study was to examine the effect of vitamin B6 on the gene expression of p21 and p53 activation.

In the second study, the upregulation in mRNA expression of p21 was confirmed in colon carcinoma HT29 cells exposed to PL by real-time PCR analysis. p21 is known as cyclin-dependent kinase inhibitor 1, and functions as a negative regulator of cell cycle progression. The expression of this gene is tightly controlled by the tumor suppressor protein p53. p21 has been suggested to be a tumor suppressor *in vitro* and *in vivo* in several studies. The effect of PL on the gene expression of p21 in other cell lines was analyzed. The results showed that PL elevated the gene expression of p21 in Caco2 cells, HEK293T cells and HepG2 cells. Adding PL significantly elevated the mRNA expression of p21 in both HT29 cells and HepG2 cells at 24 h, whereas other vitamers, including PLP, PN and PM, caused no such effect. In order to understand the mechanism of higher mRNA expression of

p21 by PL, the p53 activation was examined (the upstream factor for p21 mRNA transcription) in both HT29 cells and HepG2 cells. The results indicated that PL caused higher protein level of p-p53 (active form of p53) of whole cell lysate and nucleus in these two cell lines.

To understand the effect of vitamin B6 on the p53 activation and mRNA expression of p21 *in vivo*, two groups of mice fed with different vitamin B6 diets for 5 weeks were investigated. Western blot analysis showed that vitamin B6 deficient diet caused a trend of lower protein expression of p-p53 compared with normal vitamin B6 diet. Real-time PCR analysis indicated that the mRNA expression of p21 in colon was also significantly reduced by vitamin B6 deficient diet compare to that of the normal vitamin B6 diet. These results suggest an important role of vitamin B6 in elevating protein of p-p53 and mRNA of p21 in the cancer cell lines and colon of mice. Taken together, my study implies the anti-tumor effect of vitamin B6 via elevating p-p53 and p21.

In conclusion, the first study showed that PL caused upregulation of the mRNA expression of cystatin A, p21, IGFBP1, GADD45B and DDIT3, and downregulation of the gene expression of NOX1 in HT29 cells and HepG2 cells. As for the expression of IGFBPs, only PL of the vitamers caused elevation of gene expression of IGFBP1, IGFBP3 and IGFBP4 in HepG2 cells. The mechanistic study suggests that vitamin B6 can induce expression of these IGFBPs via a mechanism involving ERK pathway. The second study provided evidence that vitamin B6 can elevate the mRNA expression of p21 and activate p53 in both HT29 cells and HepG2 cells. Trend of higher protein of p-p53 and significant stimulation in mRNA expression of p21 was confirmed in the colon of mice fed with the normal vitamin B6 diet compared with mice fed with vitamin B6 deficient diet. Taken together, this study provides the first evidence of nutrient-stimulated the expression of IGFBP1 and mRNA expression of IGFBP4. This study further provides the first evidence of vitamin B6-stimulated activation of p53 and mRNA expression of p21. The findings in my study suggest the novel mechanisms of the anti-disease function of vitamin B6 through the modulation of IGFBPs and p21.