Hepatoprotective Effect of Vitamin B₁₂ on Dimethylnitrosamine-Induced Liver Injury

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Vitamin B₁₂ contains a cobalt complex and accumulates at high levels in the liver. Vitamin B₁₂ was examined for its hepatoprotective effect on dimethylnitrosamine-induced liver injury in mice. Vitamin B₁₂ decreased the blood levels of aspartate aminotransferase and alanine aminotransferase, and clearly inhibited the overaccumulation of collagen fibrils. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the liver showed that the gene expression of α-smooth muscle actin and heat-shock protein 47, which are markers of fibrosis, were suppressed by vitamin B₁₂ administration. Our findings indicate that vitamin B₁₂ could be an effective hepatoprotective agent.

Key words hepatoprotection; vitamin B₁₂; liver fibrosis; metal complex

The incidence of hepatoma related to hepatitis C and B continues to increase in developed countries. Chronic liver injury, including that caused by virus infection, cause persistent inflammation and fibrosis, followed by the development of liver cirrhosis and hepatoma. The use of interferon (IFN) has become the first-line treatment for viral hepatitis, but it is not effective in patients with a high viral load. Recently, investigators have begun to seek hepatoprotective agents that might facilitate the treatment of liver failure.

Vitamin B₁₂ contains a cobalt complex and is therefore also known as cobalamin. Its molecular weight is the largest of all the vitamins, and it is known to accumulate at high levels in the liver. Therefore, the concentration of vitamin B₁₂ in the blood rises in the presence of acute or chronic liver disease. Also, vitamin B₁₂ associates with many enzymes, such as adenosylcobalamin-dependent isomerases, methylcobalamin-dependent methyltransferases, and dehalogenases. Chronic feeding of a methyl-donor, vitamin B₁₂-deficient diet causes the spontaneous development of hepatocellular carcinoma. Therefore, when the liver is injured, stored vitamin B₁₂ leaks out into the blood, which causes a severe B₁₂-deficit in the liver, probably resulting in metabolic dysfunction. So far, it has been reported that vitamin B₁₂ was effective for the liver protection against the acute liver injury. However, there is no findings of the effect on chronic liver fibrosis. Therefore, we examined the effect of vitamin B₁₂ on the fibrogenesis using chronically liver-injured mice.

Dimethylnitrosamine (DMN) is a potent hepatotoxin, carcinogen and mutagen. DMN induces liver fibrosis in a highly reproducible manner, first inducing a central hemorrhagic necrosis followed by the formation of septa and establishing micronodular cirrhosis after 3 weeks of treatment. DMN-induced liver fibrosis in animals is a good and reproducible animal model for studying pathophysiological alterations associated with the development of liver fibrosis and cirrhosis in humans. In this study, we found that the treatment of a chronic liver-injury model with vitamin B₁₂ suppressed both liver inflammation and fibrosis.

MATERIALS AND METHODS

Animals BALB/c mice were purchased from SLC (Shimizu, Japan). The animals were housed in an air-conditioned room at 22°C before the experiment. Hepatic injury in mice aged 6 weeks was elicited by the intraperitoneal administration of dimethylnitrosamine (DMN; Sigma, St. Louis, MO, U.S.A.) at 5 mg/kg body weight for the first 3 consecutive days of the week for 4 weeks. Vitamin B₁₂ (Wako Pure Chemicals, Osaka, Japan) was administered intraperitoneally at 10 mg/kg body weight at the same time as DMN. After 4 weeks of treatment, the mice were anesthetized, and blood samples were taken from the orbital sinus. The animal experiments were conducted according to the ethical guidelines of the Osaka University Graduate School of Pharmaceutical Sciences.

Histological Analysis Liver specimens were fixed in 10% formaldehyde and embedded in paraffin. Sections were cut from the tissue blocks, mounted on slides, and stained with Elastica van Gieson (EG).

Assays Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using an assay kit (Iatrophyme TA-Lq; Mitsubishi Kagaku Iatron Inc., Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) The liver was excised and homogenized after removing the blood with phosphate-buffered saline. The total RNA was extracted from the liver homogenates using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). The gene expression of α-smooth muscle actin (α-SMA) was analyzed by RT-PCR using the following primers: forward 5′-CAGGGAGTAATGGTTGGAAT-3′ and reverse 5′-CGTCGATTCTCTGGTTGCTGA-3′. Heat-shock protein 47 (HSP47) gene

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expression was analyzed using the following primers: forward 5’-CCATCGACAAGAACAGA-3’ and reverse 5’-TCATATTTCCCTCCCCCATC-3’. β-Actin gene expression was analyzed using the following primers: forward 5’-CATCCCCAAAGTTTAC-3’ and reverse 5’-CCAAAGCTTCCATACAT-3’. RT was performed using 1 μg of total RNA sample with the BcaBEST RNA PCR kit (Takara, Kyoto, Japan). The PCR conditions were: 1) 94 °C for 1 min; 2) 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; 3) 72 °C for 5 min.

Statistics The data were analyzed for statistical significance by Student’s t-test.

RESULTS AND DISCUSSION

We examined the hepatoprotective effect of vitamin B12 on DMN-induced liver injury in mice. As shown in Fig. 1, after 4 weeks of DMN treatment, the activities of blood AST and ALT increased 2.9- and 3.3-fold, respectively, compared with controls, and the intraperitoneal administration of vitamin B12 significantly decreased the activities of AST and ALT. These results suggest that vitamin B12 suppresses the hepatic inflammation caused by the DMN treatment.

We next examined the effect of vitamin B12 administration on liver injury and fibrogenesis (Fig. 2). Liver sections were prepared after 4 weeks of DMN treatment and examined by EG staining. EG staining showed the significant accumulation of collagen fibrils after DMN treatment. This accumulation was clearly lower in DMN-treated mice given vitamin B12. DMN bioactivation is thought to occur through the liver mixed-function oxidases (cytochrome P450 2E1). The end result is the formation of toxic intermediates such as hydroxyl radicals, reactive oxygen intermediates. Therefore, vitamin B12 appears to protect liver from the oxidation stress caused by DMN.

To confirm the antifibrotic effect of vitamin B12, we next examined the gene expression of α-SMA and HSP47, which are markers of fibrosis, using RT-PCR analysis. As shown in Fig. 3, marked upregulation of the expression of the α-SMA and HSP47 genes was observed in the DMN-injured liver compared with the control liver. Therefore, although the liver damage was not fatal, the long-term liver injury caused inflammation and resulted in fibrosis. Vitamin B12 significantly...
suppressed the increased gene expression of both α-SMA and HSP47. Furthermore, in preliminary in vitro experiments, vitamin B<sub>12</sub> protected rat primary hepatocytes from hepatotoxic-induced cell death. Therefore, vitamin B<sub>12</sub> might suppress liver inflammation and the subsequent fibrogenesis by protecting hepatocytes from liver injury.

During liver injury, hepatic stellate cells (HSCs) are activated to transdifferentiate into myofibroblasts and overproduce extracellular matrix, which leads to fibrosis. Oxidative stress stimulates the activation of HSCs, and substances with antioxidative activity, such as vitamin E, glutathione, and L-cysteine, inhibit HSC activation, thus suppressing liver fibrosis. However, we have not found any antioxidative activity by vitamin B<sub>12</sub> (data not shown). It has been reported that the activity of glutathione reductase was found to be significantly lower in B<sub>12</sub>-deficient liver. Recently, it was reported that the interaction between vitamin B<sub>12</sub> and glutathione could protect against disease related to vitamin B<sub>12</sub> deficiency. Although vitamin B<sub>12</sub> itself does not have radical scavenging ability, it might play an important role to maintain the sulfhydryl level under oxidative conditions. Vitamin B<sub>12</sub> contains a cobalt complex and is widely used to describe compounds of the cobalamin group. It is possible that the cobalt complex of vitamin B<sub>12</sub> is involved in the inhibition of liver inflammation and fibrogenesis, but further studies are necessary to clarify vitamin B<sub>12</sub>’s mechanism of action.

We previously reported that Zn(Mal)<sub>2</sub> suppresses cytoxin-induced apoptotic and necrotic cell death in isolated hepatocytes. This zinc complex has free-radical scavenging activity. Several manganic porphyrins mimicked superoxide dismutase and had protective effect against oxidative stress. Finally, here we found that the increase in α-SMA and HSP47 gene expression caused by DMN treatment was suppressed by vitamin B<sub>12</sub>. Thus, a variety of metal complexes seem to have therapeutic potential.

In conclusion, we found that vitamin B<sub>12</sub> is potent a hepatoprotective agent. This report is the first to demonstrate the hepatoprotective effect of vitamin B<sub>12</sub> on liver fibrosis.

REFERENCES