主論文の要旨

Antifibrotic Effects of $1,25(OH)_2D_3$ on Nonalcoholic Steatohepatitis in Female Mice

（雌マウスにおける非アルコール性脂肪性肝炎に対する $1,25(OH)_2D_3$ の抗線維化効果）

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【Background】

Nonalcoholic fatty liver disease (NAFLD), a common cause of chronic liver disease, is characterized by fat accumulation in the liver in the absence of significant alcohol consumption. NAFLD patients with nonalcoholic steatohepatitis (NASH) are at high risk of progression to cirrhosis and hepatocellular carcinoma.

Along with a decrease in estrogen level, post-menopausal women remain at high risk for insulin resistance and abnormal fat distribution, which closely associates with NAFLD. Although hormone replacement therapy is the primary clinical treatment for alleviating various symptoms caused by menopause, it can lead to the risk of breast cancer and endometrial carcinogenesis.

Vitamin D is produced primarily in skin exposed to ultraviolet light and then hydroxylated to $25(OH)D_3$ in the liver. $25(OH)D_3$ is released from hepatocytes and transported to the kidney, and then hydroxylated into the fully active form, $1,25(OH)_2D_3$. Vitamin D deficiency could lead to metabolic diseases, thereby inducing fat accumulation in hepatocytes, as well as inflammation and fibrosis in the liver, and it is also an increased risk associated with developing chronic diseases. The anti-fibrotic activity of vitamin D and role in the regulation of lipid transfer have also been observed in NAFLD patients. Pre-clinical studies have revealed that supplementation with an analog of the active form of vitamin D to activate the VDR can reduce myocardial fibrosis in male mice. Also, supplementation with $1,25(OH)_2D_3$ has antiproliferative and antifibrotic effects in a mouse model of biliary fibrosis. Furthermore, the fully active form $1,25(OH)_2D_3$ can also suppress liver fibrosis and steatosis in male rats that develop NASH by consuming a choline-deficient diet. However, few studies have researched for effective therapies for elderly and post-menopausal women with NASH.

【Methods and results】

Seven-week-old C57BL/6J female mice were separated into five experimental groups: SHAM, OVX, and OVX+intraperitoneal (i.p.) injection of $1,25(OH)_2D_3$ (0.0008, 0.004, and 0.02 μg/kg). All groups were fed a CDHF diet for 8 weeks. Injections took place twice per week throughout the experimental period. Blood samples and liver tissue were collected for analyzing liver histological changes, serum biochemical indicators of hepatic function, and hepatic genes associated with fibrosis.

To detect liver steatosis and determine fibrosis stage, we performed pathological analysis of CDHF-fed mice by H&E and Sirius red staining. The fibrosis degree of the 0.0008, 0.004, and 0.02 μg/kg groups were obviously lower than that of the OVX group. Fibrosis score and Sirius red-positive area were also significantly lower in the $1,25(OH)_2D_3$ treatment groups. Supplementation with $1,25(OH)_2D_3$ slightly down-regulated the TNF-α and IL-6 levels in the treated groups, although the difference was not significant (Fig.1).

Aside from this, the NAS in the treatment and SHAM groups were all below 5, significantly lower than in the OVX group.

We continued the study by examining primary fibrotic markers such as type 1 collagen (COL1A1), tissue inhibitor of the metalloproteinase (TIMP)-1, transforming growth factor (TGF)-β1, and α-SMA. Relative mRNA levels of TIMP-1 were significantly lower in the OVX+1,25$(OH)_2D_3$ and SHAM groups than in the OVX group. In groups treated with 0.004 and 0.02 μg/kg 1,25$(OH)_2D_3$, relative mRNA expressions of COL1A1, α-SMA, and TGF-β were significantly reduced, and slightly decreased the level in the 0.0008 μg/kg group (Fig.2).

We found that SMAD2 was significantly down-regulated in the 0.004 and 0.02 μg/kg treatment groups, and that the SMAD3 level was significantly reduced in the 0.0008 and 0.004 μg/kg groups. We examined VDR on the last day of the experimental period. Expression of the VDR did not differ significantly among the five groups. Immunohistochemical staining revealed that in all five groups, VDR was expressed at a low but still detectable level in mice liver (Fig.3).

【Discussion】

In a previous study, we showed that hormone replacement therapy in OVX mice ameliorates liver injury, whereas removal of the ovaries and taken a CDHF diet for 8 weeks accelerates the progression of NASH. Compared with CDHF diet, high-fat diet (HFD) can induce the symptoms such as obesity, diabetes, hyperlipidemia, and hepatic insulin resistance in OVX mice after taken by 12–18 weeks, but it could not induce severe liver inflammation and fibrosis. It indicates that a short-term feeding of HFD may not induce stable NASH model. And also, HFD contains sufficient vitamin D, which may delay the progression of hepatic steatosis and inflammation.

Activated hepatic stellate cells (HSCs) increase the expression of TIMP-1 and α-SMA. TIMP-1 plays a vital role in promoting the progression of organ fibrosis. In our study, supplementing 1,25$(OH)_2D_3$ suppressed the expression of TIMP-1 and α-SMA in treated groups. These results suggest that the activity of HSCs was decreased, and then down-regulated the expression of TIMP-1 and α-SMA, thereby alleviating liver fibrosis. The COL1A1 gene encodes collagen type 1, a hallmark in fibrotic diseases. TGF-β accelerates the progression of organ fibrosis, both by regulating collagen type 1 and modulating matrix metalloproteases and their inhibitor TIMPs. The SMAD proteins transduce the TGF-β signal into the nucleus from the cell membrane. SMAD2 and SMAD3 have almost the same structure, and they are both phosphorylated and translocated into the nucleus after TGF-β stimulation. From the results, we found that the expressions of SMAD2 and SMAD3 were suppressed by 1,25$(OH)_2D_3$ in treated groups, especially in 0.004 μg/ kg group. Moreover, SMAD2 and SMAD3 are downstream factors of TGF-β that are required for profibrotic

gene expression in HSCs. The results indicate that $1,25(OH)_2D_3$ may ameliorate fibrosis by down-regulating SMADs.

VDR is a ligand-dependent factor. Binding of $1,25(OH)_2D_3$ results in phosphorylation of VDR, allowing it to interact with retinoid X receptor (RXR), its heterodimeric partner in the cells. This complex can modulate gene expression in the nucleus in three different ways: it can promote the expression of individual genes by binding positive vitamin D3 response elements (VDREs), it can negatively regulate genes by binding negative VDREs; or it can antagonize specific gene signaling. Phosphorylated VDR shifted the binding curve of SMADs stimulated by TGF-β, thereby reversing their activity. After HSCs are fully activated with VDR, the level of phosphorylated VDR expression is decreased. In our study, we examined the expression of VDR on the last day of an 8-week experimental period. Based on the results that the expressions of SMAD2 and SMAD3 were suppressed by $1,25(OH)_2D_3$ in the OVX treated groups, it shows that VDR expression may be once stimulated in treated groups. Thus VDRs inactivate SMADs, resulting in reduced expression of TGF-β. Hence, $1,25(OH)_2D_3$ phosphorylated VDRs may ameliorate liver fibrosis by crossing with a TGF-β-SMAD pathway. However, the physiological role of VDR in liver tissue remains unclear. In the future, to evaluate the trend in VDR expression in OVX mice, further examination including VDR levels at different times during the treatment will be needed.