主論文の要旨

Raloxifene Ameliorates Liver Fibrosis of Nonalcoholic Steatohepatitis Induced by Choline-Deficient High-Fat Diet in Ovariectomized Mice

（Raloxifeneは卵巣摘出マウスのコリン欠乏高脂肪食による非アルコール性脂肪性肝炎の線維化を改善する）

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Introduction

Nonalcoholic fatty liver disease (NAFLD), one of the most common causes of chronic liver disease, encompasses a spectrum of conditions associated with lipid deposition in hepatocytes. NAFLD ranges in severity from steatosis (simple fatty liver), to nonalcoholic steatohepatitis (NASH: fatty changes with inflammation and hepatocellular injury or fibrosis), to advanced fibrosis and cirrhosis.

Recent epidemiological reports have shown that NAFLD is more common in men than in women. In women, NASH is more prevalent in older patients, whereas in men the prevalence of NASH increases in middle ages.

NAFLD occurs less frequently in women who take hormone replacement therapy (HRT) after menopause. Estrogen also suppresses liver fibrosis in ovariectomized female rats and prevents hepatic stellate cell activation in cultured liver cells. However, circulating estrogen that engages estrogen receptors promotes breast and uterine proliferation that contributes to estrogen-responsive cancers.

We investigated the therapeutic effect of raloxifene, a second-generation selective estrogen-receptor modulator (SERM), on NASH induced by a choline-deficient high-fat (CDHF) diet in female ovariectomized (OVX) mice.

Materials and methods

Seven-week-old female C57BL/6J mice were divided into 3 groups. One group underwent sham operation; the other two groups received bilateral ovariectomy (OVX) and were maintained for 1 week during recovery. Thereafter, all mice were fed a CDHF diet or choline-sufficient high-diet (CSHF) diet as a control diet (Oriental Yeast CO., Tokyo, Japan) for 8 weeks. The OVX mice were treated with intraperitoneal injection of 3mg/kg body weight of raloxifene or vehicle daily for 10 days before killing. Serum biochemical indicators of hepatic function, hepatic TG level and liver histological changes were evaluated. Levels of mRNAs (TGF-β1, ER-α, IL-6, IL-1β, α-SMA, iNOS.) were assessed by TaqMan real-time quantitative polymerase chain reaction (qPCR).

Results

CDHF diet enhances liver injury and fibrosis in female mice

Both in SHAM and OVX mice, bodyweight were significantly decreased in CDHF diet fed mice compared with CSHF diet fed mice (Table 1), Uterus weight was significantly decreased in OVX mice. Serum triglyceride, free fatty acid, ALT and AST levels were significantly increased in CDHF diet-fed mice compared with CSHF diet-fed mice. AST levels were increased in OVX-CDHF diet compared with SHAM-CDHF diet. Histological analysis showed that fibrosis staging was significantly higher in CDHF diet fed mice than that in CSHF diet. OVX group showed significantly higher fibrosis score than SHAM

group in CDHF diet fed mice.

Administration of raloxifene ameliorates liver injury in OVX-CDHF mice

Serum AST levels were significantly higher in the OVX group than in the SHAM group and lower in the OVX+RLX group than in the OVX group, but the differences were not statistically significant. ALT levels were significantly lower in the OVX + RLX group than in the OVX group (Table 2).

OVX mice develop pathological NASH, but raloxifene treatment ameliorates liver injury in OVX-CDHF mice

CDHF-fed OVX mice exhibited pathological findings of NASH including marked steatosis, lobular inflammation, and periportal or pericellular fibrosis. The severity of infiltration and periportal fibrosis was reduced in OVX + RLX mice (Fig. 1A). No significant difference in steatosis score and hepatic triglyceride content in the three groups (Fig. 1B, 1C). Inflammation score and NAS score was significantly lower in the OVX + RLX group than in the OVX group (Fig. 1D, 1E). Inflammatory cytokines, IL-1β increased in OVX group (P=0.061), and decreased in OVX + RLX group (Fig. 2A). IL-6 mRNA expression were also significantly increased in OVX group, and significantly decreased in OVX + RLX group (Fig. 2B). Meanwhile, mRNA expression of TNF-α mRNA did not show any difference between the three groups (Fig. 2C.D).

Raloxifene treatment ameliorates liver fibrosis in OVX mice

The RNA expression of α-SMA was also increased in OVX group than that in SHAM group, and decreased in the OVX+RLX group (Fig. 3E). According to the Brunt fibrosis staging system, fibrosis stage was significantly higher in the OVX mice than in the SHAM group, and reduced in the OVX + RLX group (Fig. 3B). The proportion of Sirius red–positive area was significantly higher in the OVX group than in the SHAM group, but significantly lower in the OVX + RLX group than in the OVX group (Fig. 3C). Raloxifene significantly decreased the level of TGF-β1 mRNA (Fig. 3D).

Raloxifene treatment increased liver estrogen receptor-α expression in OVX mice.

Immunohistochemical staining for ER-α revealed that the protein was predominantly localized in the hepatocyte nucleus. ER-α staining in the liver was weaker in OVX mice than in SHAM mice, but more intense in raloxifene-treated OVX mice (Fig. 4A). We also confirmed by RT-PCR that the relative expression of ER-α mRNA was significantly lower in the OVX group than in the SHAM group (Fig. 4B), but significantly higher in the OVX + RLX group than in the OVX group.

Discussion

Recent reports reported accelerated progression of NAFLD/NASH in postmenopausal women. Postmenopausal women had an increased risk of developing more severe liver fibrosis than premenopausal women. Fischer L M *et al*. found that postmenopausal

women are more susceptible to the risk of fatty liver in response to a low-choline diet, they also reported that postmenopausal women have a higher dietary requirement for choline than do premenopausal women. Here, we demonstrated that ovariecotomized mice fed a CDHF diet for 8 weeks developed NASH with increased steatosis, inflammation, and fibrosis. We also found that raloxifene treatment mitigated liver injury and ameliorated liver histological inflammation and fibrosis in OVX mice fed by CDHF diet.

ER-α is the only one of the three major estrogen receptors (ER-α, ER-β, and ER-γ) to be expressed in mouse liver. Ovariectomy decreases the levels of ER-α protein and mRNA expression in hepatocytes from female rats, and estrogen treatment restores these levels. As a SERM, raloxifene works as an estrogen receptor antagonist in breast tissue and an estrogen receptor agonist in bone. Unlike tamoxifene, another type of SERMs, raloxifene showed estrogen receptor antagonist effect on uterus, which was constant with our finding that the uterus weight was not increased in raloxifene treated ovariectomied mice. In liver, the effect of raloxifene on estrogen receptor remains unknown. Limited data revealed that raloxifene exerts estrogen like effect to decrease liver fibrosis on chronic hepatitis C patients. In our study, we demonstrated that raloxifene increased ER-α expression, indicated raloxifene works an estrogen receptor agonist in liver.

Recent studies point to the importance of estrogen and ER-α in liver fibrosis. Estrogen prevented reactive TGF-β production in cultured rat HSCs by suppressing NADH/ NADPH oxidase activity. Hepatic ER was elevated after treatment with estradiol in carbon tetrachloride (CCl4)-induced fibrotic rats, in that experiment, estradiol treatment led to parallel increases in the levels of ER. In our study, fibrogenic gene TGF-β1 and α-SMA RNA expression were upregulated in OVX mice, and raloxifene treatment improved the TGF-β1 and α-SMA expression.

Conclusion

Raloxifene may slow or prevent the progression of liver fibrosis associated with NASH induced by CDHF diet in overietomized female mice, and up-regulation of ER-α may play an important role in these beneficial effects.