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ORIGINAL ARTICLE

Lipid deposition in liver cells: The influence of short form augmenter of liver regeneration

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Summary
Background and objective: The short form augmenter of liver regeneration (sfALR) is a novel human hepatotrophic growth factor. The aim of this study was to investigate the potential role of sfALR in NAFLD.
Methods: The free fatty acids (FFA) induced lipid accumulation in mouse liver parenchymal cells was examined by Oil Red O staining and triglyceride level determination. The cell cycle was determined by flow cytometry and the proliferation was assessed by CCK8. The expression levels of gfer, miR-122, *srebp-1c*, *fas*, *dgat2*, *acc1* and *Lrp1B* were assessed by quantitative real-time PCR. Furthermore, the MAPK pathway was detected by western blot.
Results: The results showed that sfALR could alleviate the lipid accumulation in mice both *in vivo* and *in vitro*. sfALR relieved the proliferation inhibition and G2 arrest of mouse liver parenchymal cells induced by FFAs. Free fatty acids affected *gfer* expression in a time-and dose-dependent way. And sfALR suppressed JNK activation, increased miR-122 level and reduced fatty acid synthesis-related gene expression.
Conclusion: These findings suggested that sfALR could alleviate the severity of fatty liver in mice.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasing health problem related to the rising levels of obesity around the world promoted by increasing affluence and more sedentary occupations. A spectrum of liver tissue pathology exists in NAFLD, comprising hepatic steatosis characterized by the deposition of lipid droplets in hepatocytes, through to nonalcoholic steatohepatitis (NASH) associated with hepatocyte death, inflammation and fibrosis. Advanced NAFLD disease may progress to cirrhosis and hepatocellular carcinoma (HCC) [1]. Promising treatments for NAFLD include antioxidants and lipid metabolism interference drugs, whose treatment effect is not satisfactory [2]. So it is important to further explore NAFLD drug intervention.

Augmenter of liver regeneration (ALR, encoded by GFER) is a widely distributed pleiotropic protein originally identified as a hepatic growth factor. As a recently discovered enigmatic flavin-linked sulfhydryl oxidase, ALR has been shown to protect hepatocytes from various toxins. For example, ALR gene therapy could attenuate CCl_4-induced liver injury and fibrosis in rats [3]. Knockdown of ALR exerted anti-inflammatory actions via suppressing the mitogen-activated protein kinase signaling pathway [4]. Also, ALR was showed to be required for mitochondrial function and lipid homeostasis in the liver by developing mice with liver-specific deletion of ALR [5]. While the 22 kDa recombinant ALR could not stimulate DNA synthesis in hepatocytes, the short form (15 kDa) of human recombinant shot form ALR (sfALR) was reported to be equipotent as or even stronger than TGF-α or HGF as a mitogen for hepatocytes [6].

Stimulation of the c-Jun N-terminal kinase (JNK) pathways has been hypothesized as a concurrent pro-apoptotic mechanism in NASH and lipotoxicity [7]. JNK stress signaling pathways are stimulated by the same factors that have been demonstrated to contribute to NASH, including inflammation, oxidative stress and ER stress. JNK activation has been observed in NASH patients as well as murine steatohepatitis model [8].

MicroRNAs (miRNA) are non-protein-coding, small single-stranded RNA, typically 21—23 nucleotides long that regulate gene expression via mRNA degradation and/or translational inhibition [9]. MiRNAs regulate gene expression by binding to target mRNAs and control a wide range of biological functions. Recent reports have identified specific miRNAs as major regulators of fatty acid and cholesterol homeostasis [10]. NASH is associated with altered hepatic microRNA expression [11]. Under expression of miR-122 potentially contributes to altered lipid metabolism implicated in the pathogenesis of NASH [12].

The dual objectives of this study were to determine whether sfALR is effective in alleviating the severity of fatty liver in mice and whether the observed effect is related to JNK and/or miR-122.

Materials and method

sfALR expression plasmid preparation

The short form of human ALR cDNA was subcloned into a pCDNA3.1 expression vector designed for high-level stable and transient expression in mammalian hosts with a human cytomegalovirus immediate-early (CMV) promoter. The plasmid was purified using the E.N.Z.A. Endo-fre Plasmid Maxi Kit (Omega Biotek, USA).

Isolate, culture and fat-overloading induction of mouse liver parenchymal cells

The BALB/c mouse liver parenchymal cells were isolated under the guideline of ''Isolation of Mouse Hepatocytes'' [13]. In brief, after cannulation and perfusion, the liver was carefully removed and then transferred to a 100-mm cell-culture plate containing 10 ml of ice-cold hepatocyte-washing medium. Then the cells were dispersed from the liver, the suspension was filtered and the cells were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 0.5% fetal bovine serum (FBS), and kept at 37 °C in a humidified atmosphere of 5% CO_2. All cells were plated in cell culture flasks at least 36 h before treatment. To induce FFA overloading, mouse liver parenchymal cells were exposed to a mixture of long-chain FFAs (oleic acid/palmitic acid, 2:1) in media containing 1% BSA.

Oil Red O staining

Mouse liver parenchymal cells were grown at an initial density of 10^5 cells/well in a 6-well plate and treated with different concentrations of FFAs for 40 h. Cells were then washed three times with iced PBS and fixed with 10% paraform for 30 minutes. After fixation, cells were washed three times and stained with Oil Red O solution (working solution, 0.5 g Oil Red O powder dissolved in 100 ml Isopropanol) for 20 min at room temperature. Cells were washed again with 75% ethanol to remove unbound staining and washed with PBS. The lipid droplets were observed under microscope (Olympus, Japan) [14].

Determination of triglyceride level

For the triglyceride measurement, cultured cells were scraped in PBS and disrupted by tissue lysates, placed in a 70 °C water bath for 10 min, followed by centrifugation at 3000 g/min for 5 min. Thereafter, supernatants were collected and the triglyceride content was measured using a commercial kit based on phosphoglycerol oxidase/peroxydase enzymatic reaction according to the manufacturer's instructions [15].

Cell cycle analysis

Experimental cells (5×10^4 cells in 1 ml media/well) were seeded into 12-well plates (Costar®). After 48 h treatment with FFA and sfALR, the cells were washed with PBS and then resuspended in 50 μg/ml propidium iodide containing 0.1% sodium citrate with 0.1% Triton X-100 for 20 min at 4 °C. Cells were then analyzed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems).