BASIC AND TRANSLATIONAL—LIVER

Farnesoid X Receptor Protects Hepatocytes From Injury by Repressing miR-199a-3p, Which Increases Levels of LKB1

CHAN GYU LEE,* YOUNG WOO KIM,* EUN HYUN KIM,‡ ZHIPENG MENG,§ WENDONG HUANG,§ SE JIN HWANG,‡ and SANG GEON KIM*

*College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Korea; ‡College of Medicine, Hanyang University, Seoul, Korea; and §Department of Gene Regulation and Drug Discovery, Beckman Research Institute, City of Hope National Medical Center, Duarte, California

BACKGROUND & AIMS: Hepatocyte injury occurs during liver fibrogenesis. MicroRNAs (miRNA) regulate some of these processes, and some are regulated by the farnesoid X receptor (FXR). We investigated the effect of repression of specific miRNAs by FXR in hepatocyte injury using fibrotic liver tissue from patients and hepatocytes. **METHODS:** We used immunohistochemistry or real-time polymerase chain reaction to analyze proteins and miRNAs in human and mouse liver samples. HepG2 cells were transfected with pre-miRNA, antisense oligonucleotides, small interfering RNAs, the 3′-untranslated region of liver kinase B1 (LKB1) (STK11), or constructs for overexpression, and analyzed. **RESULTS:** Liver tissue from patients with severe fibrosis had lower levels of FXR and greater amounts of hepatocyte death than samples from patients with mild disease. Levels of several miRNAs changed when FXR expression was disrupted in the liver; one of these, miR-199a-3p, was significantly up-regulated in patients with severe fibrosis. Activation of FXR by its ligand reduced the level of miR-199a-3p in HepG2 cells. LKB1 messenger RNA was identified as a target of miR-199a-3p, and its expression was reduced in human fibrotic liver tissue. Overexpression of FXR or incubation of cultured hepatocytes with the FXR ligand up-regulated LKB1; LKB1 was not induced in cells transfected with miR-199a-3p. Incubation of HepG2 cells with FXR ligand, or injection of the ligand into mice, protected hepatocytes from injury and increased levels of LKB1; levels of miR-199a-3p were reduced compared with cells that were not incubated with the FXR ligand. Activation of FXR reduced mitochondrial dysfunction and oxidative stress and increased hepatocyte survival. **CONCLUSIONS: In hepatocytes, FXR represses production of miR-199a-3p. In fibrotic livers of humans and mice, FXR expression is reduced, increasing levels of miR-199a-3p, which reduces levels of LKB1. FXR therefore protects hepatocytes from injury by repressing miR-199a-3p and thereby increasing levels of LKB1.**

Keywords: Nuclear Receptor; Noncoding RNA; Mitochondrial Protection; Fibrotic Liver Injury.

Liver fibrosis refers to the accumulation of fibrous scar tissue in the liver that is facilitated by orchestration of the complex events involving hepatocyte injury. When hepatocytes are injured because of hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, or other pathologic conditions, the immune system is activated, and the repair process occurs.[1] The injury or death of hepatocytes stimulates activation of hepatic stellate cells (HSCs) and production of extracellular matrix proteins. Until now, no drugs have been definitively shown to effectively reduce the development of liver fibrosis in humans. A limitation of the current antifibrotic approaches is that antifibrotic drugs are not efficiently taken up by activated HSCs and are often toxic to hepatic cells, producing unwanted adverse effects.[2] Because the chronic hepatocyte death is a crucial factor for the initiation and progression of liver fibrosis and cirrhosis,[3] the inhibition of hepatocyte injury or death may constitute an important strategy for the treatment of fibrosis.

Farnesoid X receptor (FXR) is highly expressed in major organs including the liver and serves as a ligand-mediated transcription factor that regulates the expression of various genes involved in liver homeostasis.[4] FXR ligand treatments have anti-inflammatory and liver-regenerating effects.[5,6] In patients with diabetes or nonalcoholic fatty liver disease, clinical trials of FXR agonists have recently been undertaken.[7] Moreover, FXR activation has an antifibrotic potential. Although FXR is expressed in HSCs and negatively controls HSC activation,[8] a recent study suggested that there is less FXR expression in human HSCs in liver fibrosis.[9] This observation indicates that these cells may not be the direct target for FXR ligands. More importantly, the regulatory role of FXR on the viability of hepatocytes during the state of fibrotic disease remains undefined.

MicroRNAs (miRNAs) negatively regulate gene expression by base pairing with the 3′-untranslated region (UTR) of their target messenger RNAs (mRNA) and may

Abbreviations used in this paper: AA, arachidonic acid; AMPK, AMP-activated protein kinase; ASO, antisense oligonucleotide; CAB39, calcium binding protein 39; CDCA, chenodeoxycholic acid; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; FGF19, fibroblast growth factor 19; FXR, farnesoid X receptor; GSH, glutathione; LKB1, liver kinase B1; mRNA, messenger RNA; miRNA, microRNA; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; ROS, reactive oxygen species; STRAD, STE20-related adaptor; UTR, untranslated region.

0016-5085/$36.00
doi:10.1053/j.gastro.2012.01.007

modulate liver physiology.[10] Thus, abnormal levels of miRNA could be one of the possible mechanisms responsible for the deregulation of protein expression during liver disease progression. However, specific miRNAs that induce hepatic cell injury responsible for the progression of liver disease and their molecular basis remained to be elucidated. Moreover, there is no information available on the regulatory role of FXR in the expression of miRNAs that affect hepatocyte viability during liver fibrosis.

In view of the lack of understanding on the miRNAs controlled by FXR, this study investigated the effect of specific miRNA repression by FXR against hepatocyte injury. We explored the miRNAs affected by FXR in the HBV patients with mild or severe liver fibrosis and elucidated the role of a specific miRNA in hepatocyte survival. In our finding, FXR and liver kinase B1 (LKB1) were both repressed in the livers of severe fibrotic patients with an increase in miR-199a-3p level. To address the complex regulation involving FXR and LKB1, we used several hepatic models to corroborate the role of FXR and miR-199a-3p in LKB1 expression and LKB1–AMP-activated protein kinase (AMPK)–dependent protection of mitochondria.

Materials and Methods

Materials

Information on the materials used in this study is given in the Supplementary Materials and Methods.

Patient Samples

Human liver tissues were obtained from patients who had been diagnosed with liver fibrosis or cirrhosis.[11]

Transient Transfection

The construct encoding for FXR was provided by Dr Bart Staels (Institut Pasteur de Lille, Lille, France).[12]

LKB1 3′UTR Luciferase Assay

The plasmid containing Luc-LKB1-3′UTR (product ID: HmiT017794-MT01; GeneCopoeia, Rockville, MD) was used in reporter assay.

Flow Cytometric Analysis of Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was measured with rhodamine123.[13] Details for in vitro, in vivo, and human studies are given in the Supplementary Materials and Methods.

Results

FXR Repression and miR-199a-3p Induction in Patients With Severe Fibrosis

In an effort to find the biologic relevance of FXR function in a clinical situation of liver injury, the level of FXR expression was compared in a group of HBV patients with mild (Ishak fibrosis score of 3 or less) or severe (Ishak fibrosis score of 5 or 6) fibrosis. Histopathologic and immunohistochemical analyses of the liver sections revealed that the levels of FXR were lower in the patients with severe fibrosis than those with mild fibrosis (Figure 1*A*). As expected, the degrees of hepatocyte death and inflammation were much higher in the patients with severe fibrotic livers, as indicated by mean increases in modified Knodell histologic activity index (Figure 1*B*). Consistently, FXR mRNA levels were significantly decreased as the disease progressed to the severe fibrotic stage, whereas those of retinoid X receptor (RXR) mRNA were not (Figure 1*C*). Hepatic HBV DNA contents were not different (Figure 1*D*). Among the human FXR isoforms, the levels of FXRα1 and FXRα2 transcripts, most abundant in the liver, were much lower in HBV patients with severe fibrosis (Figure 1*E*). FXRα3 and FXRα4 mRNAs were undetectable. Similarly, FXR levels were also lower in the severe fibrotic livers of HCV patients (Supplementary Figure 1). Thus, there exists a correlation between FXR repression and hepatocyte death in the fibrotic liver of viral patients.

Aberrant expression of miRNAs is a crucial cause of various diseases.[10] In a mouse model, FXR gene knockout affected the expression levels of certain miRNAs in the liver.[14] Using the database, the miRNAs whose levels were increased by a deficiency in FXR at the threshold of 1.5-fold or higher were chosen, and their expression levels were compared in the human liver samples. Among the miRNAs, the levels of miR-199a-3p, miR-34a, and miR-451 were significantly up-regulated in the livers of HBV patients with severe fibrosis compared with those with mild fibrosis (Figure 1*F*). In particular, the level of miR-199a-3p was most significantly increased, suggesting that FXR may regulate the miRNA expression in association with hepatocyte death.

miR-199a-3p Down-regulation by FXR Activation

Given the link between FXR and miR-199a-3p, we studied the regulatory role of FXR in the expression of the miRNA in HepG2 cell model. The activation of FXR by its ligand treatments caused a decrease in miR-199a-3p level (Figure 2*A*). In addition, overexpression of FXR enhanced the ability of FXR ligand to decrease miR-199a-3p level. Moreover, a deficiency in FXR by small interfering RNA knockdown reversed the effect of FXR ligand treatment on miR-199a-3p expression (Figure 2*B*), corroborating the inhibitory role of FXR in the expression of the miRNA. FXR ligand treatment increased not only the levels of FXR mRNA but also those of FGF19 mRNA (the transcription target of FXR) (Figure 2*A*, *right*), which was abolished by FXR knockdown (Figure 2*B*, *right*). Our results showed that the activation of FXR inhibited the expression of miR-199a-3p.

miR-199a-3p as a Repressor of LKB1 Translation

Having identified the repression of miR-199a-3p by FXR, we next explored the functional role of miR-199a-3p in the expression of protein necessary for cell survival. To search for the target of miR-199a-3p, we focused on the candidate target genes responsible for