Research Article

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The pro-inflammatory effects of miR-155 promote liver fibrosis and alcohol-induced steatohepatitis

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Background & Aims: Alcoholic liver disease (ALD) ranges from fatty liver to inflammation and cirrhosis. miRNA-155 is an important regulator of inflammation. In this study, we describe the *in vivo* role of miR-155 in ALD.

Methods: Wild-type (WT) (C57/BL6J) or miR-155 knockout (KO) and *TLR4* KO mice received Lieber DeCarli diet for 5 weeks. Some mice received corn oil or CCl_4 for 2 or 9 weeks.

Results: We found that miR-155 KO mice are protected from alcohol-induced steatosis and inflammation. The reduction in alcohol-induced fat accumulation in miR-155 KO mice was associated with increased peroxisome proliferator-activated receptor response element (PPRE) and peroxisome proliferator-activated receptors (PPAR)α (miR-155 target) binding and decreased MCP1 production. Treatment with a miR-155 inhibitor increased PPARγ expression in naïve and alcohol treated RAW macrophages. Alcohol increased lipid metabolism gene expression (*FABP4*, *LXRα*, *ACC1* and *LDLR*) in WT mice and this was prevented in KO mice. Alcohol diet caused an increase in the number of $CD163^+$ $CD206^+$ infiltrating macrophages and neutrophils in WT mice, which was prevented in miR-155 KO mice. Kupffer cells isolated from miR-155 KO mice exhibited predominance of M2 phenotype when exposed to M1 polarized signals and this was due to increased C/EBPβ. Pro-fibrotic genes were attenuated in miR-155 KO mice after alcohol diet or CCl_4 treatment. Compared to WT mice, attenuation in CCl_4 induced hydroxyproline and α-SMA was observed in KO mice. Finally, we show TLR4 signaling regulates miR-155 as TLR4 KO mice showed no induction of miR-155 after alcohol diet.

Conclusions: Collectively our results demonstrated the role of miR-155 in alcohol-induced steatohepatitis and fibrosis *in vivo*.

Keywords: microRNA; Inflammation; Alcohol; PPARα; PPARγ; Fibrosis.
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Abbreviations: ALD, alcoholic liver disease; KC, Kupffer cell; LPS, lipopolysaccharide; TLR, Toll-like receptor; miRNA, microRNA; WT, wild-type; KO, knockout; PPRE, peroxisome proliferator-activated receptor (PPAR) response element; M2, macrophage 2; TNF-α, tumor necrosis factor α; MCP1, monocyte chemo attractant protein 1; IL-1β, Interleukin-1; TGF-β, Transforming growth factor β; LMNCs, liver mononuclear cells; EtOH, ethanol; CCl_4, carbon tetrachloride.

Introduction

Alcoholic liver disease (ALD) is one of the most common causes of liver diseases in the world [1]. The clinical manifestations of ALD range from simple fatty liver to more severe forms of liver injury, including alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma. Alcoholic hepatitis has high mortality and morbidity with limited treatment options. The pathomechanism of ALD involves complex interactions between the effects of alcohol and its toxic metabolites on various cell types in the liver and gut, induction of reactive oxygen species, and upregulation of the inflammatory cascade [2–5]. Direct toxic effects of alcohol on hepatocytes, increased intestinal permeability and activation of liver macrophages (Kupffer cells; KCs) by gut-derived lipopolysaccharide (LPS) are major factors contributing to ALD [1–3,5]. LPS is recognized by the Toll-like receptor (TLR) 4 complex expressed on immune cells as well as parenchymal cells and induces proinflammatory cytokine activation [5]. Chronic alcohol exposure also sensitizes liver resident macrophages (KCs) to LPS induced inflammatory cytokine production [6].

Fatty liver (steatosis) is the first stage of response in the liver to binge drinking or chronic ethanol consumption. Accumulation of lipid products such as triglycerides in hepatocytes leads to lipid superoxidation and oxidative stress, resulting in apoptosis, and hepatic inflammation [7]. microRNAs (miRNAs) are shown to be modulated by alcohol in the liver and in hepatocytes to exert inflammatory and fat accumulation effects [6,8–10]. Among other miRNAs, miR-155 is a major regulator of immune responses [11]. Various TLR ligands are shown to induce miR-155 in macrophages [11]. Previously, we identified miR-155 as an alcohol-induced regulator of increased KC activation and TNF-α production in macrophages [6]. In recent years, the role of miR-155 in other cellular processes like fatty acid metabolism and fibrogenic events has also been emerging [12–14]. In a methionine choline-deficient (MCD) diet induced steatohepatitis, we showed that miR-155 targets genes involved in lipid metabolism (*Fab4*, *Cpt1a*) and early fibrosis (*C/EBPβ*, *Smad3*) [12].

We earlier demonstrated that miR-155 was increased in both KCs and hepatocytes isolated from alcohol-fed mice. Therefore we hypothesized that miR-155 has a role in alcohol-induced liver injury, inflammation and steatosis. To test this hypothesis, we determined the *in vivo* role of miR-155 in a mouse model of ALD. Our results revealed that alcohol-induced liver injury, steatosis and inflammation were significantly reduced in miR-155 knockout (KO) mice compared to wild-type (WT) mice. The reduction in fat accumulation in miR-155 KO mice was associated with increased peroxisome proliferator-activated receptor response element (PPRE) and peroxisome proliferator-activated receptor (PPAR) α binding after alcohol diet. The alcohol-induced increase in neutrophil leukocyte and $CD163^+$ $CD206^+$ (M2) macrophage (pro-fibrotic) infiltration observed in WT mice was prevented in miR-155 KO mice. miR-155 KO mice also showed attenuation in pro-fibrotic genes after alcohol diet as well as in a CCl_4-induced liver fibrosis. Together, these results provide *in vivo* evidence for the role of miR-155 in ALD and fibrosis.

Materials and methods

Animal studies

Animal studies were approved by the University of Massachusetts Medical School (UMMS) Institutional Animal Use and Care Committee (Worcester, MA). miR-155 KO mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and a breeding colony was maintained in the animal facility of UMMS. WT control mice, C56BL/6 were also obtained from Jackson Laboratory. For chronic ethanol feeding, female mice (n = 8–10, 8 weeks old) received 5% (v/v) ethanol (36% ethanol-derived calories) containing Lieber DeCarli diet or control (pair-fed (PF)) diet for 5 weeks. For PF diet, alcohol-derived calories were substituted with dextrin-maltose (Bio-Serv, New Jersey, USA) as described previously [6]. For CCl_4 treatment, WT or miR-155 KO male mice (n = 6, 8–10 weeks old) were treated either with corn oil (vehicle control) or CCl_4 (0.6 ml/kg i.p.) for 2 or 9 weeks, as described [15]. Mice were sacrificed 72 h after last CCl_4 injection. At the end of the experiment, blood was collected by cheek bleeding and mice were sacrificed [6]. Liver tissue was collected and snap frozen for proteins and in RNA later (Qiagen, Germany) for RNA extraction. All samples were stored at −80 °C.

Liver cell isolation and flow cytometery

Primary murine hepatocytes, KCs and liver mononuclear cells (MNCs) were isolated from WT chow fed mice by an enzyme-based tissue digestion method as described [16]. Hepatocytes were plated onto 6 well collagen-coated plates and free-floating cells were removed 3 h after plating. Both hepatocytes and MNCs were treated with LPS (100 ng/ml) for 6 h and processed for RNA extraction. Some hepatocytes were treated with MCP1 (100 ng/ml) for 36 h and nuclear proteins were used for PPRE binding. KCs isolated from WT or KO mice were treated with or without LPS (100 ng/ml), IL-4 (20 ng/ml) and IFN-γ (20 ng/ml) for 18 h.

For flow cytometry, MNCs were isolated from perfused livers of WT and miR-155 KO mice after 5 weeks of alcohol diet and processed as described [17]. For cell viability, Live/Dead Fixable Blue Dead staining was used (Life Technologies, NY, USA). Briefly, after blocking for non-specific binding to FcγRs, MNCs (~10^6 cells) were stained with CD163, CD11b-FITC, F4/80 PerCP-Cy5.5, and CD206-Alexa 647 (BioLegend, CA, USA) followed by incubation with phycoerythrin-conjugated secondary antibody for CD163 as described [17]. For neutrophils, the percentage of $CD11b^+$ PE and $Ly6G^{high}$ FITC was determined. Respective isotype-matched control antibodies were used (BioLegend). After washing with FACS buffer, cells were fixed with 1% paraformaldehyde and acquired on a BD LSR II instrument (BD Biosciences, CA, USA). Data was analyzed with FlowJo software (OR, USA).

Additional methods are in the supplementary material.

Statistical analysis

Statistical significance was determined using the non-parametric Mann-Whitney test. Data is represented as mean ± standard error and considered statistically significant at $p < 0.05$.

Results

miR-155 deficiency attenuates chronic alcohol-induced steatosis, liver injury, and oxidative stress in the liver

Inflammation, a major component in ALD, contributes to the perpetuation of liver disease and amplifies steatosis [1]. In our earlier study, we showed induction of miR-155 in the liver, isolated Kupffer cells and hepatocytes from alcohol-fed mice [6], however, the functional role of miR-155 in ALD *in vivo* is unknown. In this study, we determined the role of miRNA-155 using a miR-155 KO mouse model system. Alcohol feeding for 5 weeks in WT mice resulted in hepatic steatosis as evidenced by H&E (Fig. 1A), Oil Red O staining (Fig. 1B), histological scores (Fig. 1C) and liver triglyceride levels (Fig. 1D). Compared to WT mice, a significant decrease was observed in fat accumulation in miR-155 KO mice after alcohol feeding (Fig. 1A–D). In miR-155 KO mice we found a significant decrease in plasma ALT, a marker of liver injury, compared to WT mice after alcohol feeding (Fig. 1E). Alcohol and its metabolites increase the production of reactive oxygen species and enhance peroxidation of lipids, proteins, and DNA [1]. We found that the increased oxidative stress (measured by thiobarbituric acid reactive substances assay) observed in WT mice was prevented in miR-155 KO mice after alcohol diet (Fig. 1F), indicating that miR-155 deficiency attenuates alcohol-induced oxidative damage.

Alcohol diet results in increased PPRE and PPARα DNA binding in miR-155 KO mice

Because we found a decrease in liver fat accumulation in miR-155 KO mice after alcohol diet (Fig. 1), we sought to evaluate possible mechanisms involved. Lipid and glucose homeostasis are regulated by PPARs that belong to the nuclear hormone receptor superfamily [7,18]. There are three PPAR subtypes: PPARα, PPARγ and PPARδ [7,18]. PPARα regulates alcoholic steatosis via its effects on genes of lipid metabolism, and a decrease in PPARα has been reported in mouse models of ALD [7,18,19]. We extensively performed bioinformatics analysis and found that miR-155 has a seed region at the 3' UTR of PPARγ gene (www.microrna.org) (Supplementary Fig. 1A). A very recent study identified PPARα, a direct target of miR-155 [20] and showed that miR-155 regulates inflammation via targeting PPARα after inducing diffuse alveolar hemorrhage. We found a decrease in *PPARα* mRNA after alcohol diet in WT mice and this decease was prevented in miR-155 KO mice (Fig. 2A). We did not find a significant change in the *PPARγ* mRNA after alcohol diet in WT mice, whereas miR-155 KO mice showed increased transcription of *PPARγ* (Supplementary Fig. 1B). Next we examined PPRE binding using electrophoretic mobility shift assay (EMSA). We found a decrease in PPRE binding in WT mice after alcohol diet compared to PF mice (Fig. 2B–C). In contrast, alcohol feeding resulted in no decrease in PPRE binding in miR-155 KO mice (Fig. 2B–C). PPRE binding was similar between genotypes on PF diet. To determine the binding of PPARα in miR-155 KO mice after alcohol diet, we performed a supershift assay. Our results (Fig. 2B) indicate a decrease in PPARα binding in alcohol-fed WT mice compared to PF diet fed mice (Fig. 2B). Further, we found an increase in PPARα binding in miR-155 KO mice compared to WT mice after alcohol diet (Fig. 2B–C).