Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNA-29a mitigation of toll-like receptor 2 and 4 signaling and alleviation of obstructive jaundice-induced fibrosis in mice

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ARTICLE INFO

Article history:
Received 12 January 2018
Accepted 20 January 2018
Available online xxx

Keywords:
miR-29a
Cholestasis
Liver fibrosis
TLR2
TLR4
Proinflammatory cytokines

ABSTRACT

Cholestasis and hepatitis can cause continuous liver damage that may ultimately result in liver fibrosis. In a previous study, we demonstrated that microRNA-29a (miR-29a) protects against liver fibrosis. Toll-like receptor 2 (TLR2) and TLR4 are pattern recognition receptors of bacterial lipoprotein and lipopolysaccharide, both of which participate in activating hepatic stellate cells and liver fibrosis. The purpose of this study is to characterize the biological influence of miR-29a on TLR2 and TLR4 signaling in livers injured with bile duct ligation (BDL). We performed BDL on both miR-29a transgenic mice (miR-29aTg) and wild-type mice to induce cholestatic liver injury. Primary HSCs were transfected with a miR-29a mimic and inhibitor. In the wild-type mice, the BDL demonstrated significant α-smooth muscle actin fibrotic matrix formation and hepatic high mobility group box-1 expression. However, in the miR-29aTg mice, these factors were significantly reduced. Furthermore, miR-29a overexpression reduced the BDL exaggeration of TLR2, TLR4, MyD88, bromodomain-containing protein 4 (BRD4), phospho-p65 as well as proinflammatory cytokines, IL-1β, MCP-1, TGF-β, and TNF-α. *In vitro,* miR-29a mimic transfection reduced α-SMA, BRD4,TLR2, and TLR4 expressions in HSCs. This study provides new molecular insight into the ability of miR-29a to inhibit TLR2 and TLR4 signaling, which thus slows the progression of cholestatic liver deterioration.

1. Introduction

Liver fibrosis and cirrhosis are the end results of most types of chronic liver damage and represent a common clinical challenge throughout the world [1,2]. Hepatic stellate cells (HSC) are activated and functional trans-differentiation into contractile myofibroblastic cells responsible for producing extracellular matrix (ECM) in damaged livers [1–3]. The inhibition of HSC activation has emerged as an experimental therapy for preventing and reversing hepatic fibrosis.

MicroRNAs (miRNAs) are ~22 nucleotide single-stranded non-coding RNAs (guide strands) that suppress endogenous mRNA transcripts. Through our previous studies [4–7], we have demonstrated that the overexpression of miR-29a in cholestatic mice significantly hindered hepatocellular damage and liver fibrosis. Furthermore, miR-29a overexpression lessened hepatocellular apoptosis and HSC activation in liver injuries [4,5,8]. The miR-29a reduction of TGF-β1 [8], histone deacetylase 4 [5], and methyltransferases [7] signaling reduced profibrogenic HSC phenotypes, thus improving BDL-mediated cholestatic liver fibrosis.

The toll-like receptor (TLR) family is the best characterized class of pattern recognition receptors that signal the host in mammalian species with infection [9]. The liver is continuously exposed to infectious pathogens, a condition that is more prominent in cholestatic or cirrhotic livers [10]. Furthermore, damage caused by cellular stress may prompt the release of endogenous molecules, referred to as damage-associated molecular patterns (DAMPs), which subsequently activate the host innate immune system,

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https://doi.org/10.1016/j.bbrc.2018.01.132

including the TLRs [11]. High mobility group box-1 (HMGB1) is considered an important DAMP, and we have previously shown its importance with regard to cholestatic liver injury [12]. The receptors involved in HMGB1 signal transduction include TLR2 and TLR4 [13]. In addition, bromodomain-containing protein 4 (BRD4), a protein of bromodomain and extra-terminal member, has been clearly shown to effectively ameliorate pathological fibrotic responses [14] and inhibition of BRD4 mitigates acute myocardial infarction damage in rats via the TLR4 /NF-κB pathway (15). Therefore, we hypothesize that miR-29a interacts with TLR2 and TLR4 signaling to regulate HSC activation in liver fibrosis. In this study, we used miR-29a transgenic mice (miR-29aTg) to verify the relationship between liver fibrosis and TLR2 and TLR4 signaling in livers with obstructive jaundice and *in vitro* HSCs.

2. Materials and methods

2.1. Ethics statement

All of the animal use protocols used in this study have been reviewed and approved by Chang Gung Memorial Hospital's Institutional Animal Care and Use Committee (IACUC) (#20160092604). We obtained male C57BL/6 mice (body weight 25–35 g) from BioLASCO Taiwan Co., Ltd. All animals were housed in an animal facility at 22 °C, with a relative humidity of 55%, in a 12 h light/12 h dark cycle, with both food and sterile tap water available *ad libitum*.

2.1.1. Creation and breeding of the miR-29a transgenic mouse colony

We bred transgenic mice with an overexpression of miR-29a driven by a PGK promoter and kept them in a specific pathogen-free rodent barrier, as described in a previous study [7]. The genotype of the transgenic mice was probed with PCR and primers (forward: 5′-GAGGATCCCCTCAAGGAT ACCAAGGGATGAAT-3′ and reverse 5′-CTTCTAGAAGGAGTGTTTCTAGGTATCCGTCA-3′). The wild-type mice used were obtained from littermates without said construct.

2.1.2. Animal model and experimental protocol

To perform all of our experiments, we used six to eight C57BL/6 male mice (BioLASCO, Yilan, Taiwan) weighing 25–35 g. The mice were placed into either the “BDL” group or the “sham” group depending on whether it had received an actual ligation or a sham ligation of the common bile duct, the process of which is described in a previous study. In general, all surgical procedures were performed under ketamine (50 mg/kg) and xylazine (23 mg/kg) intramuscular injection (IM) anesthesia with clean surgical techniques. Midline laparotomy was carried out to explore the hepatic hilum and identify common bile duct (CBD). Using a dissecting microscope, we isolated CBD, doubly ligated it, and transected it between two ligatures [8]. All of the mice were euthanized one week after the surgical procedure. Liver tissues were dissected, snap-frozen, and processed to isolate total RNA and proteins, and all specimens were stored at −80 °C until we performed the biochemical analysis.

2.1.3. Real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR)

The frozen mice liver samples (0.1 g/per sample) were homogenized in 1 ml TRIzol (Invitrogen, Carlsbad, CA), and total RNA was isolated in accordance with the manufacturer's instructions. We then quantified the RNA isolates using the $A_{260/280}$ ratio of 1.7–2.0. We synthesized cDNA with M-MLV Reverse Transcriptase (Promaga, WI). GAPDH was adopted as the internal control gene in order to analyze the mRNA expression of the following transcripts: TLR2 and TLR4, myeloid differentiation primary-response protein 88 (MyD88), Interleukin-1β (IL1β), monocyte chemotactic protein 1 (MCP1), transforming growth factor beta 1 (TGFβ1), and tumor necrosis factor α (TNFα) (Table 1). QRT-PCR was carried out with SYBR Green PCR Master Mix, and each sample was analyzed twice. The mRNA of the studied genes was quantified with the LightCycler 480 System (Roche Molecular Systems, IN) using comparative methods. The quantity of mRNA was calculated using the ΔC_t method. We normalized the Ct values for each gene to the Ct value of a housekeeping gene (GAPDH) with the same reaction and presented results as $2^{-\Delta\Delta Ct}$ ($\Delta C_t = C_t^{\beta\text{-actin}} - C_t^{\text{target}}$).

2.1.4. Western blot analysis

We used approximately 20 mg of liver tissue in a 500 μl protein lysis buffer (iNtRON, Seongnam-si), homogenized by MagNA Lyser system (Roche, Germany). The protein (30 μg) obtained from the supernatant of each sample was mixed with a sample buffer and boiled for 10 min, followed by electrophoresis with 8–15% sodium dodecyl sulfate–polyacrylamide gels. The proteins in the gels were transferred to a PVDF membrane, and blots were incubated with primary antibodies against α-SMA (abcam, JHY), HMGB1 (Santa Cruz, CA), TLR2 (abcam, JHY.), TLR4 (abcam, JHY.), MYD88 (abcam, JHY), BRD4 (abcam, JHY), phospho-p65 (Genetex, TX), p-65 (Sigma, LA) and GAPDH (PROTEINTECH, IL). Once the blots were washed with TBST and incubated with horseradish peroxidase-coupled anti-rabbit immunoglobulin-G antibodies (dilution: 1:5000) and HRP anti-mouse immunoglobulin-G antibodies (dilution:1:10,000) at room temperature for 1 h, we then developed them with enhanced chemiluminescence detection (GE Healthcare Biosciences AB, Uppsala, Sweden), exposed them to film, and quantified the signals using densitometry.

2.1.5. Primary HSC isolation and culture

Primary HSCs were isolated from the C57BL/6 livers by using sequential digestion of the liver with pronase and collagenase, followed by density gradient centrifugation in 8.5% Nycodenz (Sigma-Aldrich, St. Louis, MO) as previously described in another study [16]. Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA) with 10% fetal bovine serum. The HSCs had a quiescent phenotype after spending 1 day in culture and developed an activated phenotype after 7–14 days. We carried out the passage of the cultured cells after they reached confluence and performed our experiments using cells between passages 8 and 10.

2.1.6. Culture of hepatic stellate cells and treatment with RNAi

The hepatic stellate cells (HSCs) were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA), supplemented with 10% fetal bovine serum and penicillin/streptomycin (Gibco, MA), in a humidified incubator at 37 °C with 5% CO2. Cells were seeded at a density of 8×10^5 cells per 6-cm culture dish for western blot and 2×10^4 cells/well in a 12-well plate, which was inserted into an 18 mm × 18 mm cover glass for immunofluorescence. After resting overnight, cells were transfected with a concentration of 20 nM of miR-29a precursor (mimic-miR-29a, GE Healthcare Dharmacon, IN), miR-29a antisense oligonucleotide inhibitor (inhibitor-miR-29a GE Healthcare Dharmacon, IN), or miR control (GE Healthcare Dharmacon, IN) for 24 h with Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, CA). We performed each cell culture experiment five or six times.

2.1.7. Immunofluorescence

Twenty-four hours after the previously described transfection (mimic-miR-29a, inhibitor-miR-29a, or miR control), the HSC cells

Please cite this article in press as: Y.-C. Lin, et al., MicroRNA-29a mitigation of toll-like receptor 2 and 4 signaling and alleviation of obstructive jaundice-induced fibrosis in mice, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.01.132