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Deficiency in augmenter of liver regeneration accelerates liver fibrosis by promoting migration of hepatic stellate cell

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ABSTRACT

Background: Augmenter of liver regeneration (ALR) protects liver from various injuries, however, the association of ALR with liver fibrosis, particularly its effect on hepatic stellate cells (HSC), remains unclear. In this study, we investigated the impact of ALR on the activation of HSC, a pivotal event in occurrence of liver fibrosis.

Methods: Liver fibrosis was induced *in vivo* in mice with heterozygous *ALR* knockdown (*ALR*-KD) by administration of CCl_4 or bile duct ligation. The effect of *ALR*-KD and ALR-overexpression on liver fibrosis was studied in mice and in HSC cells as well.

Results: Hepatic collagen deposition and expression of α-smooth muscle actin (α-SMA) were significantly increased in the *ALR*-KD mice compared to wild-type mice. *In vitro*, *ALR*-shRNA resulted in the activation of HSC cell line (LX-2). Furthermore, *ALR*-shRNA promoted LX-2 cell migration, accompanied by increased filamentous actin (F-actin) assembly. The *ALR*-KD-mediated increase in HSC migration was associated with mitochondrial fusion, resulting in mitochondria elongation and enhancing ATP production. In contrast, *ALR* transfection (*ALR*-Tx) decelerated HSC migration and inhibited F-actin assembly, concomitantly enhancing mitochondrial fission and reducing ATP synthesis. Mechanically, stimulation of HSC migration by *ALR*-shRNA was attributed to the increased mitochondrial Ca^{2+} influx in HSCs. Treatment of *ALR*-shRNA-cells with Ruthenium Red (RuR), a specific inhibitor of mitochondrial calcium uniporter (MCU), significantly suppressed mitochondrial Ca^{2+} influx, HSC migration, mitochondrial fusion and ATP production. *ALR*-KD-induced HSC migration was verified *in vitro* in primary mouse HSCs.

Conclusion: Inhibition of ALR expression aggravates liver fibrosis, probably via promoting HSC migration and mitochondrial fusion.

1. Introduction

Liver fibrosis is the wound-healing response following acute or chronic liver damage. Liver fibrosis is characterized by cytokine-mediated activation of hepatic stellate cells (HSCs), resulting in increased expression of α-smooth muscle actin (α-SMA), and subsequently the accumulation of extracellular matrix (ECM) [1]. The activation of HSCs is considered a pivotal step in the development of liver fibrosis [2]. Beyond the activation of HSCs by pro-fibrogenic and proliferative cytokines such as transforming growth factor (TGF-) beta and platelet derived growth factor (PDGF-) beta, several other pathways are involved in the regulation of liver fibrosis [2,3]. Although endoplasmic reticulum (ER) stress has been considered to contribute to the activation of HSCs, the links between mitochondrial stress-related phenomena, such as mitophagy and mitochondrial dynamics, and the activation of HSCs have not been explored in greater depth to date [4]. It is known that intracellular calcium plays a vital role for cell contractility and mobility [5], therefore it appears a critical task to explore the regulation of intracellular Ca^{2+} pathway in HSCs activation and migration with the aim of identifying potential intervention targets.

Hepatic stimulator substance (HSS) was first extracted from regenerating liver in weanling rats after partial hepatectomy [6]. Subsequent studies confirmed that HSS promoted hepatic proliferation only when combined with other mitogenic factors such as epidermal growth factor (EGF). As a consequence, its denomination was changed from HSS to 'augmenter of liver regeneration' (ALR) [7]. ALR is known to protect hepatocytes from several types of toxins and to promote remnant liver growth after liver injury [8]. The purified ALR protein has

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two splice forms of 15 and 23-kDa size, respectively. The smaller form of ALR is believed to act as sort of cytokine [9]. The larger ALR protein, which mainly resides in the inter-membrane spaces of mitochondria, is required for mitochondrial functions [10]. Our previous study showed that the 23-kDa ALR isoform protects hepatocytes from apoptosis induced by endoplasmic reticulum (ER) stress by improving mitochondrial function [11].

Only few studies to date have examined the links between ALR and liver fibrosis [12,13]. Considering the mitochondrial localization of 23-kDa ALR, which affects mitochondrial ATP synthesis [14,15], we speculated that ALR could impact liver fibrosis via regulation of mitochondrial dynamics of HSCs, thus affecting HSC differentiation and migration. To this end, we established heterozygous *ALR*-knockdown (*ALR*-KD) mice ($Gfer^{+/-}$) and confirmed that deficiency in ALR expression resulted in enhanced liver fibrosis following administration of carbon tetrachloride (CCl_4) or bile duct ligation (BDL). We confirmed *in vitro* that *ALR*-knockdown by shRNA promotes the migration of LX-2, a human HSC cell line. Mechanistically, *ALR*-shRNA interfered with mitochondrial fission, causing mitochondrial elongation with a marked elevation in ATP production, which favored migration of the HSCs. *ALR*-shRNA increased HSC mitochondrial calcium levels and ATP production, which could be effectively inhibited by Ruthenium Red (RuR), a specific blocker of the mitochondrial calcium uniporter (MCU). In contrast, overexpression of ALR (*ALR*-Tx) decelerated HSC migration, reducing energy supply and inhibiting mitochondrial fusion. All the data obtained from LX-2 cells were confirmed in cultured primary HSCs. Taken together, we demonstrate that the lack of ALR aggravates liver fibrosis, probably by regulating mitochondrial Ca^{2+} homeostasis and mitochondrial dynamics in HSC cells.

2. Materials and methods

2.1. Animal and cell models

C57BL/6J wild-type (WT, $Gfer^{+/+}$) mice and heterozygous *ALR*-knockdown (*ALR*-KD, $Gfer^{+/-}$) mice were bred under pathogen-free conditions at the animal care facility of Capital Medical University (Beijing, China). The methods used to generate and identify the heterozygous mice have been described previously [15]. All animals were kindly and humanely treated, and all animal experiments were approved by the Human and Animal Ethics Committee of Capital Medical University. Male mice selected for the experiments weighed 18–20 g. The CCl_4 liver fibrosis model was induced by administering a dose of CCl_4 (1 μl/g body weight, diluted with an equal volume of olive oil) and intraperitoneally injected into abdomen of mice twice a week for 4 weeks in both *ALR*-KD and WT mice. LX-2 cells, an immortalized human HSC cell line [16], were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco™, Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; ExCell Bio, Genetimes, Shanghai, China) and maintained at 37 °C under 5% CO_2–95% air.

For stable transfection, LX-2 cells were transfected with 2 μg of pcDNA3.0-*ALR*, pcDNA3.0-vector (Invitrogen™, Thermo Fisher) and pGPU6-sh*ALR*, pGPU6-scramble plasmids (GenePharma, Shanghai, China) in 6 μl of transfection reagent (FuGENE® HD, Promega, Madison, WI, USA). Cells receiving *ALR*, vector control and shRNA transfection were nominated as *ALR*-Tx, vector-Tx and *ALR*-shRNA or scramble-shRNA, respectively. Four days after transfection, the cells were supplemented with 800 μg/ml G418 (Sigma–Aldrich, St. Louis, MO, USA) for 14 days. Cells resistant to G418 were used for further colony selection. For regression tests, *ALR*-Tx cells were transfected with 25 nmol/l *ALR*-siRNA or scramble-siRNA (Dharmacon, Lafayette, CO, USA) for knockdown of ALR expressions.

Primary mouse HSCs were isolated from the livers of WT and $Gfer^{+/-}$ mice aged 6–8 weeks, as previously described [17]. Briefly, the mice were anesthetized and their abdominal cavities were opened under sterile conditions. The liver was first perfused through the portal vein with 50 ml of D-Hank's solution containing 5 μM EDTA. The liver was then perfused with 50 ml of Hank's solution containing 5 mg of collagenase IV (Sigma-Aldrich) for 15 min. Immediately after perfusion, the liver cells were dissociated and suspended in DMEM containing 10% FBS. The cell suspension was centrifuged at 50 × *g* for 5 min three times to remove hepatocytes and the supernatant was collected. Cells were washed three times via density gradient centrifugation on Nycodenz (Axis-Shield, Oslo, Norway), resuspended in DMEM containing 10% FBS and penicillin (100 IU/ml)/streptomycin (100 μg/ml), and plated. After incubation for 1 h, the medium was substituted with Stellate Cell Medium (ScienCell™, San Diego, CA, USA) to eliminate any nonadhesive cells and debris.

Primary human HSCs were purchased from ScienCell™ Company and cultured in Stellate Cell Medium (ScienCell™). Cells were transfected with *ALR*-siRNA (25 nmol/l) or scramble-siRNA (Dharmacon) for 96 h and α-SMA expressions were determined by Western blot.

2.2. Immunofluorescent staining and Western blot

2.2.1. Immunofluorescence for tissues and cells

Frozen tissue sections were denatured by heating in a microwave in 0.01 M citrate buffer (pH 6.0) for 15 min for antigen retrieval, and then cooled to room temperature. The sections were washed three times with phosphate-buffered saline (PBS) on a rotary shaker at 100 rpm and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 10 min. The sections were washed with PBS, blocked with 5% bovine serum albumin (BSA; Sigma–Aldrich) in PBS at room temperature for 30 min, and incubated overnight with the primary antibody (rabbit polyclonal antibody directed against α-SMA, diluted 1:200; Sigma-Aldrich) in PBS containing 5% BSA. Next day, the sections were washed three times with PBS and incubated with the secondary antibody (Alexa-Fluor®-594-conjugated goat anti-rabbit IgG antibody, diluted 1:200; Thermo Fisher Scientific Inc.) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:1000; Beyotime, Shanghai, China). After the sections were washed three times with PBS, they were observed with a confocal laser scanning microscope (SP8, Leica, Wetzlar, Germany). To observe the cultured cells, cells (5×10^4) were seeded on 15 mm glass-bottomed dishes (NEST Biotechnology Co., Wuxi, China) and incubated at 37 °C under 5% CO_2–95% air for 24 h. The cells were fixed in 4% paraformaldehyde (PFA) at 37 °C for 20 min, washed three times in PBS, and permeabilized with 0.1% Triton X-100 (Sigma–Aldrich) in PBS at room temperature for 10 min. After the cells were blocked with 5% BSA in PBS at room temperature for 30 min, they were incubated with iFluor® 488-conjungated-Phalloidin (1:1000; AAT Bioquest, Sunnyvale, CA, USA) and Alexa Fluor® 594-conjungated-deoxyribonuclease-1 (DNase 1) (1:1000; Thermo Fisher Scientific Inc.) in PBS containing 5% BSA at room temperature for 30 min. The cells were observed with a confocal laser scanning microscope (Leica).To observe mitochondria, cells (5×10^4) were plated on 15 mm glass-bottomed dishes and incubated under 5% CO_2–95% air at 37 °C for 24 h. After the cells were washed twice with PBS, they were incubated in 1 ml of DMEM containing MitoTracker™ Red FM (1:4000; Invitrogen™, Thermo Fisher Scientific Inc., Waltham, MA) for 45 min and analyzed with confocal microscopy. Fifty cells in each group were randomly chosen for observation. The images of the mitochondria were processed with the confocal-microscopy-specific software, Leica Application Suite X (LAS X).

For patient specimens, the liver samples of patients were acquired from Beijing You An Hospital during surgical operation and the fibrosis/cirrhosis tissues were carefully dissected. The study was approved by the Ethics Committee of Beijing You An Hospital. Immunofluorescence staining was performed and the staining protocols were described similarly as above. Tissues from liver were fixed in 4% formaldehyde for 24 h, embedded in paraffin, and serially sectioned at a thickness of 6 μm. Sections were deparaffinized and stained with anti-ALR or α-SMA.