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Knockdown of myeloid differentiation protein-2 reduces acute lung injury following orthotopic autologous liver transplantation in a rat model

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ABSTRACT

Background: Acute lung injury (ALI) is a serious complication that commonly occurs during orthotopic liver transplantation (OLT). Toll-like receptor 2/4 (TLR2/4) are the main membrane receptors that respond to inflammatory stimuli and mediate NF-kappa B (NF-κB) signal pathway. We previously showed that TLR2/4 expression on monocytes and serum cytokine levels were increased in patients with ALI induced by OLT. Myeloid differentiation protein-2 (MD-2) expresses the functional domains that combines TLRs and play a key regulatory role in TLRs activation. Therefore, we hypothesized that blocking MD-2 would inhibit the TLR2/4-mediated inflammatory response and lessen ALI induced by liver transplantation.

Method: Thirty-two Sprague Dawley (SD) rats were randomly divided into four groups. One group received a sham operation (Group S), and the other three groups underwent orthotopic autologous liver transplantation (OALT) 48 h after intratracheal administration of saline (Model group; Group M), non-targeting siRNA (negative siRNA control group; Group NC) or siRNA against MD-2 (intervention group; Group RNAi). Lung pathology, lung water content, PaO_2, and expression levels of MD-2, TLR2/4, NF-κB, TNF-α, IL-1β and IL-6 were assessed 8 h after OALT.

Results: In Groups M and NC, OALT produced marked lung pathology with decreased PaO_2 levels and increased MD-2, TLR2/4 gene and protein expression levels. Furthermore, the nuclear translocation of the NF-κB P65 subunit, was increased, as were lung concentrations of TNF-α, IL-1β and IL-6. The pathology of ALI and the severity of the above biochemical changes induced by OALT were significantly reduced in the group treated with MD-2 siRNA.

Conclusion: MD-2 gene knock-down attenuated the increase in TLR2/4 activation and reduced ALI after OALT.

1. Introduction

Acute lung injury (ALI) commonly occurs after orthotopic liver transplantation (OLT) and can result in serious complications. ALI occurs in about 34.2%–77.8% of patients undergoing liver transplantation [1–5] and is one of the main causes of death after OLT [6]. We [3,7] and others [8] have shown that serum concentration of cytokines are increased in patients with ALI after OLT, suggesting that inflammation plays an important role in ALI induced by OLT. An uncontrolled inflammatory response in the lungs is a hallmark feature of ALI [9] but the degree of inflammatory reaction induced by OLT in lung is still unclear, which ultimately makes it difficult to predict the efficacy of treatments designed to lessen ALI during OLT.

Toll-like receptor 2/4 (TLR2/4) are the most important members in the TLRs family and play a key role in the inflammatory response. TLR2/4 recognize biological or non-biological-derived endogenous stimuli and activate nuclear factor κB (NF-κB) subsequently promoting cytokine synthesis [10–13]. The inflammatory response

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mediated by TLR2/4 has been suggested to be the main mechanism of ALI induced by lipopolysaccharide (LPS) or trauma [14,15]. We previously found a significant upregulation of TLR2/4 expression on monocytes in patients with ALI, concomitant with increased levels of cytokines in the serum. Collectively, these findings indicate a tight correlation between upregulation of TLR2/4 and the occurance of ALI during OLT [3,7]. Further, our recent study shows that TLR4 gene knock down significantly attenuated the severity of ALI in a rat model of orthotopic autologous liver transplantation (OALT) [16], which suggests that TLR4 plays a critical role in OLT-induced ALI. However, the specific changes of TLR2/4 expression and inflammatory mediators in lung tissue following OLT-induced ALI remain largely unknown, and it is yet to be determined if there is a molecule that can regulate the changes of pulmonary TLR2/4 during this pathology.

Myeloid differentiation protein-2 (MD-2) contains structural and functional domains that help assemble TLRs, particularly TLR2 and TLR4, and therefore MD-2 has a unique regulatory role in the signal transduction pathway of TLR2/4 [17–20]. For example, cells transfected with TLR2 or TLR4/CD14 do not respond to LPS, whereas cells co-transfected with MD-2 and TLR2 or MD-2 and TLR4/CD14 do react to LPS [17,18,21]. Furthermore, these in vitro studies have been corroborated by in vivo studies showing a regulatory role for MD-2 in the TLR2/4 signaling pathway [22,23]. However, whether MD-2 regulates activation of the TLR2/4 pathway in response to OLT and thus contributes to ALI, is not known.

In this study, we performed the OALT model in rats and evaluated whether MD-2 knock-down via siRNA can attenuate excessive TLR2/4 activation during OLT and alleviates ALI. Our results suggest that MD-2 regulation of the TLR2/4 signaling pathway contributes to ALI and therefore may be an attractive therapeutic target for treating ALI during OLT.

2. Methods and materials

2.1. Animals

Male Sprague Dawley rats, aged 8–10 weeks, weighing 220–250 g, were purchased from *Medical Experimental Animal Center of Guangdong Province*. All the animal research protocols used in this study were approved by the institutional laboratory review board and were in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 2003) [24].

2.2. Establishment of OALT model in rats

The OLAT model performed was similar to that described by Jin C et al. [25], but with some modifications. Briefly, an open face guard was used to administer the inhalational ether anesthesia. After entering the abdominal cavity, the falciform ligament of the liver was resected and ligated, and the blood vessel along the esophagus was severed. The liver was revealed until the supra hepatic vena cava (SVC) was totally liberated. A bold line was prepared to guide the SVC for blockage use. The liver was then placed back in its original position and the inferior vena cava (IVC) was liberated until the upper region of the left renal vein was totally liberated. The first hepatic portal was dissected and the portal vein (PV) was liberated from the convergence of the inferior mesenteric and splenic veins. The hepatic artery and biliary tract were liberated together on account of their anatomic relationship. The portal hepatics were ligated. Vascular clamps were used at the convergence of the inferior mesenteric and splenic veins, the hepatic artery, and the SVC and IVC. The PV was punctured with 24-gauge needle in preparation for reperfusion and fixed in place with a vascular clamp. Pre-cooled Ringer lactate solution (4 °C) was injected during the reperfusion at 2.5 ml/min, and a 1-mm incision was made in the wall of the IVC as an outflow tract. Finally, the needle was extracted and the opening of the PV and IVC was closed using 8-0 sutures, and the PV, SVC, IVC and hepatic artery were unclamped. In total, the anhepatic phase lasted for 20 ± 1 min.

2.3. siRNA preparation, transfection and screening

Three 21-nt siRNA duplexes targeting rat MD-2 gene were designed using the siRNA Target Finder and Design Tool available at http://www.ambion.com and were commercially obtained from Ribobio (Guangzhou, China). The sequences of these siRNAs were as follows:

MD-2 siRNA1, target sequence 1: CCATCATTCACCACCATAA,

5′-CCAUCAUUCACCACCAUAA dTdT-3′ (sense)
3′-dTdT GGUAGUAAGUGGUGGUAUU-5′ (antisense)

MD-2 siRNA2, target sequence 2: CCAATGGATTTGTGCATGT,

5′-CCAAUGGAUUUGUGCAUGU dTdT-3′ (sense)
3′-dTdT GGUUACCUAAACACGUACA-5′ (antisense)

MD-2 siRNA3, target sequence 3: CCTTCGACGGAATACTATT,

5′-CCUUCGACGGAAUACUAUU dTdT-3′ (sense)
3′-dTdT GGAAGCUGCCUUAUGAUAA-5′ (antisense).

Green fluorescent protein (GFP) siRNA, which has no homology to MD-2 gene, was used as siRNA control. The efficacy of the MD-2 siRNAs was tested in a follow-up siRNA screening experiment. Hepatic stellate cells (HSC) were transfected with siRNA using Lipofectamine2000 (Invitrogen, CA) according to the manufacturer's instructions. MD-2 expression levels were determined using Q-PCR and Western blot. Compared to the blank control, MD-2 mRNA expression was suppressed by 64.7 ± 4.6, 82.7 ± 7.1, and 44.6 ± 2.8% in cells treated with sequence 1, 2, 3 MD-2 siRNA, respectively, when measured 48 h post-transfection (Fig. 1A). In agreement with the Q-PCR results, Western blot analysis of MD-2 protein expression showed dramatically decreased levels of MD-2 after transfection with MD-2 siRNAs, confirming that sequence 3 siRNA was most effective in suppressing MD-2 expression (Fig. 1B, C). Therefore, MD-2 siRNA3 was chosen for the subsequent in vivo experiments.

2.4. Animal groups and siRNA delivery

Rats were randomly divided into four groups. For the sham group (Group S, $n = 8$), rats were anesthetized and an abdominal incision was made, the portal vein was dissociated and the abdominal incision was sutured. The other three groups received one of the following three treatments 48 h prior to OALT: saline (model group Group M; $n = 8$), non-targeting siRNA (negative siRNA control group; Group NC; $n = 8$), or MD-2 targeting siRNA (intervention group; Group RNAi, $n = 8$). Endotracheal treatment, a recently developed pulmonary exposure rat model which allows homogenous exposure of the whole lung, was used to deliver saline and siRNA, according Perl M's reports [26]. Briefly, a cold light, a blunt wire, a mouth-opening device and a 16G catheter were used as follows: the anesthetized rat was suspended by its upper incisors on a wire rod at the top of a slant board (16–25 cm, 60° angle), and the cold light was placed in front of the neck area. The tongue was gently stretched using blunt forceps and the trachea (illuminated by the cold light) was visualized through the open glottis. The blunt