Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Prevention of acute liver injury by suppressing plasma kallikrein-dependent activation of latent TGF-β

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ARTICLE INFO

Article history:
Received 30 August 2018
Accepted 6 September 2018
Available online xxx

Keywords:
Plasma kallikrein
TGF-β activation
Macrophages/Kupffer cells
Acute liver injury
Proteinase inhibitor

ABSTRACT

Acute liver injury (ALI) is highly lethal acute liver failure caused by different etiologies. Transforming growth factor β (TGF-β) is a multifunctional cytokine and a well-recognized inducer of apoptotic and necrotic cell death in hepatocytes. Latent TGF-β is activated partly through proteolytic cleavage by a serine protease plasma kallikrein (PLK) between the R58 and L59 residues of its propeptide region. Recently, we developed a specific monoclonal antibody to detect the N-terminal side LAP degradation products ending at residue R58 (R58 LAP-DPs) that reflect PLK-dependent TGF-β activation. This study aimed to explore the potential roles of PLK-dependent TGF-β activation in the pathogenesis of ALI. We established a mouse ALI model via the injection of anti-Fas antibodies (Jo2) and observed increases in the TGF-β1 mRNA level, Smad3 phosphorylation, TUNEL-positive apoptotic hepatocytes and R58-positive cells in the liver tissues of Jo2-treated mice. The R58 LAP-DPs were observed in/around F4/80-positive macrophages, while macrophage depletion with clodronate liposomes partly alleviated the Jo2-induced liver injury. Blocking PLK-dependent TGF-β activation using either the serine proteinase inhibitor FOY305 or the selective PLK inhibitor PKSI-527 or blocking the TGF-β receptor-mediated signaling pathway using SB431542 significantly prevented Jo2-induced hepatic apoptosis and mortality. Furthermore, similar phenomena were observed in the mouse model of ALI with the administration of acetaminophen (APAP). In summary, R58 LAP-DPs reflecting PLK-dependent TGF-β activation may serve as a biomarker for ALI, and targeting PLK-dependent TGF-β activation has potential as a therapeutic strategy for ALI.

1. Introduction

Acute liver injury (ALI) is a symptom of hepatic illness characterized by high morbidity and development over a short duration but without an obvious previous record of liver disease [1]. Different etiological factors, such as infection with hepatic viruses, drug and alcohol use, and other unknown reasons, might cause ALI [2]. The acknowledged major processes of ALI include the apoptosis or necrosis of hepatocytes and physiological disorders in other hepatic cells [3].

During the pathogenesis of ALI, massive hepatocyte apoptosis directly destroys liver function and burdens the immune system, along with various pathological signaling factors and cytokines, including transforming growth factor β (TGF-β), which induces apoptosis in rat fetal hepatocytes or hepatoma cells through the mitochondria-dependent apoptosis pathway via Smad3. Increases in TGF-β at both the mRNA and protein levels have also been observed along with the exacerbation of liver injury in ALI murine models [4]. Overactivation of the Smad3 pathway exacerbated liver damage, while the knockdown of Smad3 relieved liver injury in the anti-Fas (Jo2) antibody-induced ALI mice model [5].

TGF-β is synthesized and released as a latent complex, in which 25-kD, active TGF-β is trapped by its propeptide region (termed latency associated protein, LAP) [6]. The latent TGF-β is activated before binding to its receptor, at least in part via proteolytic cleavage by plasma kallikrein (PLK) between the R58 and L59 residues of LAP [7]. We recently developed a specific monoclonal antibody, R58, to

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https://doi.org/10.1016/j.bbrc.2018.09.026
0006-291X/

detect the N-terminal side LAP degradation products ending at residue R58 (R58 LAP-DPs), which reflect PLK-dependent TGF-β activation in various mouse liver disease models as well as in patients [7–12]. In this study, we investigated the role of PLK-dependent TGF-β activation in Jo2 or acetaminophen (APAP)-induced mouse models of ALI. Immunohistochemical (IHC) staining for R58 LAP-DPs in the mouse livers showed that PLK-dependent TGF-β activation occurred, partly in/around macrophages, in the Jo2-induced ALI model. R58 LAP-DPs reflecting PLK-dependent TGF-β activation may serve as a biomarker for ALI. Importantly, loss-of-function analyses with chemical inhibitors, including the TGF-β receptor kinase inhibitor SB431542 [13], the serine protease inhibitor camostat mesilate (CM, *i.e.,* FOY305) [14], the PLK-selective inhibitor, trans-4-aminomethylcyclohexanecarbonyl-phenylalanyl-4-carboxymethyl-anilide, PKSI-527 [15] and macrophage-depleting clodronate liposomes [16], suggested that targeting PLK-dependent TGF-β activation and its downstream signaling pathways has potential as a therapeutic strategy for ALI.

2. Materials and methods

2.1. Animals

Seven to eight-week-old male C57BL/6 mice were used in this study. The mice were housed in individual plastic cages at a controlled temperature (25 ± 1 °C) and maintained on a reverse 12-h light/dark cycle. The animal experiments were performed in accordance with protocols approved by the Institutional Committee of Animal Experiment of RIKEN and adhered to the guidelines in the Institutional Regulations for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

2.2. Establishment of murine ALI models

The Jo2 (554254, purified NA/LE hamster anti-mouse CD95, BD Pharmingen, USA) antibodies were diluted with phosphate-buffered saline (PBS) and intravenously injected into the tail vein of each mouse at a dose of 150 μg/kg. The APAP (015–13942, WAKO Chemicals, Japan) was diluted with PBS and intraperitoneally injected into each mouse at 600 mg/kg (weight/body weight). The control mice were injected with the same volume of PBS.

2.3. Loss-of-function experiments

The mice were intraperitoneally injected with SB431542 (192–16541, WAKO chemicals, Japan) at 10 mg/kg (dissolved in 13% dimethyl sulfoxide (DMSO) in PBS) or vehicle (13% DMSO in PBS), PKSI-527 (BMLPI1470025, Enzo Life Sciences, USA) at 15 mg/kg dissolved in PBS or PBS alone, or clodronate liposomes or control liposomes (F70101C-AC; Formu Max, USA) at 200 μL/mouse or were orally administered FOY305 (035–17763, WAKO chemicals, Japan) at 400 mg/kg (dissolved in PBS) or PBS alone. The treatment time points and vehicles are indicated in the figures.

2.4. Immunostaining and hematoxylin and eosin (HE) staining

The paraffin-embedded tissues and 4-μm-thick sections were prepared as previously reported [17]. Immunostaining, including immunohistochemical (IHC) staining and immunofluorescence (IF) staining, was performed as previously described [7]. The detailed protocols are shown in the Supplementary Methods.

2.5. Plasma aspartate aminotransferase (AST) activity

Blood was collected from the inferior vena cava and mixed with ethylenediaminetetraacetic acid (EDTA, final concentration 5 mM). After the plasma was isolated by centrifugation at 3500 rpm for 15 min at room temperature, it was stored at −80 °C. Plasma AST activity was measured using an AST activity assay kit (K753-100, Bio Vision, USA) according to the manufacturer's instructions. Plasma was diluted with the corresponding buffer in the kits by a factor of 40. The absorbance was measured on an ENSIGHT multimode plate reader (PerkinElmer, USA).

2.6. Real-time RT-PCR

RNA was isolated from liver tissue stored at −80 °C. The cDNA preparation and quantitative PCR were performed as previously reported [17]. Data were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) expression. The following primer sequences were used: mouse *Tgf-β1* (forward: 5′-GGTTCATGTCATGGATGGTG-3'; and reverse: 5′-ATACGCCTGAGTGGCTGTCT-3′) and mouse *Gapdh* (forward: 5′-AACTTTGGCATTGTGGAAGG-3'; and reverse: 5′-ACACATTGGGGGT AGGAACA-3′).

2.7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed using the In Situ Cell Death Detection Kit, Fluorescein (Cat. No. 11684795910, Roche, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

The animal experiments were repeated at least twice with 3–10 mice per group, and representative results are shown. Statistical analyses were performed with GraphPad Prism 6 (GraphPad, USA) and Excel 2016. The statistical significance of differences was assessed using the Student *t*-test or Gehan-Breslow-Wilcoxon test. Relationships were considered statistically significant at $P < 0.05$. Quantitative data are shown as the mean ± standard deviation (RT-PCR and plasma AST activity).

3. Results and discussion

In PLK-dependent TGF-β activation, PLK cleaves LAP between R58 (arginine) and L59 (leucine) residues and releases active TGF-β [7]. The products of this process, R58 LAP-DPs, can be used as a specific reflection of the PLK-dependent TGF-β activation in the tissues [7]. Here, using a specific monoclonal anti-R58 antibody to detect R58 LAP-DPs, we explored whether PLK-dependent TGF-β activation might be a possible biomarker and target for the treatment of ALI. Furthermore, we examined whether both the serine proteinase inhibitor FOY305 and the selective PLK inhibitor PKSI-527 have therapeutic potential against ALI.

3.1. TGF-β is involved in the pathogenesis of Jo2-induced ALI

To establish the ALI model, the mice were injected with Jo2 at 150 μg/kg, and the liver and blood were collected at 0, 2 and 8 h (Fig. 1A). Following Jo2 injection, the levels of AST activity in the plasma (Fig. 1B) at 2 and 8 h and the mRNA levels of *Tgf-β1* in the liver (Fig. 1C) at 8 h were increased. Both the level of p-Smad3 (Fig. 1D upper panels and Fig. S1A), a TGF-β downstream transducer, and the number of TUNEL-positive cells (Fig. 1D lower panels and Fig. S1B) were increased at 8 h after treatment with Jo2. These data suggest that TGF-β is involved in the Jo2-induced ALI process.

Please cite this article in press as: M. Li, et al., Prevention of acute liver injury by suppressing plasma kallikrein-dependent activation of latent TGF-β, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.09.026