Research Article

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A novel mouse model of depletion of stellate cells clarifies their role in ischemia/reperfusion- and endotoxin-induced acute liver injury

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Background & Aims: Hepatic stellate cells (HSCs) that express glial fibrillary acidic protein (GFAP) are located between the sinusoidal endothelial cells and hepatocytes. HSCs are activated during liver injury and cause hepatic fibrosis by producing excessive extracellular matrix. HSCs also produce many growth factors, chemokines and cytokines, and thus may play an important role in acute liver injury. However, this function has not been clarified due to unavailability of a model, in which HSCs are depleted from the normal liver.

Methods: We treated mice expressing HSV-thymidine kinase under the *GFAP* promoter (*GFAP*-Tg) with 3 consecutive (3 days apart) CCl_4 (0.16 μl/g; ip) injections to stimulate HSCs to enter the cell cycle and proliferate. This was followed by 10-day ganciclovir (40 μg/g/day; ip) treatment, which is expected to eliminate actively proliferating HSCs. Mice were then subjected to hepatic ischemia/reperfusion (I/R) or endotoxin treatment.

Results: CCl_4/ganciclovir treatment caused depletion of the majority of HSCs (about 64–72%), while the liver recovered from the initial CCl_4-induced injury (confirmed by histology, serum ALT and neutrophil infiltration). The magnitude of hepatic injury due to I/R or endotoxemia (determined by histopathology and serum ALT) was lower in HSC-depleted mice. Their hepatic expression of TNF-α, neutrophil chemoattractant CXCL1 and endothelin-A receptor also was significantly lower than the control mice.

Conclusions: HSCs play an important role both in I/R- and endotoxin-induced acute hepatocyte injury, with TNF-α and endothelin-1 as important mediators of these effects.

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Keywords: Hepatic stellate cells; Depletion; Ischemia/reperfusion; Endotoxemia; Lipopolysaccharide; Hepatocytes; Liver; Injury; Necrosis; Apoptosis.

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Abbreviations: HSC, hepatic stellate cell; GFAP, glial fibrillary acidic protein; ECM, extracellular matrix; ET-1, endothelin-1; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; TNF, tumor necrosis factor; IL, interleukin; I/R, ischemia/reperfusion; KCs, Kupffer cells; WT, wild type; Tg, transgenic; HSV, herpes simplex virus; TK, thymidine kinase; GCV, ganciclovir; IP, intraperitoneal; CCl_4, carbon tetrachloride; PBS, phosphate-buffered saline; H/E, hematoxylin and eosin; Ab, antibody; qRT-PCR, quantitative real-time polymerase chain reaction; ALT, alanine aminotransferase; ROS, reactive oxygen species.

Introduction

The perisinusoidal hepatic stellate cells (HSCs) constitute 8–12% of the liver cell population, express glial fibrillary acidic protein (GFAP) and/or desmin, and are the major storage site of retinoids [1]. During liver injury, HSCs undergo activation characterized by the loss of retinoids, expression of α-smooth muscle actin and differentiation into proliferating myofibroblast-like cells. Activated HSCs produce excessive extracellular matrix (ECM) and exhibit increased expression of tissue inhibitors of metalloproteinases and reduced or unchanged expression of matrix metalloproteinases [2–4], thus becoming the major cell type responsible for hepatic fibrosis [5]. Activated HSCs are postulated to contribute to portal hypertension by their high contractility and up-regulation of the powerful vasoconstrictor endothelin-1 (ET-1) and its receptors [6,7].

HSCs express intercellular adhesion molecule-1 [8], produce various cytokines and chemokines [8–10], and thus can play an important role in hepatic inflammation. Gram-negative bacterial endotoxin (lipopolysaccharide: LPS) stimulates the synthesis of nitric oxide (NO), ET-1, tumor necrosis factor (TNF)-α and interleukin (IL)-6 in both quiescent and activated HSCs; LPS-challenged HSCs stimulate NO synthesis, inhibit DNA synthesis and cause apoptosis of cultured hepatocytes [11–14].

Recent work demonstrates that HSCs also influence hepatic immunological functions. HSCs induce apoptosis of allogeneic $CD4^+$ and $CD8^+$ T cells [8,15], present bacterial lipid antigens to NKT cells [16], expand immunosuppressive regulatory T cells [8], and render dendritic cells immunosuppressive [10].

Furthermore, HSCs secrete powerful antioxidant protein(s) that protect hepatocytes from ischemia/reperfusion (I/R) injury [17].

Thus, HSCs are highly versatile cells that can profoundly influence hepatic structure and functions in physiology and pathology. Most of the *in vivo* work confirming their role in hepatic pathology has been focused on fibrosis. A fungal metabolite gliotoxin was found to cause apoptosis of activated rat and human HSCs *in vitro*, and of rat HSCs *in vivo* resulting in resolution of fibrosis [18,19]. However, gliotoxin also induces apoptosis of KCs and endothelial cells in the fibrotic liver [20,21]. Ebrahimkhani *et al.* [22] administered gliotoxin into bile duct-ligated mice in conjugation with the single-chain antibody C1–3, which recognizes synaptophysin expressed by activated HSCs [23]; C1–3-gliotoxin caused resolution of fibrosis by selectively depleting HSCs. It was recently reported, using a similar mouse model described in the present study that concomitant treatment of B6.Cg-Tg(Gfap-Tk)7.1Mvs/J transgenic mice with ganciclovir promoted depletion of HSCs, and caused amelioration of CCl_4-induced fibrosis and hepatic injury [24]. However, the role of HSCs in acute injury to the normal liver has not yet been evaluated. Here, we show amelioration of I/R- and endotoxin-induced acute injury to otherwise normal HSC-depleted liver, suggesting HSCs' critical role in pathologies unrelated to activation-dependence.

Materials and methods

Animals

The protocols were approved by the IACUC according to NIH guidelines. Wild-type male C57BL/6 (WT-B6) and B6.Cg-Tg(Gfap-Tk)7.1Mvs/J (*GFAP*-Tg) mice were from The Jackson laboratory. *GFAP*-Tg mice express the herpes simplex virus thymidine kinase (HSV-TK) transgene under the *GFAP* promoter [25]. HSV-TK phosphorylates non-toxic ganciclovir (GCV) to GCV-monophosphate, which is converted to GCV-triphosphate by cellular guanylate kinase; phosphorylated GCV incorporates into the DNA causing death of replicating cells [25,26]. GFAP is expressed exclusively by HSCs in the liver, which are quiescent physiologically [1]. We treated the *GFAP*-Tg mice or WT-B6 mice with three CCl_4 injections (0.16 μl/g in 50 μl peanut oil; ip), 3 days apart; control mice received peanut oil. After the last CCl_4 injection, each group was divided into two subgroups, one receiving GCV (40 μg/g/day; ip) and the other vehicle (PBS) for 10 days. CCl_4 treatment activates HSCs, which enter the cell cycle thus making them susceptible to phosphorylated GCV-induced death. Mice were subjected to I/R or endotoxin the day after completion of GCV treatment (Supplementary Fig. 1).

Ischemia/reperfusion (I/R) or endotoxemia

For I/R, all structures (portal vein, hepatic artery, and bile duct) to the left liver lobes were occluded with a microvascular clamp for 60 min. The right lobes served as control. The clamps were removed and the abdominal incision closed. For endotoxemia induction, mice were administered LPS (10 mg/kg) intraperitoneally. Blood was drawn at 6 h following reperfusion or LPS administration for serum enzyme measurement. The livers were excised, washed in ice-cold PBS and portions were fixed in 10% buffered formalin or paraformaldehyde, or snap-frozen in liquid nitrogen.

Histological determinations

The sections of formalin-fixed tissue were stained with hematoxylin and eosin (H/E) for histopathological examination, with TUNEL-stain (ApopTag Peroxidase kit, Chemicon) to detect apoptotic cells, or immunostained using rat-anti-mouse F4/80 Ab (Serotec), biotinylated goat-anti-rat secondary Ab (Jackson Immunoresearch) and ABC Elite kit (Vector Laboratories) to detect KCs.

The sections of paraformaldehyde-fixed frozen tissue were immunostained with anti-desmin rabbit polyclonal Ab (Abcam), anti-GFAP rabbit polyclonal Ab (DakoCytomation), rat anti-mouse F4/80 Ab (BioLegend) or rat anti-mouse TNF-α Ab (R&D Systems) as described previously [8,10].

Neutrophils were identified immunohistochemically using Naphthol As-D Chloroacetate Esterase Kit (Sigma-Aldrich). Neutrophil accumulation was quantified in at least 4 randomly selected high-power fields (400×) of each liver section.

Table 1. **Primers used in qPCR reactions.**

Gene	Primers
GFAP	5'-ACCGCATCACCATTCCTGTAC-3'(F) 5'-TTGCCTTCTGACACGGATTT-3'(R)
IL6	5'-CCGGAGAGGAGACTTCACAG-3'(F) 5'-TCCACGATTTCCCAGAGAAC-3'(R)
TNFα	5'-CCCAGGTATATGGGCTCATACC-3'(F) 5'-GCCGATTTGCTATCTCATACCAGG-3'(R)
CXCL1	5'-CTGCACCCAAACCGAAGTC-3'(F) 5'-AGCTTCAGGGTCAAGGCAAG-3'(R)
ET-1	5'-GTGTCTACTTCTGCCACCTGGACAT-3'(F) 5'-GGGCTCGCACTATATAAGGGATGAC-3'(R)
ET_AR	5'-GATGGATAAGAACCGGTGTGAAC-3'(F) 5'-GAGCTATTGGGTTTATGCAAGAATTC-3'(R)
ET_BR	5'-ACCAGAGCAATCCACACAGG-3'(F) 5'-AGAGCGATTGGATTGATGCAG-3'(R)
GAPDH	5'-TGTTGAAGTCACAGGAGACAACCT-3'(F) 5'-AACCTGCCAAGTATGATGACATCA-3'(R)

mRNA analysis

RNA was prepared from the snap-frozen tissue using TRIzol Reagent (Invitrogen), and cDNA was prepared using high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time-PCR (qRT-PCR) was performed using the Sybr green master mix and 7500 Fast Real-Time PCR System (Applied Biosystems) with PCR primers listed in Table 1.

Statistics

Statistical significance was determined by non-parametric Kruskal-Wallis One-Way ANOVA on Ranks. A p value <0.05 was considered statistically significant.

Results

Characterization of the CCl_4 effect on WT mice

To ensure that at the end of GCV treatment of mice, there is no residual hepatic injury due to earlier CCl_4 administration before subjecting them to I/R or endotoxemia, WT-B6 mice were sacrificed a day after the third CCl_4 injection or after 10 days of GCV treatment following termination of CCl_4. There was significant hepatic injury the day after third CCl_4 administration (Fig. 1A and B, middle panels), in both centrilobular and periportal areas (Supplementary Fig. 2), accompanied by inflammatory infiltration (Fig. 1B and C), and increased serum ALT (Fig. 1D). However, at the end of GCV treatment, liver histology and inflammation (Fig. 1A and B, lower panels; Fig. 1C) as well as serum ALT (Fig. 1D) had returned to normal and were identical to that in control mice.

Depletion of HSCs from the mice

Similar to WT-B6 mice (Fig. 1), no evidence of liver injury was observed in the *GFAP*-Tg mice after completion of CCl_4/GCV treatment as determined by histopathology and serum ALT (Supplementary Fig. 3A and B). GFAP immunostaining (Fig. 1E)

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