Research Article

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Interferon regulatory factor 9 is a key mediator of hepatic ischemia/reperfusion injury

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Background & Aims: Hepatic ischemia/reperfusion (I/R) injury is characterized by anoxic cell injury and the generation of inflammatory mediators, leading to hepatic parenchymal cell death. The activation of interferon regulatory factors (IRFs) has been implicated in hepatic I/R injury, but the role of IRF9 in this progression is unclear.

Methods: We investigated the function and molecular mechanisms of *IRF9* in transgene and knockout mice subjected to warm I/R of the liver. Isolated hepatocytes from *IRF9* transgene and knockout mice were subjected to hypoxia/reoxygenation (H/R) injury to determine the *in vitro* effects of IRF9.

Results: The injuries were augmented in IRF9-overexpressing mice that were subjected to warm I/R of the liver. In contrast, a deficiency in IRF9 markedly reduced the necrotic area, serum alanine amino transferase/aspartate amino transferase (ALT/AST), immune cell infiltration, inflammatory cytokine levels, and hepatocyte apoptosis after liver I/R. Sirtuin (SIRT) 1 levels were significantly higher and the acetylation of p53 was decreased in the *IRF9* knockout mice. Notably, IRF9 suppressed the activity of the SIRT1 promoter luciferase reporter and deacetylase activity. Liver injuries were significantly more severe in the *IRF9*/*SIRT1* double knockout (DKO) mice in the I/R model, eliminating the protective effects observed in the *IRF9* knockout mice.

Conclusions: IRF9 has a novel function of inducing hepatocyte apoptosis after I/R injury by decreasing SIRT1 expression and increasing acetyl-p53 levels. Targeting IRF9 may be a potential strategy for ameliorating ischemic liver injury after liver surgery.

Keywords: IRF9; Hepatic injury; Ischemia/reperfusion.
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Abbreviations: I/R, ischemia and reperfusion; IRF, interferon regulatory factor; KO, knockout; SIRT, sirtuin; LTx, liver transplantation; ISGF3, interferon-stimulated gene factor 3; ALT, alanine amino transferase; AST, aspartate amino transferase; TNF, tumour necrosis factor; IL, interleukin; MCP, monocyte chemoattractant protein; CXCL, chemokine ligand; LDH, lactate dehydrogenase; MPO, myeloperoxidase; NPCs, nonparenchymal cells; H/R, hypoxia/reoxygenation; DKO, double knockout; NTG, non-transgene; WT, wild type; ChIP, chromatin immunoprecipitation.

Introduction

Liver ischemia and reperfusion (I/R) injuries arising from transplantation, trauma, or hypovolemic shock may cause liver failure or even multi-organ system failure [1]. Because cell death has long been viewed as a fundamental process that directly determines the extent of liver function damage in I/R injury, hepatocyte protection is a potential strategy for ameliorating I/R liver damage [2,3]. However, anti-apoptotic approaches are not widely used in existing therapies for liver I/R [4]. One possible reason is that the upstream mediators remain poorly defined in hepatocytes, even though considerable attention has been focused on apoptotic pathways [5]. The identification of novel apoptotic regulators is urgently needed.

Interferon regulatory factors (IRFs) belong to a transcription factor family that regulates the expression of genes, participating in both innate and acquired immunity [6]. Recent studies have demonstrated that IRFs contribute to the process of hepatic I/R. IRF1 is upregulated in a warm ischemia model of the liver, and IRF1-deficient mice exhibit less hepatic damage in this model than IRF1-sufficient mice [7]. In orthotopic liver transplantation (LTx) models, IRF1 deficiency in the donor liver graft, but not in the recipient, results in significantly less damage [8]. Previous research has shown that IRF3 plays a crucial role following I/R-induced TLR4 activation and that liver function is protected in *IRF3* KO mice [9]. However, a recent paper reported that liver damage is increased in *IRF3* KO mice after liver I/R [10]. Collectively, these data highlight the complexity of IRF functions in liver I/R. Although it is well established that various IRFs differ in their respective cellular localizations, structural properties, and activation triggers, suggesting that each IRF has its own function [6,11], the intricacies of IRF signalling after liver I/R remain unclear.

IRF9, also known as interferon-stimulated gene (ISG)F3γ, has long been known as the DNA-binding subunit of the IFN-stimulated gene factor 3 (ISGF3) complex [11]. IRF9 is activated by type I IFNs (IFNα/β) to induce the expression of ISGs against viral infection [6,12]. Recent work in our laboratory suggests that IRF9 attenuates inflammation, insulin resistance, and steatosis through its interaction with peroxisome proliferator-activated receptor (PPAR)α and the upregulation of PPARα target genes [13]. The role of IRF9 in liver I/R injury has not yet been reported. Furthermore, the pathophysiology of I/R injury is unlike the process of functional damage that occurs in obesity and diabetes. Therefore, we tested the hypothesis that IRF9 plays a role in hepatic I/R injury.

This study provides evidence that hepatocellular IRF9 is involved in liver I/R injury, by influencing the death of hepatocytes and the inflammatory response. These findings contribute to our understanding of the intracellular mechanisms of programmed cell death in hepatic I/R injury. The IRF9-dependent pathway is a potential target for pharmaceutical agents that prevent ischemia-induced cell death in the liver.

Materials and methods

Detailed experimental procedures are provided in the Supplementary Materials and methods.

Animals

All animals used in this study, including C57BL/6 (wild type, WT) mice; *IRF9* knockout (KO) mice, hepatocyte-specific *IRF9* transgenic (TG) mice, myeloid-specific *IRF9* transgenic (LysM-IRF9-TG) mice, hepatic-specific *SIRT1* KO mice (*SIRT1* KO), and *SIRT1*-KO/*IRF9*-KO (DKO) mice (all produced in this lab on a C57BL/6 background); and Alb-cre mice (Jackson Laboratory, Bar Harbor, ME, USA), were housed in an environment with controlled light (12 h light/12 h dark), temperature, and humidity; food and water were available *ad libitum*. Only 8- to 10-week-old (24–27 g) males were used. All animal procedures were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University, China.

Warm hepatic I/R injury mouse model

A non-lethal model of segmental (70%) warm hepatic I/R injury was used as previously described [14]. After an indicated period of reperfusion, each mouse was anaesthetised; then, blood was collected from the inferior vena cava, and the ischemic liver lobes were harvested for further analysis.

Assessment of liver damage

Serum alanine amino transferase (sALT) and serum aspartate amino transferase (sAST) activities were measured with a spectrophotometer (Chemix 180i, Sysmex Shanghai Ltd.) according to the manufacturer's instructions. For histopathological analysis, liver tissues were fixed in 10% formalin, embedded in paraffin, sectioned (5 μm per section), and stained with haematoxylin and eosin (H&E). The necrotic area was quantitatively assessed with the Image Pro Plus software (version 6.0). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining was performed using the ApopTag® Plus In Situ Apoptosis Fluorescein Detection Kit (EMD Millipore Corporation, S7111) according to the manufacturer's instructions.

Measurement of myeloperoxidase activity

The activity of myeloperoxidase (MPO), an enzyme specific to polymorphonuclear neutrophils, was evaluated in frozen tissue according to published methods [15].

Cytokine measurement

Serum cytokines, namely, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-6, IL-10, and monocyte chemoattractant protein (MCP)-1, were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Additional details are included in Supplementary Table 1.

Caspase assay

Caspase-3 activity was determined using a caspase-3 activity assay kit (Cell Signaling Technology, 5723), and the liver tissue samples were prepared according to the manufacturer's instructions.

Isolation, culture, and treatment of hepatocytes and nonparenchymal cells (NPCs)

Hepatocytes and NPCs were isolated and plated as previously described [16]. For experiments involving hypoxia, the medium was replaced with serum-free DMEM/F12 medium that had been saturated at 37 °C with 95% N_2/5% CO_2 and cells were placed in an experimental hypoxia chamber in a saturated atmosphere of 95% N_2 and 5% CO_2.

Flow cytometry

Flow cytometry was performed to assess the infiltration of Kupffer cells, inflammatory monocytes, and neutrophils into the liver as described previously [16]. We defined neutrophils as $CD11b^{hi}Ly6G^{hi}$, inflammatory monocytes as $CD11b^{int}Ly6C^{hi}$, and Kupffer cells as $CD11b^{+}F4/80^{+}$. Data were acquired with a CyAn ADP analyser (Beckman Coulter) and analysed with Summit v4.2. Each experiment was repeated a minimum of three times.

Cell viability assay

Cell viability was assessed using a MTT Cell Viability Assay Kit (KA1606, Abnova), and lactate dehydrogenase (LDH) release was assessed with a colorimetric LDH cytotoxicity assay (G1782, Promega, Madison, WI) according to the manufacturer's instructions. DNA fragmentation in primary hepatocytes was assessed with a commercially available cell death detection ELISA kit (11585045001, Roche Applied Science) that detects cytosolic mono- and oligo-nucleosomes; the kit was used according to the manufacturer's instructions. To obtain morphological evidence of apoptosis or necrosis, cells were stained with Hoechst 33258/propidium iodide (PI).

SIRT1 activity measurement

SIRT1 activity was determined using a deacetylase SIRT1 fluorometric kit (Abcam, ab156065) according to the manufacturer's protocol. Briefly, endogenous SIRT1 from liver samples was immunoprecipitated using a SIRT1 antibody (Abcam, ab7343) in RIPA buffer. The SIRT1 substrate reagent and NAD^{+} were added to SIRT1-conjugated beads and incubated at 37 °C for 80 min after a final washing. The substrate-SIRT1 mixture was placed in a 96-well plate, and the developer reagent was added to the wells at 37 °C for 20 min. The plate was read with a spectrophotometer at an excitation wavelength of 405 nm.

Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed as described in our previous report [17].

Luciferase reporter assay

SIRT1-luciferase activity was assessed using a Dual-Luciferase® Reporter Assay System kit (Promega) and a single-mode SpectraMax microplate reader (Molecular Devices), as described previously [17,18].

Statistical analysis

Data are presented as the means ± SEs. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by the least square difference (LSD) test (assuming equal variances) or Tamhane's T2 test (without the assumption of equal variances). Comparisons between two groups were performed with the two-tailed Student's *t* test. Values of $p < 0.05$ were considered significant.

Results

Increased IRF9 during hepatic I/R contributes to liver injury

Hepatic *IRF9* is transcriptionally regulated in a variety of pathophysiological processes [13,19]; however, the expression pattern