Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Iron inhibits replication of infectious hepatitis C virus in permissive Huh7.5.1 cells

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See Editorial, pages 990–992

Background & Aims: Chronic infection with hepatitis C virus (HCV) is often associated with elevated hepatic iron levels. Excess iron is known to promote oxidative stress and exacerbate liver disease. Nevertheless, biochemical studies in subgenomic HCV replicon systems showed that iron can also suppress the expression of viral RNA and proteins by inhibiting the enzymatic activity of the RNA polymerase NS5B. To explore the physiological relevance of this response, we evaluated the effects of iron during infection of permissive Huh7.5.1 hepatoma cells with HCV.
Methods: We utilized Fe-SIH (iron complexed with salicylaldehyde isonicotinoyl hydrazone), a cell permeable and highly efficient iron donor.
Results: Treatments of infected cells with Fe-SIH drastically reduced the expression of viral proteins (core and NS3) and RNA, in a dose-dependent manner. The inhibition was dramatic when Fe-SIH was administered simultaneously with the HCV inoculum or early afterwards, while pre-treatment of cells with Fe-SIH before infection failed to elicit antiviral responses. Iron chelation with SIH did not significantly alter the expression of viral proteins.
Conclusions: Our data establish a critical role of hepatic iron concentration on the progression of HCV infection, and are consistent with iron-mediated inactivation of NS5B.

Introduction

The vast majority (85%) of individuals infected acutely with HCV develop chronic hepatitis C (CHC) that may progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma [32]. CHC is a leading cause of liver failure and poses a major health care challenge, with an estimated 170 million patients worldwide [15]. The disease is often associated with mild to moderate hepatic iron overload with variable distribution among reticuloendothelial and parenchymal cells [1,25,31]. Excess hepatic iron is considered as a co-morbid factor that aggravates liver damage by promoting oxidative stress. Altered redox homeostasis disrupts organellar architecture and growth properties of hepatocytes and hepatic stellate cells [11,20,27]. Parenchymal iron accumulation in CHC patients has been linked to necroinflammation [31] and to misregulation of iron homeostasis by HCV-dependent inhibition in the expression of hepcidin [9,19,22]. A decrease in levels of this peptide hormone leads to unrestricted intestinal iron absorption and iron release from macrophages due to stabilization of the iron transporter ferroportin [21]. This phenotype is the hallmark of hereditary hemochromatosis, a disease caused by genetic defects in the hepcidin pathway [17,26].

The idea that excess iron exacerbates the clinical picture of CHC is concordant with the pathology of hereditary hemochromatosis that triggers in its own right liver fibrosis, cirrhosis, and hepatocellular carcinoma [14]. Nevertheless, biochemical experiments showed that iron can also exert antiviral effects. Thus, we previously reported that iron binds tightly to NS5B, the RNA-dependent RNA polymerase of HCV and inhibits its catalytic activity by displacing Mg^{2+} from the enzyme's active site [8]. The antiviral activity of iron was further validated in a subgenomic HCV replicon model, where the administration of exogenous iron blocked viral replication and attenuated the production of viral RNA and proteins [8]. Further data suggested that the expression of the subgenomic HCV replicon leads to an iron-poor phenotype in host Huh7 cells, possibly to bypass the iron-dependent block in viral replication [7]. Here, we employ an *in vitro* model for HCV infection based on permissive Huh7.5.1 hepatoma cells [40], and examine the effects of iron on this process. We demonstrate that exogenous iron diminishes HCV replication in these cells and inhibits the expression of viral proteins and RNA.

Materials and methods

Materials

SIH was a kind gift of Dr. Prem Ponka (McGill University). Fe-SIH was prepared as described earlier [8]. The HCV genotype 2a consensus clone JFH-1 [35], derived from a Japanese patient with fulminant hepatitis, and permissive human Huh7.5.1 hepatoma cells [40] were kindly provided by Dr. Takaji Wakita (Tokyo Metropolitan Institute for Neuroscience).

Keywords: Hepatitis C virus; Iron metabolism; NS5B; NS3; Ferritin; Transferrin receptor 1.
DOI of original article: 10.1016/j.jhep.2010.08.003
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Abbreviations: HCV, hepatitis C virus; SIH, salicylaldehyde isonicotinoyl hydrazone; NS3, non-structural protein 3; CHC, chronic hepatitis C; TfR1, transferrin receptor 1; PBS, phosphate-buffered saline; RT-PCR, reverse-transcription polymerase chain reaction; Hmox1, heme oxygenase 1.

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Cell culture

Huh7.5.1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum, 100 nM non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Transfection and inoculation of cells

In vitro transcribed JFH-1 RNA was transfected into Huh7.5.1 cells by electroporation [13]. Culture media were collected 14 days after transfection, cleared using low speed centrifugation and filtered [13]. Naïve Huh7.5.1 cells were infected by inoculation with this material for 24 h, then washed and incubated with fresh media for 1–4 days.

Western blotting

The cells were washed twice in phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1% SDS, 0.5% Na deoxycholate, 1% Triton X-100). Lysates were resolved by SDS–PAGE on 13% or 7% gels and transferred onto nitrocellulose filters. The blots were saturated with 10% non-fat milk in PBS and probed with 1:1000 diluted antibodies against NS3 (Abcam), core protein (Affinity BioReagents), TfR1 (Zymed), ferritin (Novus) or β-actin (Sigma). Dilutions were in PBS containing 0.5% Tween-20 (PBST). Following wash with PBST, the blots with monoclonal NS3, core protein, and TfR1 antibodies were incubated with peroxidase-coupled rabbit anti-mouse IgG (1:5000 dilution), and the blots with polyclonal ferritin and β-actin antibodies were incubated with peroxidase-coupled goat anti-rabbit IgG (1:10,000 dilution). Peroxidase-coupled antibodies were detected with the enhanced chemiluminescence method (Amersham), according to the manufacturer's instructions.

Quantification of HCV RNA

The cells were lysed with the Trizol reagent (Invitrogen) and RNA was prepared according to the manufacturer's recommendations. Total cellular RNA (1 μg) was retro-transcribed and HCV RNA was quantified by real time RT-PCR [34], following normalization to values of cellular β-actin.

Statistical analysis

Data are shown as means ± SD. Statistical analysis was performed by the unpaired Student's *t*-test with the Prism GraphPad Software (version 5.0c).

Results

To generate infectious HCV particles, Huh7.5.1 cells were initially transfected with JFH-1 RNA. Lysates generated at different time intervals were analyzed for expression of the virally encoded proteins NS3 and core (Fig. 1A). Both NS3 and core proteins were readily detectable by Western blotting within 5–7 days (lanes 2–3) and their expression peaked between 11 and 15 days post-transfection (lanes 5–7), in line with previous findings [35,40]. The peak in viral protein expression was accompanied by a robust increase in viral RNA levels (Fig. 1B). Under these conditions, the culture supernatant is expected to contain infectious HCV particles [35,40]. Indeed, the inoculation of naïve Huh7.5.1 cells with culture supernatant of JFH-1-transfected counterparts resulted in infection of these cells with HCV, as judged by the expression of NS3 and core proteins (Fig. 1C), as well as the expression of viral RNA (Fig. 1D).

We employed Fe-SIH, a lipophilic iron delivery vehicle [28], to address the effects of iron on the progression of HCV infection. Naïve Huh7.5.1 cells were inoculated with culture supernatant from JFH-1-transfectants containing HCV particles. The cells received increasing doses of Fe-SIH either concurrently with the inoculation (day 0) or on the following days 1–3, and the incubation was continued until day 4. The addition of 25 or 50 μM Fe-SIH together with the inoculum, or 1–2 days post-infection, dramatically inhibited the expression of the viral proteins NS3 and

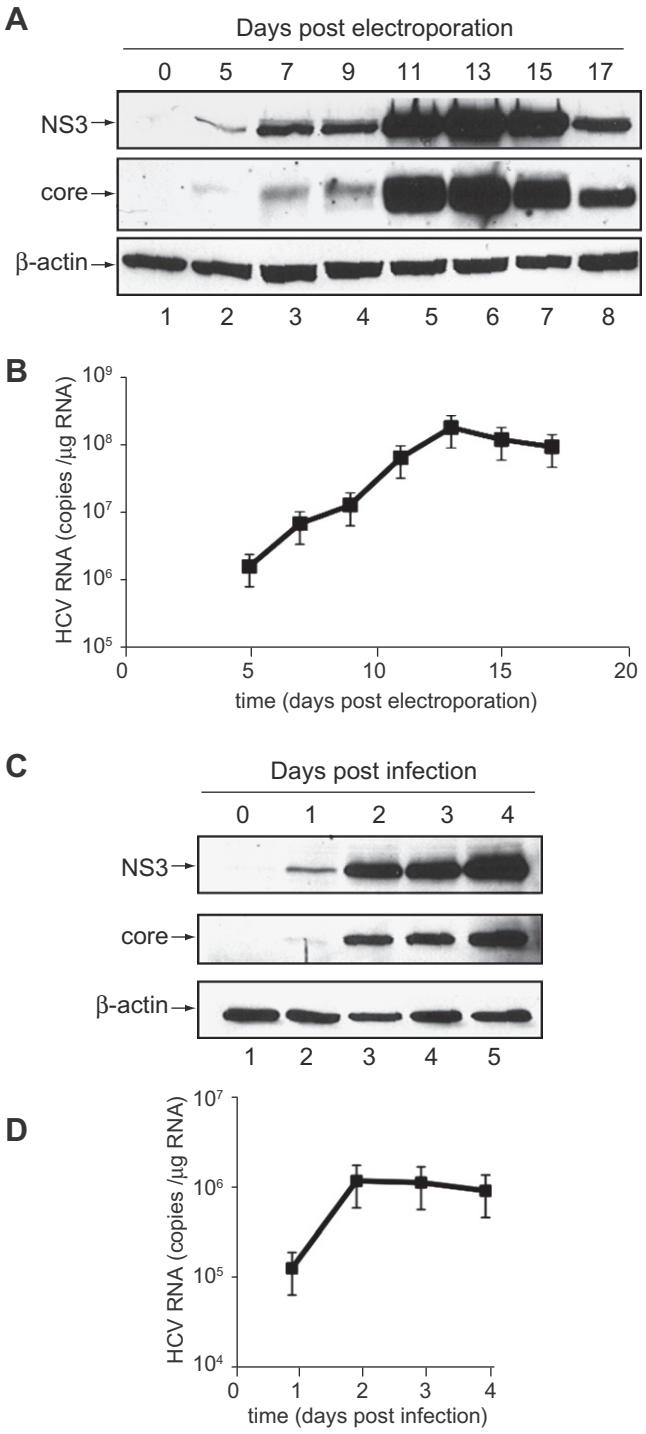

Fig. 1. Infection of permissive Huh7.5.1 cells with HCV. The cells were initially electroporated with JFH-1 RNA (A and B) and the culture supernatant was used to inoculate naïve counterparts (C and D). The expression of the viral proteins NS3 and core and of cellular β-actin was analyzed by Western blotting, and the levels of HCV RNA were determined by RT-PCR. Viral RNA from four independent experiments was quantified by RT-PCR (mean ± SD).