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Interaction of hepatitis C virus core protein with Hsp60 triggers the production of reactive oxygen species and enhances TNF-α-mediated apoptosis

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ABSTRACT

The hepatitis C virus (HCV) core protein is the primary protein component of the nucleocapsid that encapsidates the viral RNA genome. Besides its role as a viral structural protein, the core protein is implicated in HCV chronic infection-associated liver diseases by induction of reactive oxygen species (ROS) production and modulation of apoptosis. Here, we show that interaction of the core protein, through its N-terminal domain (amino acids 1–75), with heat shock protein (Hsp60) is critical for the induction of ROS production, leading to sensitization of core protein-expressing cells to apoptosis induced by tumor necrosis factor-α (TNF-α). Moreover, overexpression of Hsp60 rescued the core protein-expressing cells from cell death by reducing ROS production. Collectively, our results suggest that impairment of Hsp60 function through binding of HCV core protein contributes to HCV viral pathogenesis by ROS generation and amplification of the apoptotic effect of TNF-α.

1. Introduction

Hepatitis C virus (HCV) infection often leads to chronic viral hepatitis, which over time evolves into liver cirrhosis with a high risk of developing hepatocellular carcinoma (HCC) [1–3]. Several studies have suggested that oxidative stress, which is induced either by viral proteins or host immune responses, is one of major determinants of the HCC development [4]. Since chronic HCV infection is associated with an enhancement of TNF-α production by macrophages and cytotoxic T lymphocytes [5,6], TNF-α further induces oxidative stress by stimulating the release of ROS, which may in turn induce massive liver steatosis, and plays a central role in liver injury during elimination of virus-infected cells *via* apoptosis [7–10].

HCV core protein is the viral nucleocapsid protein that binds and packages the viral RNA genome. Besides its role as a viral structural protein, the core protein is known to induce reactive oxygen species (ROS) production in tissue cultures and animal models [11–13]. However the mechanism(s) by which the core protein induces ROS production are largely unknown. In an attempt to gain a better understanding of how HCV core protein is involved in HCV viral pathogenesis by interacting with cellular proteins, we previously used a proteomic approach and found that heat shock protein 60 (Hsp60) is one of the cellular protein that binds to the core protein [14]. Hsp60, a stress response molecular chaperon [15], is primarily but not exclusively compartmentalized in mitochondria matrix [16] and has a crucial function in folding/refolding of mitochondria-imported pre-proteins [17]. In addition to its function in folding of macromolecules in mitochondria, Hsp60 has been shown to have potent anti-apoptotic role in both the cytosol and mitochondria [18,19].

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These observations prompted us to test whether the interaction between Hsp60 and the HCV core protein is linked to ROS production and the regulation of apoptosis. We report here that the core protein directly interacts with Hsp60 and induces ROS production, which potentiates TNF-α-induced apoptotic cell death. Thus, our data reveal a novel mechanism of Hsp60 regulation through an interaction with the HCV core protein, which may contribute to liver diseases and carcinogenesis.

2. Materials and methods

2.1. Cell lines and culture

The human hepatoma cell line, Huh7, was grown in RPMI-1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin under standard culture conditions (5% CO_2, 37 °C). The human hepatoma stable cell line Huh7TR-4 expressing the tetracycline repressor and the Huh7TR-core expressing HCV core protein in a tetracycline-inducible manner [14] were maintained in the presence of blasticidin S (10 μg/ml) and Zeocine (100 μg/ml). Core protein expression in Huh7TR-core was induced by addition of 1 μg/ml tetracycline for 48 h, unless otherwise specified.

2.2. Plasmids

The pcDNA3.1-Flag-core expressing Flag-epitope-tagged full-length HCV core protein was described previously [14]. For expression of the enhanced green fluorescence (EGFP)-fused HCV core protein and its deletion derivatives, pEGFP-C(1–191), pEGFP-C(1–75), pEGFP-C(1–121), pEGFP-C(1–173), pEGFP-C(76–191) and pEGFP-C(99–191) were constructed by cloning the cDNAs encoding the amino acid sequence indicated within parenthesis. Partial HCV core gene fragments were generated by conventional PCR using Vent DNA polymerase (NEB) with pCV-J4L6S [20] as a template. The amplified PCR products were digested with EcoRI and BamHI and inserted into pEGFP-C1 (Clontech). The HA-tagged Hsp60-expression vector, pcDNA3-Hsp60-HA, was provided by Yasuo Tanaka (University of Tokyo, Japan).

2.3. Protein digestion, mass spectrometric analysis and database searching

Protein spots were excised from a stained two-dimensional gel, processed, and digested with a bovine trypsin solution (10 μg/ml) (Roche), as described previously [14]. Digested peptides were analyzed by a MALDI-TOF/TOF tandem mass spectrometer (ABI 4700 Proteomics Analyzer, Applied Biosystems). The MS/MS spectra were then used to search for a matching protein using the MASCOT search program (http://www.matrixscience.com), as described [14].

2.4. Immunoprecipitation and Western blot analysis

Cell lysates were prepared in lysis buffer (1% Triton X-100, 100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4 and 17.5 mM β-glycerophosphate) supplemented with a protease inhibitor cocktail (Roche). Cleared cell lysates were incubated with mouse monoclonal anti-Hsp60 antibody (LK-1 clone, Stressgen) for 1 h with gentle agitation at 4 °C. Immune complexes were then recovered by adsorption to Protein G-Sepharose (Qiagen) for 1 h at 4 °C. For immunoprecipitation of the Flag-tagged HCV core protein, Huh7 cells were transfected with the pcDNA3.1-Flag-core expression vector using fuGENE6 (Roche) and harvested at 48 h post-transfection. Cell lysates were incubated with an anti-Flag peptide antibody M2-conjugated agarose beads (Sigma–Aldrich) for 2 h at 4 °C. For Western blot analysis, immunoprecipitates or lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and blotted onto nitrocellulose membranes. Membranes were analyzed by immunoblotting, as described previously [14], using various primary antibodies [rabbit anti-Flag antibodies (Sigma–Aldrich), HCV patient serum (kindly provided by Dr. K. H. Han at Yonsei University School of Medicine), rabbit anti-GFP antibody (Santa Cruz Biotechnology), rabbit anti-poly (ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology) detecting full-length (116 kDa) and the cleaved fragment (89 kDa) of PARP, anti-caspase 3 antibody (Cell Signaling Technology) detecting full-length (35 kDa) and the cleaved fragment (17 kDa), and anti-vimentin antibody (V9 clone, Sigma–Aldrich)] known to detect intact vimentin (58 kDa) and its cleavage products (47, 41, 28 and 22 kDa) [21]], and appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoblots were developed with the ECL detection kit (GE Healthcare Life Sciences) according to the manufacturer's recommendations.

2.5. Confocal microscopy

Flag-tagged core protein in cells permeabilized with 0.2% Triton X-100 was visualized by immunostaining with anti-Flag and FITC-conjugated anti-rabbit IgG antibodies (Sigma–Aldrich), as described previously [14]. For the staining of mitochondria, cells were incubated in medium containing 100 nM Mitotracker Red CMXRos (Molecular Probes) for 45 min. Hsp60 was detected using a mouse monoclonal anti-Hsp60 antibody and a Texas-red conjugated anti-mouse IgG antibody (Vector Laboratories). Nuclei were visualized by staining with 1 μM 4′,6′-diamidino-2-phenylindole (DAPI) in PBS for 10 min. Confocal images were obtained using a BIO-RAD Radiance 2000 multi-photon laser scanning confocal microscope.

2.6. Subcellular fractionation and protease accessibility assay

Subcellular organelles were fractionated by differential centrifugation as described previously [22]. Mitochondrial fraction was treated with proteinase K (0–10 μg/ml) in the absence or presence of Triton X-100 (1%) for 30 min on ice. The reaction was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride. Samples were then analyzed by immunoblotting.