Research Article

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Distinct antiviral signaling pathways in primary human hepatocytes and their differential disruption by HCV NS3 protease

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Background & Aims: Molecular sensors recognize viral nucleic acids and initiate events that subsequently enable cells to control and clear infection. Hepatitis C Virus (HCV) can interfere with the innate host response and the NS3/4A protease was reported to specifically block antiviral signaling pathways, a finding that had yet to be studied in human primary hepatocytes.

Methods: Freshly isolated human primary hepatocytes, transduced with a lentiviral vector expressing HCV NS3/4A were stimulated with extracellular and intracellular double-stranded RNA (dsRNA) and the innate immune antiviral genes were quantified by quantitative PCR and microarrays analysis.

Results: We demonstrate that sensing receptors of human hepatocytes in primary cultures are stimulated following recognition of either mode of dsRNA delivery, inducing transcriptional up-regulation (over 100-fold) of multiple immune genes, either selectively or independently of recognition pathways. We also report that the intracellular dsRNA-activated innate response is severely compromised upon ectopic expression of the HCV NS3/4A protease gene in normal human primary hepatocytes, and completely restored by treatment with the NS3/4A protease specific inhibitor BILN2061.

Conclusions: The present study indicates that NS3/4A has a wider protease-dependent effect on the intracellular Pathogen Recognition Receptor (PRR)-mediated immune response than on its extracellular counterpart, which underlies the major role of cytosolic dsRNA receptors in HCV recognition by primary human hepatocytes.

Keywords: Hepatitis C virus; Primary hepatocytes; NS3/4A; Interferon stimulated genes; Viral interference.

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Abbreviations: HCV, hepatitis C virus; NS3/4A, non structural proteins 3 and 4A; PRR, pathogene recognition receptor; MOI, multiplicity of infection; edsRNA, extracellular double-stranded ribonucleic acid; idsRNA, intracellular double-stranded ribonucleic acid; TLR3, Toll-like receptor 3; RIG-I, retinoic acid inducible gene-I; IFN, interferon; ISG, interferon stimulated gene; ISRE, interferon-stimulated response element.

Introduction

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is a serious and growing threat to human health; the majority of HCV-infected individuals develop a persistent infection [1]. Chronic infection is established through a high genetic variability of HCV together with several powerful strategies to evade the host's immune response. The immediate-early phase of the host's innate response to viral infections is initiated through recognition of viral RNA by pathogen recognition receptors (PRRs). This subsequently activates transcription factors, such as nuclear factor (NF)-κB and interferon regulatory factor (IRF)-3, that regulate the expression of a set of immediate early protective genes, including antiviral genes such as type I IFN (*IFNα* and *β*), interferon stimulated genes (ISGs) (*IFIT1*, *IFIT2* and *IFIT3*), chemokines (*CCL5*) and proinflammatory cytokines (*TNFα*, *IL6*) [2]. Among PRRs that recognize viral RNAs, RIG-I and MDA5 both contain the DEXD/H box signature of RNA helicases and two caspase-recruitment and activation domains (CARD) and have been identified as dsRNA cytosolic sentinels [3]. In the context of an HCV infection, it was recently proposed that HCV poly-U/UC RNA directs stable interaction with RIG-I in a 5′PPP-dependent manner [4] and induces an activation of its signaling pathway through CARDIF (also referred to as IPS-1, MAVS or VISA), a common adaptor shared by RIG-I and MDA5. Similarly, Toll-like receptor 3 (TLR3), a membrane-bound receptor which is involved in extracellular and endosomal recognition of dsRNA, ssRNA viruses and poly(I:C), recruits the adaptor

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TRIF to activate downstream signaling pathways leading to activation of IRF3 and NF-κB [3]. However, TLR3's involvement in host defense against HCV remains controversial. HCV NS3/4A protease was shown to inhibit expression of type I IFN and ISGs [5–9] by cleaving CARDIF at a membrane-proximal site, disrupting its dimerization [10] and causing the loss of downstream signaling activity [8,11]. HCV protease inhibitor BILN2061 was shown to block NS3-mediated polyprotein processing [12]. Moreover, CARDIF cleavage by HCV NS3 protease in HCV-infected Huh7 cells is completely abrogated by BILN2061 treatment [9,13]. Additionally, TRIF was shown to be cleaved by purified single chain NS3/4A protease *in vitro* [14,15], whereas TRIF cleavage products in HEK 293 cells expressing NS3/4A have not been found. The demonstration by Dansako et al., that the TLR3/TRIF signaling pathway was not affected in the non-neoplastic human hepatocyte PH5CH8 cells expressing NS3/4A, built up the controversy over the veracity of TRIF as a target for HCV protease [16,17]. Antiviral responses have been studied in human hepatic-derived cell lines that often differ from primary cells in their ability to activate antiviral pathways in response to dsRNA or viral infection. Huh7 and its derivative Huh7.5 cells, both widely used as models, are defective in their TLR3 and RIGI signaling pathways, respectively [18,19]. The significance of these studies that decipher various antiviral signaling pathways largely requires validation in primary cultures. In this study, mRNA levels of selected antiviral genes were quantitatively analyzed and global cellular gene expression analysis was performed to characterize dsRNA-activated innate responses in primary cultures of freshly isolated human hepatocytes. We report the up-regulation of typical innate immune regulatory genes demonstrating functionality of viral sensing and signaling pathways in human hepatocytes following dsRNA stimulation. Moreover, we confirm the HCV NS3/4A protease-mediated major interference of the intracellular dsRNA-activated signaling pathway upon ectopic expression in primary culture of human hepatocytes, concomitant with a BILN2061-reversible CARDIF proteolytic cleavage.

Materials and methods

Patients, cell culture and dsRNA stimulations

Primary cultures of hepatocytes were obtained from healthy peritumoral liver tissues of 17 non-HCV, non-HBV infected patients undergoing partial hepatectomy for secondary metastatic lesions due to colorectal cancers (see Table S1). All procedures followed were in accordance with the ethical commission of the CRCHUM, Montréal, Canada and with the Helsinki Declaration. Biopsy processing, isolation by collagenase perfusion and purification of hepatocytes were carried out as previously described [20]. Hepatocytes were seeded and cultured overnight at 500,000 cells ml^{-1} in 6 well-plates precoated with rat tail collagen type I (BD Biosciences, Bedford, MA) and used within 16–24 h of isolation for stimulations experiments. Cells were stimulated with poly(I:C) (InvivoGen, San Diego CA), for 0–48 h at 37 °C with either 2–50 μg/ml of poly(I:C) in Opti-MEM (TRIF-dependent) or 0.25–15 μg/ml of poly(I:C) or 0.75 μg/ml *in vitro* transcribed HCV RNA (AB12 plasmid) complexed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in a 1:1 ratio in Opti-MEM (CARDIF-dependent). Poly(I:C) in culture medium was kept during the course of both stimulation experiments.

Quantitative PCR (qPCR) assays

Total RNA was extracted using Rneasy Mini kit (Qiagen, Mississauga, Ontario) according to the manufacturer's protocol. Reverse transcription was performed on 300 ng total RNA using the QuantiTect RT kit (Qiagen). Real time PCR DNA amplification was performed using SyBRgreen PCR master mix (Qiagen) (see primers in Table S3). Absolute copy numbers were determined on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) using standard curves established with 10-fold serial dilutions of pCR2.1-TOPO containing the gene of interest and normalized to β-actin.

Microarray data analysis

Microarray experiments were performed using Illumina Human RefSeq-8 BeadChips v2 arrays containing 22,184 probes. Illumina probe data were exported from BeadStudio (version 3) as raw data and were screened for quality. Samples failing visual inspection and control examination were removed. Analysis was performed as detailed in Supplemental material.

HCV NS3/4A-encoded lentivirus preparation and transduction of human hepatocytes

The lentiviral vector system was kindly provided by D. Trono (University of Genève, Switzerland). A PCR-amplified full-length NS3/4A genotype 1b was subcloned into pWP1. Production of lentiviruses was performed as described in protocols section at http://tronolab.epfl.ch./ with one modification: supernatants were concentrated 10-fold with Centricon plus 70 cut-off 100 kDa (Millipore Corporation, Billerica, MA). For transduction of hepatocytes, cells were incubated for 6 h with lentivirus preparations (MOI of 2) with 8 μg/ml polybrene. Culture medium was then changed and cells were maintained for 72 h in absence or presence of 2 μM of the protease inhibitor BILN2061 (provided by Virochem Pharma, Montréal, Canada) prior to poly(I:C) stimulation.

Immunofluorescence

Hepatocytes were seeded on coverslips precoated with rat tail collagen type I and fixed with formaldehyde 3.8% (Fisher Scientific, Ottawa, Ontario) in PBS for 15 min at room temperature (RT). After permeabilization with Triton X-100 0.5% (Sigma–Aldrich), PBS washes, and blockage with goat serum 10% (Sigma–Aldrich) for 1 h at RT, cells were incubated with anti-NS3 antibody kindly provided by Dr. Moradpour (CHUV, Lausanne, Switzerland). The Tyramide Signal Amplification Kit (Invitrogen) with Alexa Fluor 594 was used and nuclei were stained with DAPI (Invitrogen). Cells were observed with an Axioplan 2 Imaging Universal Microscope (Zeiss, Berlin, Germany).

Immunoblot analysis

Whole-cell extracts were prepared as previously described [21]. Membranes were incubated with rabbit anti-CARDIF (Alexis Biochemicals, San Diego, CA), rabbit anti-TRIF (Alexis Biochemicals), monoclonal anti-NS3 (Biodesign, Saco, ME) or mouse anti-β-actin antibody (Chemicon International, Temecula, CA). After washes, membranes were incubated in HRP-conjugated goat anti-rabbit or HRP-conjugated goat anti-mouse antibody (KPL, Gaithersburg, MD).

Results

Molecular sensors of extracellular and intracellular dsRNA induce an innate antiviral response in human primary hepatocytes

Most cultured hepatic cell lines radically differ from their primary counterparts in their ability to respond to both extracellular dsRNA (edsRNA) (TLR viral sensors) and intracellular dsRNA (idsRNA) (cytoplasmic viral sensors), partly due to differences in expression of pathogen recognition receptors (PRRs) [18,22]. In our preparations of human hepatocytes, in which more than 99% of freshly isolated cells positively stained for an hepatocyte antigen by immunofluorescence (see Fig. S1), mRNAs coding for *TLR3*, *IFIH1* (*MDA5*) and *DDX58* (*RIGI*) virus-sensing receptors were detected for up to 4 days in culture under unstimulated conditions (see Fig. S2). This convinced us to further explore the functionality of viral RNA recognition and the antiviral signaling response in primary cultures of human hepatocytes. In particular, we focused on the detection of the synthetic dsRNA analog