Research Article

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HCV core-mediated activation of latent TGF-β via thrombospondin drives the crosstalk between hepatocytes and stromal environment

Nassima Benzoubir[1,2], Charlène Lejamtel[1,2], Serena Battaglia[1,2], Barbara Testoni[4,5], Barbara Benassi[5], Claire Gondeau[6,7], Laure Perrin-Cocon[8,9], Christophe Desterke[10], Valérie Thiers[1,2,†], Didier Samuel[1,2,3], Massimo Levrero[4,5], Christian Bréchot[1,2,11], Marie-Françoise Bourgeade[1,2,3,*]

[1]*INSERM U785, Villejuif F-94800, France;* [2]*Univ Paris-Sud, UMR-S 785, Villejuif F-94800, France;* [3]*AP-HP Hôpital Paul Brousse, Centre Hépato-Biliaire, Villejuif, France;* [4]*Department of Internal Medicine – DMISM and LEA-INSERM U785, Sapienza University Rome, Italy* [5]*AIRC Rome Oncogenomic Center, Regina Elena Cancer Institute, Rome, Italy;* [6]*INSERM U1040, Montpellier, France;* [7]*University Montpellier 1, Montpellier, France;* [8]*INSERM U1111, International Research Center in Infectiology, Lyon, France;* [9]*Université de Lyon, Lyon, France;* [10]*SFR André Lwolf, Villejuif F-94800, France;* [11]*Mérieux Institute, Lyon F-69002, France*

Background & Aims: The mechanisms by which fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) develop during chronic hepatitis C virus (HCV) infection are not fully understood. We previously observed that HCV core protein induced a TGF-β-dependent epithelial mesenchymal transition, a process contributing to the promotion of cell invasion and metastasis by impacting TGF-β1 signalling. Here we investigated HCV core capacity to drive increased expression of the active form of TGF-β1n transgenic mice and hepatoma cell lines.

Methods: We used an *in vivo* model of HCV core expressing transgenic mice.

Results: We observed that about 50% of genes deregulated by core protein expression were TGF-β1 target genes. Active TGF-β levels were increased in HCV core transgenic mouse livers. Overexpression of core protein in hepatoma cells increased active TGF-β levels in culture supernatants and induced Smad2/3 phosphorylation, thus reflecting activation of the TGF-β signaling pathway. Moreover, our data showed the implication of thrombospondin-1 in core-dependent TGF-β activation. Finally, hepatoma cells expressing HCV core could activate stellate cells in co-culture and this activation was TGF-β dependent.

Conclusions: Collectively, these data delineate a novel paradigm where HCV may be related to liver pathogenesis through its ability to induce a local, intrahepatic TGF-β activation. They argue for a dual impact of HCV core on liver fibrosis and liver carcinogenesis: HCV core could act both as autocrine and paracrine factor modulating TGF-β responses within hepatocytes and in stromal environment through TGF-β activation.

Introduction

Chronic hepatitis C virus (HCV) infection is associated with a major risk of developing progressive liver diseases including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [1]. Despite epidemiologic evidence connecting HCV infection to HCC as well as a large body of literature from clinical and animal studies, little is known about how HCV causes fibrosis and subsequent development of cirrhosis and HCC [2]. The long latency period between HCV infection and HCC may signify indirect action of this virus. It could be postulated that persistent stimulation of cellular stress responses by viral proteins within hepatocytes may modulate the microenvironment that plays an important role in chronic infections. It is thus of interest to obtain data that provide information about host proteins of predicting importance in HCV-related liver diseases since different reports highlight the crosstalk between infected cells and the surrounding stroma as a key modulator of the processes of fibrosis, epithelial mesenchymal transition (EMT), tumor invasion, and metastasis. Several lines of investigation pointed out that, besides its role in viral RNA packaging, HCV core protein interacts with several cellular proteins leading to modulation of transcription of genes dependent on these cascades and consequently to modulation of a number of cellular regulatory functions [3] including

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* Corresponding author. Address: INSERM U785 CHB Hôpital Paul Brousse, 12 Avenue Paul-Vaillant Couturier, Villejuif 94807, France. Tel.: +33 1 45 59 60 88; fax: +33 1 45 59 60 90.
E-mail address: marion.bourgeade@inserm.fr (M.-F. Bourgeade).
† Present address: Institut Pasteur, 75015 Paris, France.

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; EMT, epithelial mesenchymal transition; TGF-β, transforming growth factor beta; ECM, extracellular matrix; TSP, trombospondin; cT, HCV core from tumor nodule; cNT, HCV core from non-tumor nodule; SMA, smooth muscle actin; HSC, hepatic stellate cell.

control of cell growth, apoptosis, oxidative stress, carcinogenesis, and immune modulation.

Overexpression of TGF-β and concomitant decrease in hepatocyte growth inhibition are frequently observed in HCC, supporting the notion that TGF-β could play a tumor promoting role in liver cancer [4]. TGF-β is a multifunctional cytokine involved in the regulation of immune response, cell cycle, differentiation, and apoptosis. TGF-β acts as a two-edged sword in oncogenesis acting as a tumor suppressor or promoter depending on the stage of tumorigenesis [5]. Moreover, in the inflammatory liver, hepatic stellate cells transdifferentiate into fibrogenic, extracellular matrix (ECM) producing myofibroblasts upon TGF-β1 signaling [6].

TGF-β1 is secreted as a latent complex that is targeted to the extracellular matrix. This latent complex is composed of mature TGF-β1, latency associated protein (LAP), and latent TGF-β binding protein (LTBP) [7]. Activation of latent TGF-β1nvolves disruption of the interaction between LAP and mature TGF-β to permit binding of the cytokine to its receptor and subsequent activation of transcriptional responses through the specific Smad pathway.

Extensive work on this process led to the identification of two classes of TGF-β activators: various proteases could activate TGF-β by degrading LAP, and other proteins such as integrins [8] and thrombospondin-1 (TSP-1) [9] were shown to alter LAP conformation allowing TGF-β1 activation. Just a minor fraction of total TGF-β produced by the cells is made available for signaling, suggesting that the balance between latency and activation represents a major regulatory step to set strength and precise localization of TGF-β activity within tissues [10]. Although clinical and experimental studies have demonstrated that TGF-β1 levels were increased in chronic HCV infection [11,12], no study has yet determined whether the balance between latent and active TGF-β levels has been modulated during chronic HCV infection.

We previously reported that HCV naturally occurring variants of HCV core isolated from tumoral and non-tumoral liver of the same patient were able to shift TGF-β1 responses from tumor suppressor effects to tumor promotion by decreasing hepatocyte apoptosis and increasing EMT through a decrease of Smad3 activation. Moreover, we observed that core protein expression was sufficient to provoke EMT induction in primary hepatocytes. This effect was reverted by addition of a specific inhibitor of the TGF-β1 receptor suggesting a TGF-β1 dependent effect of core on EMT development [13,14].

We here report that HCV drives activation of TGF-β through HCV core. Indeed, we show that mouse primary hepatocytes obtained from mice transgenic for two different core sequences or HuH7 cells stably expressing these cores exhibit a permanent activation of the TGF-β pathway and are able to activate hepatic stellate cells in co-culture.

Cumulatively, our data describe a novel paradigm for the role of a viral protein in liver fibrosis and HCC where HCV core acts both by modulating TGF-β responses in hepatocytes in an autocrine mechanism and by affecting the stellate cell activation in a juxtacrine/paracrine mechanism through TGF-β activation.

Materials and methods

Materials

Active TGF-β1 was from Abcys, and latent TGF-β1 from R&D. The TGF-β receptor inhibitor SB431542 was from Calbiochem, Leu-Ser-Lys-Leu (LSKL) peptide was from AnaSpec.

Vectors

See Supplementary Material.

Cells

HuH7, HuH7.5, FL/neo Replicon cells [15], and HuH9.13 cells expressing the HCV NS3-NS5B subgenomic replicon [16] were maintained in DMEM containing 10% fetal calf serum. HuH9.13-cured cells were obtained from HuH9.13 after 1-month treatment with 500 U/ml interferon α2a. The human stellate cell line LX-2 [17] was maintained in DMEM containing 2% fetal calf serum. Cells were transfected with the different vectors using X-treme (Roche) or siRNA using jetPRIME (Polyplus). Primary hepatocytes from mice transgenic for HCV core were isolated by *in situ* collagenase perfusion of livers as previously described [14].

HuH7.5 infection with JFH1

High titer stocks of cell culture–derived JFH1 were prepared as described elsewhere [18]. HuH7.5 cells were seeded in 6-well plates and inoculated 24 h later with increasing concentrations of cell culture–derived JFH1 (0.5, 1, 5, 20, and 50 HCV mRNA copies/cell). After 16 h at 37 °C, the inoculum was removed and cells were washed three times with DMEM. Two ml/well of fresh medium was then added. Cell supernatant was collected 72 h post-infection and harvested for HCV RNA evaluation or protein analysis by Western blotting. Usually, in these experimental conditions, 60–80% of cells are infected when 50 RNA copies/cells are applied (i.e., HCV CORE or NS5A positive cells).

Microarrays, Taqman® Low Density Arrays (TLDAs) and qRT-PCR

See Supplementary Material. Data deposition is in Gene Expression Omnibus (GEO) under GEO Accession GSE36220 at http://www.ncbi.nlm.nih.gov/geo/.

Western blotting

Cells were lysed in RIPA buffer containing 0.5% SDS and Benzon nuclease (Novagen). Proteins were quantified and extracts separated on SDS polyacrylamide gel, transferred on nitrocellulose membrane and blotted with different antibodies. Membranes were revealed with a chemioluminescence detection kit (ECL Plus, GE Healthcare) using a DCC camera (G Box Syngene).

Immunofluorescence staining

Cells fixed with a 4% PFA solution, permeabilized with PFS (saponin gelatin in PBS) were first incubated with an anti-αSMA antibody (Clone 1A4, Sigma), and after with an Alexa Fluor 488 conjugated anti-mouse antibody (Molecular Probes). They were counterstained with Hoechst and examined by fluorescence microscopy.

Luciferase reporter experiments

Cells were co-transfected with vectors coding for the gene of interest together with FoxH1 and ARE-luc (Activin Responsive Element) or SBE-Luc (Smad Responsive Element) reporter plasmids and the *Renilla* luciferase plasmid to normalize the results. They were incubated 24 h later in the absence or presence of TGF-β1 or SB431542 for another 18 h. Luciferase activity was measured with the Dual-Luciferase Reporter Assay (Promega).

Determination of active TGF-β1 levels

See Supplementary Material.

Statistical analysis

Statistical differences between conditions were validated by paired t test with significant $p < 0.05$.