# Hepatitis C Virus–Replicating Hepatocytes Induce Fibrogenic Activation of Hepatic Stellate Cells

ANJA SCHULZE–KREBS,* DOROTHEE PREIMEL,* YURY POPOV,*,† RALF BARTENSCHLAGER,§ VOLKER LOHMANN,§ MASSIMO PINZANI,‖ and DETLEF SCHUPPAN*,†

*Department of Medicine I, University of Erlangen-Nuremberg, Erlangen-Nuremberg, Germany; †Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; §Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany; and ‖Department of Internal Medicine, University of Florence, Florence, Italy

***Background & Aims:*** **The mechanism by which hepatitis C virus induces liver fibrosis remains largely obscure. To characterize the profibrogenic potential of hepatitis C virus, we used the hepatitis C virus replicon cell line Huh-7 5-15, which stably expresses the nonstructural hepatitis C virus genes NS3 through NS5B, and hepatic stellate cells as fibrogenic effector cells.** ***Methods:*** **Rat and human hepatic stellate cells were incubated with conditioned media from replicon cells, and expression of fibrosis-related genes was quantified by using real-time polymerase chain reaction, protein, and functional assays. Transforming growth factor β1 activity was determined by bioassay.** ***Results:*** **Hepatitis C virus replicon cells release factors that differentially modulate hepatic stellate cell expression of key genes involved in liver fibrosis in a clearly profibrogenic way, up-regulating procollagen α1(I) and procollagen α1(III) and down-regulating fibrolytic matrix metalloproteinases. Transforming growth factor β1 expression and bioactivity were increased severalfold in hepatitis C virus–replicating vs mock-transfected hepatoma cells. However, transforming growth factor β1 activity was responsible for only 50% of the profibrogenic activity.** ***Conclusions:*** **Hepatitis C virus nonstructural genes induce an increased expression of transforming growth factor β1 and other profibrogenic factors in infected hepatocytes. The direct induction of profibrogenic mediators by hepatitis C virus in infected hepatocytes explains the frequent observation of progressive liver fibrosis despite a low level of inflammation and suggests novel targets for antifibrotic therapies in chronic hepatitis C.**

More than 200 million people worldwide are infected with the hepatitis C virus (HCV),[1,2] which leads to chronic liver disease in 50%–70% of patients, 15%–20% of whom develop cirrhosis within 20 years. Cirrhosis is a prerequisite for the development of HCV-related hepatocellular carcinoma, which occurs at a yearly rate of 2%–3%.[3] Progression of liver disease does not correlate with serological or histological inflammation, and most infections are diagnosed by chance or in an advanced stage, when complications become apparent. Currently, the best available therapy is the combination of pegylated interferon α and ribavirin.[4–6] Although combination therapy can lead to viral elimination in up to 50% and 80% of patients with genotypes 1 and 2 or 3, respectively, less expensive and side effect–free treatments are desirable. For those patients who do not respond to antiviral therapy or who are in an advanced stage of their disease, antifibrotic treatment is urgently needed that can halt the progression of fibrosis or even reverse it.

HCV is an enveloped flavivirus harboring a plus-stranded RNA with approximately 9600 nucleotides that encode a polyprotein which is cleaved cotranslationally and posttranslationally by proteolysis into 10 structural and nonstructural proteins. The liver is the main organ of HCV replication, although hepatic levels of viral RNA and protein are usually low, with mononuclear cells apart from hepatocytes as suggested sites of viral replication. HCV exists as 6 genotypes, and type 1 is the most prominent in Europe and the United States.[7]

Liver fibrosis results from excessive accumulation of extracellular matrix (ECM) components, a down-regulation of ECM-removing matrix metalloproteinases (MMPs), and an increase of tissue inhibitors of MMPs (TIMPs), mainly TIMP-1. The fibril-forming interstitial collagens I and III and the sheet-forming basement membrane collagen IV are the most abundant ECM molecules in the liver, with an up to 10-fold increase in cirrhosis.[8] Collagens, MMPs, and TIMPs are mainly produced by myo-

*Abbreviations used in this paper:* CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HSC, hepatic stellate cell; MF, myofibroblastic cells; MMP, matrix metalloproteinase; PCR, polymerase chain reaction; ROS, reactive oxygen species; TGF, transforming growth factor; TIMP, tissue inhibitor of matrix metalloproteinases.

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fibroblastic cells (MFs), which either derive from activated hepatic stellate cells (HSCs) or activated portal or perivascular fibroblasts.[9–11] Activated Kupffer or endothelial cells, proliferating bile duct epithelia, and other mononuclear cells or even the myofibroblasts themselves are sources of fibrogenic cytokines and growth factors that can stimulate HSCs and portal or perivascular fibroblasts to become MFs.[11] A prominent profibrogenic cytokine is transforming growth factor (TGF)-β1, which can be released from almost any cell type during inflammation, tissue regeneration, and fibrogenesis.[11–13] Active TGF-β1 strongly up-regulates the production and deposition of ECM molecules and down-regulates most MMPs. Apart from causing an inflammatory response in some patients, HCV can significantly modulate the metabolism of infected hepatocytes. Thus, core, NS3, and NS5A can induce derangement of lipid metabolism,[14–16] modulate hepatocyte apoptosis, or alter signal transduction, potentially favoring hepatocarcinogenesis.[17–19] Derangement of lipid compartmentalization and metabolism by HCV may lead to the production of reactive oxygen species (ROS),[20] and ROS were shown to induce TGF-β1.[21] However, a clear profibrogenic role of HCV-infected hepatocytes has not been shown.

Therefore, we investigated whether and by what mechanisms HCV may induce liver fibrosis, independent of inflammation, by using the human hepatoma cell line Huh-7 5-15, which stably expresses the nonstructural viral genes NS3 through NS5B, and HSCs/myofibroblasts as fibrogenic effector cells. We could show that HCV-replicating, but not mock-transfected, hepatocytes express increased levels of TGF-β1, as well as other unidentified factors that induce a profibrogenic gene-expression pattern in HSCs/MFs.

## Materials and Methods

### Cells and Cell Culture

The human hepatoma cell line Huh-7 was grown in the presence of 5% $CO_2$ in Dulbecco's modified Eagle medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin). For the stably transfected cell lines, neomycin (Invitrogen, Karlsruhe, Germany) was used at a concentration of 1 mg/mL. The Huh-7 clone 5-15 harbors the nonstructural viral genes NS3 through NS5A of HCV genotype 1b, which are responsible for viral replication, whereas the mock-transfected Huh-7 clone contains only the parent vector pcDNA3.[22,23]

Human HSC/MF and rat CFSC-2G cells, an HSC line with intermediate activation (a gift of Dr M. Rojkind, Departments of Biochemistry and Molecular Biology and Pathology, George Washington University, Washington, DC), were grown under the same conditions as the human hepatoma cell line, but without neomycin. Human HSCs were isolated from wedge biopsy samples by outgrowth of normal human livers not suitable for transplantation[24] and show the characteristics of MFs.[25]

### Preparation of Conditioned Media

Huh-7 5-15 or Huh-7 pcDNA3 was grown to approximately 70% confluency and washed 3 times with phosphate-buffered saline (PBS), followed by culture in DMEM containing 0.2% FCS without neomycin for another 72 hours. Media were harvested, centrifuged at 1000 rpm for 10 minutes to remove cell debris, and stored at 4°C for no longer than 3 weeks.

### Inhibition of Bioactive Transforming Growth Factor β

Conditioned medium was supplemented with 250 μg/mL of rabbit anti–TGF-β1–neutralizing antibody (AB-100-NA; R&D Systems, Wiesbaden, Germany) or unspecific rabbit immunoglobulin G of the same concentration (Sigma, St Louis, MO) and incubated for 60 minutes on a rotation shaker at room temperature before addition to cells.

### Expression of Extracellular Matrix and Fibrosis-Related Genes

A total of 60,000 CFSC-2G cells or human HSCs were seeded into 9.6 $cm^2$-well plates and incubated in DMEM containing 10% FCS for 24 hours. After reaching 50% confluency, cells were washed 3 times with PBS and starved for 24 hours with DMEM without FCS, followed by the addition of conditioned medium of mock-transfected or replicon-containing Huh-7 cells for another 24–72 hours. For coculture experiments, equal cell numbers of stably transfected human hepatoma cells and CFSC-2G rat HSCs were seeded in 25-$cm^2$ cell-culture flasks in DMEM containing 10% FCS. After 24 hours, the medium was removed, and cells were washed 3 times in PBS, followed by culture in DMEM containing 0.2% FCS for up to 96 hours. After RNA isolation at predefined time points and reverse transcription, transcript levels of fibrosis-related messenger RNAs (mRNAs) were quantified by real-time polymerase chain reaction (PCR) on a LightCycler (Roche, Penzberg, Germany).

### RNA Isolation and Preparation of Complementary DNA

RNA was extracted from the cells by using RNAPure (PeqLab, Erlangen, Germany) according to the manufacturer's protocol. RNA concentration, purity, and integrity were verified by spectrophotometry at 260 and 280 nm and by visualization of the 18S and 28S ribosomal RNA bands after gel electrophoresis and ethidium bromide staining.

Transcription of mRNA derived from 0.5 μg of total RNA into corresponding complementary DNA (cDNA) was performed by SuperScript II reverse transcriptase (Invitrogen)

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