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Hepatitis C virus core protein induces fibrogenic actions of hepatic stellate cells via toll-like receptor 2

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Hepatic stellate cells (HSCs) represent the main fibrogenic cell type accumulating extracellular matrix in the liver. Recent data suggest that hepatitis C virus (HCV) core protein may directly activate HSCs. Therefore, we examined the influence of recombinant HCV core protein on human HSCs. Primary human HSCs and the human HSC line LX-2 were stimulated with recombinant HCV proteins core and envelope 2 protein. Expression of procollagen type I α-1, α-smooth muscle actin, cysteine- and glycine-rich protein 2, glial fibrillary acidic protein, tissue growth factor β1, matrix metalloproteinases 2 (MMP2) and 13, tissue inhibitor of metalloproteinases 1 and 2 was investigated by real-time PCR. Intracellular signaling pathways of ERK1/2, p38 and, jun-amino-terminal kinase (JNK) were analyzed by western blot analysis. Recombinant HCV core protein induced upregulation of procollagen type I α-1, α-smooth muscle actin, MMP 2 and 13, tissue inhibitor of metalloproteinases 1 and 2, tissue growth factor β1, cysteine- and glycine-rich protein 2, and glial fibrillary acidic protein mRNA expression, whereas HCV envelope 2 protein did not exert any significant effect. Blocking of toll-like receptor 2 (TLR2) with a neutralizing antibody prevented mRNA upregulation by HCV core protein confirming that the TLR2 pathway was involved. Furthermore, western blot analysis revealed HCV-induced phosphorylation of the TLR2-dependent signaling molecules ERK1/2, p38 and JNK mitogen-activated kinases. Our *in vitro* results demonstrate a direct effect of HCV core protein on activation of HSCs toward a profibrogenic state, which is mediated via the TLR2 pathway. Manipulating the TLR2 pathway may thus provide a new approach for antifibrotic therapies in HCV infection.
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Chronic hepatitis C virus (HCV) infection is a major cause of chronic liver disease leading to hepatic fibrosis und ultimately to liver cirrhosis.[1,2] Hepatic stellate cells (HSCs) are the major producers of extracellular matrix proteins and have a key role in the development of liver fibrosis.[3–6] After hepatic injury, HSCs become activated and secrete markedly increased amounts of collagen type I and III, the principal matrix proteins in hepatic fibrosis[7] and express the intermediate filament proteins α-smooth muscle actin (SMA), endoded by the *ACTA2* gene, and glial fibrillary acidic protein (GFAP). In addition, they express tissue growth factor β1 (TGF-β1, encoded by *TGFB1*), and *CSRP2*, a gene in the liver exclusively expressed by HSC coding the cysteine- and glycine-rich protein 2.[8–12] Furthermore, activated HSC produce factors involved in tissue remodelling:[13] matrix metalloproteinases 2 (MMP2, gelatinase A, collagenase IV) and 13 (MMP13, matrix metallopeptidase 13, collagenase 3) are proteolytic enzymes activated during hepatic fibrogenesis degrading extracellular matrix, thus contributing to the destruction of the physiological liver architecture and further activation of HSC.[14] On the other hand, MMPs facilitate penetration of inflammatory cells into the liver further enhancing local tissue damage. This process is controlled by specific tissue inhibitors of metalloproteinase (TIMP) acting as antifibrolytic enzymes.[15] In particular, TIMP1 and TIMP2 are activators of MMP9 and MMP2, respectively, by cleaving the precursor proteins.[16]

HCV is a single-stranded RNA virus composed of structural (core, envelope 1 and 2 (E1 and 2)) and non-structural proteins 2–5.[17] Apart from their role in viral replication, HCV proteins can affect a variety of cellular functions in

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infected cells.[18,19] HCV core protein is an RNA-binding dimeric α-helical protein that participates in the formation of the viral nucleocapsid.[19] The protein exerts a number of biological actions in many cell types, including hepatocytes.[20] These effects depend on the target cell type, and include control of cell growth, apoptosis, oxidative stress, carcinogenesis and immunomodulation.[21–25] HCV core protein can also trigger inflammatory cells and thus contribute to the immunopathology of HCV infection.[26,27]

Adenoviral infection of HSC with a HCV core protein expressing construct-induced cell proliferation and increased procollagen type I α-1 gene (*COL1A1*) expression.[28] We and others have recently shown that HCV core protein triggers inflammatory cell activation by stimulating the pattern recognition receptor toll-like receptor 2 (TLR2).[29–31]

After ligand binding, TLR2 recruits different signaling molecules that are also involved in the regulation of fibrosis. These include the adapter molecule MyD88, triggering interleukin-1 receptor-associated kinase activated by phosphorylation and leading to the recruitment of the mitogen activated kinase (MAPK) pathways jun-amino-terminal kinase (JNK), p38 and ERK1/2 that induce NFkB activation.[32]

Thus, we hypothesized that stimulation of TLR2 on HSC by HCV core protein increased fibrogenesis during HCV infection. In this study, we present evidence that HCV core protein stimulates the expression of the fibrogenic genes *COL1A1*, *ACTA2*, *MMP2*, *MMP13*, *TIMP1*, *TIMP2*, *TGFB1*, *CSRP2* and *GFAP* via triggering of TLR2 and activation of TLR2-dependent signaling pathways.

MATERIALS AND METHODS

FACS Analysis

The following antibodies were used for FACS analysis: PE-conjugated anti-TLR2 (eBioscience, San Diego, CA, USA), PE-conjugated anti-CD81 (BD Bioscience, Heidelberg, Germany). Cell viability was assessed by propidium iodide (BD Bioscience) staining. After incubation of the cells with 10 μl antibody for 20 min and washing in PBS, samples were analyzed on a FACScalibur flow cytometer using CellQuest Pro software (BD Bioscience) and Flowjo 7.2.2 (Treestar, Ashland, OR, USA).

Cell Culture

Isolated human primary HSCs (pHSC, ScienCell, Carlsbad, CA, USA) were used between passage 2 and 6, and cultured in defined medium supplemented with 2% fetal bovine serum and 50 U/ml penicillin and 50 μg/ml streptomycin (all ingredients obtained from ScienCell). The LX-2 human HSC line was kindly provided by Dr SL Friedman (Mount Sinai School of Medicine, New York, NY, USA).[33] LX-2 cells were grown and maintained in Dulbecco's modified Eagle's Medium (PAA Laboratories, Pasching, Austria) supplemented with 2% fetal calf serum (Biochrom AG, Berlin, Germany) and 50 U/ml penicillin and 50 μg/l streptomycin (PAA Laboratories). Cells were cultured in 75 cm^2 flasks (Greiner Bio-One, Frickenhausen, Germany) until 90% confluence and then trypsinized (0,05% trypsin, 0,02% EDTA, PAA Laboratories) and reseeded at a ratio of 1:3 or used for biological studies. Morphology was assessed with a Axiovert 200 M microscope (Zeiss, Jena, Germany).

Cell Stimulation

pHSC and LX-2 cells were plated in 12-well plates at 80% confluence and grown in stellate cell medium or serum-depleted Dulbecco's modified Eagle's Medium with 0.2% bovine serum albumin (Sigma, St Louis, MO, USA) for 48 h, respectively. Then either 1 μg/ml, 2 μg/ml or 3 μg/ml of recombinant full-length HCV core protein (kindly provided by M Houghton), 3 μg/ml of truncated HCV core protein (amino acids 1–115, Mikrogen, Neuried, Germany), 10 μg/ml of HCV E2 protein (kindly provided by M Houghton) or 5 ng/ml of lipopolysaccharide (LPS; Sigma-Aldrich, Taufkirchen, Germany) were added for another 20 h. For western blot analysis, LX-2 cells were stimulated with HCV core protein for 15 min before cell harvesting. As a control HCV core protein was heat inactivated at 90°C for 30 min. To block TLR2, the anti-TLR2 antibody TL2.1 or an isotype control antibody (eBioscience) was added at 2 h before stimulation with HCV core protein.

Proliferation Assay

Serum-depleted LX-2 cells in different cell densities were incubated over 48 h in 96-well microtitre plates (Nunc, Wiesbaden, Germany) in Dulbecco's modified Eagle's Medium containing 0.2% bovine serum albumin and stimulated with 3 μg/ml HCV core protein. After 20 h, stimulated HSC were labeled with 1 mCi of [3H]thymidine (Amersham, Braunschweig, Germany) at 4 h before the cells were harvested. All experiments were performed in triplicate. Stimulation indices were calculated as the ratio of [3H]thymidine uptake in relation to the culture medium control. Stimulation indices $\geqslant 2$ were considered to indicate positive proliferative responses of the LX-2 cells.

RNA Extraction and Reverse Transcription

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacture's standard protocol. The extracted RNA was eluted in 50 μl of RNAse-free water and stored at −80°C. Reverse transcription was carried out for 15 min at 42°C with 1 μg template RNA in a final volume of 20 μl using the Quantitect RT kit (Qiagen) following the manufacturer's recommendations. cDNA samples were stored at −20°C.

Real-Time Quantitative PCR

For detection of *COL1A1*, *ACTA2*, *MMP2* and *MMP13*, *TIMP1* and *TIMP2*, *TGFB1*, *CSRP2* and *GFAP* QuantiTect Primer Assays from Qiagen were used according to the manufacturer's recommendations. Primers of the housekeeping genes, *β-actin*, were purchased from TibMolbiol

(Berlin, Germany): sense 5′-TGGCATCGTGATGGACTCC-3′, antisense 5′-AATGTCACGCACGATTTCCC-3′. Quantification of cDNA transcripts was performed using a Light Cycler device (Roche Diagnostics, Mannheim, Germany). In brief, 2 μl LightCycler-FastStart DNA Master 'Plus' SYBR Green I (Roche Diagnostics), 1 μl of each primer 0.5 μM, 1 μl of cDNA preparation and 6 μl of water were mixed in a total volume of 10 μl for each PCR reaction. PCR settings were: 95°C for 2 min, 50 amplification cycles for *COL1A1*, *MMP2* and 40 cycles for *TIMP2*, *ACTA2*, *MMP13*, *TIMP1*, *TGFB1*, *CSRP2* and *GFAP* with denaturation of 1 s at 95°C, 5 s of 55°C for DNA strand annealing and extension at 55°C for 5 s. *β-Actin* PCR was carried out for 45 cycles with denaturation at 95°C for 2 s, annealing at 60°C for 5 s, extension at 72°C for 10 s and fluorescence acquisition at 89°C.

After each PCR run, a melting curve analysis was carried out. Fluorescence was measured after each elongation step at 87°C for *COL1A1*, 81°C for *MMP2* and 72°C for *TIMP2*, *ACTA2*, *MMP13*, *TIMP1*, *TGFB1*, *CSRP2* and *GFAP*. mRNA concentrations were determined semiquantitatively using purified PCR products in five log10 serial dilutions as external standards. Target gene mRNA levels were normalized with respect to mRNA levels of the housekeeping gene *β-actin*. The LightCycler software version 3.5 was used in all PCR experiments. RNA extraction, reverse transcription, PCR and post-PCR procedures were carried out in separated areas of the laboratory to prevent carry over contamination. Identity of PCR products was confirmed by melting curve analysis and 2% agarose gel electrophoresis.

Western Blot Analysis

Western blots were performed following standard protocols. In brief, cell lysates were dissolved in SDS sample buffer and 20 μg of protein were applied to a 10% polyacrylamide slab gel. After separation by electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) by electroblotting. The membrane was placed in blocking buffer (5% non-fat dry milk dissolved in 150 mM NaCl, 50 mM Tris, pH 7.5 and 0.1% Tween-20) for 1 h. Next, the membrane was incubated for 1 h at room temperature using the following antibodies: anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38, anti-JNK and anti-phospho-JNK (Cell Signaling, Danvers, MA, USA). Then the membrane was washed three times with PBS/0.1% Tween-20, and incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (Zymed, South San Francisco, CA, USA) at a dilution of 1:5 000 in PBS/0.1% Tween-20. After washing three more times in PBS/0.1% Tween-20, the membrane was soaked in ECL detection reagent and exposed to Hyperfilm MP (Amersham) for 10 s.

Statistical Analysis

Statistical analysis was performed with SPSS software version 17.0.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism for Windows version 4.00. Data were compared by two-tailed Mann–Whitney test as appropriate. Data are reported as means ± s.d. Results with $P<0.05$ were regarded as statistically significant.

RESULTS

HCV Core Protein Induces Expression of Fibrogenic Relevant Genes

We first studied the effect of HCV core protein on the expression of fibrogenic genes. As shown in Figure 1, incubation with recombinant HCV core protein (3 μg/ml) resulted in significantly upregulated expression of *COL1A1* in both pHSC (2.09-fold ($P=0.0237$), Figure 1a) and LX-2 cells (1.86-fold ($P=0.002$), Figure 1b). Furthermore, HCV core protein induced an increase in mRNA expression of various other fibrogenic marker genes *ACTA2* (1.58-fold in pHSC, $P=0.0079$; 2.47-fold in LX-2 cells, $P=0.0079$), *MMP2* (2.45-fold in pHSC, $P=0.0237$; 1.44-fold in LX-2 cells, $P=0.0028$), *MMP13* (1.51-fold in pHSC, $P=0.0079$; 1.73-fold in LX-2 cells, $P=0.0317$), *TIMP1* (2.07-fold in pHSC, $P=0.079$; 2.29-fold in LX-2, $P=0.0079$), *TIMP2*

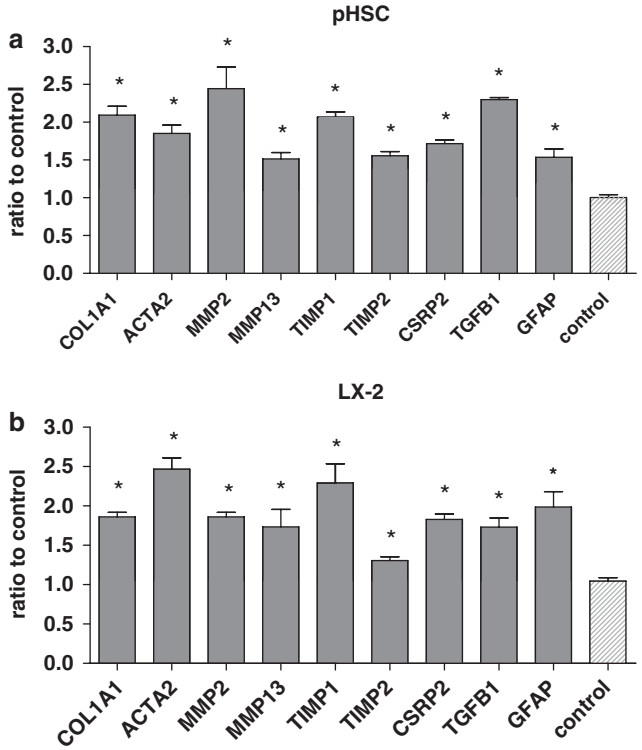

Figure 1 Upregulated expression of fibrogenic genes after stimulation with HCV core protein. This figure summarizes the analysis of mRNA levels using quantitative real-time reverse transcription PCR from six independent experiments. Stimulation of primary HSC (**a**) and serum-starved LX-2 cells (**b**) with 3 μg/ml HCV core protein resulted in a significantly upregulated expression of *COL1A1*, *ACTA2*, *MMP2*, *MMP13*, *TIMP1*, *TIMP2*, *TGFB1*, *CSRP2* and *GFAP*. Target mRNA expression was normalized with respect to mRNA levels of the housekeeping gene *β-actin*. Increase in gene expression is shown in comparison with untreated controls. *$P<0.05$ compared with untreated controls.

(1.44-fold in pHSC, $P=0.00237$; 1.31-fold in LX-2, $P=0.0028$), *TGFB1* (2.30-fold in pHSC, $P=0.0117$; 1.73-fold in LX-2, $P=0.0079$), *CSRP2* (1.69-fold in pHSC, $P=0.0073$; 1.83-fold in LX-2, $P=0.0049$) and *GFAP* (1.48-fold in pHSC, $P=0.073$; 1.99-fold in LX-2, $P=0.0079$) compared with unstimulated cells (Figure 1). HCV-induction of genes in HSCs was confirmed by the use of a truncated HCV core protein (amino acids 1–115) (Figure 2) and showed a nearly linear dose-dependent kinetics (Figure 3). HCV envelope glycoprotein E2, which interacts with HSC via CD81 but not TLR2, did not stimulate the LX-2 cells (Figure 2).

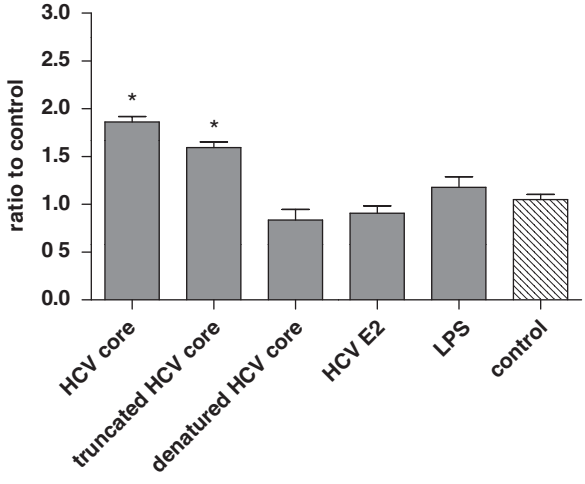

Figure 2 Full-length and truncated (aa1-115) recombinant HCV core protein but not heat-denatured HCV core protein, HCV E2 protein and the TLR4 ligand LPS upregulate mRNA expression of fibrogenic genes. The figure shows the *COL1A1* mRNA expression levels in LX2 cells from three independent experiments. In contrast to 3 μg/ml full-length (HCV core) and truncated HCV core (truncated HCV core) protein, stimulation of serum-starved LX-2 cells with 3 μg/ml heat-denatured HCV core protein, 10 μg/ml HCV E2 protein and the TLR4 ligand LPS (5 ng/ml) did not result in upregulated expression of *COL1A1* mRNA. Gene expression levels in stimulated cells are shown as ratios with reference to untreated control cells. *$P<0.05$ compared to control cells.

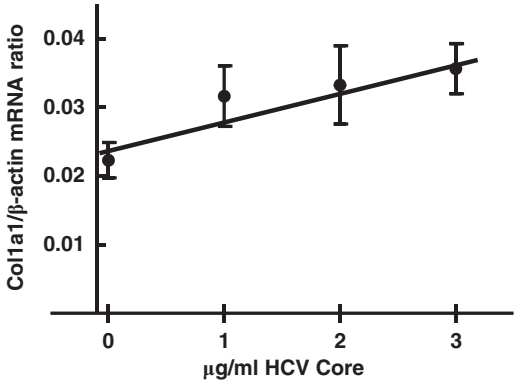

Figure 3 Induction of COL1A1 by HCV core protein shows a nearly linear dose-dependent kinetics. Serum-depleted LX-2 cells were stimulated with 0 μg/ml, 1 μg/ml, 2 μg/ml and 3 μg/ml HCV core protein. COL1A1 mRNA levels were normalized with respect to mRNA levels of the housekeeping gene *β-actin*. The figure shows average values produced by three independent experiments.

Proliferation of HSCs is not Affected by Stimulation with HCV Core Protein

We then examined the effect of HCV core protein on cell viability and proliferation. LX-2 cells were plated in different cell densities and then incubated for 48 h in serum-depleted medium (0.2% bovine serum albumin) before stimulation with HCV core protein for 24 h. On microscopic examination, morphology did not change (Figure 4). Furthermore, there was no increase in propidium iodide labeling in the FACS analysis, suggesting that cell viability of the cells was also not affected by the recombinant HCV core protein (data not shown). In addition, cell proliferation in different cell densities analyzed by 3H-thymidine incorporation did not show any relevant changes to basal proliferation (data not shown). We did not find any evidence that contact inhibition was the cause for unchanged proliferation rates.

HSCs Express TLR2

Next, we analyzed the expression of TLR2 on HSCs by flow cytometry. As described for other primary human and murine HSCs[28,29] incubation with an antibody against TLR2 resulted in considerable fluorescent staining of pHSC and LX-2 cells as compared with incubation with an isotype-matched control antibody (Figure 5a). TLR2 expression levels were stable and not altered by the duration of culture or by culture in serum-containing or -depleted media.

Induction of Fibrogenic Genes is Inhibited by Blockade of the TLR2 Receptor

As HCV core protein has been shown to interact with TLR2, we hypothesized an activation of fibrogenic genes by HCV core protein via TLR2. Therefore, LX-2 cells were stimulated with HCV core protein after preincubation with an TLR2-blocking antibody. Blocking of TLR2 prevented induction of the *COL1A1* by HCV core protein ($P=0.0264$; Figure 6), whereas a control antibody had no effect. Denaturing HCV core protein by heat inactivation abolished its stimulating effect on LX-2 cells, suggesting a conformation-dependent interaction (Figure 2). LPS, a TLR4 ligand,[30] did not induce *COL1A1* upregulation, thus excluding inadvertent activation of LX-2 cells by endotoxin contamination (Figure 2).

HCV Core Protein Stimulation Leads to Increased Phosphorylation of TLR2-Dependent Signaling Proteins

Next, we examined the signaling mechanisms downstream of TLR2 activation. Therefore, cell extracts were prepared for western blot analysis after incubation of LX-2 cells with HCV core protein. Immunoblotting with an antibody directed against the phosphorylated form of the ERK1/2 MAPK revealed that phosphorylation of these proteins was increased in HCV core protein-stimulated LX-2 cells ($P<0.05$, Figure 7a). On the contrary, blotting with an antibody

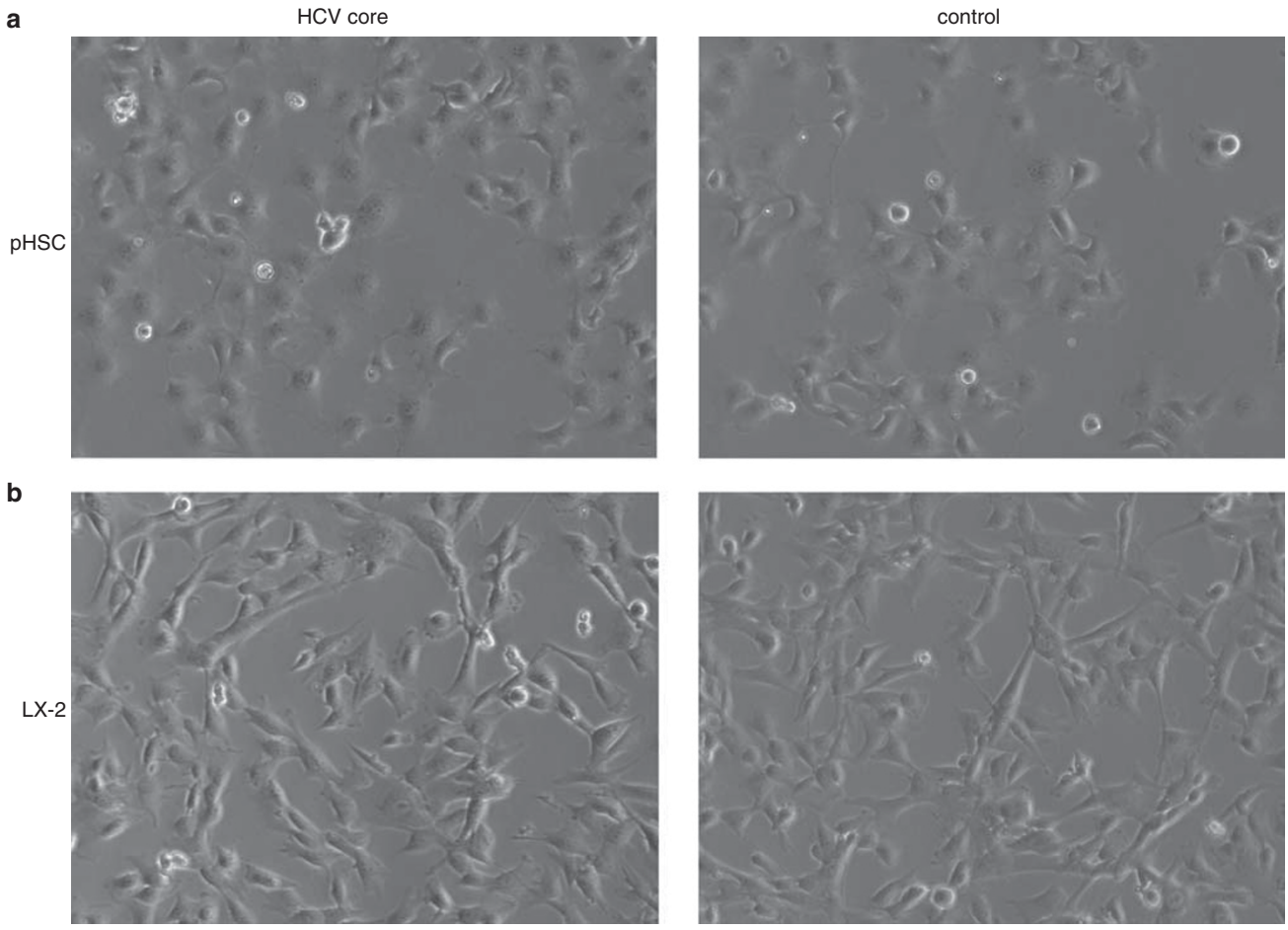

Figure 4 Morphology of pHSC and LX2 cells is not changed by stimulation with HCV core protein. The figure shows representative microscopic examinations of pHSC (**a**) and LX-2 cells (**b**) after stimulation with 3 μg/ml HCV core protein (left) compared with untreated controls (right) demonstrating no relevant morphological changes.

detecting both phosphorylated and unphosphorylated ERK1/2 indicated that the amount of total ERK protein had not changed (data not shown). Thus, the HCV core protein induced increase of ERK1/2 phosphorylation was not the result of an altered abundance of ERK1/2 protein. Similarly, HCV core protein stimulation resulted in increased phosphorylation of p38 and JNK MAPKs ($P<0.05$, Figure 7b and c). Inhibition of TLR2 activation with a blocking antibody specifically prevented phosphorylation of the MAPK signaling molecules confirming involvement of the TLR2-dependent pathways.

DISCUSSION

HSCs have a key role in the development of fibrosis in chronic liver disease because they synthesize and secrete extracellular matrix proteins, chemokines and other factors contributing to an inflammatory environment and fibrotic tissue remodeling in the liver.[34] Although chronic HCV infection is a major cause of liver fibrosis little is known about HCV-specific steps in the pathogenesis of liver fibrosis.[35] As it has been shown that HCV proteins can directly activate HSCs,[28] the aim of this study was to further characterize the activation of human HSC by HCV core protein. Here we demonstrate that the interaction of HCV core protein with TLR2 on human HSCs results in the downstream induction of TLR2-dependent intracellular signaling pathways and leads to increased expression of the profibrogenic genes *COL1A1*, *ACTA2*, *MMP2*, *MMP13*, *TIMP1*, *TIMP2*, *TGF-β1*, *CSRP2* and *GFAP*. To our knowledge, this is the first report showing a direct interaction of HCV core protein with HSCs via TLR2.

Increased expression of fibrogenic genes in pHSC and LX-2 cells induced by recombinant HCV core protein was a specific effect, because the HCV envelope protein E2 interacting with CD81 as well as LPS did not induce increased expression of fibrogenic genes. Furthermore, activation of HSC by HCV core protein showed a dose-dependent kinetics. In addition, LX-2 cells could be stimulated by a truncated core protein narrowing the critical sequence for TLR2 interactions to amino acids 1 and 115 of the HCV core protein

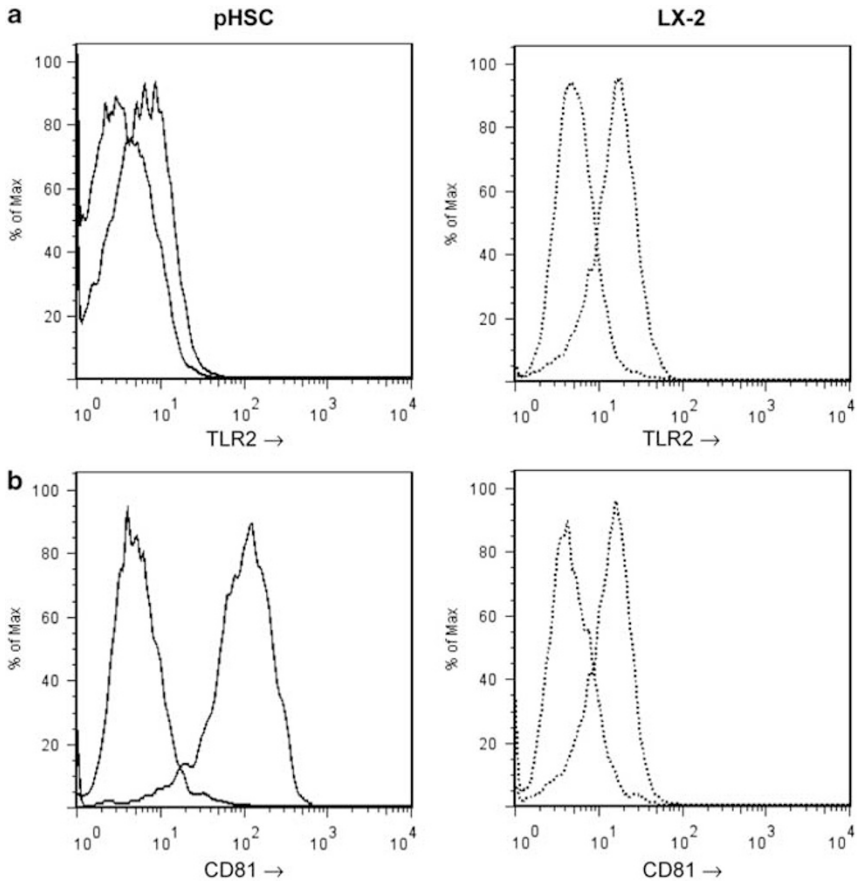

Figure 5 Human pHSC and LX-2 cells express toll-like receptor 2 (TLR2) and CD81. Flow cytometric analysis shows expression of TLR2 (**a**) and CD81 (**b**) by pHSC (black line) and LX-2 cells (dotted line) compared with the isotype-matched control antibodies.

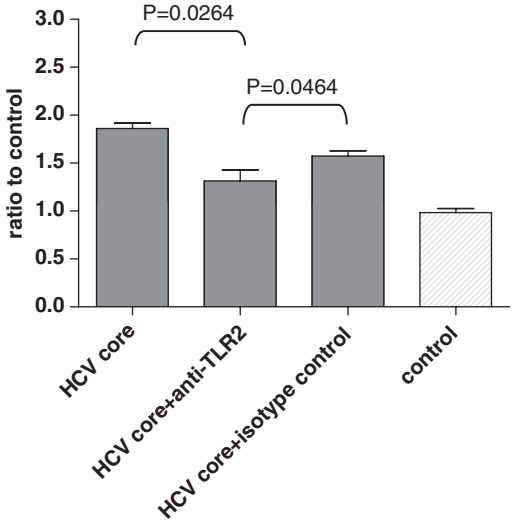

Figure 6 Induction of COL1A1-mRNA expression is inhibited by blockade of the TLR2 receptor. Serum-starved LX-2 cells were stimulated with HCV core protein alone and after preincubation with the neutralizing TLR2-antibody (TL2.1), respectively. The figure shows the mRNA-expression levels from three independent experiments. Increase in gene expression of COL1A1 is shown in comparison with untreated controls.

in line with previous data.[30] The dimension of the effects seen in our experiments correspond well with previous works analyzing the induction of *COL1A1*,[28,36] *MMP2* and *TIMP1*.[37]

The fibrogenic effects could be demonstrated for both, human primary HSCs and the permanent human HSC line LX-2. Cultured primary cells represent an authentic target to characterize physiological and pathological reactions in liver fibrosis. However, they lack unlimited access, and exhibit considerable amount of heterogeneity and a limited life span.[38] As characterized by Xu *et al*[33] immortalized LX-2 cells retain key features of human HSCs in tissue culture making them a suitable model for cell culture-based studies of human hepatic fibrosis providing a consistent state of activation in order to obtain reproducible and reliable results.[39] However, LX-2 cells constitute a constantly proliferating cell that does not fully reflect HSC in the quiescent state. For this reason we also studied primary human HSCs. Of note, primary HSC and LX-2 cells both express CD81 and TLR2 (Figure 5), surface molecules that can bind HCV proteins and exhibited similar gene activation patterns on exposure to HCV core protein. This finding strengthens the concept that HCV core protein acts directly on human HSC.

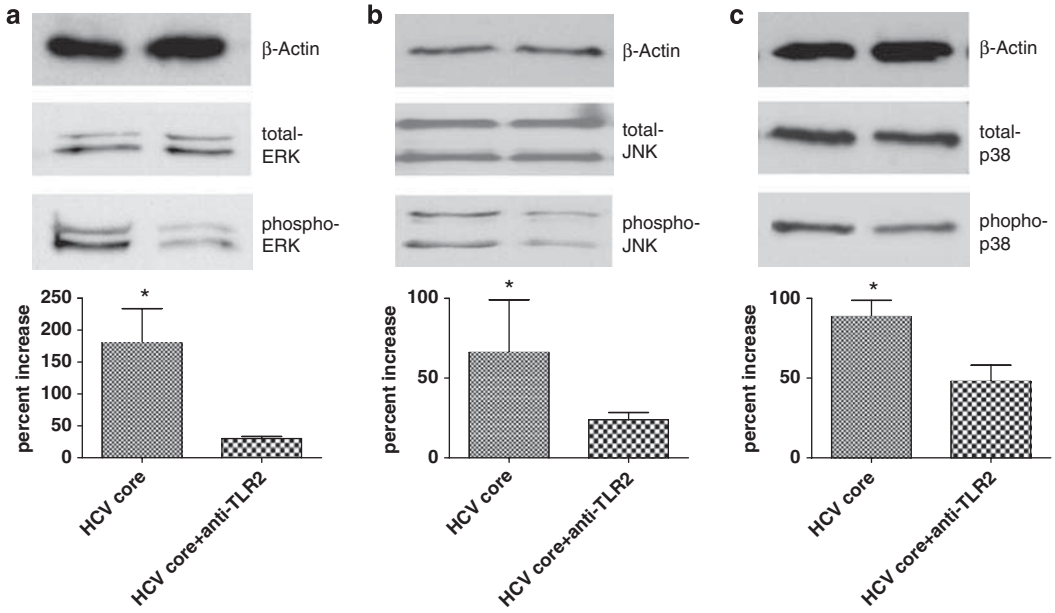

Figure 7 HCV core protein activates TLR2-dependent signaling pathways involving ERK1/2 (**a**), JNK (**b**) and p38 MAPK (**c**). Serum-starved LX-2 cells were stimulated with 3 μg/ml HCV core protein for 15 min with and without anti-TLR2 antibody respectively. Levels of phospho-ERK1/2 (**a**), phospho-JNK (**b**) and phopho-p38 MAPK (**c**) were determined by western blot analysis using 20 μg of total cellular protein. A representative blot and a densitometric analysis of three independent experiments are shown. Increase in protein levels are shown by percentage in comparison with untreated controls. $^*P<0.05$.

Previous own findings and a report published by Dolganiuc *et al*[29] also suggested activation of TLR2 by HCV core protein.[30] Human primary HSCs LX-2 both express TLR2. Of note, unlike the isotype control incubating HSC with a neutralizing TLR2 antibody prior to HCV core, protein exposure blocked mRNA upregulation of profibrogenic genes in LX-2 cells confirming that gene activation was triggered by the interaction of HCV core protein with TLR2.

Bataller *et al*[28] previously reported that HCV core protein transduced into HSC by adenoviral vectors induce profibrogenic effects in this cell type, and in particular increased expression of procollagen type I. Independently from the possibility that HCV might enter into HSC to exert the above effects, our data suggest that its core protein may cause fibrogenic effects also by interacting with TLR2 on the plasma membrane. Bataller *et al* also demonstrated activation of intracellular signal transduction pathways, but in contrast to our results failed to demonstrate upregulation of collagen mRNA in rat HSC in response to HCV core protein. Apart from species-specific differences discrepancies to our findings could also be the result of the lower concentration of 10 ng/ml of HCV core protein which they used in their experiments.

HCV core protein is detectable in the serum of chronically HCV-infected patients at concentrations of 100–200 pg/ml,[40,41] and much higher concentrations may be assumed in states of high viral replication such as for instance occurs in fibrosing cholestatic hepatitis.[42] Thus, under such conditions HSC are likely to become exposed to locally high concentrations of HCV proteins which can directly trigger fibrogenesis in HSC via TLR2-pathways independently from any tissue damage.

It has been reported that activation of TLR2 increases activity of ERK1/2, p38 and JNK MAPK. Accordingly our western blot analysis indicated that HCV core protein leads to increased phosphorylation of ERK1/2, p38 and JNK in LX-2 cells. Blocking TLR2 with a TLR2-specific antibody in turn reversed the increased phosphorylation confirming that HCV core protein exerts its effects on fibrogenic genes via TLR2 activation.

In line with our results, Bataller *et al* found increased phosphorylation of ERK1/2 in HSC. However, in contrast to our experiments, these authors did not find HCV-induced phophorylation of JNK and p38 MAPK. As stated above, such differences might reflect differences in the experimental settings, eg, differences in species. Considering the many different functions and effects of the MAPK pathways, it must be assumed that HCV core protein can exert diverse effects in the liver. Thus, the role of HCV proteins in chronic HCV infection needs further investigation. On the other hand, fibrogenic effects induced by the activation of MAPK pathways have been shown for a variety of different other stimuli that converge at the level of transcription of fibrogenic genes.[36,43] Thus, direct activation of fibrogenesis by HCV core protein may have considerable pathophysiological relevance.

In summary, we provide evidence that HCV core protein induces HSC activation in a TLR2-dependent manner. These results suggest that HCV core protein can directly contribute to the hepatic fibrogenesis in HCV-infected

patients. The results introduce a new aspect of HCV biology potentially relevant for the understanding of the pathogenesis of HCV-induced liver fibrosis and a basis for possible new antifibrotic therapy strategies.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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