Hepatitis C virus-induced furin and thrombospondin-1 activate TGF-β1: Role of TGF-β1 in HCV replication

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In this study, we demonstrated the molecular mechanisms of TGF-β1 induction as well as proteolytic activation in HCV (JFH-1)-infected cells. Our studies showed the synthesis and secretion of TGF-β1 in HCV-infected cells which was reduced in the presence of Ca2+ chelators, an inhibitor of mitochondrial Ca2+ uptake, and antioxidants. We also showed that the expression of HCV NS proteins NS3/4A, and NS5A can induce TGF-β1 by cell-based luciferase assay. Furthermore, mutational analysis revealed that the functionally active protease domain of NS3 and N-terminus domain of NS5A are required for TGF-β1 activity. Using siRNA approach we demonstrated that HCV-induced furin and thrombospondin-1 (TSP-1) are involved in the proteolytic activation of TGF-β1. Our results also suggest that TGF-β1 positively regulates HCV RNA replication. Collectively, these observations provide insight into the mechanism of TGF-β1 activation, which likely manifest in liver fibrosis associated with hepatitis C infection.

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Introduction

Hepatitis C virus (HCV) often causes persistent infection in humans, which may lead to chronic hepatitis in up to 60–80% of infected adults and can progress to liver fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (Di Bisceglie, 1997). HCV is an enveloped, single-stranded, positive-sense RNA virus which is approximately 9.6 kb in length, and encodes a single polyprotein of about 3000 amino acids (Bartenschlager and Lohmann, 2000). The viral polyprotein is cleaved by host and viral proteases, into three structural (core, E1 and E2) and seven non-structural (p7, NS2, NS3–NS5A/B) proteins (Grakoui et al., 1993; Lohmann et al., 1996). The single open reading frame (ORF) is flanked by 5′- and 3′-nontranslated regions (NTRs), which have been shown to be essential in both initiation of translation and viral RNA replication (Bartenschlager and Lohmann, 2000). Previously, the studies of molecular mechanisms of HCV replication and pathogenesis have been hampered by the lack of an efficient cell culture system and a suitable small-animal model. The development of a robust and productive HCV (genotype 2a) infection system has provided a major breakthrough which allows the production of infectious virus in cell culture (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

Calcium-mediated mitochondrial dysfunction has been suggested to play an important role in HCV-induced liver disease pathogenesis (Piccoli et al., 2007). Previously, we have shown that HCV gene expression in the endoplasmic reticulum (ER) induces ER stress with depletion of ER Ca2+ levels (Benali-Furet et al., 2005; Tardif et al., 2004, 2005). The concentration of Ca2+ released from the ER may be high enough to switch on the “low affinity” mitochondrial uniporter (Rizzuto and Pozzan, 2006). The uptake of Ca2+ by the mitochondria subsequently results in the generation of reactive oxygen species (ROS) (Waris et al., 2002). The elevated levels of ROS have emerged as a key player in the progression of HCV-induced liver disease pathogenesis (Machida et al., 2006; Pal et al., 2010). Previously, we have shown that HCV gene expression in the ER induces oxidative stress through deregulated Ca2+ signaling in the ER (Burdette et al., 2010; Gong et al., 2001; Tardif et al., 2005). Several HCV proteins including core, NS3, NS5A, and HCV subgenomic replicon have been shown to induce ROS in human hepatoma cells (Bureau et al., 2001; Gong et al., 2001; Kasprzak and Adamek, 2008; Machida et al., 2006; Okuda et al., 2002; Waris et al., 2005). ROS is known to up-regulate the synthesis of TGF-β1 and collagen gene expression, hallmarks of liver fibrosis.

The molecular mechanisms underlying liver injury and fibrosis in chronic HCV remain unclear. It has been postulated that immune-mediated damage is linked to fibrosis, where cytokines including TGF-β1 play a prominent role (Schuppan et al., 2003). TGF-β1 is a pleiotropic cytokine that plays a role in tumor suppression as well as tumor progression (Bissell et al., 2001). Most tumors progress and metastasize in the presence of high levels of TGF-β1. It has been reported that HCV infection is associated with a significant increase in TGF-β1 expression in both serum and liver (Grungreiff et al.,...
of TGF-β1 activation in the context of HCV-infection. To confirm that HCV-infected cells secrete TGF-β1, cell culture supernatant was collected from mock and HCV-infected Huh-7 cells and subjected to TGF-β1-specific enzyme-linked immunosorbent assay (ELISA) analysis. The results revealed the secretion of TGF-β1 (724 pg/ml) at day 2 postinfection (Fig. 1C) and peaked at day 3 postinfection (1453 pg/ml) compared to cell culture supernatant collected from mock-infected Huh-7 cells at days 1, 2, and 3 (Fig. 1C).

To determine whether HCV infection induces TGF-β1 mRNA expression, total cellular RNA was extracted from mock-infected and HCV-infected Huh-7 cells and the level of TGF-β1 mRNA was quantified by real-time RT-PCR. The results showed an increase in TGF-β1 mRNA levels in Huh-7 cells infected with HCV in a time-dependent manner and peaked at day 3 (about 3.6 fold) compared to Huh-7 cells mRNA collected at days 1, 2, and 3 (Fig. 1D). Taken together these results clearly indicate that HCV-infection in Huh-7 cells induces transcriptional stimulation, synthesis, and secretion of bioactive TGF-β1.

Role of HCV proteins on TGF-β1 induction

In order to identify the HCV protein(s) responsible for TGF-β1 induction, we performed TGF-β1 promoter-luciferase reporter assay. Huh-7 cells were transiently-transfected with wild-type TGF-β1 promoter-luciferase reporter (pH1G1 – 1362/+1) along with various HCV protein expression vectors and were subjected to dual-luciferase assay. We observed an increase in TGF-β1 promoter-luciferase activity by core (~2.5 fold), NS3 (~2.5 fold), NS3/4A (~4 fold), and NS5A (~4 fold) (Fig. 2A). In contrast, the expression of HCV E1/E2, NS4B, and NS5B did not show any significant effect on TGF-β1 promoter-luciferase activity.

To determine if HCV protein expression can induce TGF-β1 secretion, cell culture supernatant was collected and subjected to TGF-β1 specific ELISA analysis. The results show the increased secretion of TGF-β1 in the cell culture supernatant of Huh-7 cells transfected with core (375 pg/ml), NS3 (474.56 pg/ml), NS3/4A (774 pg/ml), NS4B (275 pg/ml), and NS5A (574 pg/ml) (Fig. 2B). The expression of E1/E2 and NS5B did not induce TGF-β1 secretion (Fig. 2B). To determine the expression of various HCV proteins in Huh-7 cells, cellular lysates were immunoblotted for respective HCV proteins (Fig. 2C). These results suggest that among HCV NS proteins NS3/4A and NS5A are critical in TGF-β1 induction and secretion.

Effect of NS3/4A mutations on TGF-β1 induction

To identify the domain(s) of NS3/4A which is responsible for TGF-β1 promoter activity, deletion and point mutations of NS3/4A were used in this study (Li et al., 2005). Huh-7 cells were transfected with wild-type TGF-β1 promoter-luciferase reporter along with the wild-type pNS3, pNS3/4A, or mutant expression vectors pNS3/4A (Δ1487–1501) and pNS3/4A (S1156A). Cellular lysates were collected and subjected to dual-luciferase assay. The results indicate approximately 4 fold increase in NS3/4A mediated TGF-β1 promoter activity which was decreased in the presence of NS3/4A deletion or point mutations (Fig. 3A). These results suggest that proteolytically active NS3/4A complex is required to activate TGF-β1 promoter.

To determine the effect of NS3/4A mutations on TGF-β1 secretion, cell culture supernatants were collected and subjected to TGF-β1 ELISA analysis. The results show the increased secretion of TGF-β1 in the cell culture supernatant of Huh-7 cells transfected with NS3 (453 pg/ml), NS3/4A (631 pg/ml), pNS3/4A (Δ1487–1501) (382 pg/ml) and pNS3/4A (S1156A) (334 pg/ml) (Fig. 3B). These results indicate that HCV NS3 alone or NS3/4A mutants were unable to induce TGF-β1 secretion as efficiently as HCV NS3/4A protein. The expressions of wild-type and mutant NS3/4A proteins were shown
by western blotting (Fig. 3C). The expression of NS3/4A (Δ1487–1501) was low and a similar expression pattern was observed previously (Johnson et al., 2007).

**Effect of NS5A mutations on TGF-β1 induction**

To determine the region(s) of NS5A which is involved in induction of the TGF-β1 promoter, various NS5A deletion mutations were used (Gong et al., 2001). Huh-7 cells were transfected with wild-type TGF-β1 promoter luciferase construct along with the wild-type or mutant NS5A expression vectors. The NS5A mutant, pCNSM1 is a N-terminal deletion mutant, pCNSM3 is a C-terminal deletion mutant. Cellular lysates were collected and subjected to dual-luciferase assay. The results indicate an approximately 4 fold increase in wild-type NS5A-mediated TGF-β1 promoter activity (Fig. 3E), which was efficiently reduced in the presence of pCNSM1; however, pCNSM3 did not affect the TGF-β1 promoter activity. These results suggest that the N-terminal 163 amino acids (aa) of NS5A are important for activation of the TGF-β1 promoter reporter.

To determine the effect of NS5A mutations on TGF-β1 secretion, cell culture supernatants were collected and subjected to TGF-β1 specific ELISA analysis. The results show the increased secretion of TGF-β1 in the cell culture supernatant of Huh-7 cells transfected with NS5A wild-type (600 pg/ml), and pCNSM3 (495 pg/ml). The NS5A mutant pCNSM1 (200 pg/ml) was impaired in inducing secreted TGF-β1 (Fig. 3F). The expression of wild-type NS5A (58 kDa), pCNSM1 (31 kDa), and pCNSM3 (18 kDa) was shown by western blotting (Fig. 3G).

**Role of HCV-induced Ca²⁺ signaling and elevation of ROS on TGF-β1 induction**

To determine if HCV-induced Ca²⁺ efflux from the ER and induction of ROS in the mitochondria play a key role in TGF-β1 induction, we first established that HCV-infection induces ROS via Ca²⁺ signaling in the ER. Mock-infected and HCV-infected cells were incubated with calcium chelators (BAPTA-AM, or TMB-8), an inhibitor of mitochondrial Ca²⁺ uptake (ruthenium red) and were assayed for ROS by flow cytometry. The results show an increase in ROS in HCV-infected cells, which was reduced in the presence of BAPTA-AM, TMB-8, or ruthenium red, (Fig. 4A). Mock-infected cells treated with these inhibitors did not show any effect. Huh-7 cells incubated with hydrogen peroxide were used as a positive control (Fig. 4B). To further confirm the induction of ROS through Ca²⁺ signaling, cells were visualized by microscopy. The results show an increase in ROS in HCV-infected cells, which was reduced in the presence of BAPTA-AM, TMB-8, or ruthenium red (Fig. 4C). Mock-infected cells treated with these inhibitors did not show any effect. Huh-7 cells incubated with hydrogen peroxide were used as a positive control (Fig. 4B). To further confirm the induction of ROS through Ca²⁺ signaling, cells were visualized by microscopy. The results show an increase in ROS in HCV-infected cells, which was reduced in the presence of BAPTA-AM, TMB-8, or ruthenium red (Fig. 4C). The expression of HCV core represents the HCV-infection (Fig. 4D, lane 2). These results suggest that HCV-mediated Ca²⁺ signaling in the ER induces ROS production in the mitochondria.
To determine the effect of Ca\(^{2+}\) signaling and elevation of ROS on wild-type TGF-\(\beta\)\(_1\) promoter luciferase activity, mock-infected and HCV-infected Huh-7 cells were transfected with wild-type TGF-\(\beta\)\(_1\) promoter-luciferase reporter. The cells were incubated with non-toxic doses of specific Ca\(^{2+}\) chelators (BAPTA-AM, and TMB-8), specific inhibitors of mitochondrial Ca\(^{2+}\) uptake (ruthenium red), antioxidants (PDTC) and an inhibitor of ROS generated through NADPH oxidase system (DPI). The results show a 5 fold increase in TGF-\(\beta\)\(_1\) promoter activity by HCV infection (Fig. 5A) which was decreased in HCV-infected cells treated with BAPTA-AM, ruthenium red, or TMB-8. However, treatment with EGTA (an extracellular Ca\(^{2+}\) chelator) did not show significant reduction of wild-type TGF-\(\beta\)\(_1\) promoter activity (Fig. 5A). Similarly, a decrease of TGF-\(\beta\)\(_1\) promoter activity in HCV-infected cells treated with antioxidant PDTC was observed (Fig. 5A). HCV-infected cells incubated with DPI did not reduce the TGF-\(\beta\)\(_1\) promoter activity. These inhibitors did not show any effect on TGF-\(\beta\)\(_1\) promoter activity in mock-infected cells (data not shown).

To further strengthen these results, we determined the effect of Ca\(^{2+}\) signaling and elevation of ROS on endogenous TGF-\(\beta\)\(_1\) mRNA expression. Mock-infected and HCV-infected cells were incubated with various inhibitors as described above. The results show an ~4.5 fold increase in TGF-\(\beta\)\(_1\) mRNA expression by HCV infection (Fig. 5B) which was reduced in HCV-infected cells treated with BAPTA-AM, ruthenium red, or TMB-8 (Fig. 5B). However, treatment with EGTA did not show a significant reduction of TGF-\(\beta\)\(_1\) mRNA expression. Similarly, a decrease of TGF-\(\beta\)\(_1\) mRNA expression in HCV-infected cells treated with antioxidants PDTC and NAC was observed (Fig. 5B) but not with DPI treatment. These results suggest that HCV-mediated Ca\(^{2+}\) signaling in the ER is critical for the generation of ROS in the mitochondria which plays a key role in the activation of TGF-\(\beta\)\(_1\) promoter and expression of endogenous TGF-\(\beta\)\(_1\) mRNA.

**Effect of HCV-induced proprotein convertases on proteolytic processing of TGF-\(\beta\)\(_1\)**

There are numerous proprotein convertases that have been shown to proteolytically activate TGF-\(\beta\)\(_1\) (Annes et al., 2003). To determine if HCV-infection induces the expression of potential proprotein convertases, total cellular RNA was harvested from mock-infected and HCV-infected cells and quantitative RT-PCR was performed using primers directed against potential proteases such as furin, thrombospondin-1 (TSP-1), matrix metalloproteinase-9 (MMP-9) and calpain. The results show the induction of furin (3 fold) and TSP-1 (5 fold) mRNA in HCV-infected cells (Fig. 6A). The induction of calpain and MMP-9 mRNA was not affected. To determine the protein expression, cellular lysates and cell culture supernatant were collected from mock-infected and HCV-infected cells and subjected to immunoblot analysis. The results showed an increase in furin protein expression (Fig. 6B) and secretion of TSP-1 in HCV-infected cells compared to mock-infected Huh-7 cells (Fig. 6B). To determine the proteolytic activation of TGF-\(\beta\)\(_1\) in HCV-infected cells, cellular lysates were immunoblotted using antibody against TGF-\(\beta\)\(_1\). The results displayed induction and proteolytic cleavage of TGF-\(\beta\)\(_1\) into mature form in HCV-infected cells (Fig. 6B, lane 2). These results show that HCV-infection induces
propoprotein convertases which are potentially involved in the processing of latent TGF-β1 into bioactive TGF-β1. To further confirm the expression of furin in HCV-infected cells, mock-infected and HCV-infected cells were also subjected to immunofluorescence analysis using furin, TGF-β1, and HCV NS3 antibodies. The results show an increased expression of furin, and TGF-β1, in a time-dependent manner (Fig. 6C). We also observed the cytoplasmic localization of TGF-β1 with furin in HCV-infected cells (Fig. 6C). These results strengthen the notion that furin is induced by HCV infection and plays an important role in the proteolytic processing of latent TGF-β1 into bioactive form.

In order to demonstrate that HCV-induced furin or TSP-1 have an effect on the proteolytic activation and subsequent secretion of TGF-β1, mock-infected and HCV-infected cells were transfected with siRNA directed against furin, TSP-1, TGF-β1, and GFP. To determine the effect of the specific siRNA on the target gene expression, total cellular RNA was collected and subjected to quantitative RT-PCR. Fig. 7A shows a 50% decrease in furin mRNA expression after siRNA transfection, an 80% decrease in TGF-β1 mRNA, and a 90% decrease in TSP-1 mRNA expression. Cell culture supernatant from these siRNA transfected cells were collected and subjected to TGF-β1-specific ELISA analysis. The results show an increase in the secretion of TGF-β1 which was reduced in HCV-infected cells transfected with siRNA against TGF-β1, furin, or TSP-1 (Fig. 7B). GFP siRNA was used as a negative control. The detection of TGF-β1 in the cell culture supernatant by this method does not differentiate between bioactive and inactive TGF-β1.

**HCV-infection induces secretion of bioactive TGF-β1**

The bioactive TGF-β1 protein in cell culture supernatant was quantified using a standard growth inhibition assay with mink lung epithelial cells (MLEC) as described previously (Abe et al., 1994; Schulze-Krebs et al., 2005). In this assay, MLEC stably transfected with the PAI/L demonstrate a dose-dependent increase in luciferase activity which indirectly corresponds to growth inhibition. MLEC were incubated with cell culture supernatant from siRNA transfected mock-infected and HCV-infected cells and subjected to luminescence assay. The results show increased luciferase activity in HCV-infected cells, which was reduced in HCV-infected cells transfected with TGF-β1, furin, or TSP-1 (Fig. 7C). These results suggest that HCV infection induces secretion of bioactive TGF-β1 through furin and TSP-1.

Effect of furin, TSP-1, and TGF-β1 on HCV replication

To evaluate the effect of furin, TSP-1, and TGF-β1 on HCV RNA replication in HCV infected cells, we used RNA interference approach as described in Fig. 7. Total cellular RNA was extracted from various

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**Fig. 3.** (A) Effect of NS3/4A on TGF-β1 promoter luciferase activation. Huh-7 cells were transfected with wild-type TGF-β1 promoter luciferase reporter (pHTG−1362/+1), and NS3, NS3/4A, NS3/4A (Δ1487−1501), and (S1156A) protein expression vectors or vector control. At 36 h posttransfection, cellular lysates were subjected to dual luciferase assay. The values represent the means±standard deviations of three independent experiments performed in triplicate. * denotes p<0.05 compared to vector control transfected cells. ** denotes p<0.05 compared to wild-type NS3/4A. (B) Secretion of TGF-β1 in Huh-7 cells transfected with wild-type HCV NS3, NS3/4A and mutant expression vectors. Cell culture supernatant was harvested and total secreted TGF-β1 protein was determined by ELISA. The values represent the means±standard deviations of three independent experiments performed in triplicate. * denotes p<0.05 compared to vector control transfected cells. ** denotes p<0.05 compared to wild-type NS3/4A. (C) The expression of wild-type and mutant NS3/4A. Cellular lysates from Huh-7 cells transfected with NS3, NS3/4A, NS3/4A (Δ1487−1501), and NS3/4A (S1156A) were subjected to immunoblot analysis using Flag antibody. (D) Schematic of NS3/4A domains. NS3 is composed of two domains; a serine protease domain and a nucleoside triphosphatase/DExD/H box helicase domain. Numbers above the drawing represent the amino acids number of the HCV polyprotein. The star on the NS3/4A S1156A construct represents the serine 1165 (of the catalytic triad) mutation to alanine, which ablates protease activity. (E) Effect of HCV NS5A on TGF-β1 induction. Huh-7 cells were transfected with wild-type TGF-β1 promoter luciferase reporter (pHTG−1362/+1), and HCV NS5A and mutant expression vectors or vector control. At 36 h posttransfection, cellular lysates were subjected to dual luciferase assay. The values represent the means±standard deviations of three independent experiments performed in triplicate. * denotes p<0.05 compared to vector control transfected cells. ** denotes p<0.05 compared to wild-type NS5A. (F) Secretion of TGF-β1 in Huh-7 cells transfected with wild-type and mutant HCV NS5A. Total secreted TGF-β1 protein in cell culture supernatant was determined by ELISA. The values represent the means±standard deviations of three independent experiments performed in triplicate. * denotes p<0.05 compared to vector control transfected cells. ** denotes p<0.05 compared to wild-type NS5A. (G) The expression pattern of wild-type and mutant NS5A proteins. Cellular lysates were subjected to immunoblot analysis using Flag antibody. (H) Schematic of NS5A wild-type and deletion mutants. The dotted lines represent the extent of deletion of NS5A sequences.
cells and subjected to quantitative RT-PCR analysis using HCV-specific primers and Taqman probe. We observed an increased replication of HCV RNA in HCV-infected cells (Fig. 8A), which was significantly reduced in HCV-infected cells in the presence of TGF-β1 siRNA, TSP-1 siRNA or furin siRNA, (Fig. 8A). However, transfection of GFP siRNA (negative control) in HCV infected cells did not show any change in HCV replication (Fig. 8A).

To evaluate the effect of the HCV RNA replication and transfection of cells with TGF-β1, furin, or TSP-1 siRNA on cell proliferation, siRNA transfected, mock-infected and HCV-infected cells were subjected to MTT assay. The results show a proliferative effect of HCV replication in Huh-7 cells, but there was no significant proliferative effect after siRNA transfections (Fig. 8B).

Discussion

The molecular mechanisms underlying liver fibrosis in chronic hepatitis C are not clearly understood. TGF-β1 is a multifunctional cytokine involved in the regulation of cell proliferation, differentiation and liver fibrogenesis (Jennings and Pietenpol, 1998; Kaminska et al., 2005). Deregulation of TGF-β1 expression has been implicated in the pathogenesis of various diseases including liver fibrosis. TGF-β1 overexpression is frequently observed in human HCC (Rossmanith and Schulte-Hermann, 2001). TGF-β1 can play a role of tumor suppression as well as tumor progression (Bissell et al., 2001). There is growing evidence that in the later stages of cancer development TGF-β1 is actively secreted by tumor cells and contributes to cell growth, invasion and metastasis (Jennings and Pietenpol, 1998). Previous studies have shown that HCV-infection is associated with significant increase in inflammatory cytokines and growth factors including TGF-β1 in both serum and liver (Blackard et al., 2006; Nelson et al., 1997). It has been postulated that the host T-cell immune response to HCV-infected hepatocytes is associated with release of cytokines and growth factors, such as TGF-β1, TNF-α, PDGF, and IL-6, which activate hepatic stellate cells to secrete extracellular matrix components.

Most of the studies related to fibrosis in the context of HCV have been conducted in HSCs. In the absence of inflammation, liver TGF-β1 is secreted from HSC and Kupffer cells but not from hepatocytes. However, upon inflammation or liver injury, hepatocytes gradually become a major source of TGF-β1 (Canbay et al., 2004; Gao et al., 1996; Jeong et al., 2004; Takehara et al., 2004). The increased levels of hepatic TGF-β1 may enhance progression of liver fibrosis in patients with HCV (Wilson et al., 2006). HCV infects only hepatocytes in the liver and induces chronic inflammation. No other nonparenchymal liver cells are targets for HCV (Bataller et al., 2004). The possibility that HCV-infected hepatocytes themselves produce profibrogenic cytokines has not been explored in detail. Previously, HCV has been shown to induce TGF-β1 gene expression in human hepatoma cell lines (Taniguchi et al., 2004). However, the molecular mechanisms of TGF-β1 induction and posttranslational processing in the context of HCV-infection are unclear. Recently, the intracellular processing of latent TGF-β1 before secretion has been reported in liver tissue and primary hepatocytes treated with carbon tetrachloride (CCL4) (Hosui et al., 2009).

In the present study, we investigated the molecular mechanisms of TGF-β1 synthesis and its proteolytic activation using HCV cell culture infection system. We observed that HCV-infection can induce and secrete TGF-β1 in a time-dependent manner. This is consistent with the previous studies in which HCV JFH-1 cell culture infection system has been shown to induce TGF-β1 (Lin et al., 2010). The HCV structural protein core and the nonstructural (NS) proteins NS3, NS4B, and NS5A have been implicated in various signal transduction pathways (Gouttenoire et al., 2001; Johnson et al., 2007; Li et al., 2009; Macdonald and Harris, 2004). Previously, HCV core protein has been shown to induce TGF-β1 in cultured cells (Taniguchi et al., 2004). However, the determinants of HCV NS proteins responsible for TGF-β1 induction have not been explored. The results described here show that HCV NS3/4A is able to induce the TGF-β1 promotor, implying that NS4A (a cofactor of NS3) is required to form a functionally active protease domain. However, pNS3/4A (Δ1487–1501) has little effect on wild-type TGF-β1 promoter activation (Fig. 3A) as the mutation is outside the protease domain, however the
pNS3/4A (S1156A) mutation showed a greater decrease of TGF-β1 promoter activity because it results in an inactive NS3/4A protease region. These results are consistent with the studies demonstrating the role of various NS3 constructs along with cofactor NS4A in inhibiting host antiviral signaling (Johnson et al., 2007).

HCV NS5A is part of the replication complex that catalyses replication of the viral genome (Bartenschlager and Lohmann, 2000). NS5A has the potential to regulate not only interferon responses but also many other cellular functions, such as mitogenic signaling, apoptosis, cell cycle and ROS signaling, by interacting with a variety of host proteins (Gong et al., 2001; Macdonald and Harris, 2004; Waris et al., 2002). Our results indicate that N′-terminal 163 amino acids are critical for the activation of TGF-β1. The N-terminal domain of NS5A forms a highly conserved amphipathic alpha helix and has been shown to associate with ER membrane and induce ER to nucleus signal transduction pathway which can lead to chronic inflammation and liver fibrosis (Brass et al., 2002; Gong et al., 2001; Waris et al., 2003). The N′-terminal deletion mutant (pCNSM1) did not associate with ER membrane and unable to activate TGF-β1, however, C-terminal deletion mutant (pCNSM3) was able to associate with ER and induce the activation of TGF-β1 (Figs. 3E–H).

HCV nonstructural proteins associate with the ER membrane in the reticular network of the perinuclear region and are believed to form a ribonucleoprotein complex along with the viral RNA genome that engages in RNA replication (Bartenschlager and Lohmann, 2000). HCV gene expression in the ER causes induction of ER stress (Gong et al., 2001). One of the consequences of the ER stress response is Ca2+ release from the ER, uptake of Ca2+ by the mitochondrial uniporter, followed by oxidative stress via elevation of ROS in the mitochondria (Burdis et al., 2010; Gong et al., 2001; Waris et al., 2007). Our results show that HCV-infection activates TGF-β1 via Ca2+ signaling and induction of oxidative stress. This is consistent with the previous observations that activation of TGF-β1 occurs under condition of oxidative stress (Lin et al., 2010). In contrast to these studies, according to our model, ROS is generated in the mitochondria through the assembly of HCV replicase complex in the perinuclear membrane of the ER. This association leads to Ca2+ efflux from the ER and generation of ROS (Fig. 9, model). Calcium-mediated mitochondrial dysfunction has been suggested to play an important role in HCV-induced liver disease pathogenesis (Piccoli et al., 2007). Our results clearly demonstrate the inhibition of TGF-β1 activity using antioxidants, PDTC and NAC but an insignificant decrease using DPI (an inhibitor of ROS generated through NADPH oxidase system), suggesting that ROS is generated through mitochondria but not through NADPH oxidase system.

TGF-β1 is synthesized as 55-kDa precursor polypeptides, which is cleaved in the cells by proteases to form bioactive TGF-β1 (Dubois et al., 1995). There are several mammalian proprotein convertases that can process pro-TGF-β1 into bioactive TGF-β1 (Dubois et al., 1995). Furin and TSP-1 are the best characterized members of the mammalian proprotein convertase family and are responsible for pro-TGF-β1 proteolytic processing (Annes et al., 2003). Furin and TSP-1 are upregulated in various cancers including hepatocellular carcinoma (Kazerounian et al., 2008; Paradis et al., 2005). In this study, we observed an increase in furin and TSP-1 expression (Figs. 6A–C). We also observed the decreased secretion of TGF-β1 from HCV-infected...
Huh-7 cells silenced with furin or TSP-1 specific siRNA, suggesting a potential role of furin and TSP-1 in TGF-β1 proteolytic processing. Furin is a Ca²⁺ dependent serine protease and in our model Ca²⁺ influx occurs from the ER in HCV-infected cells arguing that HCV-induced Ca²⁺ signaling induces furin which can lead to the proteolytic processing of latent TGF-β1 into bioactive TGF-β1 (Fig. 9, model). TSP-1 is a protein associated with tissue remodeling. In some tumor cells, a positive feedback loop between TSP-1 and TGF-β1 may exist as active TGF-β1 induces TSP-1 expression through several pathways (Kazerounian et al., 2008). Similar to furin, recent studies of a polymorphism in human TSP-1 suggest that Ca²⁺-induced conformational changes regulate some of TSP-1 physiological functions such as altered interactions of TSP-1 with various ligands (Calzada et al., 2008). These studies suggest that intracellular increases in Ca²⁺ levels can activate TSP-1 and/or furin, which can lead to proteolytic processing of TGF-β1 in HCV-infected cells.

Previous studies have shown the increase of respiratory syncytial virus and JC virus replication by TGF-β1 (McCann and Imani, 2007; Ravichandran et al., 2007). The role of TGF-β1 in HCV replication is not clearly defined. Recently, the stimulation as well as suppression of HCV replication by exogenous addition of TGF-β1 has been demonstrated in HCV replicon system (Lin et al., 2008; Murata et al., 2005). However, the regulation of HCV replication by endogenous TGF-β1 has not been studied. Recently, endogenous TGF-β1 has been shown to induce intracellular signaling pathways including activation of hypoxia inducible factor-1 (HIF-1) and direct interaction of TGF-β1 with STAT-5 leading to liver fibrosis (Hosui et al., 2009; McMahon et al., 2006). Our results show that furin, TSP-1, and TGF-β1 positively regulate HCV replication. We argue that the formation and proper processing of TGF-β1 through furin or TSP-1 in HCV-infected cells can positively regulate HCV replication that may include the activation of signaling pathways.

In summary, we show that ER stress mediated Ca²⁺ efflux followed by oxidative stress-induced stimulation and proteolytic activation of TGF-β1 in HCV infected cells positively regulate HCV replication. These studies provide greater insight into the role of HCV in liver fibrosis. The role TGF-β1 and intracellular signaling events in HCV infected cells provide the extensive knowledge regarding the mechanisms of liver fibrosis. The results of these studies open new avenues for alternative strategies in the treatment of chronic HCV infection associated with liver fibrosis.

Materials and methods

Expression plasmids

The TGF-β1 promoter luciferase reporter plasmid (pHTG1 − 1362/+ 11) was kindly provided by Dr. S. J. Kim (NCI, Bethesda, MD). The infectious JFH-1 cDNA (HCV genotype 2a) along with the replication-defective JFH-1/GND construct was obtained from Dr. T. Wakita (Tokyo, Japan). The infectious J6/JFH-1 cDNA (HCV genotype 2a) was obtained from Dr. C. Rice (Rockefeller University, New York City, NY).
The expression plasmids pFlag-NS3, pFlag-NS3/4A, pFlag-NS3/4A S1165A, and pFlag-NS3/4A Δ1487–1501 (ΔArg) were kindly provided by Dr. M. Gale (University of Washington, Seattle, WA) (Johnson et al., 2007). The wild-type HCV NS5A and various mutants, core, and NS5B expression vectors were obtained from Dr. A. Siddiqui (UC San Diego, CA), E1/E2 from Dr. J. Dubuisson (HHMI, France), and NS4B (Dr. K. Konan, Pennsylvania State University, PA).

Cell lines

The human hepatoma cell line Huh-7 was obtained from A. Siddiqui, UCSD, CA, and Huh7.5 subline was obtained from Dr. C. Rice, Rockefeller University, NY (Blight et al., 2002). These cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 U of penicillin/mL, and 100 μg of streptomycin sulfate/mL. For cells supporting subgenomic replicons, G418 (Geneticin; Gibco-BRL) at 750 μg/ml was added to the culture medium.

HCV cell culture infection system

The plasmid pFL-J6/JFH1 encoding the HCV J6/JFH-1 strain genome was transcribed and delivered into Huh7.5 cells by electroporation (Burdette et al., 2010; Zhong et al., 2005). The cell-free virus was propagated in Huh-7.5 cell cultures, as described previously (Lindenbach et al., 2005). The expression of HCV protein was analyzed using western blot assays. The HCV cell culture supernatant was collected at appropriate time points and used to infect naive Huh-7 cells at appropriate dilutions (moi = 1) for 5–6 h at 37 °C and 5% CO2 (Waris et al., 2007; Burdette et al., 2010). The viral titer in cell culture supernatant was expressed as focus forming unit (ffu) ml⁻¹, which was determined by the average number of HCV-NS5A-positive foci detected at the highest dilutions as described previously (Zhong et al., 2005).

Western blot and antibodies

Mock-infected and HCV-infected Huh-7 cells were harvested and cellular lysates were prepared by incubating in radioimmune precipitation (RIPA) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM sodium formate, and 10 μl/ml protease inhibitor cocktail) (Thermo Scientific, IL) for 30 min on ice. The samples were subjected to SDS-PAGE. Gels were electroblotted onto nitrocellulose membrane (Thermo Scientific, IL) in 25 mM Tris, 192 mM glycine and 20% methanol. Membranes were incubated for 1 h in blocking buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20, 5% dry milk], probed with primary antibody for 1 h at room temperature (RT) and washed twice for 10 min with blocking buffer without milk followed by incubation with secondary antibody for 1 h at RT. After an
Fig. 7. (A) Effect of siRNAs on target gene expression. Huh-7 cells were transfected with siRNAs against GFP, TGF-β1, furin, and TSP-1. Total cellular RNA was extracted, and quantitative RT-PCR was performed using furin, TSP-1, and TGF-β1 specific primers. The data shown here represent the means ± standard deviations of at least three independent experiments performed in triplicate. * denotes p < 0.05 compared to mock-infected cells transfected with control GFP siRNA. (B) Effect of TGF-β1, furin and TSP-1 siRNAs on TGF-β1 secretion. Mock-infected and HCV-infected Huh-7 cells were transfected with siRNA against TGF-β1, furin, and TSP-1 as described above. Cell culture supernatant was collected and the total secreted TGF-β1 protein was determined by ELISA. The data shown here represent the means ± standard deviations of at least three independent experiments performed in triplicate. * denotes p < 0.05 compared to mock-infected control cells. ** denotes p < 0.05 compared to HCV-infected cells. (C) Effect of TGF-β1, furin or TSP-1 siRNAs on bioactive TGF-β1 secretion. Mock-infected and HCV-infected Huh-7 cells were transfected with siRNA against TGF-β1, furin, or TSP-1 as described above. Cell culture supernatant was collected and subjected to growth inhibition assay using mink lung epithelial cells as described in Materials and methods. The data shown here represent the means ± standard deviations of at least three independent experiments performed in triplicate. * denotes p < 0.05 compared to mock-infected cells. ** denotes p < 0.05 compared to HCV-infected mock-transfected cells.

Fig. 8. (A) Effect of furin, TSP-1 and TGF-β1 siRNAs on HCV replication. Mock-infected and HCV-infected Huh-7 cells were transfected with furin, TSP-1, and TGF-β1 siRNAs as described above. Total cellular RNA was extracted and the levels of HCV RNA were determined by quantitative RT-PCR. The data shown here represent the means ± standard deviations of at least three independent experiments performed in triplicate. * denotes p < 0.05 compared to mock-infected control cells. ** denotes p < 0.05 compared to HCV-infected cells. (B) Effect of TGF-β1, furin and TSP-1 siRNAs on mock-infected and HCV-infected Huh-7 cell proliferation. Mock-infected and HCV-infected Huh-7 cells were transfected with siRNA against TGF-β1, furin, and TSP-1 as described above. At 48 h posttransfection cells were subjected to cell proliferation assay as described in Materials and methods. The data shown here represent the means ± standard deviations of at least three independent experiments performed in triplicate. * denotes p < 0.05 compared to mock-infected control cells.
Additional washing step with blocking buffer, immunoblots were visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

All the primary antibodies were used according to the manufacturer’s protocol: HCV NS3 (Virogen, Watertown, MA), HCV core (Affinity BioReagents, Rockford, IL), HCV E1/E2 (a kind gift of Dr. Dennis Burton, TSRI, San Diego, CA), HCV NS4B (Virogen, Watertown, MA), HCV NS5A (Virogen, Watertown, MA), HCV NS5B (Alexis Biochemicals, Plymouth, PA) Actin (Thermo Scientific, Rockford, IL), Flag (Sigma, St. Louis, MO), furin (Santa Cruz, Santa Cruz, CA), TSP-1 (Santa Cruz, Santa Cruz, CA), albumin (MBL International, Woburn, IL), and TGF-β1 (Cell Signaling, Danvers, MA).

Immunofluorescence assay

Huh-7 cells in eight-well chamber slides (Nalgene Nunc) were serum starved overnight, and incubated with HCV virion (moi = 1) for 5–6 h at 37 °C. The cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at RT, permeabilized for 5 min with 0.2% Triton X-100, and blocked for 45 min with 5% bovine serum albumin in PBS. The cells were next incubated with primary antibodies for 1 h at RT, followed by incubation with secondary antibodies (anti-mouse Alexa Fluor 633, anti-goat Alexa Fluor 546 or anti-rabbit Alexa Fluor 488) (Molecular Probes, Eugene, OR) for 1 h. After being washed with PBS, cells were mounted with antifade reagent containing DAPI (4, 6-diamidino-2 phenylindole) (Invitrogen, CA) and observed under a fluorescence microscope equipped with the Nikon MetaMorph digital imaging system.

RNA interference

Huh-7 cells were transfected with siRNAs against TGF-β1, furin, TSP-1 and GFP according to the manufacturer’s protocol (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Each siRNA consists of pools of three to five target-specific 19–25 nt siRNA designed to knockdown the target gene expression. For each transfection, two solutions were prepared. Solution A: 60 pmol of siRNA duplex was mixed with 100 μl siRNA transfection medium. Solution B: 6 μl of transfection reagent was added to 100 μl siRNA transfection medium. Solutions A and B were allowed to incubate at RT for 20 min. After 20 min, solutions A and B were combined, and allowed to incubate another 20 min at RT. The combined solutions were then added to the cells in six well plates, and then incubated for 5 h at 37 °C and 5% CO2, and the transfection solution replaced with complete DMEM growth media.

Luciferase assay

Mock-infected and HCV-infected Huh-7 cells were transfected and at 30 h post-transfection, cells were serum starved overnight followed by treatment with 20 μM of PDTC, BAPTA-AM, NAC, ruthenium red, TMB-8, EGTA, and DPI for 12 h. Cells were harvested and cellular lysates were analyzed for luciferase activity using the dual-luciferase reporter assay kit (Promega, WI). All transfections included a renilla expression vector to serve as an internal control. All the inhibitors were obtained from Calbiochem-Novabiochem Corp, San Diego, CA.

Detection of intracellular ROS

Mock-infected and HCV-infected cells were washed with PBS and then incubated with 10 μM carboxy derivative of fluorescein (CM-H2DCFDA) (Invitrogen, CA) in warm PBS for 1 h. Cells were treated with BAPTA-AM, TMB-8, and ruthenium red, at 20 μM for 12 h, harvested and washed two times with PBS and finally suspended in 300 μl of PBS. Huh-7 cells treated for 10 min with 500 μM H2O2 was used as a positive control. ROS levels were measured using a BD LSR-II flow cytometer. For microscopy, semi-confluent mock-infected and HCV-infected cells were washed with PBS and incubated with 10 μM of CM-H2DCFDA in serum free medium at 37 °C, 5% CO2 for 1 h. Cells were washed two times with PBS and visualized under the microscope. Huh-7 cells treated for 10 min with 500 μM H2O2 was used as a positive control. ROS levels were visualized at 488 nm using a Nikon Eclipse TE2000-S inverted microscope and MetaMorph software.

Cytokine array

Sub-confluent mock-infected and HCV-infected Huh-7 cells were washed 3 times with PBS, and allowed to culture in DMEM containing 0.2% FBS for 24 h. The cell culture supernatant (conditioned medium) from mock-infected and HCV-infected Huh-7 cells were harvested, and centrifuged at 1000 rpm for 10 min to remove cell debris and subjected to cytokine array using a human cytokine array kit according to the manufacturer’s protocol.
to the manufacturer's protocol (RayBiotech, GA). Densitometric analysis of the cytokine array blot was performed to determine the difference in the release of human cytokines from cells. The values were normalized to identical background levels using the Ray Bio Human Cytokines antibody array V analysis tool.

**TGF-β1 ELISA**

The cell culture supernatant from sub-confluent mock-infected and HCV-infected Huh-7 cells were harvested, and prepared by centrifugation at 1000 rpm for 10 min to remove cell debris. The secreted TGF-β1 protein in cell culture supernatant from mock-infected and HCV-infected Huh-7 cells was determined by ELISA according to the manufacturer’s protocol (Promega, WI). For activation 20 μl of 1 N HCl was added to 0.8 ml of cell culture supernatant, mixed, and incubated for 10 min at room temperature. The samples were neutralized by adding 30 μl 5 M NaOH, mixed, and used immediately for the sandwich TGF-β1 ELISA. A standard curve was constructed by using serial dilutions of human recombinant TGF-β1. TGF-β1 levels were measured in triplicate determinations.

**Measurement of bioactive TGF-β1 by mink lung epithelial cell (MLEC)**

Mink lung epithelial cells containing bioactive TGF-β1 sensitive plasminogen activator inhibitor promoter lucerase construct (PAI/L) was a kind gift from Dr. D.B. Rifkin, NYU, New York, NY. The assay is based on the ability of bioactive TGF-β1 to bind to mink lung epithelial cells receptors. This results in a dose-dependent increase in luciferase activity. Briefly, mink lung epithelial cells were plated on 96 well plates at a concentration of 2.5×10^4 cells per well and incubated for 24 h at 37 °C. These cells were grown in DMEM supplemented with 10% fetal calf serum, 100 μg of penicillin/ml, and 100 μg of streptomycin sulfate/ml. Cells were washed twice with PBS, and 50 μl of reporter lysis buffer (Promega, Madison, WI) was added to the wells. Twenty microliters of cell extract and 90 μl of lucerase assay reagent were added to 96 well white opaque flat bottom plate and light emission is measured for 10 s in a Bio-TEK Synergy HT Multi-Detection microplate reader. TGF-β1 standards were prepared by adding 500 μl of 0.2% FBS DMEM into a polypropylene tube. The standard stock solution is then serially diluted to obtain standards from 1000 pg/ml to 125 pg/ml.

**Quantitative real-time RT-PCR**

Total RNA was extracted from mock-infected and HCV-infected Huh-7 cells using TRIzol (Invitrogen, CA). HCV RNA was quantified by real-time RT-PCR using an ABI PRISM 7500 Sequence Detector (Applied Biosystems, CA). Amplifications were conducted in duplicate using the following primers and 6-carboxyfluorescein (6FAM)- and tetrachloro-6-carboxyfluorescein (TAMRA)-labeled probes (Applied Biosystems, CA): HCV Taqman probe, 5′-6FAM-CTG CCG AAC CGG TGA GTA CAC TAMRA-3′; HCV sense primer — CGG GAG AGC CAT AGT GG; HCV antisense primer — AGT ACC ACA AGG CCT TTC G. The sequences for the primers and probes were designed using Primer Express software (Applied Biosystems, CA). The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control (Applied Biosystems, CA). Amplification reactions were performed in a 25 μl mix using RT-PCR core reagents kit and the template RNA. Reactions were performed in a 96-well spectrofluorometric thermal cycler under the following conditions: 2 min at 50 °C, 30 min at 60 °C, 10 min at 95 °C, 44 cycles of 20 s at 95 °C and 1 min at 62 °C. Fluorescence was monitored during every PCR cycle at the annealing step. At the termination of each PCR run, the data was analyzed by the automated system and amplification plots were generated.

**SYBR green PCR**

The expression of cellular genes in mock-infected and HCV-infected Huh-7 cells was quantified by real-time RT-PCR using the following primers. TGF-β1, sense primer — CAA CAA TAC CTG GCG ATA CC; antisense primer — GAA CCC GTT CAT GTC CAC TT: furin, sense primer — GAG ATT GAA AAC ACC AGC GA; antisense primer — GCG GTG CCA TAG AGT ACG AG: TSP-1, sense primer — GTG TGT GAC ATC TTT GAA CTC; antisense primer — CCA AAG ACA AAT CTC ACA TTC: calpain, sense primer — CGG GGA CCT CAT ACG TGA GT; antisense primer — AGG TCC CCT GTG AAA ATG TG: MMP-9, sense primer — CGC CGG TCA CCT TCA C: antisense primer — GCG CAG GGA CCA CAA CT: 18s, sense primer — ACA TCC AAG GAA GGC AGC AG: antisense primer — TCG TCA CTA CCT CCC CGG. Total cellular RNA was extracted using TRIzol and DNase treated using RQ1 RNase-free DNase prior to cDNA production. The cDNA was reverse-transcribed from 1 μg of total RNA using oligo(dT) primers. Quantitative RT-PCR was carried out by using SYBR green master mix and specific primer sets. Amplification reactions were performed under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles for 15 s at 95 °C, and 1 min at 60 °C. Relative transcript levels were calculated using ΔΔCT method as specified by the manufacturer.

**Non-radioactive cell proliferation assay (MTT assay)**

Cell proliferation assay was performed according to the manufacturer's protocols (Promega, Madison, WI). Mock-infected and HCV-infected cells, transfected with TGF-β1, TSP-1, or furin siRNA were plated in 96-well culture plates and incubated at 37 °C, 5% CO₂ for 24 h. Following incubation, 15 μl cell proliferation dye was added to the cells and was incubated at 37 °C, 5% CO₂ for 4 h. One hundred microliter stop solution was added and absorbance was read at 570 nm and 650 nm. Absorbance was measured in triplicate.

**Statistical analysis**

Error bars show the standard deviations of the means of data from three individual trials. Two-tailed unpaired t-tests were used to compare experimental conditions to those of the respective controls. The significance level was set at α value of 0.05.

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