Hepatitis C Virus Nonstructural 5A Protein Inhibits Lipopolysaccharide-Mediated Apoptosis of Hepatocytes by Decreasing Expression of Toll-Like Receptor 4

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Background. Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) has been shown to modulate multiple cellular processes, including apoptosis. The aim of this study was to assess the effects of HCV NS5A on apoptosis induced by Toll-like receptor (TLR) 4 ligand, lipopolysaccharide (LPS).

Methods. Apoptotic responses to TLR4 ligands and the expression of molecules involved in TLR signaling pathways in human hepatocytes were examined with or without expression of HCV NS5A.

Results. HCV NS5A protected HepG2 hepatocytes against LPS-induced apoptosis, an effect linked to reduced TLR4 expression. A similar downregulation of TLR4 expression was observed in Huh-7–expressing genotype 1b and 2a. In agreement with these findings, NS5A inhibited the expression of numerous genes encoding for molecules involved in TLR4 signaling, such as CD14, MD-2, myeloid differentiation primary response gene 88, interferon regulatory factor 3, and nuclear factor–κB2. Consistent with a conferred prosurvival advantage, NS5A diminished the poly(adenosine diphosphate–ribose) polymerase cleavage and the activation of caspases 3, 7, 8, and 9 and increased the expression of anti-apoptotic molecules Bcl-2 and c-FLIP.

Conclusions. HCV NS5A downregulates TLR4 signaling and LPS-induced apoptotic pathways in human hepatocytes, suggesting that disruption of TLR4-mediated apoptosis may play a role in the pathogenesis of HCV infection.

Hepatitis C virus (HCV), a member of Flaviviridae, is a causative agent of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1, 2]. The HCV genome containing positive-strand RNA is ∼9.6 kb and encodes a polyprotein precursor of ∼3000 amino acids, which is cleaved by both viral and host proteases into structural (core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. HCV nonstructural protein 5A (NS5A) exists as 2 phospho-proteins, p56 and p58, which are both phosphorylated at serine residues after the mature protein is released from the polyprotein [3]. Other studies have shown that HCV NS5A interacts with the proteins of oncogene and interferon (IFN) signaling pathways [4–7].

The immune system provides the first line of host defenses against microbial pathogens. Toll-like receptors (TLRs) are type I transmembrane proteins that have evolved to sense structurally conserved microbial components, known as microbial-associated molecular patterns. Thus, TLRs play a primary role in host responses to infection and in bridging innate and adaptive
**METHODS**

**Plasmids, Cells, and Virus**

pCXN2, pCXN2-HCV NS5A, and pCDNA3 and pCDNA3-full-length human TLR4 vectors were generously provided by J. Miyazaki (Osaka University), N. Kato (Institute of Medical Science, Tokyo University), and Scott L. Friedman (Mount Sinai School of Medicine), respectively. The TLR4 promoter luciferase reporter vector (−518 construct) was described elsewhere [18]. Human hepatoma cell lines HepG2 and Huh-7 were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum. HepG2 cells were stably transfected with pCXN2 (HepG2 control cells) or pCXN2-HCV NS5A genotype 1b (HepG2-NS5A cells). Cells were collected after 3 weeks of G418 selection, stocked, and used for further studies. Huh-7 cells harboring HCV subgenomic replicon genotype 1b and HCV Japanese fulminant hepatitis 1 (JFH1) genotype 2a were obtained as described elsewhere [19–21].

**Treatment of Cells With TLR Ligands**

HepG2 control or HepG2-NS5A cells were plated in 6-well plates and incubated with agonists of TLR1/TLR2 (Pam3CSK4; 3HCL; 100 μg/ml), TLR3 (poly[I:C]; 50 μg/ml), TLR4 (LPS from *Escherichia coli*; 5 μg/ml), TLR5 (purified flagellin; 100 μg/ml), TLR6/TLR2 (macrophage-activating lipopeptide 2; 100 μg/ml), TLR7 (Imiquimod [R-837]; 2.5 μg/ml), and TLR9 (type B CpG ODN; 0.5 μg/ml) (all purchased from Imgenex). After 24 hours of incubation, cells were fixed for 30 minutes with methanol, washed 3 times with water, air-dried, and stained for 30 minutes with 0.1% crystal violet.

**Luciferase Assays**

HepG2 cells (5 × 10^5) were transfected with 0.2 μg of reporter plasmid pTLR4-luc and pCXN2 or pCXN2-HCV NS5A using
Effectene (Qiagen). The total amount of DNA was kept constant. Cells were lysed with reporter lysis buffer (Promega), and luciferase activity was determined by luminometer (Luminescencer-JNR II AB-2300; ATTO), as described elsewhere [22].

RNA Purification and Real-Time Reverse-Transcription Polymerase Chain Reaction
Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and 5 µg of RNA was reverse-transcribed using the First Strand cDNA Synthesis Kit (SuperArray). Quantitative amplification of complementary DNA (cDNA) was monitored with SYBR Green by real-time polymerase chain reaction (PCR) analysis. Amplification was carried out in 25 µL of ROX PCR Master Mix (SuperArray) containing each primer (0.2 µmol/L) and 1 µL of the reverse-transcription reaction mixture, using 7300 Real-Time PCR system (Applied Biosystems) according to the manufacturer’s protocol. Primers were purchased from SuperArray. Data analysis was based on the comparative threshold cycle method. The expression of the genes of interest was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western Blot Analysis
Cells were harvested using sodium dodecyl sulfate sample buffer. Proteins were subjected to electrophoresis on 10% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (ATTO). Membranes were probed with antibodies specific for TLR4 (AnaSpec); HCV NS5A and HCV core protein (Biodesign International); PARP and cleaved PARP; procaspase 3 and caspases 3, 7, 8, and 9; Bax; Bcl-2; cellular FLIP (c-FLIP; official name, CFLAR [CASP8 and FADD-like apoptosis regulator]) (Cell Signalling Technology); GAPDH and β-tubulin (Santa Cruz Biotechnology). After washing, membranes were incubated with secondary horse-radish peroxidase–conjugated antibodies. Signals were detected by means of enhanced chemiluminescence (GE Healthcare) and scanned by image analyzer LAS-1000 and Image Gauge (version 3.1) (Fuji Film) and Scion Image (Scion) software.

Cell Viability Assay
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays were performed (CellTiter 96 AQ One Solution Cell Proliferation Assay; Promega) [23]; 20 µL/well of the MTS reagent was added to 100 µL of media containing cells in each well of 96-well plates and left for 4 hours at 37°C in a humidified 5% carbon dioxide atmosphere. For analysis, absorbance at 490 nm was measured using a Bio-Rad iMark microplate reader (Bio-Rad).

Apoptosis Assay
The APOPercentage Apoptosis Assay (Biocolor) was used to quantify apoptosis according to the manufacturer’s instructions. Purple-red stained cells were identified as apoptotic cells. The number of purple-red cells per 300 cells was counted [23].

Enzyme-Linked Immunosorbent Assay
HCV core protein was quantified in HCV-infected cell culture supernatants with a commercially available enzyme-linked immunosorbent assay kit (Ortho Diagnostics). The detection limit was 44 fmol/L.

Statistical Analysis
Results were expressed as means ± standard deviations. Student t test was used to determine statistical significance.

RESULTS

HCV NS5A and Protection of HepG2 Cells From LPS-Induced Apoptosis
We and others have previously shown that retinoic acid-inducible gene I and TLR3 are the 2 major host defense pathways triggered by HCV in hepatocytes [24, 25]. In contrast, little is known about the role played by other TLRs in response to HCV infection [26]. It has been demonstrated that inhibition by HCV NS5A of TNF-mediated apoptosis may contribute to viral persistence and eventually to HCV-associated disease progression [4], in a manner similar to that seen with other viruses [27]. Moreover, some TLR ligands induced apoptosis in the liver [28, 29]. To examine the effects of HCV NS5A on TLR signaling in hepatocytes, we treated HepG2-NS5A and HepG2 control cells with TLR1-9 ligands and analyzed cell death 24 hours later (Figure 1A and 1B). Stimulation with LPS (TLR4 ligand) induced massive death of HepG2 control cells but not HepG2-NS5A cells. Quantification of apoptosis showed a significant, 3-fold increase in apoptosis in HepG2 cells, compared with HepG2-NS5A cells (Figure 1, A and B). Other TLR ligands, sensed through TLR1/2, TLR2/6, TLR3, TLR5, TLR7, and TLR9, did not significantly alter the viability of HepG2 control or HepG2-NS5A cells.

HCV NS5A Downregulation of TLR4 Expression in Human Hepatoma Cell Lines
It has been reported elsewhere that adenovirus infection enhanced TLR4 expression in wild-type but not in HCV NS5A transgenic mice [7]. Therefore, we hypothesized that HCV NS5A impaired TLR4 expression in hepatocytes. To verify this, we compared TLR4 protein levels in HepG2 and HepG2-NS5A cells. Western blot analysis showed that TLR4 expression was markedly downregulated in HepG2 cells expressing NS5A (Figure 2A).

To further substantiate this observation, we next analyzed TLR4 expression in HCV subgenomic replicon genotype 1b and its parental Hu-7 cells [18]. Western blot analyses demonstrated a significant downregulation of TLR4 expression along with stable expression of HCV NS5A in replicon cells (Figure 2B). Finally, Hu-7 cells infected for 3 days with HCV genotype 2a (JFH1) expressed strongly reduced levels of TLR4 compared with mock-infected cells (Figure 2C). Confirming effective viral replication of JFH1 in Hu-7 cells, cell
Hepatitis C virus (HCV) nonstructural protein 5 A (NS5A) protects hepatocytes from lipopolysaccharide (LPS)–induced cell death. A, B, HepG2 control (A) and HepG2-NS5A (B) hepatocytes were cultured for 24 hours with ligands of Toll-like receptor (TLR) 1/TLR2 (Pam3CSK4), TLR3 (poly[C:C]), TLR4 (LPS), TLR5 (flagellin), TLR6/TLR2 (macrophage-activating lipopeptide 2), TLR7 (Imiquimod [R-837]), TLR8 (type B CpG oligonucleotide), and control oligonucleotide, as indicated in Materials and Methods. Cells were washed and stained with crystal violet, and experiments were performed 3 times. PBS, phosphate-buffered saline. C, HCV NS5A protects hepatocytes from LPS-induced apoptosis. HepG2 control and HepG2-NS5A hepatocytes were cultured for 24 hours with LPS (5 µg/mL). Cell apoptosis was quantified using the APOPercentage Apoptosis Assay. Data are expressed as means ± standard deviations of triplicate determinations from 1 experiment representative of 3 independent experiments.

HCV NS5A Inhibition of TLR4 Transcription
To unravel the molecular mechanisms by which NS5A decreased TLR4 expression in hepatocytes, we first tested whether NS5A interacted with TLR4. Cell lysates were prepared from HepG2 cells cotransfected with FLAG-tagged NS5A and TLR4 expression constructs [30], immunoprecipitated with FLAG antibodies, and probed with TLR4 antibodies. No coprecipitation of TLR4 and NS5A was detected under our experimental conditions (data not shown). TLR4 messenger RNA levels, quantified by means of real-time reverse-transcription PCR, were dramatically reduced in HepG2-NS5A cells (35-fold), compared with HepG2 cells. In agreement, transient transfection of HepG2 cells with the pCXN2 HCV NS5A expression plasmid reduced TLR4 promoter driven luciferase activity (Figure 2E). Overall, these data suggested that NS5A reduced TLR4 expression, at least in part, by inhibiting TLR4 transcription in hepatocytes, but not by TLR4 destabilization through direct protein–protein interactions.

HCV NS5A and Expression of Numerous Innate Immune Genes
To further characterize the influence of NS5A on host defense genes in hepatocytes, we used real-time PCR to quantify the expression of several genes in HepG2 and HepG2-NS5A cells. Besides TLR4, NS5A downregulated the expression of molecules involved in the formation of the TLR4 receptor complex (MD-2 [22-fold] and CD14 [38-fold]) and the expression of downstream signaling molecules (MyD88 [>100-fold], nuclear factor–κB2 [100-fold], and IFN regulatory factor 3 [6.4-fold]).

HCV NS5A and LPS-Induced Apoptosis
It has been reported that the combined effects of HCV and alcohol on various host cell types, via reactive oxygen species production, LPS signaling, and cytokine production, produce an environment of impaired antiviral response, greater hepatocellular injury, and activation of cell proliferation and differentiation responsible for a range of diseases [31]. Thus, we examined the effects of LPS with or without ethanol on hepatocytes. Cell triggering through TLR4 has been shown to stimulate apoptotic signaling pathways [29]. Considering that NS5A sustained survival of LPS-stimulated HepG2 cells (Figure 1), we investigated whether NS5A interfered with apoptosis, using Western blot analysis to detect PARP cleavage and expression of mature caspases 3 and 7. Whereas PARP was expressed at higher levels in resting HepG2-NS5A cells (1.10 ± 0.053 vs 1.0 ± 0.026; P <.042), cleavage of PARP induced by LPS with or without ethanol was observed in HepG2 control cells but was barely detectable in HepG2-NS5A cells (LPS, 4.57 ± 0.65 vs 44 ± 1.37 [P <.001]; LPS plus ethanol, 4.71 ± 1.13 vs 43.1 ± 0.24 [P <.001]) (all n = 3) (Figure 3A). Accordingly, activation of procaspase 3 into caspase 3 by LPS with or without ethanol was strongly reduced in HepG2-NS5A cells versus HepG2 control cells (LPS, 0.96 ± 0.22 vs 3.27 ± 0.24 [P <.001]; LPS plus ethanol, 0.93 ± 0.052 vs 3.51 ± 0.29 [P <.001]) and increased expression of caspase 7 (LPS, 1.21 ± 0.18 vs 2.24 ± 0.13 [P = .0013]; LPS plus ethanol, 1.15 ± 0.20 vs 2.87 ± 0.69 [P = .014]) (all n = 3) (Figure 3B). These data demonstrated that HCV NS5A protected HepG2 hepatocytes from LPS-induced apoptosis. It has been reported that LPS may recruit extrinsic apoptotic signals, and
alcohol increases liver apoptosis predominantly through intrinsic signaling [32], but we did not observe a significant difference in PARP cleavage and the activation of caspases 3 and 7 between LPS with and LPS without ethanol, suggesting that LPS may also increase apoptosis through intrinsic signaling in hepatocytes.

**HCV NS5A and Expression of Caspases 8 and 9, Bcl-2, and FLIP**

Activation of effector caspases 3 and 7 is controlled by caspases 8 and 9, which play a central role in the activation of extrinsic and intrinsic apoptosis pathways [33]. Interestingly, the expression levels of both caspases 8 and 9 were decreased in resting HepG2-NS5A cells and those stimulated by LPS with or without ethanol versus HepG2 control (caspase 8, 0.72 ± 0.013 vs 1.0 ± 0.013 [P < .001] and 0.77 ± 0.028 vs 1.3 ± 0.013 [P < .001] or 0.77 ± 0.013 vs 1.28 ± 0.013 [P < .001]) (caspase 9, 0.71 ± 0.035 vs 1.0 ± 0.024 [P < .001] and 0.75 ± 0.017 vs 1.34 ± 0.024 [P < .001] or 0.77 ± 0.017 vs 1.35 ± 0.010 [P < .001]) (all n = 3) (Figure 4A). The activation of caspases 8 and 9 is tightly controlled by regulators, such as cellular FLICE-like inhibitory protein (c-FLIP), a cellular inhibitor of procaspase 8 cleavage into caspase 8, and members of the Bcl-2 family, including Bax and Bcl-2, which have pro- and anti-apoptotic activities, respectively [23]. Thus, we investigated whether NS5A affected the expression of apoptosis regulators in HepG2 cells. Figure 4B shows the increased levels of Bcl-2 and c-FLIP in resting HepG2-NS5A cells and those stimulated by LPS with or without ethanol, when compared with HepG2 cells (Bcl-2, 4.5 ± 0.088 vs 1.0 ± 0.13 [P < .001] and 4.47 ± 0.18 vs 1.02 ± 0.22 [P < .001] or 4.58 ± 0.26 vs 1.0 ± 0.30 [P < .001]; c-FLIP [FLIP1, plus FLIPS], 1.29 ± 0.059 vs 1.0 ± 0.059 [P = .0038] and 1.25 ± 0.049 vs 1.0 ± 0.036 [P = .0020] or 1.29 ± 0.059 vs 1.0 ± 0.013 [P = .0011]) (all n = 3).

**Overexpression of TLR4 and Apoptosis in HepG2-NS5A Cells Treated With LPS**

We also chose to overexpress TLR4 to examine whether this would alter Bcl-2 in HepG2-NS5A cells treated with LPS. First, we examined cell viabilities 1 day after transient transfection of pCDNA3 or pCDNA3-full-length human TLR4 vectors into HepG2-NS5A cells and treatment with 5 μg/mL LPS. Cell viabilities of TLR4-overexpressed HepG2-NS5A were reduced, compared with those of control (74.9 ± 11.4% vs 100 ± 16.6%; n = 4; P = .046).

Next, we compared TLR4, cleaved PARP, and Bcl-2 expression in TLR4-overexpressing LPS-treated HepG2-NS5A cells with that in control LPS-treated HepG2-NS5A cells. Figure 5...
shows the increased levels of TLR4 and PARP in LPS-stimulated HepG2-NS5A cells transfected with pCDNA3-full-length human TLR4, compared with those transfected with pcDNA3. Importantly, Bcl-2 expression was lower in LPS-stimulated HepG2-NS5A cells transfected with pCDNA3-full-length human TLR4 than in those transfected with pcDNA3. Therefore, these results confirmed that NS5A counteracted LPS-induced apoptosis of hepatocytes by favoring the expression of the anti-apoptotic signaling molecule Bcl-2.

**DISCUSSION**

Here we report that downregulation of TLR4 expression by HCV NS5A is a key step in the negative regulation of LPS-induced hepatocyte apoptosis. This process negatively influences TLR4 signaling, including caspase activation and PARP cleavage, presumably to counteract the deleterious effects of LPS on hepatocyte viability (Figure 6).

The host defense system against pathogens involves both innate and adaptive immunity. Whereas HCV-specific CD4+ and CD8+ T cells are specific for a given antigen, innate immune cells, such as natural killer and dendritic cells, recognize patterns expressed by infectious agents, thereby shaping cytokine production and adaptive immune responses. TLR4 plays an important role in apoptosis in the liver [34]. The liver is involved at the end of an immune response, and its cells experience apoptosis, a phenomenon that is impaired in mice lacking TLR4 [35]. TLR4 deletion...
was reported to attenuate pancreatitis-induced mouse liver cell apoptosis [36] and to reduce ischemia and reperfusion injury in a murine liver transplantation model [37].

Why does HCV downregulate TLR4 expression by hepatocytes? HCV induces a lifelong infection and has evolved multiple strategies to evade host immune clearance, including downregulation of major histocompatibility complex class II by HCV core protein [38], cleavage of IFN promoter stimulator-1 by HCV NS3/NS4A [39], suppression of intrahepatic IFN-γ production by HCV NS5A [7], and inhibition of TNF-mediated apoptosis by HCV core protein [41] and NS5A [15]. The results presented here suggest an additional role of HCV NS5A in targeting TLR4 signaling and inhibiting LPS-induced proapoptotic signals. Our results showing that HCV NS5A downregulated TLR4 also support an earlier report in macrophage cell lines [6].

Inflammation drives the development of hepatic fibrosis that leads to cirrhosis in patients with chronic HCV infection. Multiple variants of the TLR4 gene modulate the risk of liver fibrosis [30]. Manigold et al [41] reported that TLR4 expression was downregulated in peripheral mononuclear cells of patients with high serum endotoxin levels at Child-Pugh stage A, irrespective of the cirrhosis origin (alcoholic or viral). Machida et al [42] used transient transfection with plasmids expressing individual HCV proteins, observing that HCV genotype 1a induced TLR4 expression in Raji cells and Huh7 cells and increased the amount of IFN-β and IL-6 with the use of 10 ng/mL LPS, less than in the present study. Our findings support the previous report that HCV infection can directly interfere with TLR4 signaling in hepatocytes, peripheral blood mononuclear cells, Raji cells [42], and dendritic cells [43]. TLR4 signaling itself may regulate HCV replication [44]. TLR4 missense variants appear to be associated with the risk of liver fibrosis [45] and other diseases [46]. HTLV-I p30 also interferes with TLR4 signaling and modulates the release of pro- and anti-inflammatory cytokines from human macrophages [47]. These data suggest that TLR4 signaling plays an important role in the pathogenesis of HCV infection. We also found that upregulation of Bcl-2 and downregulation of TLR4 is important for blocking LPS-induced apoptosis in these cells.

Although HCV induced apoptosis as well as the activation of Bid cleavage and cytochrome c release [48], it remains unknown whether apoptosis helps in host cell survival or is beneficial for HCV replication. HCV NS5A may play a fundamental role during HCV-related HCC development by inhibiting apoptosis. Further studies will be needed to elucidate the significance of these results, possibly leading to the development of effective molecular-targeted treatment against HCC, which is notoriously resistant to systemic therapies, often recurring even after aggressive local therapies. Although TLR4 inhibitors are also now under preclinical and clinical evaluation for the treatment of sepsis and inflammatory diseases [49], HCV might evade the innate immune response and also interfere with the adaptive immune response by functional inactivation of TLR4.

In conclusion, HCV NS5A downregulated TLR4-related signaling pathways and blocked LPS-induced apoptosis in human hepatocytes, suggesting that it plays an additional role in the pathogenesis of chronic HCV infection.
important role in lasting chronic infection and regulation of inflammation. The enhancement of TLR4 signaling may have therapeutic value, and the development of HCV NS5A-targeting drugs could improve the pathogenesis of HCV infection [50].

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**References**


