Hepatitis C Virus Infection Causes Cell Cycle Arrest at the Level of Initiation of Mitosis[∇]

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Received 8 February 2011/Accepted 9 May 2011

Chronic infection with the hepatitis C virus (HCV) is associated with increased risk for hepatocellular carcinoma (HCC). Chronic immune-mediated inflammation is likely to be an important factor in the development of HCV-associated HCC, but direct effects of HCV infection on the host cell cycle may also play a role. Although overexpression studies have revealed multiple interactions between HCV-encoded proteins and host cell cycle regulators and tumor suppressor proteins, the relevance of these observations to HCV-associated liver disease is not clear. We determined the net effect of these interactions on regulation of the cell cycle in the context of virus infection. Flow cytometry of HCV-infected carboxyfluorescein succinimidyl ester-labeled hepatoma cells indicated a slowdown in proliferation that correlated with abundance of viral antigen. A decrease in the proportions of infected cells in G₁ and S phases with an accumulation of cells in G₂/M phase was observed, compared to mock-infected controls. Dramatic decreases in markers of mitosis, such as phosphohistone H3, in infected cells suggested a block to mitotic entry. In common with findings described in the published literature, we observed caspase 3 activation, suggesting that cell cycle arrest is associated with apoptosis. Differences were observed in patterns of cell cycle disturbance and levels of apoptosis with different strains of HCV. However, the data suggest that cell cycle arrest at the interface of G₂ and mitosis is a common feature of HCV infection.

Chronic infection with hepatitis C virus (HCV) is associated with an increased risk for hepatocellular carcinoma (HCC) (8). Typically, cancer only develops after several decades of infection. Although the incidence of newly acquired HCV infections has decreased over the past 20 years, the incidence of HCV-associated HCC is increasing significantly as the infected population ages. Liver cancer associated with chronic HCV infection will, thus, be a significant public health burden for years to come. A greater understanding of the mechanisms by which chronic HCV infection leads to HCC will be critical for the development of improved therapies.

HCV has high genetic diversity and has been classified into six major genotypes that differ in their geographical distributions and natural history (33). Globally, infection with genotype 1 is the most common. Currently, only the genotype 1 and 2 HCV genomes have been propagated in cell culture.

The mechanisms by which HCV infection leads to HCC are unclear. HCV has an RNA genome with an exclusively cytoplasmic life cycle. Since HCV-associated HCC typically develops in the setting of fibrosis and cirrhosis, HCC development may be driven at least in part by chronic immune-mediated

inflammation. However, *in vitro* studies have revealed multiple interactions between HCV-encoded proteins and host cell cycle regulators and tumor suppressor proteins (24). For example, *in vitro* studies have shown that three distinct HCV proteins, core (13), NS3 (12), and NS5A (14, 20, 29), interact with the p53 tumor suppressor. In addition, the HCV RNA-dependent RNA polymerase NS5B interacts with the retinoblastoma tumor suppressor protein (Rb), targeting it for ubiquitination and proteasome-dependent degradation (27, 28). Some studies have suggested a proapoptotic role for HCV proteins while others have suggested an antiapoptotic role. Nonetheless, despite an abundance of published studies examining the effects of HCV protein overexpression on cell cycle regulators and tumor suppressors, very few studies have involved the use of HCV strains that replicate in cell culture. Thus, there is relatively little known about the consequences of HCV infection on cell growth. We set out to determine the net effect of these interactions on proliferation and cell cycle regulation in the context of virus infection and genome replication in cultured cells.

MATERIALS AND METHODS

Cell lines. Huh7.5 cells were a gift from Charles Rice (1). Cell lines were grown in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 µg/ml streptomycin, at 37°C with 5% CO₂.

The Huh7-derived cell line 2-3 (11) contains autonomously replicating, genome-length, dicistronic, selectable HCV RNAs derived from the genotype 1b HCV-N strain and is grown in the presence of 500 µg/ml G418 (Cellgro). The

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[∇] Published ahead of print on 15 June 2011.

companion, interferon-cured progeny cell line 2-3c contains no HCV RNA and was generated and maintained as described previously (31).

Plasmids, HCV genome transfection, and virus production. Plasmids encoding full-length HCV genomic RNAs of genotype 1a strain H77Sv3 (32), genotype 2a JFH1 (37), and genotype 1a/2a chimeras HJ3-5 (41) have been described previously. *In vitro*-transcribed RNAs from these plasmids can replicate and produce infectious virus when transfected into Huh7.5 cells. Mutants JFH1/GND (37), H77S/AAG (42), and HJ3-5/GND (also referred to as HJ3-5D318N) (25) are derived from JFH1, H77S, and HJ3-5, respectively, and contain point mutations in the RNA-dependent RNA polymerase of NS5B that abolish RNA genome replication. The mutant HJ3 is defective for virus assembly and is identical to HJ3-5 except for two amino acid substitutions (18). Mutant HJ3-5/KR is derived from HJ3-5 but contains 2 mutated codons in the p7 coding region that abolish the ion channel activity of the p7 protein blocking infectious virus release (39). Plasmids were linearized with XbaI and transcribed *in vitro* using a T7 Megascript kit (Ambion, Austin, TX). For HCV genome transfection, 5×10^6 cells were mixed with 10 μ g RNA in a 4-mm cuvette and subjected to a single exponential wave pulse at 270 V, 100 Ω , 960 μ F using a GenePulser Xcell system (Bio-Rad, Hercules, CA). Following electroporation, cells were resuspended in DMEM supplemented with 10% FBS and plated to 150-cm² flasks. Virus released into cell culture supernatants was propagated and quantified as described previously (40).

Western blot analysis. Preparation of protein extracts, SDS-PAGE, and subsequent immunoblotting were done as described previously (28) using mouse monoclonal antibodies against β -actin (AC-15; Sigma-Aldrich, St. Louis, MO), Rb (G3-245; BD Biosciences, San Jose, CA), and core (C7-50; Affinity BioReagents, Golden, CO) and rabbit polyclonal antibodies against NS5B (ab35586; Abcam, Cambridge, MA), phospho-S10 histone H3 (ab5176; Abcam), securin (ab3305; Abcam), Mad2 (A300-301A; Bethyl Labs, Montgomery, TX), and β -tubulin (ab6046; Abcam) and goat polyclonal antibodies against NS3 (ab21124; Abcam). Immunoblots were visualized by direct detection of infrared fluorescence on an Odyssey infrared imaging system (Li-Cor, Lincoln, NE).

CFDA-SE labeling. Huh7.5 cells (HCV infected or mock infected) were seeded into 6-well plates at 7×10^5 cells per well. Cells were allowed to adhere to the substrate for 6 h. The cell culture medium was removed, cells were washed twice in 1 $\times$ phosphate-buffered saline (PBS), and 1 ml 10 μ M carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Invitrogen) in 1 $\times$ PBS was added per well. Following a 15-min incubation at 37°C, the CFDA-SE label was removed from cells and replaced with normal growth medium. Cells were incubated at 37°C for 30 min to allow intracellular conversion of CFDA-SE to carboxyfluorescein succinimidyl ester (CFSE). To avoid cell confluence, cells were expanded into 10-cm tissue culture dishes 1 day after labeling.

For each treatment, one well of cells was harvested immediately (time zero), followed by harvesting of samples 1, 2, 3, or 4 days after labeling. Cells were harvested by trypsinization, washed twice in 1 $\times$ PBS, and fixed for 15 min at room temperature in 2% paraformaldehyde, 1 $\times$ PBS. Cells were washed in PBS and stored in the dark at 4°C prior to immunostaining and fluorescence-activated cell sorting (FACS) analysis.

Immunostaining for flow cytometry. Cells were harvested by trypsinization, pelleted at $200 \times g$, and washed twice in 1 $\times$ PBS. Cells were fixed for 15 min in 2% paraformaldehyde, 1 $\times$ PBS at room temperature. Cells were washed in 1 $\times$ PBS before permeabilization in 0.2% Triton X-100, 1 $\times$ PBS for 10 min. Cells were washed in 1 $\times$ PBS before incubation in primary antibody diluted in 1% bovine serum albumin (BSA), 1 $\times$ PBS, 0.1% Tween 20 for 1 h at room temperature. Mouse monoclonal antibodies to HCV core protein (C7-50; Affinity BioReagents) were used at a 1:400 dilution. Rabbit antibodies to phospho-histone H3 Ser10 (Abcam), phospho-cdk1 Tyr15, cleaved caspase 3 (Cell Signaling, Danvers, MA), and cleaved poly(ADP-ribose) polymerase (PARP) p85 fragment were used at 1:250, 1:25, 1:6,000, and 1:100 dilutions, respectively. Cells were washed twice in 1 $\times$ PBS, 0.1% Tween 20 before incubation for 1 h in the dark at room temperature in allophycocyanin (APC)-conjugated goat anti-mouse immunoglobulin G antibody (Invitrogen) or fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit immunoglobulin G antibody (Southern Biotech, Birmingham, AL) diluted 1:400 in 1% BSA, 1 $\times$ PBS. Cells were washed twice in 1 $\times$ PBS, 0.1% Tween 20 and resuspended in 1 $\times$ PBS prior to analysis. For analysis of DNA fragmentation, cells were further stained using the DeadEnd fluorometric terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) system (Promega, Madison, WI). Samples were analyzed using a CyAn (Beckman Coulter, Brea, CA) or FACS Canto flow (BD Biosciences) cytometer.

Cell cycle analyses. For cell cycle analyses using 5-ethynyl-2'-deoxyuridine (EdU) labeling, cells were incubated with EdU for 4 h prior to harvest. Cells were harvested by trypsinization, washed twice in 1 $\times$ PBS, fixed for 15 min in 4%

paraformaldehyde, permeabilized, and stained for HCV antigen as described above. Incorporated EdU was labeled with Alexafluor 488 azide by using the Click-iT EdU flow cytometry kit (Invitrogen) according to the manufacturer's instructions. Following treatment with RNase A, cells were resuspended in 0.5 ml 1 $\times$ PBS containing 1 μ g 7-aminoactinomycin D (7-AAD) per 1×10^6 cells. Samples were analyzed using a CyAn (Beckman Coulter) or FACS Canto (BD Biosciences) flow cytometer.

Immunofluorescence microscopy analysis. Huh7.5 cells were infected with HCV at a medium multiplicity of infection (MOI) of approximately 0.2 to 0.5 and passaged for 3 days. At 3 days postinfection (dpi), cells were trypsinized and seeded into 8-well glass chamber slides at 6×10^4 cells per well. Cells were allowed to grow for a further 24 h. Slides were washed twice in 1 $\times$ PBS, fixed for 30 min in 4% paraformaldehyde, and washed twice for 10 min each in 1 $\times$ PBS, 100 mM glycine. Cells were permeabilized in 0.2% Triton X-100 for 12 min, washed twice in 1 $\times$ PBS, and blocked for 1 h in 1 $\times$ PBS, 10% goat serum. Cells were incubated in primary antibodies diluted in 3% BSA, 1 $\times$ PBS overnight at 4°C. Mouse IgG1 anti-HCV core antibodies (Affinity BioReagents) were diluted 1:500, and mouse IgG2 anti-cyclin B1 antibodies (BD Pharmingen) were diluted 1:200. Following incubation with primary antibodies, cells were washed 3 times for 10 min each in 1 $\times$ PBS. Cells were then incubated in Alexafluor 488-conjugated goat anti-mouse IgG1 and Alexafluor 594-conjugated goat anti-mouse IgG2, both diluted 1:200 in 3% BSA, 1 $\times$ PBS for 1 h at room temperature. Slides were counterstained with Hoechst 33342, washed 3 times for 10 min each, and mounted with Vectashield (Vector Labs, Burlingame, CA).

Caspase 3/7 assays. Cells were seeded into white 96-well plates at 5×10^3 cells per well in 100 μ l medium and grown at 37°C for 24 h. The combined enzymatic activities of caspases 3 and 7 were measured using the luminescence-based Caspase-Glo 3/7 assay kit (Promega) according to the manufacturer's instructions.

WST-1 assays. Cell proliferation was measured in a WST-1 assay (Roche, Mannheim, Germany) according to the manufacturer's instructions, using a Synergy 2 multimode microplate reader (Biotek, Winooski, VT).

Northern blot analysis. Northern blot analysis was performed as described previously (28). HCV RNA and Mad2 mRNA were hybridized to biotinylated DNA probes specific for nucleotides 8213 to 8821 of HCV-N genomic RNA and nucleotides 124 to 469 of human Mad2 coding sequence, respectively.

RESULTS

HCV infection reduces cellular proliferation. To determine the effect of HCV infection on cellular proliferation, cell division was monitored by CFSE dilution in human hepatoma (Huh7.5) cells electroporated with synthetic HCV RNA genomes HJ3-5 or HJ3-5/GND (Fig. 1A). HJ3-5 is a genotype 1a/2a chimeric virus that replicates and produces infectious virus when introduced as RNA into Huh7.5 cells (41). HJ3-5/GND is identical to HJ3-5 except for a point mutation in the RNA-dependent RNA polymerase that abolishes replication. Thus, cells that are transfected with HJ3-5/GND will show any effects on cell proliferation that stem from the electroporation procedure *per se* but will not be subject to any impact from replication of the viral RNA. Cells proliferated more slowly when electroporated with HJ3-5 than the nonreplicating HJ3-5/GND genome (Fig. 1B and C). Over the 4-day experiment, cells electroporated with HJ3-5 had an average doubling time of 35 h, compared to 21 h for control cells.

Further analysis of genotype 2 virus (JFH1)-infected cells showed a similar slowing of proliferation (Fig. 2A). To investigate whether there was a relationship between intracellular levels of viral antigen and cellular proliferation, a more detailed CFSE dilution analysis was performed in which cell cultures that had been inoculated with HCV(JFH1) at an MOI of 0.25 were gated according to levels of HCV core protein expression. These analyses revealed that the HCV-induced slowdown in cellular proliferation was greatest in those cells with the highest level of viral core protein expression (Fig. 2B).

FIG. 1. Analysis of proliferation based on CFSE labeling in mock- or HCV(HJ3-5)-infected cells. (A) Schematic diagram showing the experimental design. (B) Analysis of mean CFSE labeling intensity in cells at different time points following transfection with HJ3-5 or HJ3-5/GND genomic RNA. Data shown represent the averages of three replicate electroporations, with error bars showing standard deviations. (C) Analysis of core expression (top panel) and CFSE labeling intensity (bottom panel) in Huh7.5 cells at the indicated time points following transfection with HJ3-5 (thick line) or HJ3-5/GND (thin line) genomic RNAs.

Cell cycle analysis of HCV-infected cells. The reduced proliferation of HCV-infected cells led us to ask whether HCV infection leads to cell cycle arrest. To investigate this possibility, we monitored incorporation of the nucleotide analog EdU while determining nucleic acid content by using 7-AAD labeling, thereby identifying cells in each phase of the cell cycle in Huh7.5 cells transfected with HJ3-5/GND or HJ3-5 RNA. At 5 days postelectroporation, cells were incubated for 4 h with EdU, harvested, and fixed. One-fifth of the cells were stained for core protein to determine the proportion of infected cells (Fig. 3A). For the remainder of the cells, EdU was labeled using an Alexafluor-conjugated probe and DNA was labeled using 7-AAD. EdU-positive cells represent those in S-phase at the time of harvest, while EdU-negative cells represent those in other phases. DNA content distinguished cells in G_1 phase

from cells in G_2/M phase. In cells transfected with HJ3-5, a dramatic increase was observed in the proportion of cells in G_2/M phase compared to HJ3-5/GND-transfected cells (Fig. 3B). The increase in G_2/M phase in the HJ3-5-transfected cells was accompanied by a decrease in the proportion of cells in G_1 phase (Fig. 3B).

To allow analysis of core positive and core negative cells within the same population, Huh7.5 cells were infected with HJ3-5 virus at a low MOI (0.05). At 4 dpi, cells were harvested and stained for core protein, EdU incorporation, and DNA content. Approximately two-thirds of the cells appeared core negative, while one-third were core positive (Fig. 3C). After gating for core expression, the core-negative and core-positive populations were further analyzed for EdU and DNA content to distinguish the G_1 , S, and G_2/M phases of the cell cycle. The

FIG. 2. Effects of viral antigen levels on cellular proliferation. (A, top panel) Analysis of core expression in HCV(JFH1)-infected cells at different time points following infection. Cells were gated for core expression according to whether they showed high levels (++), low levels (+), or no (–) core expression. (Bottom panel) Analysis of CFSE labeling intensity in cells following infection with JFH1. Histograms show CFSE labeling for populations with high (green lines), low (red lines), or no (blue lines) core expression. (B) Analysis of CFSE labeling in cells at different times following mock or HCV(JFH1) infection. HCV-infected cells were gated into three subpopulations: cells with no core expression, low levels of core expression, or high core expression. Mean CFSE fluorescence intensity was plotted against time for each of the populations. Each data point represents the mean of three independent infections, with error bars indicating standard deviations.

dot plots in Fig. 3D show DNA content plotted against EdU for core-negative and core-positive populations within the HCV-inoculated culture. Quantification of several experiments showed that HCV-infected (core-positive) cells accumulated preferentially in the G_2/M phase (Fig. 3E).

These data are in contrast to a previous report that concluded that HCV infection results in G_1 -phase arrest (prior to S phase), because the proportion of infected cells in S phase, determined by incorporation of the nucleotide analog EdU, was reduced (38). However, since cellular DNA content was not examined in this study, it was not possible to distinguish arrest at the G_1 versus G_2 phase.

Strain-specific differences in cell cycle perturbation. To determine whether different HCV strains have similar effects on cell cycle phase distribution, Huh7.5 cells were analyzed on day 4 following electroporation with full-length genomic RNAs (Fig. 4A) of genotype 1a strain H77Sv3 (32), genotype 2a strain JFH1 (16), the genotype 1a/2a chimera HJ3-5 (41), and as a negative control, HJ3-5/GND. With each of the replicating viral RNAs, we observed a significant increase (up to 2-fold) in the proportion of core-positive cells in G_2/M phase compared to core-negative cells (Fig. 4B to E). However, we also observed important differences between the various HCV strains. Core-positive cells transfected with HJ3-5 or H77Sv3 RNAs showed decreases in the proportion of cells in G_1 , a small increase or no change in the proportion in S phase, and increases in the proportion of cells in G_2/M phase. In contrast, core-positive cells transfected with JFH1 RNA showed a dramatic decrease in the proportion of cells in S phase, as reported by Walters et al. (38), and increases in proportions of cells in both the G_1 and G_2/M phases. These data are consistent with G_2/M -phase arrest in cells infected with all three virus genomes and an additional G_1 -phase arrest in JFH1 cells. The intensity of core staining was higher in cells infected with JFH1

than with H77Sv3 and HJ3-5 (Fig. 4C to E). In the case of H77Sv3, this is likely because JFH1 replicates to higher levels (42), while in the case of HJ3-5 this may be because HJ3-5 contains tissue culture adaptive mutations that facilitate virus assembly and release, so viral structural proteins (including core protein) do not accumulate to high levels within the cell (41).

We hypothesized that the differences in cell cycle phase distribution between cells infected with JFH1 and cells infected with the other two viruses were a consequence of differing intracellular levels of viral proteins. This would be consistent with the correlation between core protein expression and slowing of cellular proliferation we observed in JFH1-infected cells (Fig. 2). To examine the relationship between the intracellular levels of viral proteins and cell cycle arrest, we analyzed cells electroporated with two mutants related to HJ3-5: HJ3 and HJ3-5KR. HJ3 is identical to HJ3-5 except for two amino acid changes in E1 and NS3: it is defective in virus particle assembly (18). HJ3-5KR is also identical to HJ3-5 except for two amino acid substitutions in the virus-encoded ion channel p7, which render the genome defective for release of infectious virus from the cell (39). Both viral genomes replicate in Huh7.5 cells and express viral proteins, but neither produces infectious virus, leading to increased intracellular accumulation of the core protein. The absence of viral spread also contributed to a smaller number of viral antigen-positive cells in cultures transfected with these RNAs. Interestingly, core-positive cells transfected with either HJ3 or HJ3-5KR RNA demonstrated both G_1 and G_2/M arrest, similar to that observed in JFH1-infected cells (Fig. 4F and G). Thus, while a G_2/M phase-specific arrest is common to all virus genomes tested, a G_1 phase-specific arrest was observed only for those viral RNAs that produce high intracellular levels of core. Confirming this assertion, a more detailed analysis of JFH1-trans-

FIG. 3. Analysis of cell cycle phase distribution in HCV-infected cells. (A and B) Cell cycle phase analysis of Huh7.5 cells electroporated with HJ3-5/GND or HJ3-5 full-length genomic RNA. At 5 days after electroporation, cells were labeled with EdU for 4 h, harvested, fixed, and divided into two aliquots. One aliquot was stained using mouse anti-core protein followed by APC-conjugated goat anti-mouse IgG and analyzed by FACS (A). The second aliquot was analyzed for cell cycle phase distribution (B) by using an Alexafluor 488-labeled probe to detect EdU incorporation (S phase) and the nucleic acid stain 7-AAD to distinguish G₁ and G₂ phases. The percentages of cells in G₁, S, or G₂/M phases of the cell cycle were determined using FlowJo software. (C to E) Cell cycle analysis of Huh7.5 cells infected at an MOI of 0.05 with HJ3-5 virus at 4 dpi. Cells were pulse-labeled for 4 h with EdU prior to fixation. (C) Cells were immunostained using anti-HCV core protein followed by an APC-conjugated secondary antibody and gated according to whether they were core negative (–) or core positive (+). (D) Representative cell cycle distributions for core-negative and core-positive cells. EdU was detected with an Alexafluor 488-conjugated probe. DNA was stained with 7-AAD following RNase treatment. (E) Percentages of cells in each phase of the cell cycle for core-negative and core-positive populations within HJ3-5-inoculated populations (means and standard deviations for 8 independent infections). Significance was determined using a paired *t* test. *, *P* = 0.0003; **, *P* < 0.0001.

ected cells gated for core expression revealed a greater degree of cell cycle arrest in cells showing the highest levels of viral protein (Fig. 5).

Quantitative differences in induction of apoptosis following infection with different HCV strains. Previous microarray studies had suggested that HCV-mediated cell cycle perturbations may be associated with apoptosis (38). This observation led us to ask what happens to infected cells following cell cycle arrest. Other viruses that cause G₂ arrest (e.g., bocavirus) may induce apoptosis (4), and a number of studies have suggested that infection of cells with either JFH1 virus or chimeric genotype 2 viruses that are based on JFH1 is associated with apoptosis (7, 22, 38). The intragenotypic J6/JFH1 chimera has previously been shown to induce higher levels of apoptosis than the parent JFH1 virus (22). J6/JFH1 was also demonstrated to induce apoptosis through a mitochondrion-mediated pathway that activates downstream effectors, such as caspase 3 (7). Consistent with this, we found that caspase 3/7 activity was readily detectable in Huh7.5 cells infected with JFH1 at a high MOI (Fig.

6A). However, it is not known whether other HCV strains, particularly genotype 1 strains, induce apoptosis. Since we had observed virus-specific differences in cell cycle regulation, we compared the effects of transfection of the various viral RNAs shown in Fig. 3 on induction of cleaved caspase 3. Replication of H77Sv3, JFH1, and the chimera HJ3-5 resulted in elevated levels of caspase 3/7 compared to the nonreplicating control, HJ3-5/GND. HJ3-5 replication resulted in the greatest levels of caspase 3/7 activity, followed by JFH1 and finally H77Sv3 (Fig. 6C). However, since more cells were infected in the culture electroporated with HJ3-5 than JFH1 RNA, it appears that JFH1 replication results in greater levels of caspase 3/7 activity per infected cell (Fig. 6B). To directly assess this, we determined the presence of cleaved caspase 3 on a single cell basis by flow cytometry, allowing us to relate apoptosis to core protein expression levels (Fig. 6D). These results revealed that the proportion of cells staining positively for cleaved caspase 3 was greatest among cells transfected with JFH1 RNA, followed by those transfected with HJ3-5 RNA. Core-positive H77Sv3-

FIG. 4. Cell cycle analysis of Huh7.5 cells electroporated with genomic RNA of HJ3-5, HJ3-5/GND, H77Sv3, JFH1, HJ3, or HJ3-5-KR. (A) Simplified schematic representations of H77Sv3, HJ3-5, and JFH1 virus genomes. Coding sequences derived from H77S and JFH1 are represented as shaded or unshaded boxes, respectively. (B to G) Expression of HCV core protein (left panels) and cell cycle phase distributions for core-positive and core-negative populations (right panels) for Huh7.5 cells electroporated with HJ3-5 (B), HJ3-5/GND (HJ3-5 containing a mutation in the NS5B RNA-dependent polymerase that abolishes viral RNA replication) (C), H77Sv3 (D), JFH1 (E), HJ3 (identical to HJ3-5 except for two amino acid changes that prevent virus assembly) (F), and HJ3-5/KR (HJ3-5 containing KR33/35AA mutations in the coding region for the HCV p7 ion channel) (G). Analyses were performed 4 days after electroporation. Cell cycle distributions represent the means of three or four independent electroporations.

FIG. 5. The degree of cell cycle arrest is related to intracellular levels of viral antigen. Results shown are from quantitative analysis of core protein expression (A) and cell cycle distribution (B) in Huh7.5 cells infected with HCV JFH1. Cells were gated according to whether they showed no core expression (i), low (ii), or high (iii) levels of core expression.

transfected cells were not significantly more likely to contain cleaved caspase 3 than core-negative cells. Although this assay may be less sensitive than the luminescence-based assay, it does illustrate that these HCV strains differ in their capacities to induce apoptosis.

We had observed greater cytopathic effect following infection than after transfection with HCV RNA genomes. Therefore, we compared apoptosis markers in Huh7.5 cells at 6 dpi following infection with HJ3-5 or JFH1 at an MOI of 1 or mock infection. Levels of cleaved caspase 3, the p85 fragment of cleaved PARP or fragmented DNA (measured by fluorometric TUNEL assay) were analyzed. Both JFH1 and HJ3-5 infection could promote apoptosis compared to mock-infected cells. However, JFH1 infection induced higher levels of all three markers of apoptosis compared to HJ3-5-infected cells (Fig. 6E).

HCV infection blocks entry to mitosis. For each of the viral genomes tested, our data clearly show an accumulation of HCV-infected cells in G_2/M phase. However, the data do not allow a determination of whether HCV-infected cells are arresting in G_2 and failing to enter mitosis or if cells arrest in M phase and fail to complete mitosis and undergo cytokinesis. Key players in the transition from the G_2 to M phase are cyclin B1 and cdk1. Cyclin B1 levels accumulate through the G_2 phase, reaching a peak in M phase, after which cyclin B1 is rapidly degraded (2). Cyclin B1 forms a complex with cdk1 and can shuttle between the nucleus and cytoplasm. Through G_2 phase, net phosphorylation of cdk1 promotes nuclear export, keeping cyclin B1 predominantly cytoplasmic. However, during

late G_2 phase, the release of the active cdc25 phosphatase allows dephosphorylation of cdk1, resulting in activation and nuclear import of the cdk1:cyclinB1 complex, triggering mitotic entry (35). Several viruses or virus-encoded proteins have been shown to block entry to mitosis by preventing accumulation of hypophosphorylated cdk1 or by otherwise blocking nuclear localization of cyclin B1, including parvoviruses, simian virus 40, human papillomavirus type 16 E2 protein and E4 proteins, Borna disease virus p40 protein, and human immunodeficiency virus Vpr protein (6).

As a first approach to focus on the point at which HCV induces cell cycle arrest, we used flow cytometry to assess the abundance of cdk1 phosphorylated at Tyr15 in cells infected with HCV HJ3-5. A 2-fold increase in the levels of phospho-cdk1(Tyr15) was observed in core-positive cells compared to core-negative cells within the same culture or compared to mock-infected cells (Fig. 7A), suggesting that HCV infection may influence phosphorylation of cdk1 at Tyr15.

To determine whether HCV can block nuclear accumulation of cyclin B1, we compared the proportion of mock- and HCV(HJ3-5)-infected cells showing cytoplasmic versus nuclear cyclin B1 by immunofluorescence microscopy. Individual cells were scored as HCV core positive or negative and then subsequently scored according to whether they showed no cyclin B1, cytoplasmic cyclin B1, or nuclear cyclin B1. Representative immunofluorescence images are shown in Fig. 7C. Significantly fewer infected cells displayed nuclear cyclin B1 than did uninfected cells (Fig. 7B). This difference may also reflect a reduced proliferation rate, since the num-

FIG. 7. Detection of Cdk1 phospho-Tyr15 and localization of cyclin B1 in mock- or HCV-infected Huh7.5 cells. (A) Relative Cdk1 phospho-Tyr15 abundance is shown for HCV(HJ3-5)-infected Huh7.5 cells compared to mock-infected cells. Huh7.5 cells were mock or HCV(HJ3-5) infected and harvested at 4 dpi. Cells were fixed and stained for HCV core protein (APC conjugate) and Cdk1 phospho-Tyr15 (FITC conjugate). Mean phospho-Cdk1 levels were determined by flow cytometry for mock- and HCV-infected (core-negative and -positive) populations. (B) Localization of cyclin B1 in HJ3-5-infected Huh7.5 cells. Huh7.5 cells were infected with HJ3-5 at an MOI of 0.1 and cultured for 4 days. On day 4, cells were seeded to chamber slides at 6×10^4 cells per well and allowed to adhere to the substrate overnight before fixation. Cells were stained for core protein and cyclin B1. Images of random fields were recorded. Cells were scored as core positive or negative and subsequently scored according to whether they showed cyclin B1 expression and whether cyclin B1 was present in the nucleus or cytoplasm. Significantly fewer core-positive cells showed nuclear cyclin B1 compared to core-negative cells ($P = 0.0483$; χ^2 test). (C) Representative immunofluorescence images showing cells with no cyclin B1 or cytoplasmic (C) or nuclear (N) cyclin B1.

ber of infected cells displaying cytoplasmic cyclin B1 was also slightly reduced. In aggregate, these data show that HCV infection results in a small increase in cdk1 phosphorylation and an almost 25% reduction in nuclear localization of cyclin B1. This suggests a fraction of cells do not complete G₂ and enter M phase.

Another approach to examine the effect of HCV infection on initiation of mitosis is to analyze abundance of the M-phase-specific marker phospho-histone H3(Ser10). Phosphorylation of histone H3 at Ser10 is associated with condensation of the chromosomes during mitosis (9). Figure 8A shows phospho-histone H3(Ser 10) was progressively reduced in HCV(HJ3-5)-infected cells compared to mock-infected cells, with a greater reduction

over time. These data also suggest infected cells fail to complete G₂ phase or enter M.

To determine whether HCV-infected cells can initiate mitosis, cells infected with HCV(JFH1) or mock infected for 3 days were treated with the microtubule inhibitors nocodazole or paclitaxel for 24 h and then analyzed by bright-field microscopy and immunoblotting. Typically, treatment of cells with nocodazole or paclitaxel inhibits spindle formation and arrests cells in M phase through activation of the mitotic spindle checkpoint by unattached kinetochores (23). Following drug treatment, striking differences in cell morphology were observed between mock- and HCV-infected cells. Mock-infected cells arrested in M phase appeared rounded when examined by bright-field

FIG. 6. HCV infection causes apoptosis in Huh7.5 cells. (A) Caspase 3/7 activity in Huh7.5 cells that were mock infected or infected with HCV(JFH1) at an MOI of 0.2 or 2. Caspase 3/7 activity was determined in an assay in which an inactive luciferase fusion was cleaved by caspase 3 to release active luciferase (Promega, Madison, WI). Luciferase activity was normalized to cell number, which was estimated from a WST-1 assay (Roche, Palo Alto, CA). (B) HCV core expression in Huh7.5 cells transfected with HJ3-5/GND, HJ3-5, H77Sv3, or JFH1. (C) Caspase 3/7 activity in Huh7.5 cells transfected with different genomes (same transfected cells as shown in panel B). Normalized caspase 3/7 activity was determined as described for panel A. (D) Detection of cleaved caspase 3 in Huh7.5 cells electroporated with different HCV genomes. Cells were electroporated with indicated genomes, incubated for 4 days, stained for HCV core protein and cleaved caspase 3 protein, and analyzed by flow cytometry. (E) Detection of different markers of apoptosis following infection with HCV HJ3-5 or JFH1 at an MOI of 1 or mock infected. At 6 dpi, cells were harvested and aliquots were stained for HCV core protein, cleaved caspase 3, cleaved PARP (p85 fragment), or fluorometric TUNEL assay. Stained cells were analyzed by flow cytometry. The percentages of cells positive for each of the markers of apoptosis are shown.

FIG. 8. HCV-infected cells do not accumulate in M phase when treated with microtubule inhibitors that activate the mitotic spindle checkpoint. (A) Schematic diagram showing experimental design (left) and immunoblot analysis (right) of phospho-histone H3(Ser10) abundance in Huh7.5 cell lysates that were mock infected (M) or infected with HCV HJ3-5 at an MOI of 2 for 3, 4, or 5 days. Blots for core and NS5B expression are shown to demonstrate HCV infection, and β-tubulin is shown as a loading control. (B) Bright-field microscopy images of Huh7.5 cells that were either mock infected or infected with HCV(JFH1) (MOI, 2) for 3 days prior to treatment with nocodazole (Noc; 266 nM), paclitaxel (Pac; 1 μM), or dimethyl sulfoxide (DMSO [0.1%, vol/vol]) for 24 h. (C) Immunoblot analysis of markers of mitosis in lysates of mock- or HCV-infected Huh7.5 cells. Cell lysates were prepared from the cells shown in panel B and subjected to SDS-PAGE and immunoblot analysis using specific antibodies to detect the indicated proteins. (D) Flow cytometry analysis of phosphorylated histone H3(Ser10) abundance. Cells were electroporated with the full-length HCV genomic RNA of JFH1 or H77Sv3 or their cognate nonreplicating mutants, JFH1/GND or H77S/AAG, cultured for 4 days, and treated with 266 nM nocodazole for different times before harvest. Cells were stained for HCV core protein and phospho-histone H3. The percentages of cells positive for phospho-histone H3 are shown at different time points following nocodazole treatment.

microscopy. In contrast, HCV-infected Huh7.5 cells did not appear rounded in response to treatment with spindle poisons (Fig. 8B). Immunoblot analysis was used to analyze levels of mitosis-specific proteins in mock- or HCV-infected cells in response to microtubule inhibitor treatment. In the mock-infected cells, there was a sharp increase in the levels of the mitosis marker phospho-histone H3(S10) in response to treatment with nocodazole or paclitaxel, indicating cell cycle arrest in M phase. However, in the HCV-infected cells, there was no increase in the levels of phospho-histone H3(S10) following microtubule inhibitor treatment (Fig. 8C). Levels of phospho-histone H3(S10) were reduced in HCV-infected cells compared to uninfected cells regardless of whether or not they were treated with drugs that cause M-phase arrest.

The accumulation of phospho-histone H3(S10) was also monitored by flow cytometry in Huh7.5 cells treated with nocodazole following electroporation with different HCV genomes. Cells were electroporated with H77Sv3 (genotype 1) or JFH1 (genotype 2) RNA genomes. As negative controls, cells were electroporated with nonreplicating versions of both genomes (H77S/AAG and JFH1/GND). The rate of phospho-histone H3 accumulation was slower in nocodazole-treated

HCV-positive cells than in HCV-negative cells (Fig. 8D). Furthermore, JFH1-transfected cells showed lower levels of phospho-histone H3 accumulation than H77Sv3-transfected cells.

We also documented reduced levels of two other mitosis-associated proteins, Mad2 and securin, in HCV-infected cells (Fig. 8C). We showed previously that Rb is targeted for proteasome-dependent degradation in HCV-infected cells through an interaction mediated by the NS5B RNA-dependent RNA polymerase (27, 28). This leads to stimulation of E2F-dependent promoters, including the promoter for Mad2, which is activated by NS5B expression (28). Consistent with this, we found that Mad2 mRNA levels were upregulated by HCV RNA replication (Fig. 9A), despite the lower level of Mad2 expression observed in infected cells (Fig. 8C). A recent report suggested that HCV infection can lead to increases in Mad2 protein levels. Therefore, we sought to investigate Mad2 levels in HCV-infected cells in more detail. Immunoblot analyses confirmed that Mad2 protein levels are downregulated in HCV replicon-bearing 2-3 cells compared to the interferon-cured companion cell line 2-3c (Fig. 9B). Mad2 protein levels were also downregulated in HCV(HJ3-5)-infected cells compared to mock-infected cells (Fig. 9C to E). Collectively, these data

FIG. 9. Changes in Mad2 abundance following HCV RNA replication. (A) Northern blot analysis of Mad2 mRNA in 2-3 and 2-3c cell lines. 2-3 cells carry a selectable, autonomously replicating genome-length HCV RNA. 2-3c cells were generated from 2-3 cells by interferon treatment to cure the cells of the HCV replicon. (B) Western blot analysis of Mad2 protein abundance in 2-3 and 2-3c cells. (C) Diagram showing the experimental design for the analyses presented in panels D and E. (D) Western blot analysis of Mad2 abundance in HCV(HJ3-5)-infected cells. (E) Quantitative Western blot analysis of Mad2 abundance normalized to β -actin levels in mock- or HCV(HJ3-5)-infected cells. Blots were visualized and analyzed by direct detection of infrared fluorescence using an Odyssey infrared imaging system (Li-Cor, Lincoln, NE).

indicate that Mad2 is downregulated posttranscriptionally by HCV infection.

Collectively, the data shown in Fig. 8 indicate that HCV-infected Huh7.5 cells do not accumulate in M phase in response to microtubule inhibitor drugs, likely because they are blocked at a point prior to M phase.

When considered together with the cell cycle analyses in Fig. 3 and 4, the data indicate that HCV-infected cells are impaired for mitotic entry and progression through mitosis and accumulate at the interface of G_2 and M phases.

DISCUSSION

Although chronic HCV infection is associated with cancer, the impact of HCV infection on the hepatocyte cell cycle is poorly characterized. Prior overexpression studies have demonstrated both pro- and antiproliferative activities of HCV proteins, but these studies have questionable relevance to the situation existing within infected cells in which multiple viral proteins are expressed and interact with each other as well as numerous host proteins to promote amplification of the ge-

nome and production of infectious virus. Data presented here show that Huh7.5 cells infected with HCV proliferate more slowly than uninfected cells, accumulate in the G_2 phase of the cell cycle, and fail to enter M phase. The degree of cell cycle arrest correlates with the level of viral protein accumulation.

In common with other studies, we demonstrated here that HCV-infected Huh7.5 cells undergo apoptosis, but we also showed that the frequency with which this occurs varies among different HCV strains. In particular, the genotype 1 strain H77Sv3 and the genotype 1/2 chimera HJ3-5 show a reduced potential to induce apoptosis compared to JFH1. In support of these findings, previous studies demonstrated that an intragenotypic (type 2) chimera, J6/JFH1, is a more potent inducer of apoptosis than the parent JFH1 virus (22). Both J6/JFH1 and HJ3-5 consist of JFH1 sequence with the coding sequence of core to p7 (and NS2 in the case of HJ3-5) of other viruses. Thus, it is likely that sequences that determine the potency of apoptosis induction lie within this region.

Our finding that HCV slows proliferation of Huh7.5 cells is in agreement with a previous study that reported a doubling time of 32 h for naïve Huh7.5 cells compared to 34 h for JFH1-infected cells (36). Here, we show that the rate of proliferation in HCV-infected cells inversely correlates with viral antigen levels.

Previous studies that examined the effects of HCV infection on the cell cycle have suggested that infection with the JFH1 strain of HCV results in a G_1 -phase-specific cell cycle arrest (21, 38). Walters et al. (38) argued that JFH1 infection results in accumulation of cells in G_1 phase, based on the observation that fewer infected cells accumulate in S phase, as determined by immunofluorescence analysis of EdU-labeled cells (38). However, our more detailed analyses demonstrate that the decrease in the proportion of JFH1-infected cells in S phase is accompanied by increases in the proportions of cells in both G_1 and G_2 phases. Moreover, in cells transfected with genotype 1a H77S RNA, we observed significant decreases in G_1 accompanied by a slight increase in cells in S phase (Fig. 4). It is noteworthy that both H77Sv3 and HJ3-5 show different effects on the host cell cycle compared to JFH1. Genotype 1 infections are most frequently associated with cancer, likely reflecting the higher prevalence of genotype 1 infection (17). Furthermore, studies of HCV pathogenesis that use the genotype 2a JFH1 virus may not be representative of other HCV strains, particularly since this viral RNA replicates to such high levels within the cell. Levels of viral antigen are much lower in infected liver (15). Thus, it is likely that observations made with the genotype 1a H77Sv3 virus may more closely approximate events *in vivo*.

Few studies have specifically examined the effect of HCV infection at initiation of mitosis. In contrast to our findings, one report suggested that expression of HCV core protein in HepG2 cells promotes nuclear import of cyclin B1, thus promoting mitotic initiation (34). However, the physiological relevance of this observation is questionable, since core protein was overexpressed in the absence of RNA replication and other viral proteins.

Very little is known about the effects of HCV infection on cell cycle regulation within hepatocytes *in situ*. One study examined cell cycle markers in liver biopsies from HCV-infected individuals and demonstrated accumulation of cells in G_1 phase (21). Greater numbers of cells were actively cycling in

HCV-infected livers than in uninfected liver, which was almost entirely quiescent (G_0). G_1 -specific markers were elevated, but there were reduced numbers of cells with S-, G_2 - and M-phase-specific markers in liver samples from HCV-positive patients compared to samples from regenerating liver following ischemic reperfusion. However, it is difficult to interpret these results, since multiphoton microscopy suggests that only a minority of hepatocytes (typically 5 to 20%) are infected with HCV (15). Thus, it is uncertain whether the cells identified by Marshall et al. (21) that displayed G_1 -phase markers were infected. Hepatocytes are also subject *in vivo* to the effects of interferon, either produced by the host or administered therapeutically as antiviral treatment (30). Interferon is known to cause G_1 -phase-specific cell cycle arrest (5).

What difference does it make to the host hepatocyte whether HCV causes arrest in the G_1 or G_2 phase of the cell cycle? Apoptosis could result from either, but the cell will replicate its chromosomal DNA prior to arrest in the case of G_2 -phase arrest. This will occur in an environment of oxidative stress and, through decades of chronic infection, it is possible that the occasional infected hepatocyte may acquire one or more mutations that allow it to escape G_2 arrest and apoptosis and proceed through the cell cycle, providing an apoptosis-resistant clone of cells with altered cell cycle regulation.

The changes we observed in Mad2 expression (Fig. 8C and 9) may also be relevant to the development of cancer. Unscheduled overexpression of Mad2 can lead to defects in the spindle checkpoint, aneuploidy, and cancer (10). A recent study suggested that HCV core protein can cause transcriptional downregulation of Rb, leading to unscheduled overexpression of Mad2 and aneuploidy (19). Contrary to this report, our data are clear in suggesting that Mad2 protein levels are downregulated by HCV infection (Fig. 8C and 9B to E), along with several other proteins that are involved in mitosis, such as phospho-histone H3(Ser10). Furthermore, our data indicate that Mad2 is downregulated posttranscriptionally (Fig. 9), despite increases in Mad2 transcript levels, consistent with NS5B-mediated degradation of Rb (27, 28). Reduced Mad2 expression has also been linked to defects in the mitotic spindle checkpoint in Mad2 haploinsufficient mice (26). However, since our data suggest that HCV-infected cells do not enter mitosis, any defects in the mitotic spindle checkpoint that result from the activities of HCV proteins are unlikely to lead to aneuploidy and cancer, since heritable changes would not be passed to daughter cells. An important caveat to this assertion is that the current study was performed in derivatives of the human hepatoma cell line Huh7, which expresses high levels of a mutant p53 protein (3) and may differ significantly in aspects of cell cycle regulation compared to primary hepatocytes. Future studies should be aimed at understanding HCV disturbances of cell cycle regulation in primary hepatocytes.

ACKNOWLEDGMENTS

This work was supported in part by U19-AI40035 and P50-CA127004 to S.M.L. and pilot project P30-ES006676 to D.R.M.

We thank MinKyung Yi (UTMB) for HCV genome plasmids and Mark Griffin, Jean Niles, and Jennifer Timpe for technical advice and assistance with flow cytometry.

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