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Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Hepatitis C Virus Replication in Transfected and Serum-Infected Cultured Human Fetal Hepatocytes

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Understanding the pathogenesis of hepatitis C requires the availability of tissue culture models that sustain viral replication and produce infectious particles. We report on the establishment of a culture system of nontransformed human fetal hepatocytes that supports hepatitis C virus (HCV) replication after transfection with full-length *in vitro*-transcribed genotype 1a HCV RNA without adaptive mutations and infection with patient sera of diverse HCV genotypes. Transfected and infected hepatocytes expressed HCV core protein and HCV negative-strand RNA. For at least 2 months, transfected or infected cultures released HCV into the medium at high levels and usually with a cyclical pattern. Viral replication had some cytotoxic effects on the cells, which produced interferon (IFN)-β as a component of the antiviral response. Medium from transfected cells was able to infect naïve cultures in a Transwell system, and the infection was blocked by IFN-α and IFN-λ. Viral particles analyzed by sucrose density centrifugation had a density of 1.17 g/ml. Immunogold labeling with antibody against HCV envelope protein E2 decorated the surface of the viral particles, as visualized by electron microscopy. This culture system may be used to study the responses of nontransformed human hepatocytes to HCV infection, to analyze serum infectivity, and to clone novel HCVs from infected patients. ***(Am J Pathol 2007, 170:478–489; DOI: 10.2353/ajpath.2007.060789)***

An estimated 170 million people worldwide, including 1.5 to 2% of the U.S. population, are infected with hepatitis C virus (HCV).[1] Although some infected patients clear HCV by mounting a successful immune response, a chronic carrier state is established in the great majority of cases, resulting in liver injury that ranges from minimal to varying degrees of hepatic inflammation and fibrosis. After 20 to 30 years, 15 to 20% of patients develop liver cirrhosis that may lead to hepatocellular carcinoma. HCV-induced liver disease is the leading indication for liver transplantation in most U.S. medical centers.[2–4]

Many systems have been used in attempts to establish HCV replication in culture.[5–9] Most of theses systems are permissive for HCV infection but did not sustain efficient virus production. The lack of suitable HCV cell culture systems has been a serious impediment for progress in understanding the relationships between the virus and its natural host, nontransformed human hepatocytes. A major advance for culturing HCV was the development of a stable subgenomic replicon system,[10–12] which was able to replicate autonomously and at a high level in the human hepatoma line Huh-7. A breakthrough occurred in 2005, following the isolation of the genotype 2a HCV JFH-1 virus from a patient with fulminant hepatitis. This virus replicates well in Huh-7 cells without adaptive mutations.[13–15] Wakita et al[16] obtained virus production in cells transfected with the cloned JFH-1 genome, and Zhong et al[17] established a highly efficient system for production of infectious virus in Huh-7.5.1 cells. Lindenbach et al[18] have constructed full-length chimeric genomes J6/JFH and produced infectious particles in the Huh-7.5 cell line.

Viral production in these systems relies on the transfection into transformed cells of a single virus, a genotype 2a HCV cloned from a rare case of fulminant hepatitis C. Very recently, the construction of intragenomic and intergenomic hepatitis C virus chimeras using JFH-1-derived sequences,[19] the recovery of infectious JFH-1 virus from infected chimpanzee,[20] and the transfection of Huh-7.5 cells with genotype 1a H77-S virus with five adaptive

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mutations have been reported.[21] Nevertheless, the development of a system that can sustain the replication of HCV of various genotypes in nontransformed hepatocytes, after either transfection of nonchimeric virus or exposure to serum of patients infected with HCV virus, remains a challenging priority.[22]

Iacovacci et al[8] had reported the detection of replicative forms of HCV in human fetal hepatocytes (HFHs) exposed to serum of HCV-infected patients, indicating that HFHs are permissive for HCV replication. We have established and characterized long-term, serum-free primary and passaged cultures of nontransformed hepatocytes from human fetal liver, and recently isolated multipotent progenitor cells from these cultures.[23,24] We have used the HFH culture system developed in our laboratory to determine whether HCV replication can be sustained after either transfection of these cells with cloned virus or infection with patients' sera. We show that HFHs can sustain HCV replication after transfection with genotype 1a HCV or infection by patient sera of HCV genotypes 1, 2, and 3. After transfection or infection, high HCV titers were detected in the medium for at least 2 months, generally with a cyclical pattern, and viral-like particles were released into the medium. Viral infection could be transmitted to naive cells in a Transwell culture system, and the infection was abolished by exposure of the cells to interferon (IFN)-α or IFN-λ.

Materials and Methods

Cell Isolation and Culture

Livers at 90 to 117 days of gestation were obtained from the Central Laboratory of Embryology at the University of Washington as approved by the University of Washington Institutional Review Board. Cells were cultured on collagen plates as previously described[23] for a minimum of 5 days and as long as 3 months before transfection with *in vitro*-transcribed RNA or infection with patient sera.

Preparation of HCV RNA

HCV genomic strand RNA (referred to as WT HCV RNA) was transcribed from the full-length HCV cDNA construct p90/HCVFLpU of genotype 1a, following the procedure of Kolykhalov et al.[25] The ratio of RNA to DNA in the purified transcripts was 100,000:1 as determined by 10-fold serial dilution and amplified in the presence or absence of the reverse transcription step. The amount of the purified transcript was measured using RediPlate 96 RiboGreen RNA quantitation kit (Molecular Probes, Inc., Eugene, OR) and Packard Fusion Universal Microplate analyzer (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). The 3′-UTR mutant RNA, in which the entire 3′-UTR and 52 amino acids of the C-terminal region of NS5B was deleted (428-bp deletion), was transcribed by digesting the full-length cDNA template with *Not*I that has a single recognition site near the 3′ end of HCV cDNA. The NS5B mutant RNA was transcribed from the full-length construct deleted of 12 amino acids (CTMLVCGDDLVV) in the NS5B polymerase active site.

Transfection Procedures

For transfection of wild-type (WT) or mutant HCV RNA, cells were rinsed with Opti-MEM medium (Invitrogen Corporation, Carlsbad, CA) and incubated with Lipofectin-RNA complex containing 1 μg of RNA per 35-mm dish. After incubation for a minimum of 5 hours at 37°C, the cells were rinsed 6 to 10 times with Hanks' balanced salt solution (HBSS). The final wash was collected, and the medium was changed to feeding medium. The final wash contained negligible or no detectable amounts of HCV RNA. The medium was completely replenished at each feeding daily during the first 10 days, every 2 days from 12 to 20 days, and every 4 days thereafter.

Infection of HFHs with Sera from HCV-Infected Patients

HCV-positive serum was obtained from patients with chronic or post-transplant HCV infection at the University of Washington, as approved by the Institutional Review Board of the University of Washington. Sera from individual patients and pooled sera from multiple HCV-infected donor of the same genotype were included.

For serum infection, 50 μl of patient serum diluted in 1.5 ml of medium was added to cells plated in 35-mm dishes. After overnight incubation, the cells were rinsed 6 to 10 times with HBSS and 2 ml of fresh growth medium was added. The schedule of medium changes was the same as that for transfection experiments. The final wash was collected for HCV testing (shown as time 0 in the figures).

Infection by Culture Medium Using a Transwell System

For these experiments, cultures transfected with WT HCV or the NS5B mutant RNA were cultured on collagen-coated (Vitrogen; Cohesive Technologies, Palo Alto, CA) transparent high-pore density polyethylene terephthalate track-etched membranes and in deep well dishes (Becton Dickinson Labware, Franklin Lakes, NJ). A 3.0-μm pore density was used to permit virus diffusion. At 16 and 37 days after transfection, the cell inserts were removed and placed in six-well plates that contained naïve HFHs from a different isolate. Transfected and naïve cells were cultured for 2 days in the same medium and then separated. Medium was collected from the infected cultures using the same collection schedule as described above.

In some experiments, naïve cells were treated with 1 IU/ml recombinant human IFN-α (Biosource, Camarillo, CA) or 100 ng/ml recombinant human IFN-λ1 or IFN-λ2 (PreproTech Inc., Rocky Hill, NJ) beginning 1 day before co-culture. After the separation of the cultures, the infected cells were maintained in medium containing the appropriate IFN.

RNA Extraction and Quantification of HCV RNA

HCV RNA was isolated from culture medium using a QIAamp Virus BioRobot MDx kit (Qiagen, Valencia, CA). HCV-positive sera and unused culture medium were processed along with the samples to serve as the positive and negative controls, respectively. Extracted RNA was amplified using Taqman EZ RT-PCR Core reagents, and the amount of product was quantified by monitoring the increase in fluorescence of a FAM-labeled oligo probe using ABI PRISM 7700 or 7900HT real-time sequence detection system (Applied Biosystems, Foster City, CA). The amplification primers are located in HCV 5′-untranslated region and their sequences are 5′-CATGCCCCCG-CAAGA-3′ (129F) and 5′-ACCCTATCAGGCAGTACCA-CAAG-3′ (199R). The sequence of the probe is 6-FAM-CATGCCGAGTAGCGTTGGGTTGCG-6-TAMRA. The thermal cyclic profile was 50°C for 2 minutes, 60°C for 30 minutes, 95°C for 2 minutes, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. Along with the sample RNAs, 10-fold serial dilutions of HCV WT RNA were amplified to serve as standards. The standard WT RNA was digested with DNase I to remove DNA and quantified by the RediPlate 96 RiboGreen RNA quantitation kit. The copy number of WT RNA was calculated using the concentration and the molecular weight of WT RNA.

HCV RNA extraction by a QIAamp Virus BioRobot MDx kit and amplification using the Taqman method was calibrated in international units (IU)/ml. Serial dilutions of HCV standard serum (OptiQual HCV RNA High Positive Control, 2,000,000 IU/ml; AcroMetrix, Benicia, CA) were extracted and amplified. The linear range of this quantitative assay is from 40 to 200,000 IU/ml; 1 IU is approximately equivalent to 2.2 copies of WT RNA prepared by our laboratory. The detection limit of this assay is 10 IU/ml, which corresponds to approximately 25 copies per ml.

Strand-Specific in Situ *Hybridization*

To detect HCV RNA in transfected or infected cells, HFHs were cultured on collagen-coated chamber slides (Nalgen Nunc International, Naperville, IL). The hybridization procedures as well as the controls have been previously described in detail.[26]

Immunohistochemistry (IHC) and Immunofluorescence

To detect HCV proteins in transfected or infected cells, HFHs were cultured on collagen-coated chamber and fixed with paraformaldehyde. HCV core protein was detected by IHC using the C7-50 monoclonal antibody (mAb) (subtype IgG1; Affinity Bioreagents, Golden, CO) and the ABC kit. Substitution of the primary antibodies with mouse IgG (Vector Laboratories, Burlingame, CA) was used as a control for the staining.

For immunofluorescence, NS3 antibody (subtype IgG2b; Austral Biologics, San Ramon, CA) and fluorescent Alexa Fluor 594 goat anti-mouse IgG2b (Molecular Probes) were used. Nuclei were stained with blue fluorescent 4,6-diamidino-2-phenylindole (DAPI). A Nikon Eclipse E600 microscope with a QImaging Retigia EX CCD camera was used to capture black and white images of fluorescent signals. Green and blue colors were assigned to the images of NS3-positive signals and nuclei of cells, respectively.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) and Viability Assays

TUNEL assay (*in situ* cell death detection kit; Roche Diagnostics, Indianapolis, IN) was performed according to the manufacturer's directions. Cell viability assays were performed using the Live/Dead Viability Cytotoxicity kit (Molecular Probes). The kit contains fluorescent calcein AM and ethidium homodimer-1. In viable cells, intracellular esterases hydrolyze calcein AM to calcein (green fluorescence). Ethidium homodimer-1 penetrates the membrane of dying cells and binds to DNA (red fluorescence). Cells in sterile glass slides were stained for 10 minutes, and the slides were examined in a fluorescence microscope.

Equilibrium Density Gradient Centrifugation

Sucrose solutions (60, 50, 40, 30, and 10% w/v) prepared in NTE buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 1 mmol/L EDTA) were sequentially loaded into Beckman polyallomer centrifuges tubes. One milliliter of culture supernatant was layered on the sucrose solutions, and a density gradient was generated by centrifuging at 315,000 rpm for 16 hours in an Optima ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA). HCV measurements were done in sequential collections of 500 μl. The sugar content of each fraction was measured using a Leica ABBE Mark II refractometer (Reichert Analytical Instruments, Depew, NY).

Electron Microscopy

For all experiments, 400-mesh Formvar carbon-coated electron microscope nickel or copper grids (Electron Microscopy Sciences, Ft. Washington, PA) were glow-discharged before use. HCV cultures, filtered through a 1.0-micron membrane, were deposited onto grids by ultracentrifugation using a Beckman Airfuge with an EM 90 rotor (Beckman, Palo Alto, CA) at 26 lb/in^2 for 30 minutes. Goat antibody against HCV 1a envelope protein E2 (Biodesign International, Saco, MA), diluted 1:10, and 10 nm of colloidal gold conjugate anti-goat IgG at a 1:25 dilution (Aurion, Wageningen, The Netherlands) were used for immunogold labeling. The controls included samples treated with goat anti-mouse IgG (Vector Laboratories) and omission of the primary antibody. Viral particles were negatively stained with 1% uranyl acetate and examined in a JEOL JEM 1230 transmission electron microscope (JEOL Inc., Peabody, MA).

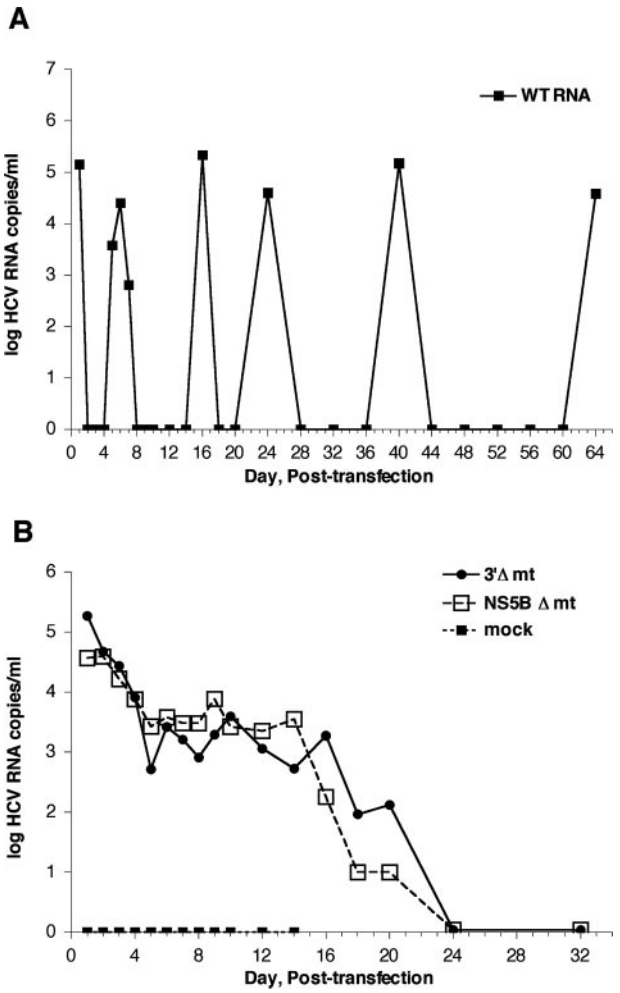

Figure 1. Virus production by HFHs transfected with WT and mutant HCV RNA. HFHs were transfected with WT (**A**), 3′-UTR mutant, and NS5B mutant HCV RNA's (**B**) using Lipofectin. Mock transfection (**B**) consisted of exposure of the cultures to Lipofectin. After 5 hours of incubation, the cells were extensively washed and the medium replaced. Little or no HCV RNA was detected in the last wash. Culture medium was collected daily up to 10 days, at 2-day intervals up to 20 days, and every 4 days thereafter. At each indicated time, medium was completely removed for the quantitative HCV RNA assay and entirely replaced with fresh growth medium. Day 1 designates culture media collected 24 hours after transfection. The detection limit of the assay was 25 copies/ml.

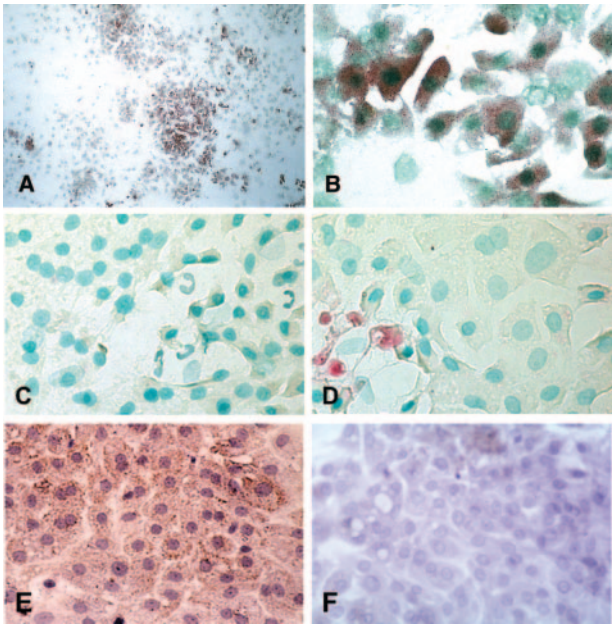

Figure 2. Detection of HCV negative-strand RNA by strand-specific *in situ* hybridization and expression of core protein in transfected HFHs. HFHs transfected with WT RNA (**A** and **B**), NS5B mutant RNA (**C**), or 3′-UTR RNA (**D**) were fixed on slides 11 days after transfection for detection of HCV negative-strand RNA by strand-specific *in situ* hybridization. Digoxigenin-labeled riboprobes were detected using an antibody conjugated to alkaline phosphatase with Vector Red as the substrate. Cells were counterstained with methyl green. HCV negative-strand RNA (red staining) was detected in clusters of cells (**A**) and was localized to the cytoplasm (**B**). Little or no staining was detected in cultures transfected with 3′-UTR or NS5B mutant HCV RNAs (**D** and **C**, respectively). Core protein expression demonstrated by immunohistochemistry was localized to the cytoplasm with a punctated distribution in cultures transfected with WT RNA (**E**). Cells transfected with 3′-deleted mutant HCV showed very faint staining (**F**). Original magnifications, ×4 (**A**); ×100 (**B–D**); ×40 (**E–F**).

Results

Viral Replication in HFHs Transfected with HCV RNA

After transfection with WT RNA, HFHs shed HCV into the culture medium for 64 days in a cyclical pattern, with peaks at 6, 16, 24, 40, and 64 days after transfection (Figure 1A). Fluctuation on HCV levels have been observed both in infected chimpanzees and in Huh-7.5 line infected with a chimeric JFH1 genome[18,20] and may reflect the effect of host responses to the virus, as discussed below. Although in our experiments the cyclical pattern of virus detection was most commonly observed after transfection of WT HCV RNA, virus persistence with a continuous pattern occurred occasionally (data not shown). In either the cyclic or the continuous pattern of virus detection, HCV levels in the medium reached high concentrations ranging from 10^5 to 10^7 copies/ml during the 2-month culture period. In marked contrast, in HFH cultures transfected with mutant HCV RNAs, either deleted of 3′-UTR or the NS5B catalytic motif (see Materials and Methods), HCV RNA levels progressively declined, and virus was no longer detectable in the medium 24 days after transfection (Figure 1B). The progressive decline of virus levels after transfection of HCV mutant viruses reported here is almost identical to the pattern described by Wakita et al[16] for Huh-7 cells transfected with JFH1 mutants. Measurements of viral levels in cells and the culture medium revealed that nonreplicating viruses are slowly released from the cells into the medium for up to 30 days.[16]

We used strand-specific *in situ* hybridization to detect the presence of negative-strand HCV RNA in cells transfected with WT and mutant HCV.[26] *In situ* hybridization for HCV negative-strand RNA in cultures transfected with WT HCV RNA revealed clusters of cells with strong cytoplasmic staining (Figure 2, A and B). In contrast, staining was barely detectable in cells transfected with NS5B mutant RNA (Figure 2C), and only a few weak positive cells and cell debris were detected in the cultures transfected with 3′-UTR mutant RNA (Figure 2D). We speculated that truncated, functional RNA polymerase proteins might have been generated after 3′-UTR mutant RNAs were

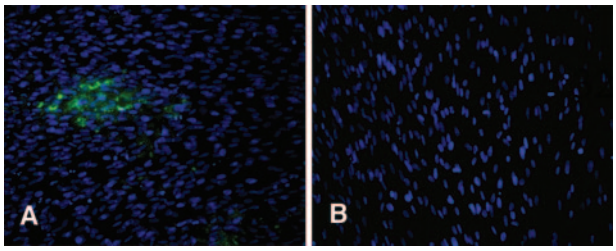

Figure 3. Staining HCV NS3 proteins in transfected HFHs. HFHs were transfected with WT HCV RNA and examined for the expression of NS3 proteins 12 days after transfection. **A** shows the superimposed images of DAPI nuclear staining and fluorescent staining of NS3 protein; **B** is a negative control without NS3 antibody.

transfected,[27,28] and produced a small amount of negative-strand RNAs in a nontemplated manner.[29,30] Core protein expression occurred in clusters of HFHs transfected with WT RNA surrounded by cells that exhibited little or no immunoreactivity and was localized to the cell cytoplasm in a punctated pattern (Figure 2E). However, no staining of core protein was detected in cultures that were transfected with the 3′-UTR mutant RNA (Figure 2F). Core staining in cells transfected with WT RNA was not detectable until 4 days after transfection, but high expression was present at days 8 and 16, demonstrating a fluctuating pattern (not shown). Cytoplasmic NS3 was detectable by immunofluorescence in clusters of cells transfected with WT HCV RNA (Figure 3).

Characterization of HCV Recovered from Cell Culture Supernatants

To determine whether HCV virions could be recovered from the culture medium of HFHs transfected with WT HCV RNA, we examined the buoyant density distribution of HCV-RNA in two samples of medium collected at days 5 and 40 after transfection. At these times, viral levels in the medium were in the range of 10^4 HCV copies/ml (Figure 4A). Sucrose gradient density centrifugation showed that the day 5 sample contained HCV distributed at varying densities, with one of the main fractions having a density of 1.12 g/ml (Figure 4B). By contrast, in the medium collected 40 days after transfection, particles had a homogeneous distribution at a density of 1.17 to 1.18 g/ml (Figure 4C), similar to the value reported by Wakita et al[16] for the JFH-1 virus.

To determine whether transfected HFH cells produce infectious HCV virions that can propagate HCV infection into noninfected cells, we designed a Transwell system that allowed naïve HFHs to be cultivated, without direct contact and separated by a membrane with a porosity of 3 μm, with transfected HFHs (see Materials and Methods). At either 16 or 37 days after transfection (Figure 1A), inserts containing the transfected cells were placed in six-well collagen-coated plates that contained naïve HFHs from a different isolate. Fresh medium was added to the two-layer cell system, and after 2 days of cocultivation, the inserts containing the transfected HFH cells were removed, and the newly infected cells were cultured independently. Medium was collected from

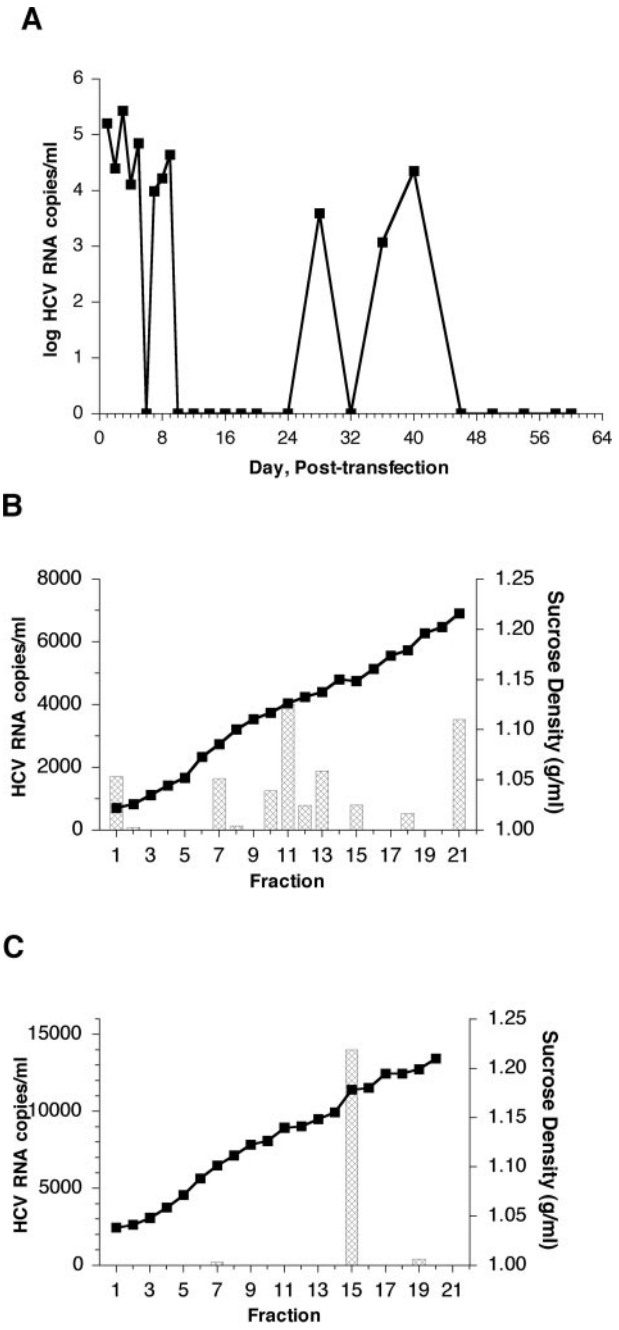

Figure 4. Sucrose density gradient analysis of HCV from culture media. HFHs were transfected with WT HCV and maintained for 60 days (**A**). One-milliliter samples of media collected 5 days (**B**) and 40 days (**C**) after transfection were layered into sucrose solutions (10 to 60% sucrose solutions; see Materials and Methods) and centrifuged for 16 hours at 315,000 rpm in an Optima centrifuge. HCV RNA amounts in each fraction and the sucrose density of the fraction measured in a refractometer are shown. Note the multiple fractions containing HCV sequences in **B** (5 days after transfection, main fraction with a density of 1.12 to 1.13 g/ml), and the homogeneous distribution of HCV particles with a density of 1.17 to 1.18 g/ml in **A** (40 days after transfection).

these cultures for HCV measurements by Taqman real-time RT-PCR assay, as shown in Figure 5, A and B. HFHs exposed to the medium of transfected cultures at days 16 to 18 after transfection were maintained for 40 days after infection (Figure 5A), and HFHs exposed at days 37 to 39 after transfection were maintained for 24 days after infec-

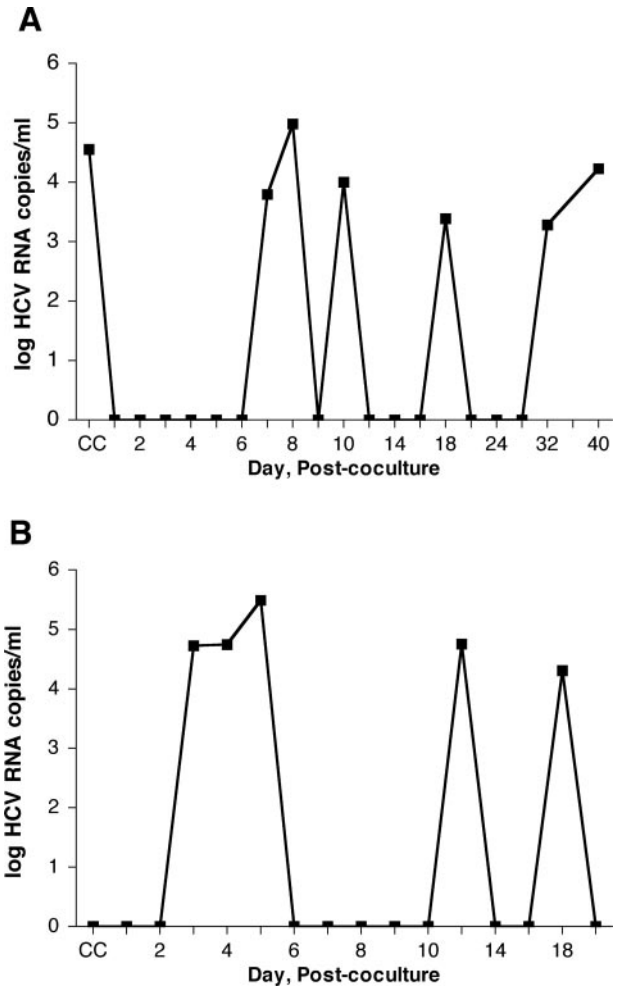

Figure 5. Infection of naïve HFHs with culture media from transfected cells. Naïve HFHs were infected by medium of transfected HFHs obtained at 16 (**A**) or 37 (**B**) days after transfection. After 2-day co-cultivation (see Materials and Methods), the inserts containing transfected HFHs were removed, and the naïve HFHs were cultured in fresh medium. Viral levels in the medium collected at the end of the 2-day co-cultivation period is labeled as CC in **A** and **B**. Media were then collected for 40 days (**A**) or 20 days (**B**) for HCV quantification.

tion (Figure 5B). In both cultures HCV was detected with cyclic fluctuations, reaching a concentration of 10^4 to 10^5 copies/ml at peak expression. Thus, virus released by HFH cultures transfected with WT HCV RNA infected naïve HFH cultures and released HCV in a cyclical pattern during a 3- to 6-week period. In similar experiments using HFH cultures transfected with the NS5B mutant HCV RNA, no virus could be detected in the naïve cultures for a period of 3 weeks after co-cultivation (data not shown).

Suppression of HCV Production by Exogenous Interferons

IFN-α is currently the only effective therapy to eliminate HCV from infected patients. IFN-λ inhibits HCV replication in an *in vitro* replicon system.[31] Here, we demonstrate that treatment of infected HFHs with either IFN-α or IFN-λ suppresses HCV replication. For these experiments, we used the co-culture system described above. One day before co-culture, recombinant human IFN-α or IFN-λ1 was added to the medium of the naïve cells (untreated cultures were not exposed to IFN at any time). The inserts containing transfected HFHs were removed after 2 days, and medium of infected cells was collected for HCV measurements. At this time, the medium of the infected cultures contained HCV at approximately 10^4 copies/ml. In untreated cultures, HCV was maintained at approximately 10^3 copies/ml, with a sharp dip at day 14 (Figure 6A). In marked contrast, virus could not be detected in cultures treated with IFN-α during an 18-day period (Figure 6B). In cultures treated with IFN-λ1, HCV was detected on day 3 after infection but was not detectable for a subsequent 30-day period (Figure 6C). Similar inhibition of HCV growth was obtained by exposing cultures to IFN-λ2 (not shown).

Electron Microscopy of Viral Particles

To visualize particles produced by HFHs infected by medium of transfected cultures, we examined the media of the infected cultures by electron microscopy. Media containing at least 3,000,000 copies/ml of HCV RNA were deposited on grids by ultracentrifugation and negatively stained with 1% uranyl acetate. The medium of infected HFHs contained virus-like particles ranging in size from 50 to 90 nm in diameter (Figure 7A). Those particles were not found in the grids containing media from cultures exposed to nontransfected cultures. To confirm the identification of the particles, media collected from transfected cultures were deposited on grids and stained with goat anti-HCV E2 antibody. Multiple gold particles decorated viral particles, as shown in Figure 7, B and C. Panels D and E show the controls for the immunogold staining. Figure 7D is from grids exposed to nonspecific primary antibody and shows few and scattered gold particles. No label was found in grids in which exposure to the primary antibody was omitted (Figure 7E; this panel also shows a viral particle in which the nucleocapsid, surrounded by an envelope, is labeled by uranyl acetate).

Infection of HFH Cultures with Patient Sera of Diverse HCV Genotypes

We first infected HFHs with HCV serum of genotype 1a collected from a post-transplant patient. HCV produced by the infected HFHs exhibited a fluctuating pattern during a period of 28 days. The highest viral titer, 4.2×10^6 HCV RNA copies/ml, was reached at 18 days in culture. By contrast, HCV could not be detected in cultures inoculated with heat-inactivated serum (Figure 8A). RT-PCR analysis of culture media collected 6 and 18 days after infection demonstrated the presence of NS5A RNA (Figure 8B).

We next tested whether HFH cultures could be infected with sera from patients carrying HCV of genotypes 1b, 2a, 2b, and 3 (Figure 8, C–F). Cultures exposed to serum from a post-transplant patient infected with HCV

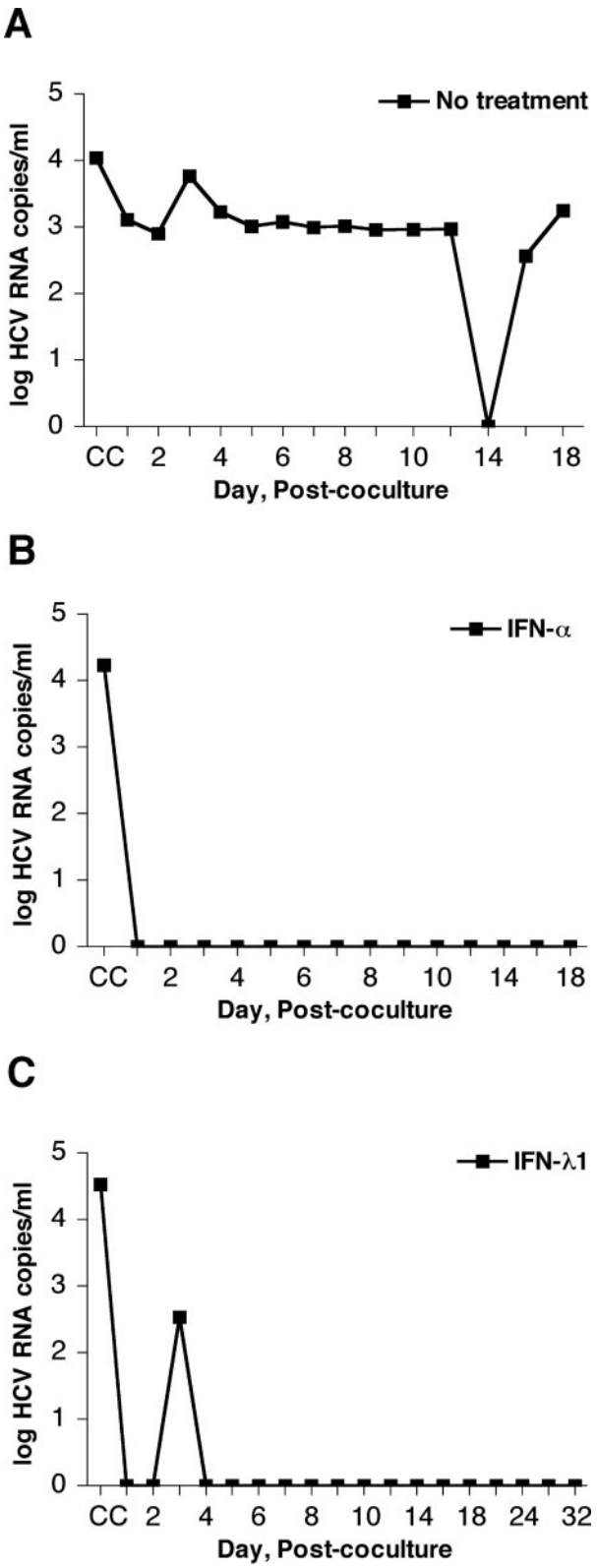

Figure 6. Inhibition of HCV replication in infected cells by human IFN-α and IFN-λ. Naïve HFHs were infected by medium of transfected cells as described in Figure 5. **A** shows the HCV levels in cultures maintained without IFN during an 18-day period after infection. **B** and **C** show HCV levels in infected HFHs maintained in medium containing human 1 IU/ml IFN-α and 100 ng/ml IFN-λ1, respectively. Interferons were added to the naïve HFHs 1 day before the start of the 2-day infection period. Virus levels at the time of infection are labeled as CC (see legend to Figure 5 and Materials and Methods).

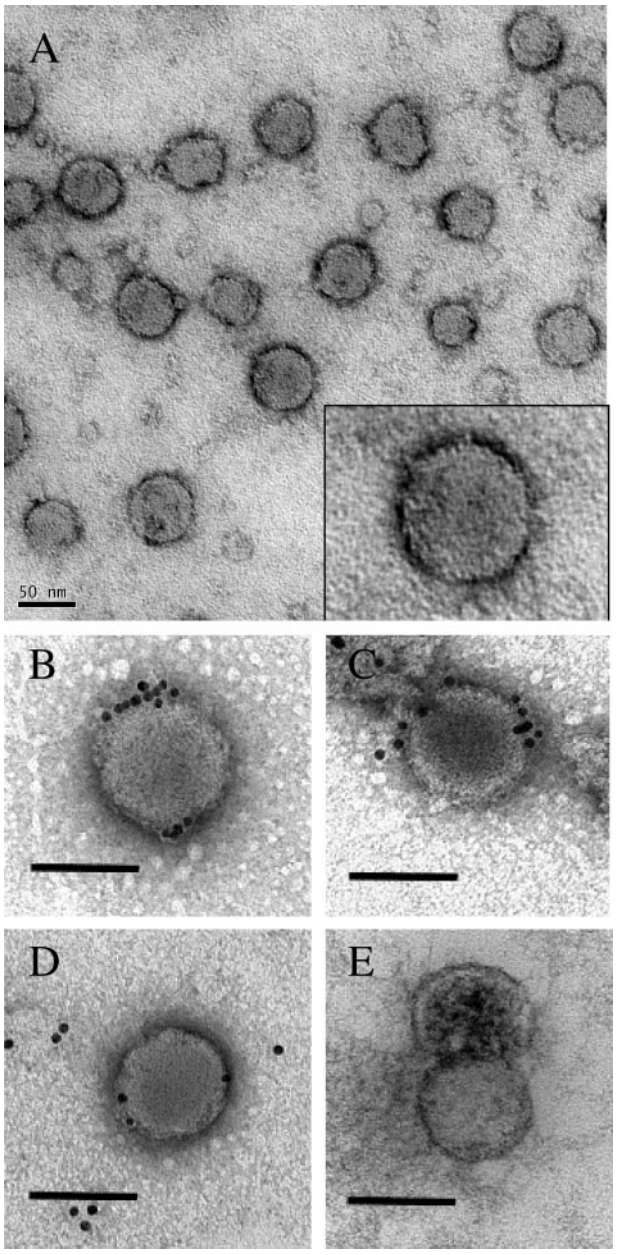

Figure 7. Electron microscopy of virus-like particles from media of infected cultures. A sample of medium, filtered through a 1-μm membrane, containing approximately 10^6 HCV copies/ml was deposited by ultracentrifugation on the grids and negatively stained with 0.1% uranyl acetate (**A** and high magnification **inset**). The medium was obtained from the infected cultures shown in Figure 5B 5 days after infection. For immunogold staining, the grids were incubated with goat polyclonal antibody against HCV genotype 1a E2 (**B** and **C**), goat antibody against mouse IgG (**D**), or no primary antibody (**E**). Rabbit anti-goat IgG conjugated with 10-nm gold particles was used as the secondary antibody for all of the samples. Note the distribution of gold particles decorating a viral particle in **B** and **C**, scattered gold particles in **D,** and no gold labeling in **E**. In **E**, the nucleocapsid of a viral particle surrounded by an envelope is stained by uranyl acetate, probably as a consequence of the high-pressure ultracentrifugation used to deposit the particles into the grid. Scale bars: 50 nm (**A**); 100 nm (**B–E**).

genotype 1b generated three cycles of viral production that lasted for several days (Figure 8C). In other experiments, pooled genotype 2a sera, pooled genotype 2b sera, and genotype 3 serum from a single individual were used to infect HFH cultures (Figure 8, D–F). In all cases,

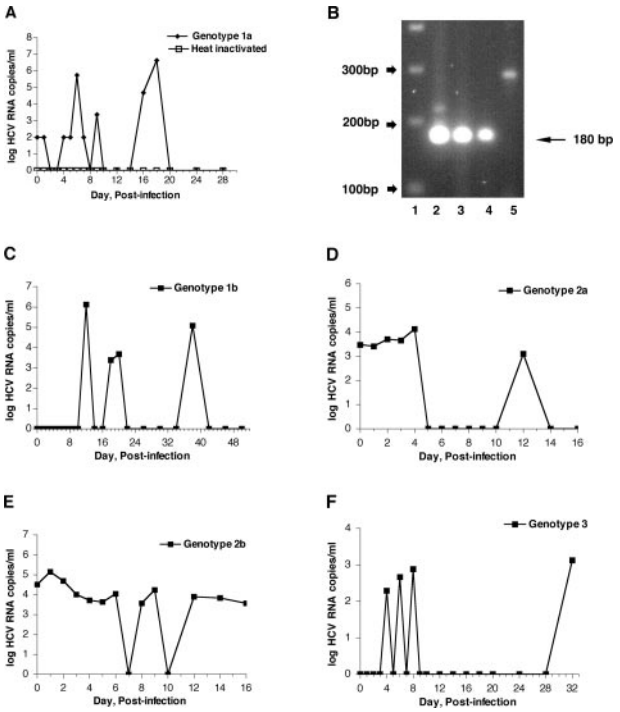

Figure 8. Infection of HFHs with patient sera of genotypes 1, 2, and 3 HCV. For infection, 50 μl of patient serum diluted in 0.5 ml of medium was added to cells plated in 35-mm dishes. **A** displays the levels of HCV released into the cultured media by HFHs infected with genotype 1a serum (**solid diamonds**; inoculum 7.9×10^6 IU of HCV RNA) and heat-treated serum (**open squares**). **B** shows the detection of HCV NS5A gene in the medium of cultures shown in **A**. The 180-bp DNA fragment is the RT-PCR product amplified from the NS5A region of extracted RNA from the culture medium.[44] **Lanes 2** and **3** are from culture media collected at 6 and 18 days after infection, respectively. **Lane 1** contains markers, **lane 4** is an HCV-positive serum control, and **lane 5** is from HCV-negative serum. **C–F** show HCV levels in cultures infected with genotype 1b serum from a single donor (**C**; inoculum 1.5×10^6 copies HCV RNA), genotype 2a serum from pooled samples of the same genotype (**D**; inoculum 6.0×10^5 copies HCV RNA), genotype 2b serum for pooled samples of the same genotype (**E**; inoculum 5.9×10^6 copies HCV RNA), and genotype 3 serum from a single donor (**F**; inoculum 1.7×10^3 copies HCV RNA). The detection limit of the HCV RNA quantitative method used for this experiment is 25 to 100 copies/ml.

the cultures released virus into the media in a cyclic pattern, although they generated different viral amounts and differed in the frequency and timing of HCV release. The same serum added to HFH cultures obtained from different cell isolates generally produced a similar but not identical cyclical pattern (data not shown).

HFHs infected with the serum of genotype 1a, grown on chamber slides, were used to visualize the expression of intracellular HCV RNA by strand-specific *in situ* hybridization. Clusters of stained cells were present in localized areas in the cultures, similar to the pattern described for transfected cultures, shown in Figure 2. The expression of negative-strand RNA was first detected in infected HFHs 1 day after infection and reached highest level at 4 days and was elevated during a 2-week period in culture (Figure 9). Core protein expression detected by IHC was first observed 24 hours after infection and showed cyclical variations over the 28-day period (not shown).

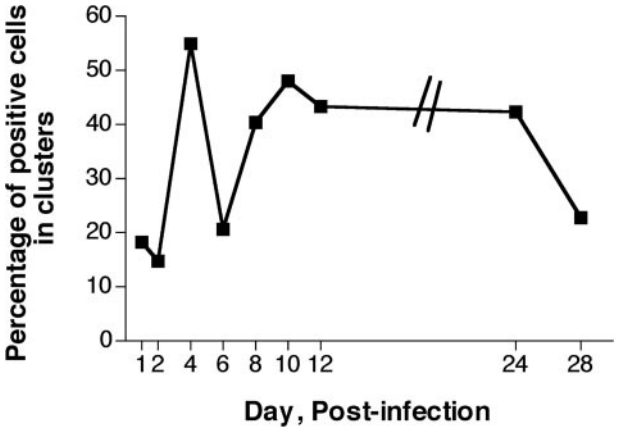

Figure 9. Detection of HCV negative-strand RNA in serum-infected cells. HFHs infected by genotype 1a HCV were grown on chamber slides and processed at various times after infection to detect intracellular HCV negative-strand RNA by *in situ* hybridization. Positive cells were located in scattered clusters; their morphology was similar to those shown in Figure 2, A and B. The percentage of cells stained positive in the clusters was determined by counting a minimum of 100 cells in three random clusters.

Production of IFN-β and Evidence of Cytotoxicity in Transfected Cultures

IFN-β is a key component of the cell defense against HCV infection, and its paracrine effects limit cell-to-cell viral spread.[32] Using an enzyme-linked immunosorbent assay (ELISA) method, IFN-β was first detected in culture medium of transfected HFHs about 2 weeks after transfection and was present for the 2-month culture period (Figure 10A). IFN-α, another antiviral agent produced by infected cells, was not detected at any time. Release of

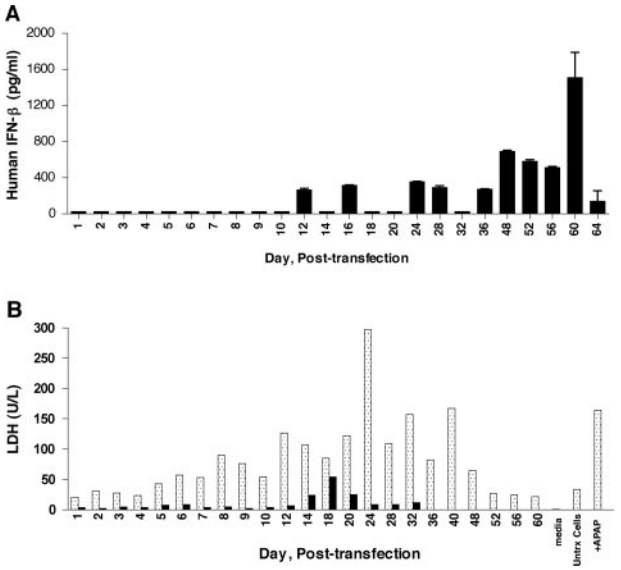

Figure 10. Production of IFN-β and LDH release by transfected HFHs. Cultures were transfected with WT HCV RNA and maintained for 64 days. Medium was collected at the days indicated in the panels, and 100 μl was used for the determination of IFN-β (**A**) by a specific ELISA (human IFN-β; PBL Biomedical Laboratories, Piscataway, NJ) and LDH (**B**, **stippled bars**) using the SYNCHRON LX system (Beckman Coulter). Media from cultures of cells transfected with NS5B mutant RNA are also shown in **B** (**black bars**). For reference, **B** also includes LDH measurements in media from untransfected cultures (Untrx) and cultures treated for 2 days with 10 mmol/L acetaminophen (APAP). All samples were tested in duplicate.

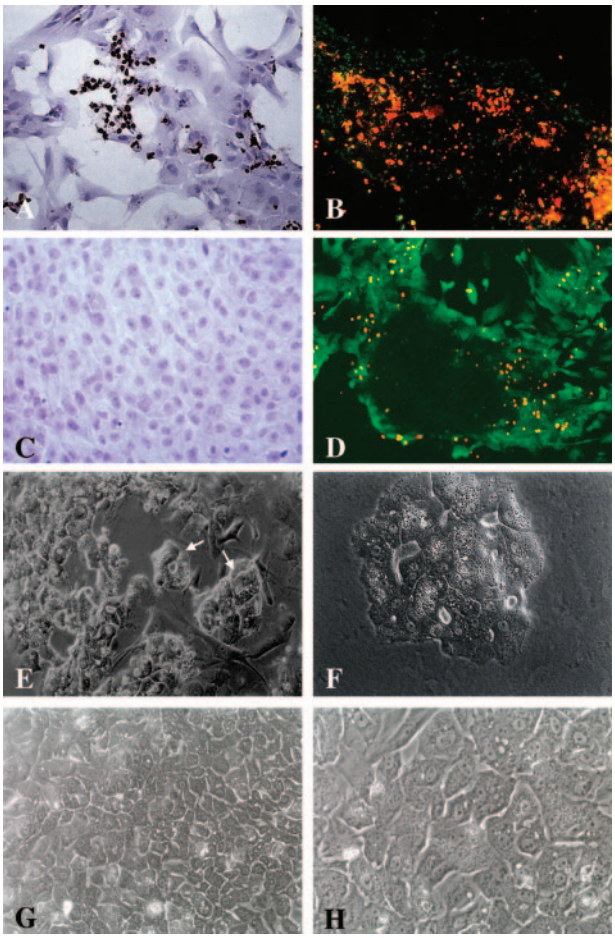

Figure 11. Evidence of cytotoxicity in transfected cultures. Cells were transfected with WT HCV or the 3′-UTR mutant, and cultures were examined for morphological alterations and presence of dead cells. **A** and **B** show the detection of dead cells in cultures transfected with WT HCV by, respectively, TUNEL and cell viability ("live/dead" assay: red, dead cells; green, viable cells) assays. **E** and **F** show morphological disorganization of focal areas of the cultures visualized by phase contrast microscopy. Cultures transfected with mutant HCV showed no dead cells by the TUNEL assay, a small proportion of cell death by the cell viability assay (**C** and **D**), and no disruption of the cell monolayer (**G** and **H**).

lactate dehydrogenase (LDH), an indication of cell injury, was particularly high at 24 days after transfection (Figure 10B). These data suggest that HCV has some cytotoxic effects on hepatocytes and that the production of IFN-β is a component of the response of HFHs to HCV infection.

Evidence of cytotoxic effects by the virus in HFH cultures transfected with WT HCV was also obtained by morphological examination of the cultures and performance of TUNEL and cell viability assays (Figure 11). The detection of dead cells indicated by the TUNEL and cell viability assays (Figure 11, A and B) and the morphological disorganization of cultures, as visualized by phase-contrast microscopy (Figure 11, E and F), occurred only in focal areas and were not widespread through HCV-transfected cultures. Nevertheless, cultures transfected with mutant virus contained few dead cells and little, if any, disorganization of the cell monolayer (Figure 11, C, D, G, and H).

Discussion

Although human hepatocytes are the natural targets for HCV infection, it has been difficult to establish culture systems that sustain HCV replication. Rapid and impressive progress has been obtained in the recent studies of the replication of the JFH-1 virus in Huh-7 cells. Nevertheless, JFH-1 is an unusual virus isolated from a patient with fulminant hepatitis C; it would be highly desirable to develop culture systems that can sustain the replication of HCV of various genotypes by either transfection or serum infection of nontransformed human hepatocytes. We show that HCV replication is sustained for weeks or months in HFH cultures transfected with HCV genotype 1a, the most common genotype in United States, or infected with patient sera containing genotype 1a, 1b, 2a, 2b, and 3. Virus produced by transfected cells was capable of infecting naïve cultures, and the infection was blocked by treatment with IFN-α and IFN-λ.

After transfection or serum infection, HCV levels in the medium fluctuated with a cyclical pattern that persisted through culture periods of 1 to 2 months. Clusters of cells contained HCV negative-strand RNA and expressed core protein. Although the majority of the cells in these clusters expressed viral proteins and contained negative-strand RNA, the overall proportion of cells expressing viral products in the cultures was approximately 10 to 20%. Work in progress seeks to optimize the conditions for viral transfection in these cultures. The culture system we have described differs from systems that rely on Huh-7 cells and transfection of JFH 1 virus. HFH cultures remain stable in prolonged culture.[23,24] These cells maintain their hepatocyte phenotype for several months and express low-density lipoprotein receptor and CD81 that function as HCV receptors.[33–35] However, we have not examined whether these receptors are required for HCV infection in these cells. The HCV virus used in our experiments is unmodified and does not contain adaptive mutations, and in contrast to Huh-7 cells, HFHs can be maintained in primary culture without passaging for more than 1 month, with complete replacement of culture medium at each medium change.

Sucrose gradient centrifugation of culture medium obtained 5 days after transfection revealed the presence of HCV RNA at variable densities. However, 40 days after transfection, practically all of HCV RNA was recovered as an homogeneous fraction at a density of 1.17 g/ml, which is similar to the density of JFH-1 virus reported by Wakita et al.[16] These data suggest that shortly after transfection there is production of particles of multiple sizes, including incomplete viruses that are the product of incomplete synthesis or viral degradation, perhaps as a consequence of cell death. However, we did not determine whether the particles of variable densities isolated from these cultures are infective. Iacovacci et al[8] reported that 30 days after infection of HFHs, the culture medium contained particles distributed between heavy (1.180 to 1.360 g/ml) and light (1.105 to 1.05 g/ml). In our experiments, at 40 days of culture, HCV appears to be produced as homogeneous particles with a density of 1.17 g/ml. Virus-like particles exposed to gold conjugated

anti-HCV E2 antibody and examined by electron microscopy showed decoration of the particles with gold staining.

The HCV WT RNA used in our experiments was transcribed from a HCV cDNA clone (p90/HCVFLpU) constructed based on the consensus sequence of HCV H77 strain of genotype 1a.[25] Chimpanzees infected with this infectious RNA developed acute hepatitis and released HCV in sera.[25,36,37] However, this HCV genome has failed to establish productive HCV replication in Huh-7 cells.[38] Some adaptive mutants of these HCV genomes were able to replicate in Huh-7.5, a derivative of Huh-7, but no HCV particles were released from the cells.[39] More recently, Yi et al[21] were able to produce infectious viral particles from Huh-7.5 cells transfected with H77-S with five adaptive mutations. In our system, we obtained infectious viral particles in nontransformed human hepatocytes transfected with unmodified H77 clone. So far, to our knowledge, infection of Huh-7 cells with sera from infected patients has not been successful.[16]

We demonstrated that HFH cultures can be infected by patient serum of various HCV genotypes, and, as was the case after transfection, release HCV into the medium in a cyclical pattern. Serum infection of HFH cultures was successful in about 80% of samples tested using patient sera from many different donors. We do not know why infection was not obtained with some cultures, but future analysis of the factors associated with serum infectivity of HFH cultures may uncover important biological features regarding HCV infectivity. Preliminary data suggest that in HFHs infected with pooled sera of mixed genotypes, genotype 1a HCV became dominant after prolonged culture.

An interesting aspect of the HFH culture system is the cyclical nature of virus release into the medium, consisting of short bursts of high HCV levels alternating with periods at which virus is not detectable in the medium. This pattern was detected in most cultures, either after transfection or infection. However, in a few cases, virus concentration was continuously high with little variation for several weeks, although we could not ascertain the factors that were responsible for this pattern of expression. Interestingly, at times, recipient naïve HFHs could be infected in the Transwell system (as shown in Figure 5B) even when HCV was not detected in the culture medium, suggesting that virus release may be continuous but is variable in its quantity during a prolonged culture period. It should be noted that we collected samples of culture medium every 4 days or less and that at each time the medium was completely changed. This procedure differs from that of Iacovacci et al,[8] who collected samples at 10-day intervals between 10 and 30 days after infection, without changing the medium completely at each collection. Fluctuations of HCV levels have been attributed to innate and adaptive immunity anti-viral mechanisms.[40] In culture systems, cyclical patterns of virus release may be a consequence of a cyclical production of HCV associated perhaps with the synthesis of antiviral agents by the cells. Chimpanzees inoculated with strain FL-J6/JFH virus showed cyclical peaks of HCV viremia during a 16-week postinfection period. The fluctuations in viremia were attributed to the innate and adaptive immune response of the host.[20] Recent findings have revealed that multiple proteins interact with HCV RNA, leading to activation of interferon regulatory factor IRF-3 and subsequent IFN-β production and secretion from infected cells.[32,41,42] The production of IFN-β by infected cells can act through a paracrine effect to limit viral spread to noninfected cells.[32] IFN-β was detected in culture media of HCV-transfected HFHs. Although a comparison between the timing of IFN-β secretion and HCV levels in the media shown in Figures 1A and 10A does not provide conclusive evidence that the production of IFN-β is responsible for the cyclical pattern of virus detection, our results demonstrate that human hepatocytes produce IFN-β as a response to HCV infection. HCV could have been released in the medium by periodic bursts of cell injury or death. Although cell death occurred in HFH-infected cells, indicating that HCV has some cytotoxic effects in these cells, we could not establish a correlation between cell death and the fluctuating HCV levels in the medium. It is possible that while virus release into the medium has a cyclical pattern, the actual production of virus in the cells proceeds continuously. However, core protein expression in transfected or serum-infected cells also displayed a fluctuating pattern during a 30-day period of culture. Further studies are required to study the possible interrelationships among virus production, viral release, cell death, and antiviral responses in HFH cultures.

It should be noted that infection of Huh-7 cells with the JFH1 virus does not induce an interferon response because the virus inactivates the adaptor molecule IPS-1, which is necessary for the interferon response.[43] In addition, Huh-7 cells are deficient in Toll-like receptor 3 (TLR 3) signaling, which recognizes double-stranded RNA viral intermediates. We suggest that HFHs produce IFN-β in response to HCV infection because the level of viral infectivity of the system is low compared with that of the Huh-7 infection by JFH 1 and because TLR3 signaling is intact in HFHs (W.T., N.F., unpublished data).

Chronic infection by HCV and the development of liver disease in infected patients are primarily a consequence of the host's immune response against infected hepatocytes. We show here that HCV may have a direct cytotoxic effect on hepatocytes. Highest release of LDH in HCV-transfected HFH cultures was detected between 12 and 40 days after transfection. Abnormalities in hepatocyte morphology and presence of apoptosis were also detected in focal areas of these cultures but were absent in cultures transfected with mutant HCV. Although we do not know whether the virus has a direct cytotoxic effect on hepatocytes in human infection, it is conceivable that such effect could occur transiently in acute infections and could help initiate the immune response.

In summary, we report the establishment of a culture system using nontransformed fetal human hepatocytes that sustains HCV replication for at least 2 months after transfection or patient serum infection. The system should be useful for uncovering the interactions between HCV and nontransformed human hepatocytes and for studies on the infectivity of sera from patients with HCV-

induced liver disease. Further studies should determine whether virus produced in HFH cultures is infective after inoculation into animals.

Acknowledgments

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References

1. Wasley A, Alter MJ: Epidemiology of hepatitis C: geographic differences and temporal trends. Semin Liver Dis 2000, 20:1–16
2. Seeff LB: Natural history of hepatitis C. Am J Med 1999, 107:10S–15S
3. Williams I: Epidemiology of hepatitis C in the United States. Am J Med 1999, 107:2S–9S
4. Alter MJ: Hepatitis C virus infection in the United States. J Hepatol 1999, 31(Suppl 1):88–91
5. Kato N, Shimotohno K: Systems to culture hepatitis C virus. Curr Top Microbiol Immunol 2000, 242:261–278
6. Shimizu YK, Iwamoto A, Hijikata M, Purcell RH, Yoshikura H: Evidence for in vitro replication of hepatitic C virus genome in a human T-cell line. Proc Natl Acad Sci USA 1992, 89:5477–5481
7. Noguchi M, Hirohashi S: Cell lines from non-neoplastic liver and hepatocellular carcinoma tissue from a single patient. In Vitro Cell Dev Biol Anim 1996, 32:135–137
8. Iacovacci S, Manzin A, Barca S, Sargiacomo M, Serafino A, Valli MB, Macioce G, Hassan HJ, Ponzetto A, Clementi M, Peschle C, Carloni G: Molecular characterization and dynamics of hepatitis C virus replication in human fetal hepatocytes infected in vitro. Hepatology 1997, 26:1328–1337
9. Loriot MA: Permissiveness of human biliary epithelial cells to infection by hepatitis C virus. Hepatology 1999, 29:1587–1595
10. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R: Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999, 285:110–113
11. Lohmann V, Korner F, Dobierzewska A, Bartenschlager R: Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J Virol 2001, 75:1437–1449
12. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, Strand D, Bartenschlager R: Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002, 76:4008–4021
13. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, Wakita T: Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 2003, 125:1808–1817
14. Date T: Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. J Biol Chem 2004, 279:22371–22376
15. Kato T: Non-hepatic cell lines HeLa and 293 cells support efficient replication of hepatitis C virus genotype 2a subgenomic replicon. J Virol 2005, 79:592–596
16. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ: Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005, 11:791–796
17. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV: Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci USA 2005, 102:9294–9299
18. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM: Complete replication of hepatitis C virus in cell culture. Science 2005, 309:623–626
19. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R: Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci USA 2006, 103:7408–7413
20. Lindenbach BD, Meuleman P, Ploss A, Vanwolleghem T, Syder AJ, McKeating JA, Lanford RE, Feinstone SM, Major ME, Leroux-Roels G, Rice CM: Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc Natl Acad Sci USA 2006, 103:3805–3809
21. Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM: Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc Natl Acad Sci USA 2006, 103:2310–2315
22. Berke JM, Moradpour D: Hepatitis C virus comes full circle: production of recombinant infectious virus in tissue culture. Hepatology 2005, 42:1264–1269
23. Lázaro CA, Croager EJ, Mitchell C, Campbell JS, Yu C, Foraker J, Rhim JA, Yeoh GCT, Fausto N: establishment, characterization and long-term maintenance of cultures of human fetal hepatocytes. Hepatology 2003, 38:1095–1106
24. Dan YY, Riehle KJ, Lazaro C, Teoh N, Haque J, Campbell JS, Fausto N: Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. Proc Natl Acad Sci USA 2006, 103:9912–9917
25. Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM: Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 1997, 277:570–574
26. Chang M: In situ distribution of hepatitis C virus replicative-intermediate RNA in hepatic tissue and its correlation with liver disease. J Virol 2000, 74:944–955
27. Ferrari E, Wright-Minogue J, Fang JW, Baroudy BM, Lau JY, Hong Z: Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in Escherichia coli. J Virol 1999, 73:1649–1654
28. Lohmann V, Korner F, Herian U, Bartenschlager R: Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J Virol 1997, 71:8416–8428
29. Ranjith-Kumar CT, Gajewski J, Gutshall L, Maley D, Sarisky RT, Kao CC: Terminal nucleotidyl transferase activity of recombinant Flaviviridae RNA-dependent RNA polymerases: implication for viral RNA synthesis. J Virol 2001, 75:8615–8623
30. Ranjith-Kumar CT, Sarisky RT, Gutshall L, Thomson M, Kao CC: De novo initiation pocket mutations have multiple effects on hepatitis C virus RNA-dependent RNA polymerase activities. J Virol 2004, 78:12207–12217
31. Robek MD, Boyd BS, Chisari FV: Lambda interferon inhibits hepatitis B and C virus replication. J Virol 2005, 79:3851–3854
32. Gale Jr M, Foy EM: Evasion of intracellular host defense by hepatitis C virus. Nature 2005, 436:939–945
33. Agnello V, Abel G, Knight GB, Muchmore E: Detection of widespread hepatocyte infection in chronic hepatitis C. Hepatology 1998, 28:573–584
34. Pileri P: Binding of hepatitis C virus to CD81. Science 1998, 282:938–941
35. Cormier EG, Tsamis F, Kajumo F, Durso RJ, Gardner JP, Dragic T: CD81 is an entry coreceptor for hepatitis C virus. Proc Natl Acad Sci USA 2004, 101:7270–7274
36. Major ME, Mihalik K, Fernandez J, Seidman J, Kleiner D, Kolykhalov AA, Rice CM, Feinstone SM: Long-term follow-up of chimpanzees inoculated with the first infectious clone for hepatitis C virus. J Virol 1999, 73:3317–3325
37. Hong Z, Beaudet-Miller M, Lanford RE, Guerra B, Wright-Minogue J,

Skelton A, Baroudy BM, Reyes GR, Lau JY: Generation of transmissible hepatitis C virions from a molecular clone in chimpanzees. Virology 1999, 256:36–44
38. Blight KJ, Kolykhalov AA, Rice CM: Efficient initiation of HCV RNA replication in cell culture. Science 2000, 290:1972–1974
39. Blight KJ, McKeating JA, Marcotrigiano J, Rice CM: Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J Virol 2003, 77:3181–3190
40. Arase Y, Ikeda K, Chayama K, Murashima N, Tsubota A, Suzuki Y, Saitoh S, Kobayashi M, Kobayashi M, Suzuki F, Kumada H: Fluctuation patterns of HCV-RNA serum level in patients with chronic hepatitis C. J Gastroenterol 2000, 35:221–225
41. Loo YM, Owen DM, Li K, Erickson AK, Johnson CL, Fish PM, Carney DS, Wang T, Ishida H, Yoneyama M, Fujita T, Saito T, Lee WM, Hagedorn CH, Lau DT, Weinman SA, Lemon SM, Gale Jr M: Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc Natl Acad Sci USA 2006, 103:6001–6006
42. Li K, Chen Z, Kato N, Gale Jr M, Lemon SM: Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-β production in hepatocytes. J Biol Chem 2005, 280:16739–16747
43. Cheng G, Zhong J, Chisari FV: Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. Proc Natl Acad Sci USA 2006, 103:8499–8504
44. Hofgärtner WT, Polyak SJ, Sullivan DG, Carithers Jr RL, Gretch DR: Mutations in the NS5A gene of hepatitis C virus in North American patients infected with HCV genotype 1a or 1b. J Med Virol 1997, 53:118–126