GASTROENTEROLOGY 2010;139:1355–1364

BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Production of Infectious Hepatitis C Virus in Primary Cultures of Human Adult Hepatocytes

PHILIPPE PODEVIN,* ARNAUD CARPENTIER,*,‡ VÉRONIQUE PÈNE,* LYNDA AOUDJEHANE,‡ MATTHIEU CARRIÈRE,‡ SAKINA ZAÏDI,* CÉLINE HERNANDEZ,* VANESSA CALLE,* JEAN–FRANÇOIS MÉRITET,*,§ OLIVIER SCATTON,‖ MARLÈNE DREUX,¶ FRANÇOIS–LOÏC COSSET,¶ TAKAJI WAKITA,# RALF BARTENSCHLAGER,** SYLVIE DEMIGNOT,‡‡ FILOMÉNA CONTI,‡ ARIELLE R. ROSENBERG,*,§ and YVON CALMUS‡

*INSERM, Equipe Avenir "Virologie de l'Hépatite C," Institut Cochin, Paris, France; ‡Université Paris Descartes, UPRES 1833, Paris, France; §Université Paris Descartes; AP-HP, Groupe Hospitalier Cochin, Service de Virologie, Paris, France; ‖AP-HP, Groupe Hospitalier Cochin, Service de Chirurgie, Paris, France; ¶INSERM, U 758; IFR 128, Biosciences Lyon-Gerland; Ecole Normale Supérieure de Lyon, Department of Human Virology, Lyon, France; #National Institute of Infectious Diseases, Department of Virology II, Tokyo, Japan; **University of Heidelberg, Department of Molecular Virology, Heidelberg, Germany; ‡‡INSERM; Université Pierre et Marie Curie; Université Paris Descartes; Centre de Recherche des Cordeliers, UMRS 872; EPHE, Laboratoire de Pharmacologie Cellulaire et Moléculaire, Paris, France

See editorial on page 1090.

BACKGROUND & AIMS: Although hepatitis C virus (HCV) can be grown in the hepatocarcinoma-derived cell line Huh-7, a cell-culture model is needed that supports its complete, productive infection cycle in normal, quiescent, highly differentiated human hepatocytes. We sought to develop such a system. **METHODS:** Primary cultures of human adult hepatocytes were inoculated with HCV derived from Huh-7 cell culture (HCVcc) and monitored for expression of hepatocyte differentiation markers and replication of HCV. Culture supernatants were assayed for HCV RNA, core antigen, and infectivity titer. The buoyant densities of input and progeny virus were compared in iodixanol gradients. **RESULTS:** While retaining expression of differentiation markers, primary hepatocytes supported the complete infectious cycle of HCV, including production of significant titers of new infectious progeny virus, which was called *primary-culture–derived virus* (HCVpc). Compared with HCVcc, HCVpc had lower average buoyant density and higher specific infectivity; this was similar to the characteristics of virus particles associated with the very-low-density lipoproteins that are produced during in vivo infection. These properties were lost after re-culture of HCVpc in poorly differentiated Huh-7 cells, suggesting that authentic virions can be produced only by normal hepatocytes that secrete authentic very-low-density lipoproteins. **CONCLUSIONS: We have established a cell-culture–based system that allows production of infectious HCV in physiologically relevant human hepatocytes. This provides a useful tool for the study of HCV interactions with its natural host cell and for the development of antiviral therapies.**

Keywords: Antiviral Drug Assay; Cell Permissiveness; Hepatocellular Physiology; Virus Propagation.

Hepatitis C virus (HCV) is a leading cause of chronic liver disease worldwide. This positive-strand RNA virus was identified by molecular cloning in 1989, and classified into 7 genotypes within its own genus. Twenty years later, however, the only approved treatment, pegylated interferon-alfa combined with ribavirin, is far from being fully satisfactory, hence there is a clear need for a better understanding of the virus life cycle and the rational development of novel therapies to interrupt it.[1] The availability of relevant cell-culture–based models is essential to achieve these goals, but culturing HCV has turned out to be a notoriously difficult task.[2,3] Because replication of this virus in vivo occurs mainly in highly differentiated, nondividing hepatocytes, primary cultures of human adult hepatocytes (PHH)[4] provide the closest in vitro model for the natural host cell of HCV. However, despite numerous attempts to infect PHH using sera from HCV-infected patients as sources of virus, replication of the viral genome was difficult to detect and production of measurable titers of progeny virus has not been achieved.[5–9] Accordingly, uncertainties persist as to whether these PHH-based systems faithfully reflect pro-

Abbreviations used in this paper: ApoB, apolipoprotein B; CYP, cytochrome P450; ffu, focus-forming units; HCVcc, hepatitis C virus derived from culture in Huh-7 cell line; HCVpc, hepatitis C virus derived from primary culture of human adult hepatocytes; HCVrecc, hepatitis C virus derived from re-culture in the Huh-7 cell line of hepatitis C virus derived from primary culture of human adult hepatocytes; MOI, multiplicity of infection; NS3, HCV nonstructural protein 3; PHH, primary human adult hepatocytes; VLDL, very-low-density lipoprotein.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.06.058

cesses involved in a productive, and thus authentic, infection cycle, and studies remain limited by the very low efficiency.[2] A breakthrough in HCV research was achieved in 2005 when it became possible to grow the virus in cell culture thanks to the cloning of JFH1, a genotype 2a-HCV isolate with exceptional efficiency at viral genome replication.[3] Highly permissive subclones of the human hepatocellular carcinoma–derived cell line Huh-7 have been shown to produce workable titers of cell-culture–derived virus (HCVcc) when replicating the full-length genome of JFH1, or chimeras consisting of the nonstructural genes (encoding the viral proteins sufficient for genome replication) of JFH1 fused to the structural genes (encoding the viral core protein and envelope glycoproteins E1 and E2) of other HCV isolates.[3,10–13] However, the transformed, rapidly dividing, and poorly differentiated Huh-7 cells differ significantly from normal human adult hepatocytes, raising doubts as to the in vivo relevance of this now widely used in vitro system.[2-4] Thus, it has remained a challenging priority to develop a cell-culture–based model supporting the complete, productive infection cycle of HCV in the context of normal, and thus physiologically relevant, human liver cells.

These considerations prompted us to investigate whether HCVcc could be used as a source of virus to achieve productive infection in PHH. With this strategy we have established a simple yet robust system supporting all steps of the HCV infection cycle in highly differentiated hepatocytes. Indeed, this system is shown here to support not only replication of the viral genome but also production of highly infectious progeny virus, which has unique properties compared with HCVcc, and which will be termed *primary-culture–derived HCV* (*HCVpc*).

Materials and Methods

Isolation and Primary Culture of Human Adult Hepatocytes

Normal-appearing liver tissue was obtained from adult patients undergoing partial hepatectomy in Cochin Hospital for the therapy of metastases or benign tumors and seronegative for HCV, hepatitis B virus, and human immunodeficiency virus. Experimental procedures were performed in accordance with French laws and regulations. Immediately after surgical resection, the liver pieces were stored in Celsior solution (IMTIX-SangStat, Lyon, France), and dissociation of cells was performed no more than 3 hours later by a 2-step perfusion method essentially as described previously[14] with some modifications, as follows. Visible vessels were first perfused for 15 minutes with Liver Perfusion Medium (Invitrogen, Cergy Pontoise, France) at 37°C to eliminate blood cells. A second perfusion then was performed with collagenase- and dispase-containing Liver Digest Medium (Invitrogen) at 37°C, at a flow rate of 10 mL/catheter/min (Masterflex peristaltic pump; Fisher Scientific, Illkirch, France), until the tissue was fully digested (typically, 30 min). Liver fragments were shaken gently in Hepatocyte Wash Medium (Invitrogen) to free loose cells, which then were filtered through a 70-μm nylon mesh before centrifugation at 200 $\times$ g for 1 minute. The fibroblast- and Küppfer cell–containing supernatant was discarded, and hepatocytes were washed a second time before assessing viability by trypan blue dye exclusion. Cells were resuspended in complete hepatocyte medium consisting of Leibovitz's L-15 medium (Invitrogen) supplemented with 26 mmol/L $NaHCO_3$, 100 IU/L insulin (Novo Nordisk, Puteaux, France), and 10% heat-inactivated fetal calf serum (Biowest, Nuaillé, France), and seeded at a density of 1.2–1.6 $\times$ 10^5 viable cells/cm^2 onto 6- or 12-well plates that had been precoated with a solution (1 mg/mL in 0.1 mol/L acetic acid) of Bornstein and Traub type I collagen from calf skin (catalog #C8919; Sigma-Aldrich, St. Louis, MO) between 1 and 10 hours previously (plates were covered with the minimal volume of collagen solution, then left to dry at room temperature). The medium was replaced 16–20 hours later with fresh complete hepatocyte medium supplemented with 1 μmol/L hydrocortisone hemisuccinate (SERB, Paris, France), and cells were left in this medium until HCV inoculation. The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

HCV Inoculation

High-titer stocks of JFH1- or Con1/C3-HCVcc and HCVpc were prepared as described in the Supplementary Materials and Methods section. PHH were inoculated 3 days after seeding. Unless otherwise stated, the multiplicity of infection (MOI) was 0.1 or 0.04 focus-forming units (ffu) per cell for HCVcc and HCVpc, respectively. The medium was replaced with the inoculum diluted in the smallest volume of fresh complete hepatocyte medium sufficient for covering the cells. After a 6- to 16-hour incubation at 37°C, the inoculum was removed, and monolayers were washed 3 times with phosphate-buffered saline. The cultures then were maintained during 15 days in complete hepatocyte medium. Half of the medium volume was changed every third day.

For drug inhibition assay and for E1 and CD81 neutralization assays, the conditions of HCV inoculation and culture are described in the Supplementary Materials and Methods section.

Phenotypic Characterization of PHH

To characterize the differentiation status of PHH, the expression of hepatocyte-specific genes and the inducibility of cytochrome P450 (CYP)3A4 expression were analyzed by a quantitative reverse-transcription real-time polymerase chain reaction technique, as described in the Supplementary Materials and Methods section.

Quantification of HCV RNA and Core Antigen and Titration of Infectivity

Intracellular levels of positive- and negative-strand HCV RNA were quantified by a strand-specific reverse-