

Expression of hepatitis C virus proteins in epithelial intestinal cells *in vivo*

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Previous work on hepatitis C virus (HCV) led to the discovery of a new form of virus particle associating virus and lipoprotein elements. These hybrid particles (LVP for lipo-viro-particles) are enriched in triglycerides and contain at least apolipoprotein B (apoB), HCV RNA and core protein. These findings suggest that LVP synthesis could occur in liver and intestine, the two main organs specialized in the production of apoB-containing lipoprotein. To identify the site of LVP production, the genetic diversity and phylogenetic relationship of HCV quasispecies from purified LVP, whole serum and liver biopsies from chronically infected patients were studied. HCV quasispecies from LVP and liver differed significantly, suggesting that LVP were not predominantly synthesized in the liver but might also originate in the intestine. The authors therefore searched for the presence of HCV in the small intestine. Paraffin-embedded intestinal biopsies from 10 chronically HCV-infected patients and from 12 HCV RNA-negative controls (10 anti-HCV antibody-negative and two anti-HCV antibody-positive patients) were tested for HCV protein expression. HCV NS3 and NS5A proteins were stained in small intestine epithelial cells in four of the 10 chronically infected patients, and not in controls. Cells expressing HCV proteins were apoB-producing enterocytes but not mucus-secreting cells. These data indicate that the small intestine can be infected by HCV, and identify this organ as a potential reservoir and replication site. This further emphasizes the interaction between lipoprotein metabolism and HCV, and offers new insights into hepatitis C infection and pathophysiology.

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INTRODUCTION

Hepatitis C virus (HCV) infects over 170 million people worldwide and is a major cause of chronic liver infection, often leading to chronic liver disease, cirrhosis and hepatocellular carcinoma. HCV possesses a single-stranded, positive-sense RNA genome encoding a single polyprotein, and the virus has been classified in the family *Flaviviridae* with the flaviviruses and pestiviruses (Pringle, 1999). Although the flaviviruses infect many tissues in a large number of hosts from insects to primates, and replicate in numerous cell lines *in vitro*, HCV replication is extremely limited and occurs mainly in the human liver (Hoofnagle, 2002; Rice,

1996). Studies on the virus replication and biology have been hampered by the lack of an efficient and reliable cell culture system, and because chimpanzee is the only animal model. HCV may be envisioned as a flavivirus whose replication relies on specific cellular functions present in specific organs such as the liver. A recent report on replication of HCV subgenomic replicons, without production of virus particles, in non-hepatic epithelial human and mouse hepatoma cells indicates that the specific cellular factors required for translation and replication of HCV RNA are not restricted to hepatocytes or cells of human origin (Zhu *et al.*, 2003). Therefore, the apparent tropism of HCV for hepatocytes is probably also determined at the level of virus entry or assembly.

Several reports have revealed the density heterogeneity of ill-defined HCV RNA-containing particles. HCV RNA-containing particles were found at density of between 1.03 and 1.25 g ml⁻¹ in the serum of chronically infected

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A figure showing how mAb 4F3H2 stains NS5A-expressing cells is available from JGV Online.

individuals (Miyamoto *et al.*, 1992; Prince *et al.*, 1996; Thomssen *et al.*, 1993). Titration of infectivity in chimpanzee established a relationship between density of particles and infectivity, the highest infectivity of plasma being associated with the majority of HCV RNA in low-density fractions ($d < 1.06 \text{ g ml}^{-1}$), while HCV RNA found in higher density fractions appeared to be poorly infectious (Bradley *et al.*, 1991; Hijikata *et al.*, 1993). The unusually low density of some HCV RNA-containing particles suggested an association of the virus with plasma lipoproteins (Thomssen *et al.*, 1993). Low-density lipoproteins (LDL, $d < 1.06 \text{ g ml}^{-1}$) are particles which consist of a hydrophobic core of neutral lipid surrounded by a monolayer of amphipathic phospholipids and free cholesterol in which apolipoproteins reside (Fisher & Ginsberg, 2002; Rustaeus *et al.*, 1999). Hepatocytes produce and secrete very low-density lipoprotein (VLDL) containing apolipoproteins B and E (apoB and apoE). Transformation of VLDL in the circulation gives rise to particles of smaller size, with intermediate to low density (intermediate-density lipoprotein, IDL and LDL; Fisher & Ginsberg, 2002). Chylomicrons are another form of circulating VLDL that are secreted by intestinal epithelial cells: they resemble VLDL but contain truncated apoB, are larger, and are enriched in triglyceride. Transformation of chylomicrons in the circulation also leads to particles of smaller size and higher density (Hussain *et al.*, 1996; Yu & Cooper, 2001). In an attempt to understand better the relationship between HCV and lipoprotein metabolism and to unveil specific factors required for virus replication and assembly, we recently conducted a study of the nature and infectivity of HCV particles in the low-density plasma fractions (Andre *et al.*, 2002). Low-density HCV RNA-containing particles (lipo-viro-particles, LVP) were rich in triglycerides, contained at least apoB, HCV RNA and core protein, and appeared as large spherical particles over 100 nm in diameter with internal structures. Delipidation of these particles resulted in capsid-like structures recognized by anti-HCV core protein antibody. These findings suggested that LVP synthesis could occur in organs specializing in production of apoB-containing lipoproteins. Because HCV replicates in the liver, this organ is probably an important source of LVP. Alternatively, because of the very high triglyceride content of LVP, intestine may also contribute to the production of these particles. To address this question we conducted a study to identify the site of LVP production.

METHODS

Blood and liver samples. Volunteers attending the Liver Unit at Necker Hospital, Paris were selected in accordance with the hospital's Ethics Committee statements; they were chronically HCV-infected patients with chronic active hepatitis and had not been given antiviral therapy for more than 6 months. Screening for HBV or HIV infection was negative. EDTA (1 mM final concentration) was added to 40 ml peripheral blood, and samples were sent to the laboratory at ambient temperature, where plasma and serum were immediately processed for density fraction separation and RNA

extraction with the NucleoSpin RNA virus kit (Macherey-Nagel) and eluted in 50 μl RNase-free water. Aliquots were stored at -80°C . Liver biopsies performed for regular clinical follow-up of three patients were retrospectively included in the study. Frozen liver tissue stored in liquid nitrogen was disrupted and homogenized with a rotor-stator homogenizer. RNA was extracted with the RNeasy kit (Qiagen) and eluted in 40 μl RNase-free water.

Small intestine and liver biopsies. Intestinal and liver biopsies were conserved at the Laboratoire d'Anatomie Pathologique, centre hospitalier Edouard Herriot, Lyon, France. Formalin-fixed liver biopsies from one HCV-seronegative patient and from four HCV RNA-positive patients were selected as negative and positive controls. Formalin-fixed and paraffin-embedded biopsies of the duodenum from 12 HCV-seropositive patients (for one patient a biopsy of the jejunum was also available) and from 12 HCV-seronegative patients were studied. Intestinal biopsies were taken for diagnostic purposes during endoscopic examinations performed for symptoms not related to HCV infection: dyspepsia in five cases, abdominal pain in five cases or suspicion of malabsorption in one case, melaena (one case) or evaluation of HCV-related vasculitis (one case). In all but two cases, the histological status of biopsy samples was normal. Aspergillosis was detected in one patient after liver transplantation for HCV-related cirrhosis, and chronic non-specific duodenitis was observed for the other patient. Ten of the 12 HCV-seropositive patients were also HCV RNA-positive by the VERSANT HCV RNA Qualitative Assay (TMA) (Bayer). For RNA extraction, biopsies were sliced into small pieces, deparaffinated and rehydrated as described below for immunostaining. Then 150 μl of 6 mg ml^{-1} proteinase K (Sigma) complemented with 20 μg yeast tRNA was added to the sections, and digestion was performed overnight at 45°C . Total RNA was further extracted with the NucleoSpin RNA virus kit and eluted in 50 μl RNase-free water.

Preparation of low-density fractions. Plasma from infected patients was separated by ultracentrifugation to obtain one low-density fraction composed of the apoB-containing lipoproteins including chylomicrons, VLDL, IDL and LDL. Density of the plasma was adjusted to $d = 1.055 \text{ g ml}^{-1}$ with NaBr (Sigma). The low-density fraction ($d < 1.055 \text{ g ml}^{-1}$) was obtained by centrifugation of plasma for 4 h at 4°C and 543 000 g with the TLA100.4 rotor and TL100 ultracentrifuge (Beckman Instruments). The top fraction was collected and extensively dialysed at 4°C against 150 mM NaCl, 0.24 mM EDTA pH 7.4 buffer, filtered through 0.22 μm filters (Millipore) and stored at 4°C in the dark.

Purification of LVP. This procedure (Andre *et al.*, 2002) has been extensively described and leads to the purification of HCV LVP. Briefly, 10 μl Protein A-coated magnetic beads (Miltenyi Biotec) were incubated at room temperature with 1 ml of the low-density fractions in PBS with gentle rocking for 30 min. Beads were then passed through a magnetic column (Miltenyi Biotec), washed and collected in 500 μl PBS or DMEM/0.2% BSA (Gibco-BRL). Samples with high lipid content were first diluted with 2 vols of normal human serum to inactivate PCR inhibitors, and RNA was extracted from 10 μl LVP with the NucleoSpin RNA virus kit, eluted in 50 μl and stored at -80°C .

HCV RNA quantification. HCV positive-strand RNA quantification was performed by real-time PCR in the 5' HCV non-coding region as described previously, with minor modifications (Komurian-Pradel *et al.*, 2001). Briefly, RNA (4 μl) was reverse-transcribed with the Thermoscript Reverse transcriptase kit (Gibco-BRL) using the RC21 primer. Real-time PCR was carried out with 2 μl cDNA and RC1 and RC21 primers using the LC FastStart DNA Master SYBR Green 1 kit and the LightCycler apparatus (Roche Diagnostics).

An index of HCV RNA association with low-density fractions was

determined including apoB as an internal standard of the lipoprotein compartment (Andre *et al.*, 2002). The apoB concentration in fractions and sera was determined using an immunochemical kit following the manufacturer's procedure (Apo B kit; bioMérieux). The concentration was determined from a calibration curve established with the Apo B kit standard.

The index of HCV RNA association with LDF was calculated as follows:

$$(\text{RNA copy number per mg apoB in LDF}/\text{RNA copy number per mg apoB in serum}) \times 100$$

Quantification of negative-strand HCV RNA was performed following the same procedure, except that a tag-RC1 primer was used instead of RC1 to initiate the reverse transcription (5'-ggc cgt cat ggt ggc gaa taa GTC TAG CCA TGG CGT TAG TA-3'), and the tag was used as primer during the amplification reaction. Details of the negative-strand quantification method are described elsewhere (Komurian-Pradel *et al.*, 2004).

Sequencing and genotyping of the 5' HCV non-coding region. PCR amplification products that were synthesized during the HCV RNA quantification process with the LightCycler were sequenced directly in both directions using the PRISM Ready Reaction AmpliTaq FS BigDye Terminator Cycle Sequencing Kit (PE/Applied Biosystems) with the Applied Biosystem 377 and 373A automated DNA sequencers. The HCV genotype was determined by comparison of nucleotide sequences with those of the Hepatitis C Virus DataBase (<http://hepatitis.ibcp.fr>).

Cloning of the virus quasispecies. The HCV core and envelope 2 (E2) regions were targeted for amplification by nested RT-PCR. Virus RNA (8 µl) was reverse-transcribed in a volume of 20 µl using the antisense primers 5'-CAT CAT ATC CCA AGC CAT-3' for core and 5'-ACG GTC GAG GTG CGT ART GC-3' for E2 genes, then amplified for 35 cycles (denaturation at 94 °C for 45 s, hybridization at 55 °C for 30 s and primer extension at 72 °C for 60 s) using the sense primers 5'-GCT TGC GAG TGC CCC GGG AGG TCT-3' and 5'-GTA ACA GGT CAC CGC ATG GC-3' for core and E2 genes, respectively. One-tenth of the volume of the first PCR products was re-amplified for 35 cycles with internal primers 5'-ATG AGC ACG AAT CCT AAA CC-3' and 5'-GGT ATC GAT GAC CTT ACC CA-3' for core gene and 5'-GCA TGG CTT GGG ATA TGA TG-3' and 5'-GCA GTC CTG TTG ATG TGC CA-3' for E2 gene. Fragments 375 and 285 bp, respectively, were obtained for the core and E2 genes, which includes the hypervariable region 1 (HVR1). The amplified PCR products of core and E2 regions were purified using the QIAquick PCR purification kit (Qiagen), cloned into the TOPO TA Cloning Kit and transformed into *Escherichia coli* strain One Shot TOPO 10 competent cells (Invitrogen). At least 10 clones were selected for each individual and sample. Plasmid DNA was extracted with the Qiagen plasmid kit (QIAprep Miniprep; Qiagen) and sequenced using the PRISM Ready Reaction AmpliTaqFS BigDye Terminator Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyser (PE/Applied Biosystems).

Quasispecies sequence analysis. Nucleotide sequences were aligned using CLUSTAL W 1.74 (Thompson *et al.*, 1994) and refined by visual inspection with SEAVIEW (Galtier *et al.*, 1996). DNA distance matrix and phylogenetic trees were computed with PHYLO_WIN (Galtier *et al.*, 1996). Distances between sequences were computed under the Kimura two-parameter model (Kimura, 1980). Trees were built using the neighbour-joining method (Saitou & Nei, 1987) and tree topologies were tested with 1000 bootstrap sampling replicates.

In order to determine whether sequences from a given compartment shared more genetic identity with each other than with sequences from other compartments, we used Mantel's test (Mantel, 1967). This

test was performed using ADE-4 (Thioulouse, 1989). The method consists of comparing a DNA distance matrix to a compartmentalized reference distribution matrix of the same dimensions, where the (i,j) value of the matrix is set to 0 if sequence i is from the same compartment as sequence j and the (i,j) value is set to 1 in the other case. The Pearson correlation coefficient r^2 was computed for all pairs (observed r^2). The null distribution was constructed by permuting the rows and columns of the reference matrix 10 000 times. From this distribution, the number of times when the observed r^2 was exceeded gave the exact P value of the correlation observed.

Normalized Shannon entropies were calculated as described (Roque Afonso *et al.*, 1999; Wolinsky *et al.*, 1996). Differences between compartments' genetic distances were assessed using the non-parametric Mann-Whitney test (STATVIEW II; Abacus Concept, Berkeley, CA, USA).

Immunostaining of biopsies. Sections of formalin-fixed paraffin-embedded biopsies of the liver and small intestine were deparaffinated and rehydrated in two baths of methylcyclohexane, two baths of 100 % ethanol, one bath of 70 % ethanol and one bath of PBS for 10 min each. Sections were placed in 0.01 M citrate buffer pH 6.0, treated for 16 min in a microwave oven at 650 W and then allowed to cool slowly to room temperature. Endogenous peroxidase was inhibited by incubation with 1 % H₂O₂ in PBS for 10 min, and sections were placed in PBS/0.2 % BSA for 30 min. mAbs to HCV NS3 (clone 4G10H4), NS5a (clone 4F3H2) and an irrelevant mAb (clone 17D1C11) were provided by bioMérieux and were of isotype IgG1. They were applied for 1 h at a final concentration of 0.07 mg ml⁻¹ at room temperature. After four washes in PBS/0.2 % BSA, sections were incubated with Envision+System HRP Mouse (Dako) for 45 min. Following four washes in PBS/0.2 % BSA and two washes in PBS, staining was developed for 10 min with a DAB peroxidase substrate kit (Vector). Sections were counterstained with Mayer's haematoxylin (Dako) and mounted in 90 % glycerol. Images were recorded with a Sony Power HAD digital camera. Monoclonal anti-NS5A antibody 4F3H2 was tested and titrated on cells stably transfected and expressing NS5A provided by D. Moradpour (Polyak *et al.*, 1999) (see supplementary figure in JGV Online).

RESULTS

LVP quasispecies distribution in blood

To determine whether LVP quasispecies are evenly distributed within the plasma HCV population, or whether they represent a particular subset, we analysed the genetic diversity of two HCV genome regions in paired LVP/plasma samples from three patients with histologically proven chronic hepatitis C. Patients were infected with HCV genotype 1b and had not been given antiviral therapy for at least 6 months. LVP were present in patients' plasma with an index of HCV RNA association to LVP ranging from 12 to 19 % (Table 1). Using RT-PCR, cloning and sequencing, we analysed 73 capsid and 80 E2 clones (including the E2 hypervariable region, HVR1) obtained from unfractionated plasma and purified LVP. A mixture of genetically distinct but closely related variants was present in each compartment. The genetic diversity was estimated by calculating the nucleotide and amino acid normalized Shannon entropy which can vary from 0 (no diversity) to 1 (maximum diversity) (Table 2). Entropy values of the capsid, E2 and HVR1 sequences were higher

Table 1. Clinical characteristics of patients with chronic hepatitis C

All three patients were male.

Patient (HCV genotype)	Age (years)	ALT/AST (IU l ⁻¹)*	HCV RNA load		Liver histology		Index of RNA association (%)†
			Plasma (copies ml ⁻¹)	Liver (copies µg ⁻¹)	Metavir	Knodel	
B (1b)	47	64/58	4.1 × 10 ⁶	1.6 × 10 ⁶	A1F1	1111	12
C (1b)	47	201/140	1.7 × 10 ⁶	0.6 × 10 ⁶	A3F4	4334	19
G (1b)	43	39/27	4.1 × 10 ⁶	2.0 × 10 ⁶	A1F2	3133	14

*Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels.

†Index of HCV RNA association to lipoproteins (see Methods).

in plasma than in LVP for the three patients. The degree of genetic diversity was determined as the mean of within-sample genetic distances based on a Kimura two-parameter matrix. Mean distances were significantly lower in the LVP fractions than in the plasma for at least one virus genome region for each patient (Table 2). Finally, bootstrapped phylogenetic trees obtained from the virus gene sequences indicated significant grouping according to the origin of the clones for each patient (an example of such grouping is shown in Fig. 1). Together, these data suggest that LVP quasispecies segregated in a distinct subset within the HCV RNA plasma population. LVP could thus originate from a particular site of virus replication.

Comparison of HCV quasispecies from LVP and liver

We then analysed the genetic relationship between liver and LVP quasispecies in patient B from whom liver and blood samples had been collected simultaneously. Seventeen

capsid and 13 envelope clones were obtained from the liver and were compared to the corresponding LVP clones. Bootstrapped phylogenetic trees constructed with the capsid and envelope regions indicated that liver and LVP quasispecies segregated in distinct clusters with little or moderate overlapping (Fig. 2A, B). Statistical significance was calculated using Mantel's test to search for a relation between pairwise Kimura two-parameter distances and compartment distribution. Significant genetic differences were observed between liver and LVP quasispecies in the capsid region ($P < 0.0001$) and in the E1/E2 region ($P < 0.04$). Evolutionary constraints acting on E1/E2 quasispecies from plasma, LVP and liver were also determined for this patient by analysing E1/E2 and HVR1 nucleotide substitution patterns (Table 3). There was a selection for conserved amino acid sequences of E1/E2 (excluding the hypervariable region HVR1) with non-synonymous versus synonymous mutation ratios ranging between 0.59 and 0.79 in the three compartments studied. Peptide variability of the HVR1 region was favoured in the plasma and liver

Table 2. Genetic distances of capsid and E1/E2 (including HVR1) quasispecies from plasma and LVP in three chronically infected patients

Patient and compartment	No. of clones*		Normalized nucleotide entropy†			Normalized amino acid entropy†			Mean genetic distance (SD)‡	
	Capsid	E1/E2	Capsid	E1/E2	HVR1	Capsid	E1/E2	HVR1	Capsid	E1/E2
Patient B										
Plasma	10	10	0.76	0.88	0.58	0.41	0.88	0.35	0.0072§ (0.0049)	0.0359 (0.0313)
LVP	12	14	0.54	0.87	0.35	0.34	0.59	0.31	0.0042§ (0.0059)	0.0436 (0.0387)
Patient C										
Plasma	14	18	0.92	0.80	0.50	0.54	0.79	0.40	0.0097§ (0.0046)	0.0265§ (0.0213)
LVP	15	13	0.63	0.21	0.11	0.39	0.21	0.11	0.0056§ (0.0043)	0.0014§ (0.0017)
Patient G										
Plasma	10	14	0.88	0.89	0.71	0.53	0.74	0.45	0.0092§ (0.0064)	0.0488§ (0.0433)
LVP	12	11	0.81	0.87	0.64	0.44	0.61	0.36	0.0083§ (0.0043)	0.0286§ (0.0359)

*Number of independently analysed clones.

†Shannon entropy values were compared for the three patients and compartments by the Wilcoxon test ($P < 0.05$).

‡Within-sample genetic distances were calculated with PHYLO_WIN based on a Kimura two-parameter matrix with a transition to transversion ratio of 2.

§Mean genetic distances between plasma and LVP for each patient were compared by the Mann-Whitney test (§ indicates $P < 0.01$).

Table 3. Genetic diversity of E1/E2 quasispecies from plasma, LVP and liver in patient as determined by E1 and HVR1 nucleotide substitution analysis

d_N and d_S , mean and SD (in parentheses) of proportions of non-synonymous substitutions per non-synonymous sites, and of synonymous substitutions per synonymous sites.

Compartment	E1/E2			HVR1		
	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
Plasma	0.036 (0.036)	0.045 (0.029)	0.79	0.144 (0.177)	0.051 (0.058)	2.80
LVP	0.038 (0.041)	0.066 (0.049)	0.59	0.173 (0.193)	0.135 (0.100)	1.28
Liver	0.017 (0.025)	0.025 (0.032)	0.69	0.082 (0.113)	0.027 (0.069)	3.02

in the crypts or in the lower part of the villi (Fig. 3E,G). Interestingly, HCV protein expression was observed in enterocytes which synthesize and secrete chylomicrons (Field & Mathur, 1995), whereas mucus-secreting cells were not stained (Fig. 3G,H). For one patient, ileal and jejunal biopsies were also stained positive. Biopsies for another patient taken 2 years apart were both positive for HCV proteins. Presence of HCV in intestine was further confirmed by HCV RNA detection from positively stained biopsies. Total RNA was extracted from two formalin-fixed tissues and HCV RNA was amplified by strand-specific RT-PCR. Despite the very low yield of RNA extractable from formalin-fixed tissue, positive HCV RNA was detected from one of the two NS3-positive biopsies tested. The intestinal sequence of the HCV 5' non-coding region matched the plasma genotype 1b sequence (GenBank accession no. X61596). Epithelial intestinal cells could thus be envisioned as an HCV reservoir which expresses virus proteins and replicates HCV RNA.

DISCUSSION

The existence of extrahepatic reservoirs for HCV replication has long been suspected, but remains controversial. Several authors have reported the presence of HCV negative-strand RNA, the replicative intermediate, in peripheral blood mononuclear cells (PBMCs) and in various autopsy tissues of chronically infected patients (Laskus *et al.*, 1998b; Yan *et al.*, 2000). These data are sometimes not considered conclusive, as the RT-PCR used to detect the negative strand could lack strand specificity, and virus protein production has not been demonstrated (Lanford *et al.*, 1995; Laskus *et al.*, 1997). However, infection of extrahepatic tissues is supported by the finding that HCV quasispecies composition differs according to the cellular or tissue origin (Cabot *et al.*, 2001; Laskus *et al.*, 1998a, 2000; Maggi *et al.*, 1997; Roque Afonso *et al.*, 1999). In particular, genome variants present in plasma and not in liver or in PBMCs suggest the existence of other virus compartments. Here we showed that LVP quasispecies defined a subpopulation of the total plasma quasispecies, and that liver and LVP quasispecies were different. These data indicated that the liver is not the only source of LVP and that another organ must contribute to their

production. Taking into consideration that LVP are apoB-containing particles enriched in triglyceride, it was assumed that LVP should be synthesized and secreted by cells producing apoB-containing lipoproteins. This identified small intestine enterocytes as a potential reservoir and replication site for HCV. Indeed, HCV NS3 and NS5A protein were detected in enterocytes from four out of ten chronically infected HCV RNA-positive patients and in no non-infected or non-viraemic patients. Infected enterocytes were grouped in foci of infected villi, leaving numerous villi uninfected. Therefore, 40% of positive intestinal biopsies is noteworthy, suggesting that the small intestine is generally infected in chronically infected patients. A similar percentage of NS3-positive liver biopsies from HCV RNA-positive patients has been reported (Wolk *et al.*, 2000). Although we could detect low levels of positive-strand HCV RNA, we failed to detect negative strand RNA, probably for technical reasons. We found that HCV negative-strand RNA in liver is several hundred times less abundant than the positive strand (Komurian-Pradel *et al.*, 2004). Accordingly, negative-strand HCV RNA has been found to replicate at a slower rate than the positive strand *in vitro* (Reigadas *et al.*, 2001). Because of the small amount of positive-strand RNA that could be extracted from formalin-fixed tissue, the detection of negative-strand RNA was not expected. Together, the data show that the small intestine can be infected by HCV and strongly suggest that this organ may be a reservoir for HCV and a source of LVP. To our knowledge, a search for HCV in intestine has been performed only once, in a study of post-mortem tissues of HCV-infected patients (Yan *et al.*, 2000). However, in that study HCV RNA was detected in many tissues, including small intestine, and the nature of infected cells was not determined.

LVP enter hepatoma cell lines through the LDL receptor, which recognizes apoB and apoE on the particle. Binding of purified LVP to HepG2 cells is very efficient and is competed out by native lipoproteins, explaining the poor binding of non-purified HCV from infected serum (Andre *et al.*, 2002). The LDL receptor is expressed *in vivo* by intestinal cells (Levy *et al.*, 2000). The entry route in enterocytes is therefore likely to be similar to that of hepatocytes. However, besides the LDL receptor, CD81 and the scavenger

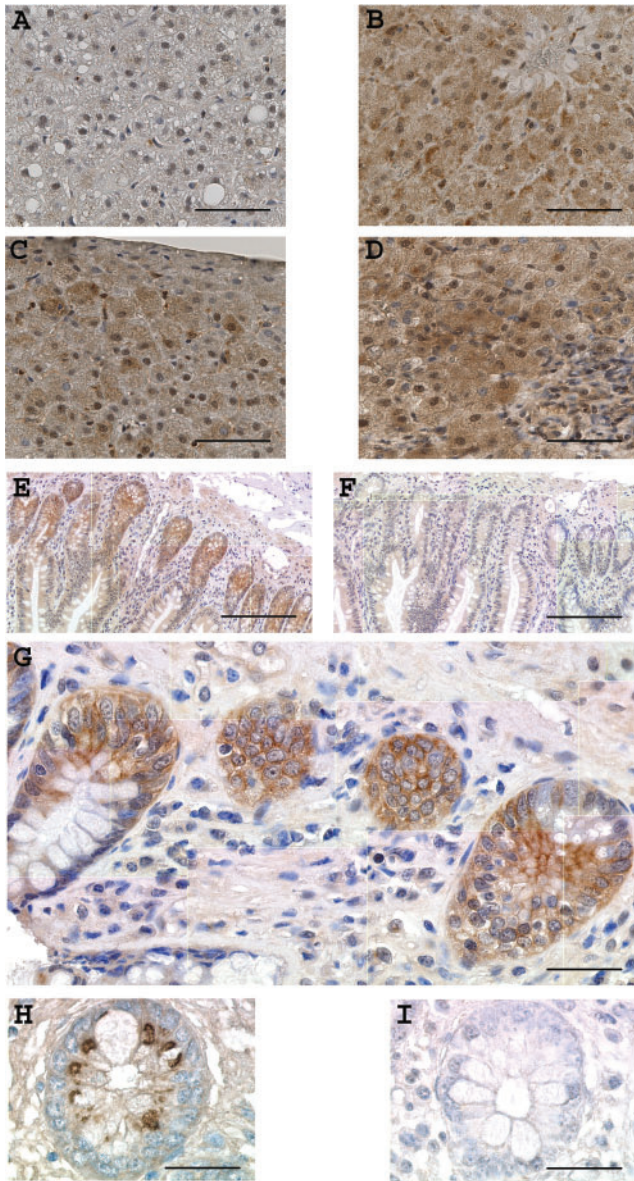


Fig. 3. Immunohistochemical staining of HCV proteins in small intestine. Anti-NS3 mAb stains HCV-infected liver biopsies: sections of paraffin-embedded liver biopsies were stained as described for NS3 (Methods). Biopsies from one HCV-negative patient (A) and from three HCV RNA-positive patients (B–D). Detection of NS3 and NS5A HCV proteins in intestinal biopsies of viraemic patients: sections of paraffin-embedded biopsies of intestine were stained as described in Methods for NS3 (E, G) and NS5A (H) HCV proteins, or with an irrelevant isotype-matching mAb (F, I) (see Methods). Bars: 200 μm (E, F); 30 μm (G–I).

receptor B class 1 (SR-B1) have been described as putative receptors for the viral envelope proteins (Pileri *et al.*, 1998; Scarselli *et al.*, 2002). CD81 is not expressed in the intestine (Okochi *et al.*, 1999), but SR-B1 was detected at the apical and basal poles of enterocytes and could be

an alternative entry pathway for E1/E2-containing virus particles (Altmann *et al.*, 2002; Cai *et al.*, 2001). However, analysis of LVP quasispecies showed that the binding site of E2 (HVRI) to SR-B1 does not appear to be subjected to any selection. It is thus likely that no constraint is exerted by this receptor on the envelope protein, and that LVP do not predominantly enter the intestinal cells via SR-B1.

Thinking of the intestine as a reservoir for HCV-producing low-density infectious virus particles modifies the current understanding of the pathogenesis of HCV infection and raises several questions. First, HCV infection of small intestine was, surprisingly, not accompanied by inflammatory response, as mild duodenitis was observed in only one case. We recently showed that the lipid composition of apoB-containing lipoproteins and of lipid emulsions has important consequences for dendritic cell maturation and function (Coutant *et al.*, 2004; Perrin-Cocon *et al.*, 2001). It is conceivable that the lipid moiety of the low-density HCV particles induces a state of immune unresponsiveness. The mechanism of such inhibition is under investigation. Second, because most chylomicrons and chylomicron remnants are captured by hepatocytes (Hussain *et al.*, 1996; Yu & Cooper, 2001), small intestine might provide a source of infectious particles responsible for continuous liver infection and for infection of liver grafts. Such a mechanism would have important consequences for antiviral therapy. Efficient drugs should target intestinal cells, and analysis of drug metabolism in these cells would be an important step in the development of antiviral therapy. Third, infection of intestinal cells *in vitro* may provide opportunities to understand virus replication and assembly better. The Caco-2 cell line is used as a model of human intestinal cells to study apoB metabolism and lipoprotein synthesis (Levy *et al.*, 1995). The possibility of inducing the differentiation of Caco-2 *in vitro* and modulating lipoprotein secretion should allow a detailed analysis of the relationship between LVP and lipoprotein assembly, leading to a cell-based HCV replication system. Synthesis of chylomicron and VLDL depends on apoB synthesis and on its translocation into the lumen of the endoplasmic reticulum. If nascent lipoproteins are not loaded with triglyceride, apoB is retro-translocated into the cytoplasm where it is degraded by the proteasome (Hussain *et al.*, 1996; Luchoomun & Hussain, 1999; Yu & Cooper, 2001). Because triglycerides are synthesized by enterocytes from dietary fatty acids and because chylomicron synthesis and secretion follow lipid digestion, it would be of interest to assess the influence of starving and of lipid-rich meals on HCV viraemia. In support of this idea, a negative correlation has recently been reported between plasma apoB concentration and HCV virus load, particularly for patients infected with genotype non-1 (Petit *et al.*, 2003).

In conclusion, the presence of HCV proteins in enterocytes further emphasizes the interaction between lipoprotein metabolism and HCV, and modifies our current understanding of hepatitis C.

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