Review

Hepatitis C virus biology

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Abstract

Hepatitis C virus infection represents a major problem of public health with around 350 millions of chronically infected individuals worldwide. The frequent evolution towards severe liver disease and cancer are the main features of HCV chronic infection. Antiviral therapies, mainly based on the combination of IFN and ribavirin can only assure a long term eradication of the virus in less than half of treated patients. The mechanisms underlying HCV pathogenesis and persistence in the host are still largely unknown and the efforts made by researchers in the understanding the viral biology have been hampered by the absence of a reliable *in vitro* and *in vivo* system reproducing HCV infection. The present review will mainly focus on viral pathogenetic mechanisms based on the interaction of HCV proteins (especially core, NS3 and NS5A) with host cellular signaling transduction pathways regulating cell growth and viability and on the strategies developed by the virus to persist in the host and escape to antiviral therapy. Past and recent data obtained in this field with different experimental approaches will be discussed.

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Abbreviations: HCC, hepatocellular carcinoma; CAH, chronic active hepatitis; STAT, signal transducer and activator of transcription; Grb-2, growth factor receptor-bound protein 2; ERK, extracellular signal-regulated kinase; NF- κ B, nuclear factor κ B; TNF α , tumor necrosis factor α ; MAPK, mitogen activated protein kinase; JNK, c-jun N-terminal kinase; NF-AT, nuclear factor of activated T cells

Introduction

Hepatitis C Virus (HCV) is a major pathogen given its extremely high prevalence (around 350 millions of chronically infected individuals worldwide), the high rate of chronic infection and the significant risk of severe chronic active hepatitis and cirrhosis among chronically infected subjects.

HCV infection induces chronic infection in up to 60–80% of infected adults. The pathogenic effects of chronic HCV infections is highly variable; some patients will only show minimal liver lesions while others (around 20%) will develop after 5–10 years follow-up severe fibrosis and cirrhosis. Finally, 30–50% of patients with cirrhosis, whatever the etiological factor, develop HCC after a 10 year follow-up.

Acute and chronic hepatitis induced by most hepatitis viruses clearly implicate the host immune response to viral proteins. The development of molecular biology has led to realize that some hepatitis viruses also directly modulate liver cell proliferation and viability. This issue has important implications for understanding the molecular bases of the liver lesions and liver carcinogenesis, as well as for developing new therapeutical strategies. The present review will focus on the viral pathogenetic mechanisms based on the interaction of HCV proteins with host cellular signaling transduction pathways regulating cell growth and viability and on the strategies developed by the virus to persist in the host and escape to antiviral therapy.

Hepatitis C virus biology

HCV belongs to the genus Hepacivirus and is a member of the Flaviviridae, together with the Pestiviruses and Flaviviruses. Its genome is a positive, single strand RNA molecule which includes two untranslated regions at the 5' and 3' ends, and a large open reading frame encoding for a 3010–3030 amino acid polyprotein. This polyprotein is post-translationally processed into structural and non-structural proteins, the cleavage being dependent on host and viral encoded enzymes (Figure 1). The structural proteins, encoded in the N-terminal region, include the core protein followed by two envelope glycosylated proteins: E1 and E2. The non-structural domain encodes for six proteins: NS2, 3, 4A, 4B and 5A, 5B (see article from Dr Bartenschlager in this Special Issue).

HCV RNA shows significant genetic variability, and exhibits an estimated rate of nucleotide change of approximately 10^{-3} substitutions/site/year (see article from Dr Simmonds in this Special Issue). This genetic variability can be seen in all domains of the HCV RNA and the concept of 'quasi-species' has been introduced. In fact, HCV RNA circulates, in most cases, as a population of RNA molecules which also differs in serum, liver and PBMC. Some 'hot spots' of mutation have been identified in the E2 envelope protein encoding sequences where there are two hypervariable sequences (HVR 1 and 2), located in the 5' part of this domain, with a high rate of non-synonymous mutations (i.e. leading to amino acid changes). Some of the non-structural (NS5A in particular) as well as the capsid encoding sequences show a lower, yet significant, rate of variability. In contrast, the 5' untranslated region is highly conserved among different

isolates although mutations can be identified at some sites. At present three major HCV types are distinguished: one, two and three as well as three to seven other types, according to the classification proposed. There is a general agreement that the response to interferon therapy depends on the infecting HCV type, while the association between certain types and liver lesions severity is highly debated.¹ The existence of a population of HCV RNA quasispecies may favor the selection of RNA molecules that are 'resistant' to anti-viral factors and some evidence have been provided for a time-dependent selection of some of these HCV species (reviewed in ²).

The progress in HCV research has been hampered by the lack of an efficient and reliable model supporting viral entry, replication and secretion of viral particles. However, some *in vitro* approaches have been recently developed such as the HCV replicons. This represents presently the most relevant model to investigate HCV RNA replication, HCV protein synthesis and maturation, and should allow new approaches for the discovery and characterization of potential antiviral molecules. The HCV replicons consist in genomic HCV-RNAs capable of autonomous replication exclusively in a particular hepatoma cell line (Huh7). First HCV replicons consisted in bicistronic constructs with a

Figure 1 HCV genome organization and processing of viral polyprotein

Figure 2 The mechanisms of HCV persistence in liver cells. The figure illustrates the various mechanisms employed by HCV to establish chronic infection. It shows in particular the association of mechanisms involving immune response to the virus and direct interactions with cellular signaling

selection gene (neomycin) downstream the HCV internal ribosome entry segment (IRES) followed by encephalomyocarditis (EMCV) IRES driving the translation of a second cistron consisting in a subgenomic HCV–RNA including the non-structural genes of the virus (NS3 to NS5B). Such constructions only allowed the persistence of the replicon in cells where HCV IRES was active. The following generation of replicons has now included the complete HCV open reading frame, encoding for both structural and nonstructural proteins. Subgenomic and genome-length replicons were able to express HCV proteins and to persist for years in the cells, but no encapsidation or secretion of viral particles has been shown in this model. The amount of replicon RNA in the cells was dependent to the degree of confluence of cell culture, rapidly decreasing at high confluence and indicating that HCV RNA replication or translation is tightly linked to host cell metabolism.^{3–5} The replicon model confirmed *in vitro* the capacity of HCV replication machinery to adapt to the cellular context by point mutations with some hot-spots along the genome. In fact, some point mutations in key regions of HCV genome (namely NS3- and mostly NS5A-encoding sequences) strongly increased HCV replication, unmasking the role of some HCV proteins in HCV-RNA transcription and translation processes.^{3,4,6}

The absence of small animal models for HCV infection has been also partially addressed by Mercer *et al.*⁷ The authors transplanted normal human hepatocytes in severe combined immunodeficient mice carrying the urokinase-like plasminogen activator transgene. Transplanted human cells, taking advantage to the toxicity of the transgene expressed in resident mice hepatocytes, extensively repopulate mouse liver and support productive HCV infection. Similar animal models hosting human hepatocytes have been previously proposed as an infection model for other hepatitis viruses.^{8,9} Finally, a different approach to study HCV infection *in vivo* has been recently developed; laser capture microdissection (LCM) technique allows the isolation and analysis of single infected cells chosen by histological and immunohistochemical criteria from liver sections.¹⁰ This strategy will open new avenues in the comprehension of HCV related pathogenesis, directly focusing on the analysis of infected cells.

Infection by HCV of non-hepatocytic cells

There is now evidence that HCV also infects extrahepatic cells. *In vivo*, HCV RNA sequences have been detected in fresh peripheral blood mononuclear cell (PBMC) preparations from HCV infected patients.^{11,12} However, such an approach does not allow distinction between absorption of serum particles and true infection of PBMC in viremic patients. Still, several observations support this hypothesis including: (a) short-term cultures of PBMC yield a significant increase in the amount of viral RNA on stimulation by PHA and PMA;¹¹ (b) *in situ* hybridization showed viral RNA in a limited percentage (around 1%) of circulating PBMC and lymph nodes;^{13,14} (c) PBMC from normal individuals can be infected by HCV;¹⁵ (d) there is evidence that Epstein–Barr virus-immortalized B-

cells and T-cell clones from HCV carriers show long-term persistence of HCV genomes (Bronowicki, personal communication and⁵); and (e) we have presented strong evidence for the persistence of HCV RNA in PBMC obtained from HCV-positive subjects and injected into SCID mice. Artifacts due to contamination with serum particles were excluded by this approach which showed infection of these mononuclear cells.¹⁶

A second issue concerns the detection of replicative forms of HCV RNA in PBMC. This point has been discussed in regards to the specificity of negative HCV RNA detection. Thus, although such negative strands are clearly detected in some patients and on stimulation with mitogens, the real prevalence of this phenomenon remains unsettled.¹⁷ Interestingly, it has been suggested that HCV type 1 might show a distinct lymphotropism, in comparison to other frequent HCV types.¹⁷ Also, using a highly strand-specific method of RT–PCR, Shimizu *et al.*¹⁸ were able to demonstrate HCV RNA of negative polarity in PBMC samples from infected chimpanzees. In addition, they found that the capacity of infected PBMC and/or liver cells in chimpanzees, as well as in human lymphocyte cell lines infected *in vitro*, varied among different HCV strains suggesting the existence of lymphotropic HCV strains.¹⁸ The possibility of specific cell subsets harboring HCV sequences has also been discussed. HCV negative strand RNA, possibly reflecting viral replication, was found in all cell subsets in some studies¹¹ and in specific cell subpopulations (B lymphocytes and monocytes) in other ones.^{17,19}

Finally, the possibility of PBMC infection by HCV has recently been confirmed in studies indicating that HCV infection of lymphoid cells may favor selection of distinctive viral variants. *In vitro* and *in vivo* studies, based on HVR1 sequence analysis, showed different quasispecies patterns between serum, liver and PBMC.^{20,21} Thus, these observations support the concept of a ‘compartmentalization’ of some HCV genomes. The differences in quasispecies composition between these tissues may arise from cellular factors in the liver and PBMC, which would favor the growth of certain variants over others.²⁰ It is not established, however, that distinct cellular tropism might be shown by some HCV variants.

Other cell types are permissive to HCV infection; we have recently demonstrated the possibility to infect, at a low level, biliary cells in primary culture.^{22,23} Biliary lesions are frequently observed during chronic HCV infection²⁴ and HCV infection of the biliary cells might be involved in this phenomenon. Overall, infection of non-hepatocytic cells might constitute a ‘reservoir’ which would favor selection of HCV variants and viral persistence. The almost universal recurrence of HCV infection after orthotopic liver transplantation corroborates the existence of extra-hepatic sites where HCV can persist and replicate.^{25,26} PBMC and bone marrow cell infection by HCV might also induce specific cell alterations that can be interpreted as the potential pathogenetic strategies of the wide range of lymphoproliferative disorders frequently appearing during the course of chronic hepatitis C virus infection (for review see^{15,27}). Some mechanisms underlying the lymphoproliferative

disorders associated to HCV chronic infection have been recently proposed and involve the anti-apoptotic protein BCL2. In fact, a chromosomal rearrangement, t(14;18), resulting in the translocation of the bcl-2 open-reading frame under the transcriptional control of the immunoglobulin heavy-chain gene (IgH) with consequent over-expression of bcl-2, has been found with high prevalence in chronic HCV infected subjects. The presence of t(14;18) translocation in B-cells was highly prevalent in HCV positive subject developing cryoglobulinemia and non-Hodgkin's lymphoma suggesting that bcl-2 overexpression may play a relevant role in extending B-cell clone life and increasing the probability of additional mutational events driving B-cells to malignancy.^{27–29} The relative contribution of the strong polyclonal B-cell stimulation during HCV infection and of putative direct signals triggered by HCV proteins on cellular membrane molecules (CD81, the presumed HCV receptor) remain to be investigated. Finally, the actual impact of PBMC infection on the quality of the immune response is unclear, even if some recent reports described a contribution of viral proteins in disturbing host immune response.

The interactions between HCV and major cellular metabolic networks

HCV interactions with interferon signaling

Chronically HCV-infected patients show an inappropriate interferon production and this has provided rationale for the use of interferon α in treating, now in combination with ribavirin, patients with HCV-related chronic active hepatitis. There is also emerging evidences that HCV might, as for many other viruses, evolve mechanisms to directly inhibit the interferon signaling in the infected cells. HCV polyprotein expression in an osteosarcoma cell line inhibits the antiviral effect of interferon α on vesicular stomatitis (VSV) and encephalomyocarditis (EMCV) viruses and decreases STAT3 DNA binding;³⁰ this study did not identify, however, the viral protein(s) involved.

NS5A is a candidate HCV protein, which is possibly involved in both the inhibition of the antiviral effects of interferon and the control of cell growth and viability (Figure 2). The pioneering studies conducted by Drs Enomoto^{31,32} and Katze *et al.*^{33,34} raised the hypothesis that NS5A might directly modulate the interferon transduction pathway by associating to and inhibiting the kinase activity of the double strand, RNA-stimulated protein kinase (PKR). In Japan, Enomoto *et al.* first demonstrated that mutations in a region of NS5A, which they referred to as the 'Interferon Sensitive Determining Region: ISDR' correlated with the response to interferon treatment. Thus, patients whose major circulating HCV genomes exhibited mutations in this region achieved the best response to treatment. Others,^{35,36} but not all,³⁷ studies from Japan supported these findings. However, certain caveats should be borne in mind in this respect, which markedly complicate analysis of these findings. Several reports have shown that the strict correlation between ISDR mutations and treatment efficacy reported in Japan could not be demonstrated in Europe or

the United States.^{38,39} The reasons for differences between clinical studies performed in Japan and Europe are not clear. It has been suggested that different interferon doses and regimens could affect the outcome of such comparative studies. However, this point has not been proved. It is also important to consider several virological parameters. The impact of NS5A mutations is dependent on the HCV type. Studies in Japan have mostly been performed in patients infected with HCV 1b or, to a lesser extent, HCV type 2, but NS5A mutations are not or only rarely identified in HCV type 3 genomes. NS5A variability in domains located outside the ISDR, and in particular in the C terminal part of the protein, may also be implicated in the control of therapeutic efficacy.⁴⁰ The conclusions of these different studies may also differ because of methodological problems: other results have indeed been obtained when analysing only the major form of the HCV genome or complete quasispecies complexity.^{41,42} Moreover, a recent study suggested that 'European' and 'Japanese' HCV 1b genomes could exhibit different biological properties, a correlation between ISDR mutations and therapeutic efficacy only being demonstrated for the 'Japanese' isolates.⁴³ These findings clearly need to be substantiated by further analyses. Finally, other major virological factors, such as the viral load, should also be taken into account.³⁵

Several reports have now reinforced these clinical observations. *In vitro*, NS5A expression is sufficient to inhibit the antiviral effect of interferon α on VSV and EMCV viruses.^{44,45} We have in particular established this point by expressing several 'natural' NS5A mutants, isolated from patients with or without response to interferon, in the well differentiated hepatocytic cell line HuH7.⁴⁶ The present data do not show, however, a correlation with the *in vivo* response to therapy.

Several important and fundamental problems remain to be solved regarding the molecular basis for the biological effects of NS5A. NS5A is located in the perinuclear area, and is a 56–58 Kd phosphoprotein. The kinase(s) involved are not identified, although casein-kinase II may be involved.⁴⁷ Both a basal and 'hyperphosphorylated' status of NS5A have been suggested, and expression of the complete, non-structural HCV region is necessary in cis for the full phosphorylation of NS5A.⁴⁸

Some reports suggest that NS5A might interact with at least two major cellular signaling pathways. NS5A might bind the catalytic domain of PKR, inhibiting its dimerisation and thus its activation.^{34,49} Furthermore, the domain involved in this interaction in NS5A might include the ISDR region and a domain adjacent to its 3' extremity. Taken together, these data suggest an elegant model whereby expression of the NS5A protein may abolish the antiviral effect of PKR on interferon stimulation, this effect being abrogated by mutations which would then favor treatment efficacy. The implication of NS5A in the resistance to antiviral therapy has been recently confirmed, by the same group, expressing the non-structural HCV proteins in a subgenomic replicon model.⁵⁰ It is important to note, however, that recent studies conducted by independent laboratories could not confirm the role of PKR in NS5A-mediated IFN resistance. In fact, by transiently expressing

full length NS5As or in the context of a subgenomic replicon system, both groups could not show any physical interaction between NS5A and PKR or a change in its kinase activity.^{46,51}

N-terminally-deleted forms of NS5A also show transcriptional activity, but it remains to be established whether such truncated NS5A molecules do exist *in vivo* and how they might participate in NS5A biological properties. Importantly, a correlation between ISDR mutations and NS5A transcriptional activity has been reported.⁵²

It has been also proposed that NS5A may partially inhibit the IFN-induced antiviral response transactivating the Interleukin-8 (IL-8) promoter and increasing IL-8 levels in HeLa cells.⁵³ NS5A might bind the Grb-2 adaptor protein and modulates the ERK1/2-dependent signaling⁵⁴ or induces the activation of NF- κ B and STAT-3 via oxidative stress originated by intracellular calcium level disturbance.⁵⁵

NS5A is not the only HCV protein implicated in the interference with IFN antiviral signaling; in fact, it has been proposed that the E2 envelope protein might also interact with PKR and inhibit its actions.⁵⁶ Interestingly, recent studies of our group demonstrated, *in vivo* and *in vitro*, the physical interaction between PKR and HCV core proteins isolated from tumor tissue of HCV-related HCC patients. These natural mutants present in HCC tissue showed only few point mutations in comparison to HCV 1b reference sequence, but revealed different biological properties including the enhancement of PKR auto-phosphorylation and the phosphorylation of its physiological target, the translation initiation factor eIF2 α .⁵⁷ Along this line, it has been also shown that HCV core protein specifically activated the 2'-5'-oligoadenylate synthetase (2'-5'-OAS) gene promoter in a dose-dependent manner in different human hepatocyte cell lines.⁵⁸

HCV interactions with cellular pathways regulating cell proliferation and viability

A large number of studies have shown that in most parts of the world, HCV constitutes a major risk factor for HCC. However, the figures remain sketchy in some regions. In Japan and Southern Europe, anti-HCV prevalence levels respectively of 80 and 60% have been found, in Northern Europe, recent data suggest that approximately 30% of cases of HCCs are associated with chronic HCV infection.^{59,60} In Sub-Saharan Africa and South-East Asia, where HBV-associated HCC is common, the impact of HCV is much less marked (around 10%). Prospective studies have also emphasized the role of HCV in various geographical areas.⁶⁰

Much data has accumulated over the past few years concerning the mechanisms which may be involved in HCV-related HCC.⁶¹ Clearly, chronic hepatitis, with its combination of chronic inflammation, liver cell necrosis and regeneration, and extensive fibrosis, constitutes a major step in this process.⁶² Some cases of HCV-related HCC, however, develop in the absence of associated cirrhosis and chronic hepatitis; interestingly, most of these tumors contain HCV genomes classified as 1b.⁶³ Viral multiplication is sustained throughout the long-term course

of chronic HCV infection and the development of cirrhosis and HCC. HCV genomes persist in tumor cells and can replicate, albeit at a lower rate than in non-tumoral livers.^{64,65} In contrast with HBV, the HCV genome does not integrate into cellular DNA and replication is thus necessary to its persistence. HCV proteins can be also detected in tumor cells, testifying to HCV genome expression in these cells (B Lebail *et al.* unpublished observations). A certain amount of evidence, indicates that, beside inducing chronic liver inflammation, some HCV proteins may modulate liver cell proliferation and viability by interfering directly with the major cellular transduction networks. Some studies have recently focused on core, NS3 and NS5A proteins.

HCV core (Figure 3) In addition to its role in the packaging of viral RNA, the HCV core can indeed modulate cellular transduction pathways (reviewed in⁶⁶). HCV core protein is mainly cytoplasmic, located on the endoplasmic reticulum membranes and around lipid vesicles.^{67,68} HCV core has been found to be associated with TNF-receptor I and TNF-related lymphotoxin-receptor, and this interaction modulates signaling by these cytokines.^{69,70} As discussed below, HCV core protein also associates with apolipoprotein AII. The viral core actually binds a number of cellular proteins, also including a cellular helicase⁷¹ and an heterogeneous nuclear ribonucleoprotein K.⁷² However, the relevance of these various interactions is still to be fully established. The HCV core transactivates a number of cellular promoters, including *c-myc*, and activates NF- κ B, AP-1 and SRE elements, interacting with c-JNK, ERK and MAPK signaling.⁷³ The interference exerted by HCV core on Raf/MAPK/Erk pathways has been also established by several groups in different cell types with different expression systems, which could partially justify some discrepancies in the obtained results.^{74–78}

Expression of HCV core protein in Jurkat T-lymphocytic cell line activated Interleukin-2 promoter through the NF-AT pathway⁷⁹ and suppressed the induction of Th-1 immunity by inhibition of Interleukin-12 and nitric oxide production from activated macrophages.⁸⁰ In addition, it suppressed the p53 and p21 promoters.^{81–83}

Whether or not a fraction of HCV core may also act in the nucleus during *in vivo* infection is still a matter for debate. C-terminally truncated core translocates to the nucleus and may exert distinct biological effects;^{67,68,84} it has not been, however, shown that such sequences are actually present during natural HCV infection. Some reports have nonetheless suggested that such truncated cores might be identified in tumor tissues from patients with HCC.^{85,86}

In the last years, an increasing number of reports depicted HCV core as a pleiotropic modulator of cell growth and viability. In cooperation with v-Ha-ras, HCV core can induce the transformation of immortalized Rat1 fibroblasts and BALB/3T3 cells^{87,88} and, possibly, primary rat fibroblasts.⁸⁹ It also interacts with and inactivates a bZIP nuclear transcription protein, LZIP, triggering cell transformation.⁹⁰ It has been also shown that HCV core can bind to Retinoid-X-Receptor- α (RXR- α) and modulate RXR- α con-

Figure 3 Schematic representation of HCV NS5A interplay with cellular signaling cascades. The present scheme summarizes the wide range of interactions described between HCV NS5A and several cellular signal transduction pathways regulating cell proliferation, apoptosis and cytokine response

troled gene expression.⁹¹ HCV core can also modify cell cycle acting on different regulatory proteins such as pRb and cyclin E.^{92,93}

Such *in vitro* results have been substantiated by *in vivo* studies in transgenic mice, which demonstrated the induction of HCC on expression of the core sequence only⁹⁴ or of the full-length genome;⁹⁵ as discussed in a previous section the development of HCC in these animal models is preceded by steatosis in the absence of liver inflammation. However, other reports on HCV core-expressing transgenic mice did not demonstrate such changes, and the reasons for these discrepancies remain unknown.⁹⁶

Oxidative stress has been indicated as one of the possible mechanisms of HCV-induced hepatocarcinogenesis. *In vivo* data, obtained in HCV core expressing transgenic mice, provided evidence of elevated peroxide products in older animals, in which scavenger systems are less effective, potentially contributing to the development of HCC in these animals.⁹⁷ Along this line, HCV core protein showed a mitochondrial localization and induced a marked increase in reactive oxygen species (ROS) levels in hepatoma cells. In addition, in mice expressing the structural HCV proteins, an augmented intrahepatic lipid peroxidation was found. Thus, the oxidative injury induced by HCV core protein may contribute to HCV pathogenesis.⁹⁸

In line with these results, HCV core also modulates cell viability, although the results obtained are still under debate. It has been suggested that HCV core protein may inhibit or enhancing programmed cell death induced by different agents both *in vitro* and *in vivo*. In fact, HepG2 cells are sensitized to apoptosis induced by HCV structural proteins⁹⁹ or by Fas¹⁰⁰ and TNF α ⁶⁹ or in contrast, there is inhibition of the apoptosis induced in HepG2 and Jurkat

cells by Fas, in HepG2, HEK-293 and MCF7 cells by TNF, and in HeLa, BRK and CHO cells by cisplatin and *c-myc* or has no effect on HepG2, Ramos, Wil2-NS and BL2 apoptosis.^{101–106} Recent *in vivo* data support the inhibitory effect of HCV structural proteins on Fas-mediated Cytochrome *c* release.¹⁰⁷

Thus, HCV core appears to be a multifunctional, cytosolic protein, capable of modulating several major cellular transduction pathways, its eventual overall effect on cell proliferation and viability being dependent on its intracellular concentration, cell differentiation and the cellular environment.

HCV NS3 The expression of NS3 may also be important, in addition to its major role in viral protein maturation and genome replication. *In vitro*, the stable expression in NIH3T3 cells of the N-terminal part of NS3, encoding for one of the two viral serine-proteases implicated in HCV polyprotein processing, can induce a transformed phenotype.¹⁰⁸ A similar transforming activity, dependent on its serine protease enzymatic activity, has been obtained expressing a full-length NS3 in rat fibroblasts.¹⁰⁹ It has been also suggested that the N- and C-terminal moieties of NS3 may regulate the catalytic activity of Protein kinase A as well as the localization of p53 (reviewed in¹¹⁰). NS3 is also responsible for a p53-dependent specific suppression of p21^{waf1} promoter activity in NIH3T3 fibroblasts.¹¹¹ A recent report indicates that NS3 may induce reactive oxygen species (ROS) production in human monocytes via activation of the NADPH oxidase.¹¹²

HCV NS5A This is another candidate protein which, besides its effects on the antiviral properties of interferon we have discussed in a previous section, might also regulate cell

proliferation and viability. An inhibitory effect of NS5A expression on cell apoptosis as well as a transforming effect in NIH3T3 has been reported.^{49,113} This effect might depend on the NS5A/PKR interaction⁴⁹ and transactivation and repression of cellular promoters.^{45,114} PKR indeed does not only show antiviral activity but also regulates cell proliferation and viability.¹¹⁵ As stated in the previous section, the NS5A/PKR interactions have not been, however, yet confirmed. Recently, the regulatory potential of NS5A of cell proliferation and viability has been further investigated; stable and transient expression of NS5A in hepatic and non hepatic cell lines induced perturbation of cell cycle by targeting Cdk1/2-cyclin complexes.¹¹⁶ It has been also shown that NS5A may repress p21 promoter activity binding to p53¹¹⁷ and modulate cellular transcription interacting with SRCAP transcription factor.¹¹⁸

Still, collectively, the interaction between HCV proteins and interferon signaling brings us back to the general and most important issue of the potential dual effect of interferon in HCV infection which is antiviral on the one hand and anti-proliferative on the other. This issue has major therapeutical implications since it would reinforce the concept raised from some clinical investigations of a protective effect of Interferon- α on HCC development, even in the absence of a detectable virological response to therapy.

The biological effects of HCV proteins on both antiviral and antiproliferative signaling also raise the hypothesis that the influence of some HCV types on Interferon response (a well documented observation) might not be dissociated from the potential, and highly debated, effect of some genotypes on the intensity of liver lesions and, possibly, liver carcinogenesis (reviewed in¹¹⁹). The impact of HCV genotypes in the risk of developing HCC is indeed an important issue to analyse. A number of recent studies point to the severity of HCV type 1 associated liver lesions, including HCC. Several, but not all, cross-sectional analysis indeed have shown higher relative prevalence of HCV 1 in patients with cirrhosis than in those with moderate CAH. These findings are however difficult to interpret since the molecular epidemiology of HCV is presently changing with the recent introduction, at least in areas such as France and Italy, of other types such as HCV 3 by intravenous drug users; in these conditions, duration of HCV infection will differ from one type to the other and markedly influence the risk of cirrhosis.^{1,120} There is still evidence for a more aggressive course of type 1 induced CAH. After liver transplantation in Europe, reinfection of the liver graft by HCV 1 induces a more rapidly progressive CAH than other HCV types and this is not related to epidemiological factors nor to the level of viremia.^{26,121} In some recent prospective studies on the risk of developing HCC in patients with HCV related cirrhosis, infection by genotype 1 has emerged as an independent risk factor;^{122,123} along the same line, HCV 1b was the most prevalent type among the patients we reported with HCV associated HCC in the absence of cirrhosis.⁶³ Clearly these are still indirect evidences, the interpretation of which is complicated by intervening factors, several of them (such as alcohol and HBV coinfections) being underestimated in most studies; altogether, they are suggestive of a particular profile of HCV 1 infection. *In vitro*

studies are now mandatory to analyze comparatively the different biological properties of various HCV isolates. In particular, as discussed for HBV, a major technical limitation to these studies is the analysis of isolated domains of the viral genomes; given the size of the HCV RNA, studies on full length clones are feasible but will require extensive efforts.

HCV infection and liver lipid metabolism

There are several observations, which point out the potential importance of such association. Circulating serum HCV particles show heterogeneous density, which reflect in part the binding of a fraction of the virions to very low density lipoproteins (VLDL) and low density lipoproteins (LDL).^{124,125} Moreover, the LDL receptor has been proposed as one of the potential viral receptors.^{126,127} A further important hallmark of chronic HCV, and not HBV, infection is its association to steatosis in at least 50% of infected subjects.²⁴ The pathogenic importance of steatosis in the course of HCV infection is not established; it has been, however, recently suggested by several studies that, in some patients with steatosis from various etiologies, liver lipid overload might contribute in the development of fibrosis.^{128,129} Several groups showed that HCV core, (and more recently NS5A) is present on the surface of lipid droplets and may alter lipid metabolism *in vitro* interacting with cellular proteins involved in lipid accumulation and storage in liver cells.^{67,130,131} This specific feature of the core protein seems to be attributed to a proline-rich domain, absent in the core protein of flavi- and pestivirus, but present in GBV-B core and in other proteins responsible for lipid storage in plants.¹³² In addition, expression of HCV core in some transgenic mice induces steatosis;⁹⁴ interestingly, these mice eventually develop HCC in the absence of detectable chronic inflammation. Consistent with this result, transgenic mice containing the complete HCV genome⁹⁵ also show steatosis followed by HCC. In contrast, however, other core-expressing transgenic mice do not show liver lesions⁹⁶ emphasizing the importance of the core expression levels, the mice genetic as well as likely environmental parameters.

In this context, we have provided direct *in vitro* evidence for the role of HCV core expression in steatosis induction.⁶⁷ Moreover, we have shown the association of HCV core to Apolipoprotein AII (Apo-AII) and the impact of this binding in the *in vitro* secretion of the viral protein.¹³⁰ We have recently investigated further this issue *in vivo* by using HCV core-expressing transgenic mice as well as mice expressing both HCV core and the human Apo-AII. Our data demonstrate that HCV core expression does not affect beta-oxidation but, instead, directly inhibits VLDL assembly and secretion. Furthermore, ectopic *in vivo* ApoAII expression induces core secretion in serum, decreases its intrahepatic level, and thus abrogates its effects on VLDL secretion.¹³³ Collectively, these data lead to propose a model whereby HCV/apolipoprotein AII interactions might regulate core accumulation, the viral protein in turn impairing liver triglyceride secretion (Figures 4 and 5).

Recent data confirm the presence of non-enveloped nucleocapsid in the serum of HCV infected patients.¹³⁴ The

Figure 4 Schematic representation of the mechanisms involved HCV core-related pathogenesis. The figure resumes schematically the principal effects induced by HCV core expression in mammalian cells. This simplified description wants to point out the pleiotropic action of core protein on cellular metabolism

Figure 5 A model for the interactions between HCV core and liver lipid metabolism. **(a)** The figure suggests that binding of HCV core to apolipoprotein AII (AII) might trigger core secretion by a presently unknown mechanism. Thus, upon translation of the core-expressing sequence, core protein might follow different pathways: (1) As described in the literature, it will be located on the cytosolic face of the endoplasmic reticulum (ER); (2) a proportion of core will be located around triglyceride (TG)-containing vesicles; and (3) binding of core with AII will, in case of high AII expression, drives core secretion. **(b)** Increased expression of ApoAII, such as that obtained by fibrate treatment or, in transgenic mice, ectopic ApoAII expression, would therefore lower intracellular core concentration (right panel). On the other hand, core inhibits VLDL and thus triglyceride secretion (left panel) and this effect is a major factor in steatosis induction; the consequences of such triglyceride accumulation remain to be explored

presence of HCV core molecules in bloodstream of infected subjects may contribute in the establishment and/or maintenance of virus persistence in the host by interacting with the complement receptor (gC1qR) and reducing T-lymphocyte responsiveness.¹³⁵

Further studies will now aim to elucidate the molecular bases of these observations and their potential impact in liver lesions development; it will be also interesting to investigate whether or not, as suggested,¹³⁶ HCV type 3 does specifically disturb liver lipid metabolism.

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