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Promotion of Hepatocellular Carcinoma by Hepatitis C Virus

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Hepatitis C virus · Hepatocellular carcinoma · Liver cancer · Steatosis · ER stress

Abstract
Persistent infection with the hepatitis C virus (HCV) is a major global health problem. Around 2–3% of the world's population are chronically infected, and infected individuals are at high risk of developing steatosis, fibrosis, and liver cirrhosis. The latter is a major predisposing factor for the development of hepatocellular carcinoma (HCC). It is generally accepted that an inflammatory response triggered by persistent HCV infection leads to increased cell proliferation and fibrogenesis that in turn promotes cirrhosis and ultimately HCC development. This indirect mechanism of tumor induction would explain the long incubation period from primary HCV infection to HCC and the requirement for additional cofactors such as toxins or drugs (most notably alcohol), metabolic liver diseases, steatosis, nonalcoholic liver disease, or diabetes. With the advent of adequate cell culture systems for HCV it is, however, becoming increasingly clear that the virus also contributes directly to HCC formation. Examples are the continuous induction of stress response or the massive accumulation of intracellular lipids. Moreover, viral proteins can bind to and sequester cell cycle control factors such as the retinoblastoma protein or the tumor suppressor DDX3. Thus, HCV-associated liver cancer is most likely promoted by the combined action of long-term chronic inflammation and targeted perturbations of cellular key pathways involved in metabolic homeostasis as well as cell cycle control.

Introduction

Persistent infection with the hepatitis C virus (HCV) is a major global health problem. Around 170 million people are infected with this virus, corresponding to ~3% of the world's population. These people are at high risk of developing serious liver damage; ~20% develop liver cirrhosis within 20–30 years after infection and people with HCV-associated cirrhosis develop hepatocellular carcinoma (HCC) in 1–6% of cases per year. Although for many types of cancer the incidence rates are stable or even declining, in liver cancer they are rising. In fact, HCC, which accounts for 70–85% of liver cancers [1], is the most frequently diagnosed cancer worldwide in men and the second most frequent cause of cancer death. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death [2]. Remarkably, HCV infection accounts for a large proportion of these liver cancers. The prognosis of patients with HCC is poor and the 1-year survival remains less than 50% in the USA [3].

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In the absence of adequate in vivo/animal models, analyses of HCV-induced pathogenesis are difficult. Thus, most studies are based on the use of engineered in vitro culture systems that are inherently prone to artifacts caused, for example, by ectopic overexpression of single HCV proteins. Moreover, in many studies heterologous cell systems of nonhepatic or nonhuman origin have been used. Some of these limitations have been overcome with the implementation of more authentic cell culture systems recapitulating the HCV life cycle in parts (e.g. the replicon system) or in total [reviewed in 4]. The latter is based primarily on a particular HCV isolate, designated JFH1 (an acronym derived from Japanese fulminant hepatitis), that replicates to exceptionally high levels in the human hepatoma cell line Huh7. Importantly, HCV particles produced in these cells are infectious in vivo (i.e. immunodeficient transgenic mice with human liver xenografts) and particles isolated from these in vivo samples are infectious for Huh7 cells [5]. While these are important achievements, the primary restriction to Huh7 human hepatoma cells is a serious limitation.

Chronic inflammation induced by viral infection appears to be a major predisposing condition for liver cancer. In the case of hepatitis C it is assumed that a persistently activated immune reaction targeting infected liver cells leads to increased cell proliferation and fibrogenesis, thus enhancing cirrhosis and HCC development [6, 7]. This indirect mechanism of tumor induction by HCV infection could explain why tumors most often develop only 10–30 years after primary infection and require additional risk factors such as alcohol consumption, metabolic liver diseases, or diabetes [6]. Nevertheless, there is increasing evidence that HCV itself, or specific viral proteins, contribute directly to HCC formation. In the following sections, we will focus on some of the possible direct contributions of HCV to the development of HCC and the molecular mechanisms underlying this process.

HCV Replication Cycle

Understanding the molecular mechanisms underlying the development of HCV-associated HCC requires detailed knowledge of the viral replication cycle and the interaction of HCV with its host cell. With the advent of robust culture models and molecular biological approaches, the principles by which HCV replicates have been unraveled (fig. 1) [8–12]. The virus enters the host cell, which is primarily the hepatocyte, via receptor-mediated endocytosis. It is achieved by interactions between the virus particle and numerous host cell molecules presumably in a consecutive manner. Virus particles are probably trapped on the surface of the hepatocyte by interaction with glycosaminoglycans and low-density lipoprotein (LDL) receptors that likely interact with the lipoprotein moiety of the HCV particle. Subsequent interactions involve CD81, scavenger receptor B1 (SR-B1), claudin-1 and occludin. The efficiency of entry is enhanced by direct or indirect interaction between HCV and the Niemann-Pick C1-like 1 (NPC1L1) cholesterol uptake receptor and the epidermal growth factor receptor (EGFR), respectively [11, 13, 14]. Upon HCV entry, the viral genome is released into the cytoplasm. This plus-strand RNA has a length of ~9,600 nucleotides and contains one long open reading frame that is flanked by two terminal nontranslated regions that form higher-order RNA structures. The 5′ nontranslated region contains an internal ribosome entry site (IRES) mediating the translation of the RNA genome and giving rise to a polyprotein that is cleaved by host proteases and two viral proteases into 10 different products. The N-terminal region of the polyprotein is composed of the structural protein core, envelope glycoprotein 1 (E1) and E2. These proteins are the major building blocks of the virus particle. The following two auxiliary proteins are required for the assembly of infectious HCV particles, but it is unclear whether they are also part of the virus particle: p7, a small hydrophobic protein that may act as an ion channel, and nonstructural protein 2 (NS2), that appears to contribute to some step of virus formation. The remainder of the NS proteins is sufficient for replication of the viral genome [15]. NS3 contains in its N-terminal domain a serine-type protease that is tightly associated with and activated by NS4A. The C-terminal NS3 domain contains NTPase/helicase activity. NS4B is a protein inducing the formation of the so-called membranous web that probably harbors the replicase complex. NS5A is an important regulator of RNA replication and virus assembly and NS5B is the RNA-dependent RNA polymerase (RdRp), the key enzyme of viral genome amplification.

HCV RNA is amplified via a minus-strand RNA template and the newly synthesized plus-strand RNA is either used for RNA translation and replication or packaged into progeny HCV particles. Viral replication occurs exclusively in the cytoplasm and leads to massive rearrangements of intracellular membranes mostly induced by NS4B. The assembly and release process of progeny virus is closely associated with the lipid metabolism of the cell [reviewed in 9]. As a result, infectious HCV particles have a unique composition and contain, in

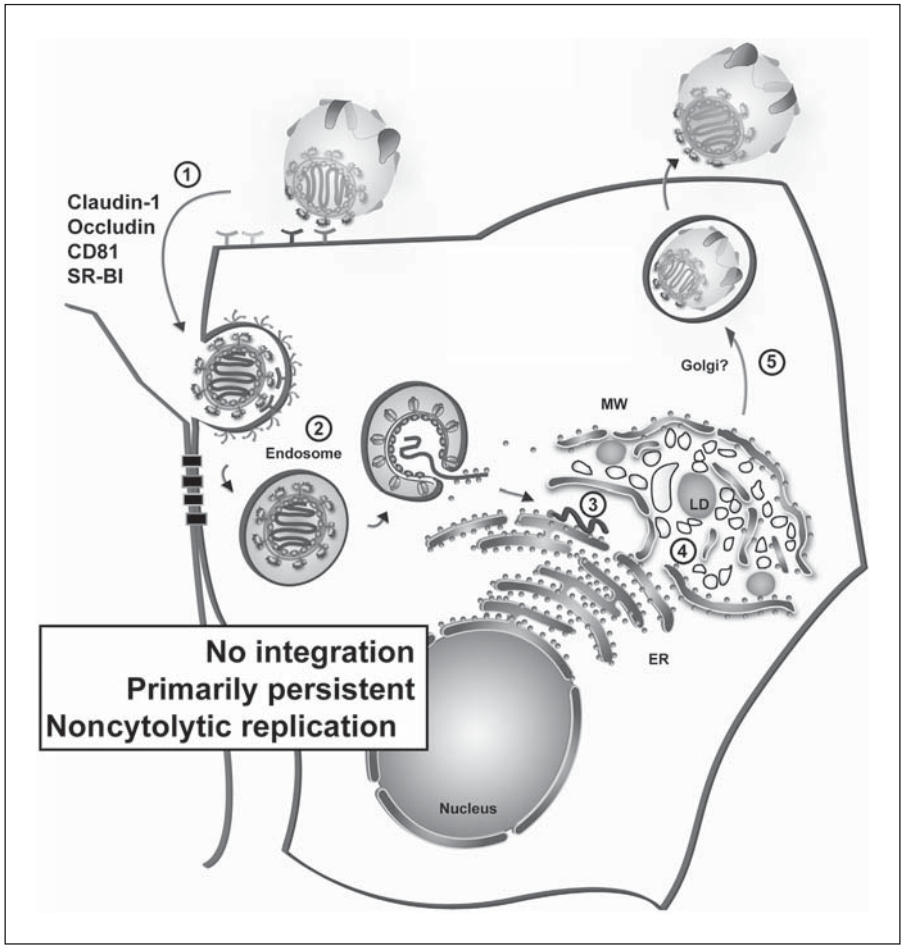

Fig. 1. Schematic of the HCV replication cycle. Numbers refer to the individual steps of the cycle. For details, see text. MW = Membranous web.

addition to E1 and E2, components of the 'very LDL' (VLDL) system, most notably apolipoprotein E (ApoE). Moreover, HCV particles have a unique lipid composition that is very distinct from all other viruses analyzed so far and from the human liver cells in which the virus replicates.

Pathogenesis of Chronic Hepatitis C: Implications for HCC Development

Since HCV replicates exclusively in the cytoplasm without integration of the viral genome into the host cell chromosome, persistence can only be achieved by continuous viral replication. HCV also triggers an immune response that is, however, inefficient; it does not clear the virus, but sustains a chronic inflammation. This is exemplified, among other things, by: (i) the correlation between the strength of the immune response and clinical manifestation of hepatitis C, (ii) the lack of correlation between the level of viremia and the course of diseases, and (iii) the observation that patients treated with ribavirin monotherapy normalize liver histology even though viremia is not affected. Thus, an inefficient immune response unable to eradicate HCV appears to sustain a chronic inflammation (fig. 2). This in turn likely increases cell turnover and thus the chance for manifestation of alterations affecting the control of cell growth. Such alterations are promoted by various conditions including risk factors of DNA damage (e.g. aflatoxins), cirrhosis (e.g. alcohol consumption), or steatosis (e.g. diabetes) [for detailed reviews, see 16–19]. Apart from these indirect and host-determined effects, it is becoming increasingly clear that the virus itself also contributes to tumor progression (fig. 2, table 1). For instance, it has been shown that the core protein binds to and sequesters the tumor suppressor protein DDX3 [20]. Along the same lines, it has been convincingly demonstrated that nonstructural

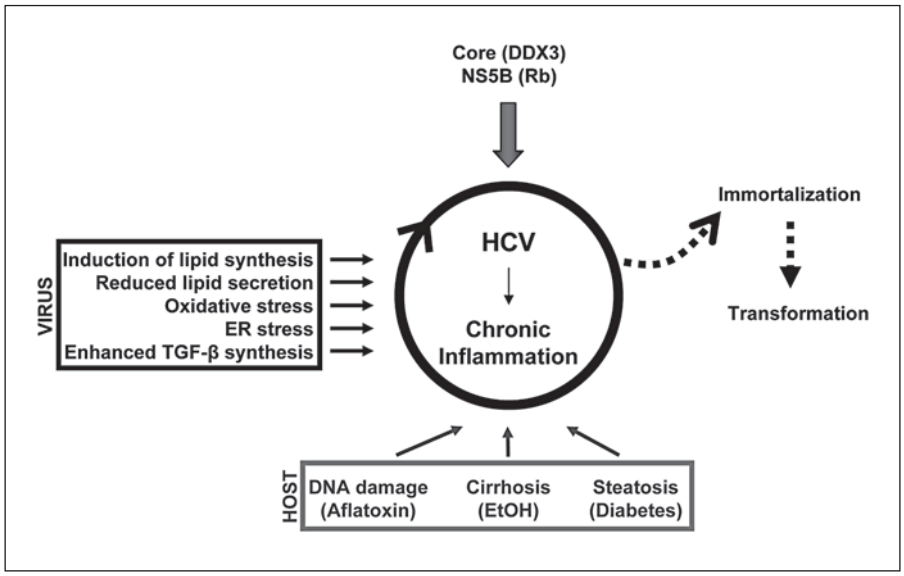

Fig. 2. Schematic representation of the different conditions promoting the development of HCV-associated liver cancer. Proliferation of hepatocytes is affected by chronic inflammation. Control of the cell cycle (black circle) is affected by multiple external cues that depend either on the host (grey box) or viral infection (black box). In addition, two HCV proteins (core and NS5B) might affect the cell cycle directly by sequestration of cell cycle control factors. For further details, see text.

protein 5B (NS5B), which is the RNA-dependent RNA polymerase, binds to and sequesters the retinoblastoma protein (Rb) [21]. Although experimental proof so far is missing, it is tempting to speculate that DDX3 or Rb sequestration by core protein or NS5B, respectively, might affect cell cycle control and thus contribute rather directly to tumor progression.

Another viral protein that received much attention in this context is NS5A. It is a highly phosphorylated protein required for HCV RNA (table 1) replication and virus production. NS5A is composed of an amino-terminal amphipathic α-helix that tethers the protein to intracellular membranes, an RNA-binding domain 1 that forms homodimers, and two natively unfolded protein domains that can form multiple complexes with host cell proteins [22]. It is this structural property of domains 2 and 3 that explains why so many NS5A-interacting host cell factors have been identified, in most cases, in rather artificial systems such as the yeast-two-hybrid system. Moreover, in several cases artificially truncated NS5A variants have been used that display biochemical properties not found with the authentic full-length proteins. Thus, even though some of the cellular NS5A interactants have been confirmed in more authentic cell-based replication systems, in most instances this is not the case and, therefore, the physiological relevance of NS5A for alterations of cellular homeostasis and the contribution of this protein to tumor formation remain to be determined.

With the advent of authentic cell culture systems it is becoming clear that, apart from individual viral proteins, HCV infection and replication per se induces numerous cellular and metabolic alterations that likely contribute to tumor progression. Examples are the induction of oxidative and ER stress, the enhanced production and secretion of TGF-β, and the enhanced accumulation of lipids. Two of these alterations will be described below. The reader interested in more details is referred to excellent recent reviews [16, 17, 23, 24].

Induction of Stress Response and Impact on Viral Persistence and Cell Survival

It is known that HCV infection triggers various stress responses [reviewed in 23, 25]. Recently, we were able to show that in cells treated with interferon (IFN)-α, HCV potently induces the highly dynamic assembly and disassembly of cytoplasmic stress granules (SGs). These structures represent sites of polyA+ mRNA sorting and storage. Unlike P-bodies, mRNAs in SGs are not degraded but rather stabilized ('stored') within this compartment [26]. By employing live-cell imaging techniques we characterized the mechanism by which HCV triggers SG formation and dynamics and found that SG assembly and disassembly correlates with phases of stalled and active RNA translation, respectively. Importantly, SG formation correlates with delayed cell division and prolonged cell survival, suggesting that oscillation of HCV-induced stress response contributes to persistence by suppressing cell death [48]. While it is likely that this strategy facili-

Table 1. HCV proteins and their possible contribution to pathogenicity and liver cancer development

HCV protein	Role in the replication cycle	Possible role in pathogenicity
Core	Nucleocapsid	Insulin resistance/steatosis/oxidative stress Interference (direct or indirect) with p53, p73, and pRb Interference with host cell signaling pathways (NF-κB, Raf1/MAPK, Wnt/β-catenin) Interference with TGF-β signaling Transcriptional activation of cellular genes
NS2	Protease/assembly	Modulation of apoptosis
NS3	Protease/helicase	Interference with innate immune response Interference with NF-κB Interference with p53
NS4B		Induction of ER stress
NS5A		Interference with protein ubiquitination Inhibition of PKR activity Induction of oxidative stress Modulation of transcription of cellular genes Activation of signaling pathways (STAT-3, NF-κB, etc.) Activation of phosphatidyl inositol-4-kinase Accumulation of β-catenin by indirect mechanisms
NS5B		Sequestration of Rb

tates persistent infection by HCV, it remains to be determined whether this indirect mechanism of suppression of cell death plays a role in HCV-associated liver cancer.

Association of HCV Infection with Steatosis and Development of Liver Cancer

One of the hallmarks of HCV infection is the induction of steatosis, which is a condition that contributes to tumor development. In fact, using the JFH1-based cell culture system we observed a ~3-fold higher amount of lipids in HCV-infected Huh7 cells versus noninfected control cells. This elevated lipid synthesis might be brought about, among other things, by the induction of a massive ER stress response that in turn could activate the SREBP (sterol regulatory element-binding protein) pathway. SREBP is an ER-resident inactive transcription factor that under conditions of ER stress is transported to the Golgi. There SREBP is cleaved by proteases liberating a cytosolic protein fragment that is transported into the nucleus and that activates transcription of genes required for the synthesis of cholesterol and for fatty acid homeostasis [27].

Why does HCV cause such profound alterations of intracellular lipid pools? On one hand, enhanced lipid synthesis appears to promote viral RNA replication and virus production; on the other hand, it might reflect a 'collateral damage' caused by viral exploitation of key enzymes involved in lipid synthesis and intracellular lipid storage. With respect to the first scenario, one has to keep in mind that HCV replication occurs on ER-derived membranes. Thus, efficient assembly of viral replication complexes requires the rearrangement of existing membranes but probably also their novel synthesis. Early studies by Egger et al. [28] described that profound membrane alterations are mainly induced by NS4B. Overexpression of this highly hydrophobic protein induced the formation of single membrane vesicles forming clusters and appearing as a 'membranous web'. Subsequent studies suggest that this web is the site of HCV RNA replication [29]. Using electron microscopy and tomography in combination with 3D reconstructions, we observed in HCV-infected human hepatoma cells numerous membrane alterations [Romero-Brey et al., unpubl. data]. Most prominent were double membrane vesicles that were most abundant and that might be sites of RNA replication. In addition, at late stages after infection we detected multi-membrane vesicles that probably reflect cellular stress-induced reactions including autophagic responses.

The interaction of the HCV core protein with lipid droplets (LDs), which are intracellular storage organelles for neutral lipids (mainly triacylglycerol and/or cholesterol esters) [reviewed in 30–32], is another example illustrating how this virus alters lipid homeostasis of the cell. We and others could demonstrate that assembly of HCV particles is tightly linked to LDs, which are sites where structural and nonstructural proteins accumulate during assembly [12, 33–35]. Importantly, HCV core protein impacts lipid metabolism in several ways: (i) by inhibition of LD mobility, (ii) by decreasing the lipid turnover in core-coated LDs, (iii) by inhibition of MTP (microsomal triglyceride transfer protein), (iv) by inhibition of VLDL secretion, and (v) by inhibition of DGAT-1 (diacylglycerol acyltransferase 1) [36–41]. These alterations appear to play important roles in pathogenesis. For instance, in an elegant recent study it was shown that mice lacking DGAT-1 are protected from HCV core-induced lipid accumulation [42]. This could be ascribed to a decrease in lipid turnover in core-coated LDs. Thus, HCV promotes steatosis both by decreasing lipid mobilization and by reducing lipid release via interference with VLDL secretion.

In vivo Studies on the Role of HCV in Promoting Liver Cancer

Studies of the role of HCV in promoting liver cancer development are limited by the lack of an HCV-permissive and immunocompetent animal model. Apart from humans, only chimpanzees are susceptible to HCV infections, but owing to ethical concerns and high costs their use is virtually impossible. To overcome these limitations, two alternative attempts are pursued: first, the generation of transgenic animals expressing HCV protein(s), and second, the development of an immunocompetent and HCV-permissive mouse model. With respect to the first approach, most studies have focused on transgenic mice expressing constructs containing the core protein or NS5A. It appears that, depending on the used mouse strain, expression construct, or experimental conditions, steatosis and occasionally HCC develop [reviewed in 16]. While these results support the notion that HCV proteins can promote tumor development, with one exception, only polyprotein fragments or isolated protein (core or NS5A) have been expressed and tumors have only been found in the C57BL/6 mouse background. Thus, the relevance of these observations for infections in humans remains to be determined.

With respect to the second approach, in the last few years several transgenic mouse models have been established that are, however, immunodeficient and rely on the transplantation of human liver xenografts [43–45]. The first model, originally described by Kneteman and coworkers used a upa/SCID mouse strain that expresses the toxic urokinase plasminogen activator (upa) selectively in the liver [43]. Soon after birth, the upa transgene is expressed and a high proportion of mouse hepatocytes are destroyed. During this phase, animals can be rescued by transfer of primary human hepatocytes that migrate into the liver and form human liver cell 'nests' in an otherwise murine liver architecture. Engrafted human hepatocytes can be infected productively with HCV, but since these animals are immunodeficient to avoid graft rejection, this system is not useful for studies of HCV-associated carcinogenesis. An improvement of this mouse model was recently described by Washburn et al. [46]. They generated AFC8-hu HSC/Hep mice by co-transplanting human $CD34^+$ hematopoetic stem cells (HSC) and hepatocyte progenitors (Hep) into mice that were transgenic for caspase 8 that was expressed under control of the albumin receptor promoter (ACF8). Expression of caspase 8 in liver cells is toxic, thus creating 'space' for transplantation of human liver cells. The authors could show that these AFC8-hu HSC/Hep mice supported HCV infection in the liver and mice developed an HCV-specific T cell response that was, however, very weak [46].

More recently, Dorner et al. [47] established a genetically humanized mouse model. By adenoviral transfer of the HCV entry factors CD81 and occludin into mouse liver cells, the animals became infectable but did not support HCV replication to a directly detectable level. To overcome this limitation, the authors made use of the Rosa26-Fluc mouse strain that expresses an inactive luciferase reporter gene that can be activated upon expression of the CRE recombinase. Upon infection of these animals with an engineered HCV expressing this recombinase, a small but detectable fraction of inoculated liver cells started to fluoresce, indicating infection of the cells and expression of the (HCV-encoded) CRE recombinase. Although this mouse model is still very inefficient, for the first time HCV replication in an inoculated immunocompetent transgenic mouse was detected. It is obvious that much more work will be required to improve this mouse model to a level that supports the complete HCV replication cycle, but only with the availability of such an immunocompetent in vivo system will it become possible to study the contribution of (persistent) HCV infection to the development of liver cancer.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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