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Hepatocarcinogenesis associated with hepatitis B, delta and C viruses

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Abstract

Globally, over half a billion people are persistently infected with hepatitis B (HBV) and/or hepatitis C viruses. Chronic HBV and HCV infection frequently lead to fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Co-infections with hepatitis delta virus (HDV), a subviral satellite requiring HBV for its propagation, accelerates the progression of liver disease toward HCC. The mechanisms by which these viruses cause malignant transformation, culminating in HCC, remain incompletely understood, partially due to the lack of adequate experimental models for dissecting these complex disease processes *in vivo*.

Introduction

As the second highest cause of cancer-related death worldwide, liver cancer is a substantial global health problem [1]. More than 90% of the primary liver cancers diagnosed are specifically hepatocellular carcinoma (HCC), which has an especially poor prognosis with 700,000 new cases and 600,000 deaths occurring every year [2]. A multitude of factors contribute to the development of HCC, including viral, immune, chemical and genetic etiologies. For 80% of all HCCs, infections with the viruses HCV, HBV and/or HDV — all of which have a high propensity for progressing to chronicity — are considered the root cause. Worldwide, billions of people have been exposed to HBV, HCV and HDV, resulting in approximately 170 million chronic HCV carriers and 350 million chronic HBV carriers of which 15–20 million are co-infected with HDV (Table 1). Between 15% and 40% of those chronically infected with HBV or HCV will develop serious liver disease, including cirrhosis and/or HCC. HBV/HDV co-infected patients experience rapid progression of liver disease and also have the highest mortality rate (20%) of any of the viral hepatitises [3–5] (Table 1). The chronic damage inflicted on the liver by the immune system in its (unsuccessful) attempts to clear these viruses is generally considered to be the main contributor to liver disease progression. However, it is less clear how these viruses trigger hepatocarcinogenesis. Different mechanisms by which HCV, HBV and HDV contribute to the development and progression of HCC have been proposed. These include continuous

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antiviral inflammatory responses, ongoing immune clearance of infected cells and hepatocyte regeneration which all occur during chronic infection and lead to genetic and epigenetic changes that put patients at increased risk for HCC establishment [6,7].

In the following sections, we briefly discuss the biology of HBV, HCV and HDV, highlight oncogenic mechanisms that lead to development of HCC in individuals infected with these viruses and provide an outlook of the tools needed to more efficiently study virally induced HCC.

Hepatitis C, B and delta viruses

HCV is an enveloped, positive-sense, single-stranded RNA virus of the *Hepacivirus* genus in the *Flaviviridae* family. Following a complex entry mechanism into hepatocytes, which involves numerous cellular host factors (reviewed in [8]), the HCV genome is released from the nucleocapsid into the cytoplasm. Here, HCV's 9.6 kb RNA genome is translated into a ca. 3000 amino acid polyprotein that is processed by viral and cellular proteases to release the viral structural proteins — core, E1, E2 — and non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. In addition, a short +1 open-reading frame (ORF) exists that produces a genome product referred to as minicore. Minicore currently has no ascribed functions, but mutations in codons 70 and 91 are associated with the development of liver cancer and lead to increased expression of this protein [9]. The structural and non-structural proteins carry out various functions in the viral life cycle, including viral genome propagation, assembly and egress of infectious virions. Although several reports have suggested the existence of extrahepatic reservoirs, including peripheral blood mononuclear cells [10,11], epithelium [12] and even tissues of the central nervous system [13], it is generally thought that HCV productively replicates exclusively in hepatocytes. HCV's highly restricted tissue range is governed by a higher — albeit not exclusive — expression of essential HCV entry factors and the microRNA, miR122, which is solely expressed in hepatocytes. In contrast to HBV and HDV, the HCV life cycle does not have a nuclear stage and is completed wholly in the cytoplasm of infected hepatocytes.

HBV belongs to the *Hepadnaviridae* family and has a very compact 3.2 kb partially double-stranded DNA genome that is organized in four partially overlapping ORFs. These encode for the HBV surface polypeptides, which form three proteins, namely the small (S), medium (M), and large (L) surface antigens that are inserted into the viral envelope. Additional virally encoded components include HBV core protein which comprises the viral nucleocapsid; the X protein which has many suggested functions including epigenetic regulation of the viral genome; and the viral polymerase which is involved in viral replication and packaging [14,15]. Like HCV, HBV is a uniquely hepatotropic virus. Although transfection experiments in tissue culture suggest that HBV is able to replicate in non-hepatic cells [16,17], viral entry is dependent on the bile acid transporter sodium-taurocholate cotransporting poplypeptide (NTCP) exclusively expressed in hepatocytes [18•, 19••]. Interaction of the N-terminal region of preS with NTCP initiates entry during which the virus is internalized via receptor-mediated endocytosis (reviewed in [20]). Following uncoating, the nucleocapsid is transported to the nuclear membrane where the genome is then released as a relaxed circular genome (rcDNA) into the nucleus. In the nucleus, the

partially double-stranded viral genome is repaired, forming covalently closed circular DNA (cccDNA), which serves as the transcriptional template for all four viral gene products as well as pre-genomic RNA (pgRNA). HBV transcripts are transported into the cytoplasm where the viral mRNAs are translated and pgRNA packaged in a nucleocapsid. Here, pgRNA is reverse transcribed into rcDNA that can either be imported into the nucleus to replenish the cccDNA pool or nucleocapsid-encased rcDNA can be enclosed by lipid bilayer containing the HBV envelope proteins and then released from the host cell [21].

HDV is a negative-sense RNA virus independent of any viral family and the sole member of the *Deltaviridae* genus. The small HDV circular genome is only ca 1.7 kb in length and is stabilized by extensive intramolecular base pairing. HDV encodes a single ORF that encodes the delta antigen (HDAg), which exists as two isoforms, the small and large HDAg [22]. HDV is considered a subviral satellite as it requires the HBV envelope proteins to form infectious virions. Consequently, the early steps of HDV entry into hepatocytes follow the same mechanism as HBV. Once within the hepatocyte, a nuclear localization signal on HDAg-L triggers the translocation of the HDV nucleocapsid to the nucleus where the viral genome is replicated. The small size of the HDV genome results in the virus' reliance on host enzymes, including cellular RNA polymerases, to successfully replicate (reviewed in [23]). The incoming RNA serves as the template for transcripts longer than the size of the virus' genome. Such multimeric, linear RNAs contain at least two copies of the antigenomic ribozyme, releasing unit-length linear RNA following self-cleavage. Antigenomic RNA is circularized and forms the template for new genomic RNAs following similar intermediate steps.

Mechanisms of HCV-associated hepatocarcinogenesis

HCV-associated hepatocarcinogenesis is a protracted process that is usually the result of decades-long persistent infection. The mechanisms driving hepatocarcinogenesis during chronic HCV infection remain incompletely understood. HCV is known to trigger strong cell-intrinsic responses mediated largely by type I and III IFN which induce a plethora of IFN stimulated genes. A variety of evasion mechanism enable HCV to blunt antiviral signaling in some cells, thereby creating a cellular environment which is more conducive for viral persistence. This antiviral innate response precedes the activation and infiltration of a variety of lymphocyte subsets, including NK cells, CD8 and CD4 lymphocytes, aiming at clearing HCV infected cells. In chronically infected patients these antiviral defenses fail as HCV rapidly acquires mutations allowing it to evade immune pressure. Clearly, the inflammatory context established in the liver during persistent HCV infection plays an important role, as HCC only develops following stages of fibrosis and cirrhosis. In efforts to clear virally infected cells, immune-mediated liver injury subsequently induces proliferation of hepatocytes, which are usually quiescent. This compensatory hepatocyte proliferation results in propagation of oncogenic mutations, leading to clonal expansion of transformed cells that eventually give rise to HCC. These oncogenic mutations may be introduced by, for example, reactive oxygen, nitrogen intermediates, and damage-associated molecular pattern molecules released by inflammatory immune infiltrates. Additionally, the expression of viral proteins in infected cells can alter the sensitivity of intracellular signal transduction

pathways (JAK-STAT, EGF-β and TGF-β), activate cellular oncogenes (such as Ras, c-Myc and E2F1), dysregulate cell cycle control and/or inactivate tumor suppressor genes [24–27].

Unlike HBV, HCV has no nuclear phase of replication, no genome integration into host DNA and also no direct oncogenic activity of its genes. However, several HCV proteins have been directly implicated in promoting HCV pathogenesis and ultimately hepatocarcinogenesis.

Most striking are the effects exerted by HCV core, exemplified by the age-dependent development of hepatic steatosis and HCC in mice with liver-specific expression of the core gene [28] which appears to require activation of peroxisome proliferator-activated receptor alpha [29]. This striking phenotype can possibly be explained by HCV core's ability to modulate intracellular pathways and cellular metabolism while dysregulating cell cycle control [30–32]. The core protein was also reported to upregulate several cellular proteins (such as IL-6, gp130, leptin receptor and STAT3) whose dysregulation can induce transformational changes in hepatocytes. Several studies also reported that HCV core results in continuous cellular proliferation by increasing telomerase activity [33–36]. Studies also suggest a role for the HCV non-structural proteins. NS3/4A can accelerate cellular proliferation by increasing phosphorylation of extracellular signal-regulated kinases and inhibiting apoptosis by forming complexes with p53 and inhibiting p21 promoter activity [37,38]. NS5A can disrupt the balance between TNF-α-triggered survival and death pathways by inhibiting TNF-α mediated NF-κB activation which in turns increases the susceptibility of hepatocytes to carcinogenic death [39,40]. The surviving cells, on the other hand, start to proliferate in response to inflammatory cytokines (such as TNF-α and IL-6) and intracellular JNK activation [40]. Similar to NS3/4A NS5A also has a suppressive effect on p53-transactivation of the p21 promoter [41]. Cytoplasmic arrest and degradation of the retinoblastoma tumor-suppressor protein (Rb) by NS5B has also been shown to promote proliferation of hepatocytes and may play a role in the genomic instability induced by dysregulation of Rb pathways [42].

However, such published data analyzing the transformative potential of individual HCV proteins are based on overexpression studies, which do not adequately reflect the actual expression levels in a virally infected hepatocyte and also do not take into account the inflammatory milieu of the infection.

HCV is dependent on microRNA-122 for replication and stabilization and one recent study shows that during HCV infection, HCV reduces argonaute binding to predicted miR-122 binding sites and functionally de-represses miR-122 targets during HCV infection. Prior work in mir-122 knockout mice which develop HCC suggests that miR-122 functions to maintain liver homeostasis and tumor suppressor activity [43,44]. Examination of miR-122 targets were shown to be upregulated in HCV infected cells and in patients with chronic HCV infection [45–47], HCV induced cirrhosis and in HCC.

Mechanisms of HBV/HDV-associated hepatocarcinogenesis

In contrast to HCV, hepatitis A and hepatitis E viruses, HBV is considered a 'stealth virus' capable of effectively interfering with antiviral inflammatory responses [48]. This is illustrated by the fact that in liver biopsies from experimentally infected chimpanzees and chronic HBV carriers few if any host cellular gene transcription is induced early during infection [49]. More recent studies have challenged this view demonstrating that interleukin (IL-) 6-mediated rather than IFN-mediated response may facilitate early control of HBV infection [50]. Likewise, it was shown that NK and NKT cells are triggered early during infection. However, these antiviral defenses seem to be transient and are efficiently antagonized by HBV infection. These seemingly contradictory observations cannot be fully explained currently but are clearly dependent on the timing of the analysis during infection and the experimental system used for the analysis. However, undoubtedly, HBV induces an intrahepatic inflammatory milieu, which is distinct from that of HCV.

Long-term HBV infection is associated with increased chronic oxidative damage of hepatocytes, immune-mediated inflammation of the liver and consecutive mutation accumulation and cancer development in patients [7,51]. Unlike HCV, HBV DNA is present in the nucleus and HBV DNA integration in the host genome is frequently observed in tumors and directly correlates with patients' survival and disease progression [52]. A variety of genomic perturbations near viral integration sites have been observed, including direct gene disruption, viral promoter-driven human transcription, viral-human transcript fusion, and DNA copy number alteration [52,53]. A combination of these processes results in an increased HCC risk in states of minimal fibrosis and no evidence of cirrhosis. This stands in stark contrast to the situation in chronic HCV carriers in which HCC only develops in patients who have progressed to cirrhosis.

Increased levels of HBV proteins, due to continuous expression of wild type and truncated HBx or truncated preS/S polypeptides, contribute to HCC by transactivation of transcription factors, stimulation of inflammatory responses, induction of oxidative damage and accumulation of mutations in the host genome [54–56]. HBxAg is known as an oncoprotein due to its multiple *trans*-acting roles in cell cycle regulation, signaling pathways and DNA repair. It does not bind directly to DNA but rather induces its effects on cellular promoters or signaling pathways via its interactions with other proteins [56,57]. In spite of humoral and cellular responses against X antigen, integration of HBV DNA into the host genome provides continuous expression of this protein even in the absence of active HBV replication [58,59]. HBV X protein has also been shown to inhibit apoptosis and promote HCC through mitochondrial translocation of Raf-1 mediated by HBx-induced oxidative stress as well as the cytoplasmic arrest of p53 and inhibition of its nuclear-translocation [60,61].

HBV also contributes to HCC development by hypomethylation and hypermethylation of host DNA as well as increased histone deacetylation via post-translational modifications of histone molecules. In this way, the expression of cellular oncogenes and tumor suppressor genes can be dysregulated [62]. Immortalization of host cells by HBV DNA integration in the human telomerase reverse transcriptase gene has also been observed, leading to

constitutive overexpression of telomerase and thus contributing to HBV persistence in hepatocytes and HCC development [7,63].

Co-infection with HBV and HDV frequently causes more rapid and exacerbated disease progression [64] and has been reported to increase patients' risk for developing HCC [3]. The mechanisms underlying this process are not well understood. HDAg expression alone is not cytopathic and does not seem to have any oncogenic potential. However, a high level of liver inflammation has been observed with intrahepatic expression of L-HDAg and through activation of NF-κB signaling and the immune response against delta infection [65,66]. Moreover, HDV replication, which does take place in the nucleus, has been shown to result in dysregulation of pathways, increased oxidative stress, and epigenetic changes that can trigger transformative events (reviewed in [67]).

Host genetic contributions to virally induced hepatocarcinogenesis

It has become increasingly clear from genome-wide association studies (GWAS), whole genome and exome sequencing that specific variations in the host genome within a population correlate with a greater risk of progressing to chronic hepatitis and/or development of HCC. Single nucleotide polymorphisms (SNPs) within the MICA [68••] and DEPCD5 [69••] loci have been reported to affect the susceptibility to HCC amongst Japanese individuals with chronic HCV infection. MICA encodes a ligand for NKG2D, which can activate cytolytic anti-tumor responses of T cells and NK cells. SNPs in EGF gene are also associated with higher EGF levels in serum and liver and the emergence of HCC in HCV infected patients [70]. Likewise, polymorphisms in the IL28B locus which result in expression of a pseudogene (IFN-λ3) [71] were identified that correlate with spontaneous clearance of an acute HCV infection and predict to a certain extent how likely patients with a given combination of IL28B alleles are to respond to peg-IFN/RBV therapy [72–75]. In both HBV and HCV infections SNPs in TGF-β1 are associated with elevated TGF-β1 expression and increased risk of HCC through inhibition of multiple tumor suppressors [76,77]. Genetic variants in chromatin regulators [78•,79] are increased in patients with HCV-associated and HBV-associated HCC. Mutations in the STAT4 confer enhanced risk for HBV-related HCC. Reduced expression of STAT4 impairs IFN-γ activation and decreases its antiviral and antitumor functions. Depending on the type of SNPs, changes in HLA-DQ gene may either cause HBV clearance or HCC development due to the effects on HBV specific CD4+ T-cell responses [80•,81]. But, what still remains a major challenge is to functionally evaluate the impact of most of these mutations on the pathobiology of HCC.

In addition to those defined genetic predispositions it has been shown that men are at greater risk for HCC when all other issues being the same (e.g. HCV genotype, HCV treatment, diabetes, and body mass index) [82]. Moreover Asian and Black (African) patients are at a much greater risk than Non-hispanic Whites [83]. While etiology is obviously genetic the mechanisms underlying this apparent racial and gender bias are not known.

Challenges for studying HCC

Gaining deeper insights into the complex biology of virally induced hepatocarcinogenesis has been hampered by the scarcity of disease models for these three viral infections. HCV, HBV and HDV share a narrow host range, infecting and robustly replicating only in humans and chimpanzees (Table 2). Considerable efforts have been undertaken to create small animal models permissive to infection, but few qualify as bona fide disease models (reviewed in [84,85]). As discussed in previous sections, expression of individual HCV, HBV or HDV gene products does not adequately recapitulate the unique inflammatory environment created by these viruses. Additionally, the observed pathogenesis in these models has to be interpreted with caution due to the nonphysiological high expression of viral proteins. Transgenic expression of a 1.3x HBV genome in mice results in the release of infectious HBV virions and — if tolerance is broken — in the induction of virus-specific immune infiltration but without HCC development [86]. Similar approaches have not (yet) been successful for HCV.

To render animals susceptible to these viruses, both host and viral adaptation approaches are being pursued. Transplantation of human hepatocytes into immunodeficient liver injury mouse strains gives rise to so-called human liver chimeric mice that are highly susceptible to HCV [87–90], HBV [88,91] and HDV [92] infection. However, due to their immunodeficient background, chronically infected liver chimeric animals do not develop liver disease. More recent efforts have focused on achieving co-engraftment of human liver tissue and components of a human immune system [93–96]. While this approach holds great potential, there is little evidence for virally induced liver pathogenesis [94,97••,98•]. Furthermore, generation of such xenotransplantation models is costly, requires considerable technical skills, is low in throughput and the results are significantly influenced by donor-to-donor variability. A mouse model with inbred susceptibility to infection would overcome these caveats but requires in-depth understanding of the barriers of interspecies transmission. The discovery of human-specific factors for HCV [99] and HBV/HDV [18•,19••] entry into the hepatocytes, led to the development of genetically humanized mouse models supporting aspects of these viruses' life cycles, albeit at low efficiencies [100,101••]. It remains to be seen whether future versions of inbred models can be refined to a point where virally induced liver disease, including HCC, can be effectively modeled.

So-called non-primate hepaciviruses, which resemble HCV, have been identified in dogs, rats, outbred mice and horses, but it is not known whether these viruses cause liver disease similar to that observed in HCV patients (reviewed in [102]). Similarly, hepadnaviruses have been found in a variety of species, including bats, ducks, primates and woodchucks (reviewed in [84]). The woodchuck model is of particular importance as infection with woodchuck hepatitis virus (WHV) causes aggressive liver disease that always culminates in HCC [103•]. HDV virions pseudotyped with WHV envelope proteins can infect woodchuck hepatocytes, causing liver disease and HCC [104]. However, a caveat of these surrogate models is that while infection is caused by viruses similar to the pathogen causing disease in humans, they are still not identical. Furthermore, mechanistic dissection of disease progression in less commonly used species such as woodchucks is hampered by their limited availability and the scarcity of species-specific reagents necessary for in-depth analysis.

Ultimately, the development of better animal models is necessary in order to further study the mechanism of virally induced hepatocarcinogenesis. In addition, new immunocompetent HBV, HCV and HDV infection models could be used to test the efficacy of novel antiviral and possibly anti-tumor therapies. Treatment options for HCV have drastically improved over the last 5 years. While previous standard of care resulted in sustained virologic responses in 50% of patients enrolled in clinical trials, now almost any chronic HCV carrier can be cured with combinations of directly acting antiviral which have very little side-effects. Eliminating HCV early during an infection will presumably minimize the risk of developing HCC. However, longer term follow up studies in patients who were cured of their HCV infection but had already progressed to advanced fibrosis showed an increased risk for HCC. Likewise, chronic HBV carriers whose viremia is efficiently suppressed–but not cured–by antiviral therapy have an elevated risk of developing HCCs. The mechanisms underlying these processes are not understood and their elucidation may be greatly aided by better experimental systems.

Conclusion

HCC development and progression is a complicated process affected by various etiological factors. Upon encountering oncogenic hepatotropic viruses such as HBV, HCV, and HDV, the immune system acts as a double-edged sword, working to clear the invading virus but at the cost of the liver's health and integrity due to the stress of inflammatory responses. Because of the varying nature of these viruses, their oncogenic effects may also undergo different mechanisms. Thus, it remains of critical importance to enhance existing models and generate novel platforms for studying viral infection and the resultant tumorigenesis in a physiologically relevant setting.

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Table 1

Prevalence and severity of chronic viral hepatitis

	HCV	HBV	HBV/HDV
Prevalence	170 million	240 million	20 million
Fibrosis	20–39% [105,106]	34% [107]	23.4% [108]
Cirrhosis	20–30% [109]	15–20% [110]	14–77% [111,112]
HCC	1–3% [110]	0.01–5.4% [113]	10–15% [114]
Time to HCC	≥30 years [109]	10–30 years	≥10 years [115,116]

Table 2

Animal models and their application in studying viral hepatitis

Virus	Animal group	Model		Phenotype
HCV	Primates	Chimpanzees		Chronic and mild infection [117]
		Rhesus Macaque		Viral entry and infection with viremia in immunocompromised xenorecipient model [118]
		Tupaia		Acute infection with mild to severe liver injury
		Other Non-human Primate Models		Mostly support viral entry but resistant to infection
	Rodents	Tg mice	Whole genome	Persistent infection with complete replication cycle [119]
			Individual proteins	No damage to severe liver injuries due to the expression of core and NS5A proteins, depending on the controlling promoters [120]
		Genetically Humanized Models		Completion of the entire viral life cycle at low levels [101]
		Xeno-transplantation models	Liver engrafted	Viral infection and evidence of fibrosis [87]
			Liver and immune system engrafted	Viral infection and fibrosis but no viremia [121]
	Surrogates	Tupaia		Acute infection with mild to severe liver injury
		Marmoset		HCV/GB virus B chimeric establishes productive infection [122]
HBV	Primates	Chimpanzees		Acute and chronic infection with liver fibrosis, cirrhosis and HCC [123]
		Rhesus Macaque		Acute infection with mild liver injury [124]
		Tupaia		Persistent HBV infection with liver necrosis and inflammation [125]
	Rodents	Tg mice	Whole genome	Supported viral replication but no liver damage [126]
			Individual proteins	No damage due to the tolerance to expressed proteins
		Genetically Humanized Models		No infection [127]
		Xeno-transplantion models	Liver engrafted	Chronic HBV infection [92]
			Liver and immune system engrafted	Persistent viral infection and fibrosis followed by viral clearance through neutralizing antibodies [98•]
	Surrogates	Woodchucks		WHV infection with severe liver disease and HCC
		Ground Squirrel		Persistent infection of GSHV and HCC
		Wooly Monkey		Acute not persistent WMHBV infection [128]
		Duck		Acute and chronic DHBV infection [129]
HBV/HDV	Primates	Chimpanzees		Acute and chronic infection with different outcomes of chronic disease compared to humans [130,131]
	Rodents	Tg mice	Whole genome	Replication but no liver damage [132]
			Individual proteins	No histopathological liver damage from S,LHDAg expression [65]
		Genetically Humanized Models		Acute HDV infection for about two weeks [133••]

Virus	Animal group	Model		Phenotype
		Xeno-transplantion models	Liver engrafted	Established HDV infection for several months [92]
			Liver and immune system engrafted	Not done yet
	Surrogates	Woodchucks		Transient acute HDV infection and HCC due to chronic WHV infection [104]
		Duck		DHBV-surface proteins do not support HDV propagation and infection [129,134]

Tg: transgenic, WHV: woodchuck hepatitis virus, HCC: hepatocellular carcinoma, GSHV: ground squirrel hepatitis virus, WMHBV: Wooly Monkey HBV, DHBV: Duck hepatitis B virus; S,LHDAg: small and large hepatitis Delta antigens.