The hepatitis delta virus: replication and pathogenesis

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List of abbreviations: HDV, hepatitis delta virus; RNP, ribonucleoprotein; HBV, hepatitis B virus; HDAg, hepatitis delta antigen; RdRp, RNA-dependent RNA polymerase; ADAR, adenosine deaminase acting on RNA; ORF, open reading frame; S-HDAg, small-HDAg; L-HDAg, large-HDAg; DIPA, delta-interacting protein A; NES, nuclear export signal; SVP, subviral particle; HSPG, heparan sulfate proteoglycan; AGL, antigenic loop; NTCP, sodium taurocholate cotransporting polypeptide; HCC, hepatocellular carcinoma.

Key words: chronic hepatitis, cirrhosis, hepatocellular carcinoma, viroids

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Key points

HDV biological characteristics do not fulfill the definition of a virus

HDV is a subviral infectious agent and obligate satellite of HBV

HDV RNA replication is dependent upon cellular RNA polymerase(s) and independent of HBV

HDV propagation depends upon the provision of the large and small HBV envelope proteins

HDV replication interferes with the helper HBV replication

HDV infection leads to the most severe forms of acute or chronic viral hepatitis in humans

Abstract

Hepatitis delta virus (HDV) is a defective virus and a satellite of the hepatitis B virus (HBV). Its RNA genome is unique among animal viruses, but it shares common features with some plant viroids, including a replication mechanism that uses a host RNA polymerase. In infected cells, HDV genome replication and formation of a nucleocapsid-like ribonucleoprotein (RNP) are independent of HBV. But the RNP cannot exit, and therefore propagate, in the absence of HBV, as the latter supplies the propagation mechanism from packaging of the HDV RNP with the HBV envelope proteins for cell egress to delivery of the HDV virions to the human hepatocyte target. HDV is therefore an obligate satellite of HBV; it infects humans either concomitantly with HBV or after HBV infection. HDV affects an estimated 15 to 20 million individuals worldwide, and the clinical significance of HDV infection is more severe forms of viral hepatitis - acute or chronic -, and a higher risk of developing cirrhosis and hepatocellular carcinoma in comparison to HBV mono-infection. This review covers molecular aspects of HDV replication cycle, including its interaction with the helper HBV and the pathogenesis of infection in humans.

Introduction - life cycle overview

Discovered almost 40 years ago in the liver of individuals chronically infected with the hepatitis B virus (HBV), the hepatitis delta antigen (HDAg) was first considered as a new HBV antigen [1]. It was soon demonstrated that the HDAg protein was associated to a small RNA as a component of a transmissible agent, or defective virus, coated with the HBV envelope proteins (Fig. 1), which could subsist as an obligate satellite of HBV. The cloning and sequencing of the HDAg-associated RNA, now known as the hepatitis delta virus (HDV) genome, revealed that it was unique among known

animal virus genomes but sharing common features with some plant subviral agents called viroids [2, 3]. The HDV characteristics do not fulfill the criteria for the definition of a virus, and it is actually classified as the prototype and sole member of the *Deltavirus* genus. The genome is the smallest among known mammalian viruses, and it bears no homology with that of the helper HBV DNA. It is a single stranded circular RNA of 1700 nucleotides in size, which, unlike plant viroids, encodes a protein (Fig. 2). Since the HDAg protein has no RNA-dependent RNA polymerase (RdRp) function, HDV must rely mostly on cellular enzymes for replication of its genome (Fig. 3), and on the assistance of its helper HBV for propagation. Similar to some plant viroids, HDV RNA replication in the cell nucleus is carried out by cellular RNA polymerase(s), and both genomic HDV RNA and its replication intermediate, the antigenomic RNA strand, include autocatalytic self-cleaving elements, referred to as ribozymes (Fig. 4). Remarkably, HDV manages to produce two forms of HDAg proteins from a single open reading frame (ORF); this is achieved using a cellular adenosine deaminase acting on RNA (ADAR) to edit an amber codon on the antigenomic RNA (Fig. 4). The two forms of hepatitis delta proteins undergo extensive post-translational modifications to fulfill diverse functions in the processes of genome replication, assembly of the HDV RNP and packaging of the latter with HBV envelope proteins. The larger form of hepatitis delta proteins manages to establish physical interaction between the RNP and the HBV envelope proteins leading to assembly HDV virions coated with the helper envelope proteins (Fig. 5). Bearing the same infectivity determinants, HBV and HDV virions also share a common tropism to human hepatocytes (Fig. 6).

The HDV genome

The HDV RNA is approximately 1680 nucleotides in size and the smallest genome of the known mammalian viruses [3]. It is present in abundance in infected cells, mostly in the nucleus, along with its replication intermediate, the antigenomic RNA. Both are circular single-stranded molecules that adopt a quasi-double-stranded conformation upon self-annealing with 74% of their nucleotides - 60% of which are G or C - forming Watson-Crick base pairs (Fig. 2). The circular nature and self-annealing properties of HDV RNAs are reminiscent of some plant viroids and are important for both genome replication and RNP assembly upon interaction with HDAg proteins [4-9]. The genetic organization of the HDV genome includes several ORFs; one, located on the antigenomic HDV RNA strand, is known to be translated into HDAg proteins [10]. A second ORF coding for a polypeptide referred to as

peptide-K, has been reported to be expressed in infected cells, but there is no information about a possible function [11]. The two HDAg proteins are referred to as small-HDAg protein (S-HDAg) of 195 amino acids in length, and large-HDAg (L-HDAg) that differs from S-HDAg by 19 additional amino acid residues at the carboxyl terminus. The difference in size is the consequence of an RNA editing event that occurs on the antigenomic HDV RNA strand during replication (Fig. 4) [12]. S-HDAg is essential to HDV RNA replication but does not possess an RdRp activity, whereas L-HDAg inhibits genome replication and is required for assembly of HDV particles. Some authors have chosen to divide the rod-like structure of the HDV genome in two domains: a small, viroid-like domain, and a larger "coding domain", including the HDAg ORF sequence and its complementary strand in the rod-like structure (Fig. 2) [13].

Since the initial sequencing of the HDV genotype-1 genome [3], seven additional genotypes have been identified [14], which display a surprisingly extensive divergence of their nucleotide sequences. For instance, the most distant HDV genotype-3 sequence is approximately 40% divergent from that of genotype-1 [15].

HDV RNA replication

Unlike most RNA viruses that replicate their genome by encoding an RdRp, but like some plant viroids, HDV RNA recruits what is normally a host DNA-directed RNA polymerase for replication. HDV and viroid RNAs have structural similarities, and their respective replication mechanisms share common features [3, 16-19]. HDV RNA replicates in mammalian cells, as efficiently as viroids do in plants [17, 20], and no helper function for HDV RNA synthesis or RNP assembly is provided by HBV. When delivered into the cell nucleus upon infection, the genomic HDV RNA is considered to be replicated through a rolling-circle mechanism of antigenomic RNA synthesis carried out by the RNA polymerase II - a normally DNA-dependent enzyme - that is diverted by HDV to use its RNA as a template. The rolling-circle mechanism of RNA synthesis, initially described for replication of some viroids, initiates with the synthesis of multimeric linear transcripts from the circular genomic template. Although multimeric forms of *de novo* synthesized RNA are indeed detected in infected cells, they do not accumulate for being cleaved into monomers by an autocatalytic self-cleaving sequence, referred to as ribozyme, on the neo-synthesized RNA (Fig. 4). The linear cleavage product is then subjected intramolecular ligation to form a circular antigenomic molecule, which in turn serves as a template for

synthesis of genomic RNAs through a similar rolling circle mechanism [21]. The HDV rolling circle model differs however from that of viroids in including the synthesis of a messenger RNA - of antigenomic polarity - for translation into HDAg proteins [22]. Details of the HDV RNA replication mechanism by Pol II are still missing, but it is clear that S-HDAg is required in this process, and the HDV RNA rod-like conformation is important to substitute for DNA as a substrate for Pol II [23, 24]. Note that Pol II is also the enzyme that amplifies the HBV genome by transcribing the latter into multiple copies of pregenomic RNA that serves as replication intermediate. It has been suggested that the interference generally exerted by HDV on HBV replication may result, at least in part, from the two viruses sharing cellular Pol II as replicase [25, 26]. Surprisingly, HDV RNA replication by Pol II is very efficient, leading to the accumulation of up to 300,000 copies of genomic HDV RNA per cell, 50 000 copies of antigenomic species and approximately 1000 copies of HDAg mRNA [23]. The details of how Pol II is redirected to replicate HDV RNA is still unclear, but the process requires the assistance S-HDAg at an early stage in the infection cycle. S-HDAg would co-localize with the polymerase and the splicing factor SC35 in structures, or speckles, of the nucleoplasm that have been described as transcription factories [26]. S-HDAg was found to bind directly to Pol II in reconstituted *in vitro* transcription assays leading to transcription stimulation upon displacement of the negative elongation factor (NELF) [27]. In the same setting, S-HDAg could also interact with the clamp of Pol II, to ensure transcription fidelity [28]. More details of the HDV RNA synthesis mechanism by Pol II have started to emerge from studies conducted mostly in *in vitro* transcription assays to characterize HDV RNA/Pol II transcription initiation complex [29], or using cells actively replicating HDV RNA in which promoter sequences on genomic or antigenomic strands could be identified [30, 31]. It is assumed that Pol II would recognize as promoters, the quasi double-stranded hairpins at one extremity of the genomic and antigenomic, rod-like structures. Although Pol II has been shown to play a major role in HDV RNA replication, it remains possible that other host RNA polymerases, such as Pol I or Pol III, could carry out antigenomic RNA synthesis in the nucleoli [32, 33]. Whatever cellular RNA polymerase is engaged, it has been interpreted that replication of HDV RNA would segregate to nucleoplasm for synthesis of genomic RNA and messenger HDV RNAs, and to the nucleolus for that of antigenomic RNA (Fig. 3) [34].

Crucial to the HDV RNA replication mechanism are two ribozymes, the only viral enzymes, one on the genomic strand and the other on the antigenomic strand. The two HDV ribozymes display related

sequences and share a similar predicted secondary structure. Initially, it was asserted that HDV ribozymes were quite different from those of so-called hammerhead or hairpin ribozymes of viroids or satellites of plant viruses [35, 36], but more recently, this assertion has been somewhat modulated [37]. The auto-cleavage reaction in HDV RNAs is a trans-esterification modulated by divalent metal ions, which converts a 3'-5' phosphodiester bond to a 2'-3' cyclic monophosphate group and a 5' hydroxyl group. Ribozyme activity by definition is observed *in vitro* in the absence of cellular proteins, and the minimal size for HDV ribozymes self-cleavage was established at approximately 85 nucleotides. Interestingly, many cellular HDV-like ribozymes have been found to play a role in a variety of biological events in different branches of life [36, 38], raising the possibility of HDV RNA originating from the cell transcriptome. This hypothesis was supported by the recent observation that circular RNA species are relatively abundant in cells [39]. Therefore, HDV RNA could have emerged in HBV infected hepatocytes after cellular RNA species processed into circular forms, became replicative [40]. Furthermore, it has been reported that a protein termed delta-interacting protein A (DIPA) [41] initially identified as an HDAg ligand in a yeast two-hybrid interaction assay, could represent a cellular ancestor of the HDAg protein. Although there was no significant sequence homology between DIPA and HDAg, this finding led to the speculation that HDV could have emerged from the capture of a cellular transcript by a self-replicating circular RNA, a scenario in which both the ribozyme and HDAg RNA coding sequences would have arisen from the human transcriptome [42].

Beside Pol II, the HDV replication cycle depends on another important cellular enzyme, namely the adenosine deaminase acting on RNA 1 (ADAR1) [43-45], the function of which is to edit the HDV RNA as a means to produce two versions of the HDAg protein, S- and L-HDAg. ADAR1 deaminates adenosine to convert it to inosine that is then recognized as guanosine by the polymerase. ADAR1 does not act directly on the HDAg mRNA, but on the circular antigenomic strand to convert UAG to UIG (Fig. 4). After a complete round of RNA-directed replication, the UAG amber codon of the S-HDAg ORF (195 codons in length) is converted to tryptophan codon UGG for L-HDAg (214 codons) on the mRNA [46]. S-HDAg is expressed an early step of replication, and upon ADAR1 editing, L-HDAg appears gradually at a later stage, the L-/S-HDAg ratio increasing with time during the HDV RNA replication process. The HDAg proteins differ from each other by only 19 amino acid residues at the carboxyl terminus of L-HDAg, but this small modification has important functional consequences. S-HDAg appears to activate HDV RNA replication/accumulation in the nucleus and to participate with L-

HDAg and genomic RNA to HDV RNP assembly. L-HDAg has been claimed to act as a dominant negative inhibitor of S-HDAg [47], but it may not act as a direct inhibitor of HDV RNA replication [48]. Undoubtedly, L-HDAg is an essential element of virion assembly [49]. ADAR1 thus plays a critical role in the regulation of the HDV life cycle by controlling the switch between early phase of HDV RNA amplification and late phase of virion assembly [12].

In addition to size modification upon RNA editing, HDAg proteins are subjected to several post-translational modifications, some of which may confer diverse functions essential to HDV RNA replication, RNP formation and virion assembly [50, 51]. Many of these modifications, including methylation at Arg-13 [52], acetylation at Lys-72 [53], phosphorylation at Ser-177 [54] and sumoylation at multiple lysine residues [55], have been claimed to directly participate to replication of HDV RNA, RNP assembly and trafficking between nucleoplasm, nucleolus and cytoplasm [50]. As discussed below the posttranslational farnesylation of L-HDAg is most essential to HDV virion assembly.

Since extracellular HDV virions contain exclusively the genomic HDV RNA strand, a selection process must exist that excludes antigenomic RNA in virion assembly. All forms of HDV RNA are synthesized in the nucleus, but soon after synthesis, a significant amount of genomic RNA is detected in the cytoplasm [7, 56], whereas most of the antigenomic RNA is retained in the nucleus. Genomic RNA export appears to proceed through the same mechanism as that used for the splicing-dependent export of cellular mRNAs [56], consistent with genomic RNA being synthesized by Pol II.

The HDV particle assembly and release

Before being packaged with the HBV envelope proteins, HDV RNA and HDAg proteins interact with each other to form the HDV RNP of approximately 20 nm in diameter. The molar ratio of HDAg proteins to HDV RNA in a virion-associated RNP was estimated at 70 [5, 9]. The molecular details of RNP assembly are still missing, but it is likely that HDAg proteins are first subjected to antiparallel coiled-coil mediated dimerization by virtue of a coiled-coil domain (residues 13-48), before dimers multimerize to form octamers that in turn can bind to HDV RNA in its rod-like conformation [9, 57]. The RNA binding function in both S- and L-HDAg was initially assigned to two arginine-rich motifs located in the middle of the protein sequence [58], but a recent study [59] provided data supporting that RNA binding would rather depend upon multiple positions in HDAg, with the amino-terminal region playing a dominant role [60, 61]. A nuclear export signal (NES) is present in the carboxy-terminal 19-amino acid

sequence unique to L-HDAg [62], which is in agreement with genomic RNA-containing RNPs being assembled in the cytoplasm [56].

For cell egress of its RNP, HDV does not rely on cellular functions but on the assistance of the helper HBV for provision of a budding mechanism (Fig. 6). It is important to remark that the envelope proteins are the only HBV contribution to the HDV life cycle. In fact, HDV could propagate in the liver in which HBV replication has been totally eliminated but not the expression of HBV envelope proteins - at least L- and S-HBsAg - from integrated viral sequences [63]. Furthermore, HDV RNA can survive in HBV-uninfected cells for at least 6 weeks before HDV propagation is rescued upon subsequent HBV infection [64]. The distinctive feature of the HBV life cycle that makes this virus the ideal partner for HDV, resides in the unique properties of its envelope proteins. Three envelope proteins, S-HBsAg, M-HBsAg and L-HBsAg are synthesized by HBV infected cells in amounts far exceeding the need for HBV virion assembly, and due to their capacity for self assembly, they are secreted in abundance as empty subviral particles (SVPs) [65]. HBV virion assembly is a rather rare event in comparison with that of SVPs, and this translates into an average infectious serum containing approximately 10^{12} to 10^{13} SVPs per ml for only 10^8 to 10^9 virions. In comparison, at the onset of an acute HDV infection, titers of 10^{10} to 10^{11} HDV virions per ml are observed. Therefore, HDV particles are more efficiently coated with the HBV envelope proteins and secreted than are HBV virions.

The HBV envelope proteins are membrane-spanning glycoproteins that differ from each other by the size of their N-terminal ectodomain (Fig.5) [65]. L-HBsAg contains a N-terminal pre-S1, central pre-S2 and C-terminal S domains. M-HBsAg is shorter than L-HBsAg in lacking pre-S1, whereas S-HBsAg lacks both pre-S1 and pre-S2 domains. Synthesis occurs at the endoplasmic reticulum (ER) membrane, and SVPs assemble at a pre-Golgi membrane before export through the secretory pathway [66]. Assembly of mature HBV virions requires L-HBsAg in addition to S-HBsAg, since its pre-S region (pre-S1+pre-S2) contains a matrix domain for nucleocapsid envelopment. HBV virions are then exported through the late endosomal multivesicular bodies (MVBs) [67-69].

The assembly determinant on the HDV RNP is located in the C-terminal 19 amino acids long polypeptide of L-HDAg (Fig. 2). It includes a C-terminal CXXQ signal for farnesylation [70-73]. The farnesyl group linked to L-HDAg is likely to anchor the RNP to the ER membrane where the HBV envelope proteins are synthesized [74]. Then binding of HDV RNP to the envelope proteins is thought

to involve a proline-rich sequence located just upstream of the farnesylation site [73, 75]. Being central to the HDV/HBV interaction and essential to HDV propagation, farnesylation has been considered as a potential antiviral target. Hence the existing farnesyl transferase inhibitors that have been developed as anti-cancer drugs are now being tested in HDV infected patients [76, 77]. Surprisingly, beside the C-terminal CXXQ farnesylation signal sequence and the presence of least 4 diversely scattered proline residues, the 19-amino acid C-terminus of L-HDAg is not well conserved among the different HDV genotypes (Fig. 2) [78].

What are the determinants of HDV RNP envelopment on the HBV envelope proteins? Clearly, S-HBsAg is sufficient for HDV assembly and export. However S-HBsAg-coated virions are non infectious for their lacking L-HDAg [79]. Within S-HBsAg, the signal for binding to HDV RNP, referred to as HDV matrix domain, maps to the carboxyl terminus of S-HBsAg, and consists of aromatic residues W196, W199, Y200 and W201 that are conserved in the envelope proteins of all *Orthohepadnaviruses* [75, 80]. Although in nature, HDV has only been found in association with HBV, it has been shown experimentally that *Orthohepadnaviruses* such as woodchuck hepatitis virus, the tent-making bat HBV, and the woolly monkey HBV, can provide helper functions to HDV because their respective envelope proteins are competent for HDV RNP envelopment [81-84]. In contrast, the envelope proteins of the Duck hepatitis B virus, are lacking a HDV matrix domain and therefore unable to package the HDV RNP [73].

Interestingly, the fact that the tryptophan-rich motif that acts as a HDV matrix domain is conserved in the envelope proteins of all HBV genotypes, and even all *Orthohepadnaviruses*, suggested that it might fulfill an essential function for HBV. Yet, it was shown dispensable to the HBV life cycle since substitution of alanine to tryptophane 196, 199 or 201 is permissive to HBV assembly and infectivity [85]. The likely explanation is that the HDV matrix domain is conserved as a consequence of the overlap between open reading frames for polymerase (*pol*) and envelope proteins (*env*) in the HBV genome. Precisely, the DNA coding sequence for the HDV matrix domain in *env* also encodes the catalytic domain (YMDD) in *pol*; in other words, the requirement for the catalytic YMDD sequence in *pol* would impose a conserved tryptophan-rich domain in *env*, which is useless to HBV but opportunistically essential as a matrix element for HDV virion assembly.

The HDV virions that are released in the extracellular space have an average diameter of 36 nm and a buoyant density of 1.25 g/cm³ in CsCl. The viral envelope is identical to that of HBV particles,

consisting of cell-derived lipids associated to HBV envelope proteins surrounding the inner RNP of approximately 20 nm in diameter [7, 86-88].

The HDV RNP delivery to human hepatocytes

The HBV envelope proteins at the surface of HDV virion then ensure that the HDV RNP is targeted to HBV susceptible cells, the human hepatocytes, and the mechanism of entry is expected to engage the same host factors for cell surface attachment and receptor binding as those used by HBV itself. For this reason, and because it presents several practical advantages in comparison to HBV, the HDV *in vitro* infection model has been used to study the HBV envelope proteins functions at viral entry [89], and it has been an essential tool for the recent identification of a receptor for HBV/HDV [90]. The initial docking of HDV particles to human hepatocytes is mediated by cell surface heparan sulfate proteoglycans (HSPGs) [91-94]. The ligand to cell-surface HSPGs in the HBV envelope proteins has been identified in the surface-exposed antigenic loop (AGL) that also bears the conserved immunodominant 'a' determinant [95, 96]. The 'a' determinant is defined by a specific conformation of the AGL polypeptide at the surface of subviral, HBV or HDV particles. It elicits the most effective neutralizing antibody response upon vaccination or infection [97]. It was the first identified HBV marker (HBsAg) [98], yet a function in the HBV life cycle had never been assigned to it until the demonstration of its requirement for infectivity [99]. Interestingly, the AGL bears a N-linked glycosylation site at N146, which is somehow only partially utilized, leading to a near-to 1/1 ratio of glycosylated/nonglycosylated isoforms in the envelope proteins. N146 glycosylation is not required for SVP or HDV assembly, and nor is it for infectivity. It was recently proposed that the systematic coexistence of nonglycosylated and glycosylated N146 would correspond to a dual function of the AGL determinant: nonglycosylated N146 would be essential for infectivity, while the glycosylated form would provide immune escape by shielding the AGL from neutralizing antibodies [100].

After low affinity attachment to HSPGs, the pre-S1 domain of L-HBsAg binds with high affinity to the HBV specific receptor, the sodium taurocholate cotransporting polypeptide (NTCP), a bile acid transporter expressed at the basolateral membrane of differentiated hepatocytes [90, 101]. The critical role of pre-S1 at viral entry was first demonstrated using HDV in an *in vitro* infection assay [79], and it was later estimated that a small number of pre-S1 polypeptides - approximately 3 to 4 per virion - is sufficient for infectivity [102]. Several studies conducted with HBV or HDV in *in vitro* infection assays

established that the N-terminal 75 amino acid residues of pre-S1, including N-terminal acylation with myristic acid, were essential for infectivity [103-109]. It led to the demonstration that synthetic myristoylated pre-S1 peptides could block infection *in vitro* [106, 110]. Pre-S1 lipopeptides were very useful to characterize the early step of HBV/HDV entry and crucial to the identification of NTCP as a receptor. In tissue culture, pre-S1 lipopeptides block HBV or HDV infections at nanomolar concentrations, and they are active upon a short preincubation with the target cells, suggesting their rapid and efficient targeting to NTCP [90, 106, 111]. More importantly, it has been proven a potent HBV/HDV entry inhibitor *in vivo* [112], and it constitutes a promising anti HBV/HDV drug (Myrcludex) currently in clinical trials.

The mechanism of HBV entry post binding is still undetermined; there are studies in the literature supporting the use of caveolae, clathrin or macropinocytosis [113-115]. The HDV entry pathway and post entry trafficking of the HDV RNP to the nucleus also remain unclear.

HDV interference with HBV

In humans and the animal models, superinfection of HBV chronic carriers with HDV leads to suppression of HBV markers in both liver and serum during the acute phase of HDV replication [20, 116, 117]. The underlying mechanism of interference remains poorly understood, but one can suspect the participation of a direct repression of HBV expression in coinfecting cells since *in vitro*, S-HDAg exerts a strong inhibition of HBV mRNAs synthesis or stability [117], while both S- and L-HDAg proteins can repress HBV enhancers 1 and 2, and activate the interferon (IFN)- α -inducible MxA gene [118].

HDV pathogenesis in humans

HDV replicates only in the liver. Thus, pathologic changes are limited to this organ. In addition, due to the peculiar dependence of HDV life cycle on HBV, HDV infection is always associated with HBV infection. Thus, any discussion on the pathogenesis of HDV-associated liver damage cannot be oblivious to the fact that a dual infection is invariably present, even accounting for the mutual interference that may occur between the two viruses.

HDV can induce the most aggressive form of hepatitis, and yet the severity of liver damage may vary from totally asymptomatic cases to acute liver failure. The clinical course of acute hepatitis D is, on

average, indistinguishable from an acute hepatitis B [119]. Symptoms are not specific, and include fatigue, nausea, and hyporexia and are accompanied by the elevation of serum ALT and AST levels. The icteric phase, which is not always observed, is characterized by elevation of serum bilirubin. Acute hepatitis D may present two sequential peaks of liver enzymes, and occasionally a cholestatic course. Acute hepatitis D may be complicated by acute liver failure, especially in case of HDV superinfection of patients with chronic hepatitis B, with massive necrosis of hepatocytes leading to death in the vast majority of patients unless liver transplantation is carried out. The standardized relative risk of acute liver failure is 2.36 (95% CI 1.48 – 4.76) [119]. A study conducted in Taiwan reported that acute liver failure might occur less often in patients infected with HDV genotype-2 than in those with genotype-1 (2/35 vs. 4/6, respectively) [120]. On the other hand, HDV genotype-3, which prevails in South America, may increase the risk of acute liver failure [15, 121, 122]. This aggressive course has been associated with a specific HDAg sequence [123], and it remains an isolated observation that requires confirmation in other isolates.

In patients simultaneously coinfecting with HDV and HBV, the progression to persistent, dual infection with HDV and HBV occurs at a similar rate as in acute hepatitis B, i.e. less than 5% [124]. In patients who are chronically infected with HBV, superinfection with HDV progresses to chronic HDV infection in the vast majority of patients [125]. Once chronic HDV infection is established, preexisting liver disease worsens, even though HBV replication is typically, but not invariably, suppressed [126-128]. Chronic hepatitis D is characterized by inflammatory infiltrate and progressive deposition of fibrosis leading to cirrhosis through a typical wound-healing process, as in other chronic viral hepatitis. Accordingly, ALT and AST levels are persistently elevated in most cases. Longitudinal studies have shown that chronic hepatitis D has a 3-fold higher risk of progressing to cirrhosis in comparison to HBV mono-infection [129]. Thus, chronic hepatitis D patients may develop cirrhosis in 15% of cases within 1-2 years [130], and in 70-80% of cases within 5 to 10 years [131, 132]. In a comprehensive review, the risk of cirrhosis was estimated to increase over time, up to 77% after 30 years of infection [133]. Risk factors of disease progression include the HDV genotype, with genotype-1 and -3 being associated with more severe disease than genotype-2 and -4 [15, 134]. Although HBV replication is usually suppressed, high levels of HBV replication are associated with more severe liver damage [135]. HBV genotypes may also play a role in the course of chronic hepatitis D [133].

Cirrhosis due to HDV may remain stable for long periods of time before progressing to decompensation or developing a hepatocellular carcinoma (HCC). This may be due to a decline in HDV replication over time [132]. The risk of clinical decompensation in HDV-related cirrhosis is more than 2-fold compared to cirrhosis due to HBV [130], and the probability of survival at 5 and 10 years is 49 and 40%, respectively [132]. Whether HDV infection increases the risk of developing HCC vs. HBV mono-infection remains controversial. Experiments conducted in transgenic mice expressing HDAg proteins seem to exclude a direct carcinogenic role of HDAg [136]. Nonetheless, the early retrospective study from the Eurohep cohort reported a 5-year risk of developing HCC of 13%, with a 3.2-fold increase compared to HBV-related cirrhosis [130], while the risk of death was increased two-fold. Recent studies identified liver decompensation and death, rather than HCC, as the major complication of chronic hepatitis D [137]. In particular, in a large multicenter study from Italy on 127 cirrhotic patients, the 5- and 10-year event-free survival were 83.9% and 59.4%, and outcome was worse among males. The cumulative probability of episodes of liver decompensation was 75% at 17 years, with an incidence rate of complication of 2.47 per year. Only 17 cirrhotic patients developed a HCC, with a cumulative rate of 0.55 at 17 years, and an annual incidence rate of 1.0 [138]. In another large, single-center study from Milan, conducted on 299 patients with chronic HDV (186 with cirrhosis), the main cause of death was liver failure, accounting for 59% of fatalities, with HCC occurring at a relatively low rate of 2.8% per year. Interestingly HDV replication was an independent predictor of histological progression, HCC development and mortality [139, 140]. In addition, a study from Sweden showed a HCC standardized incidence ratio of 6.11 (95% CI = 2.77 to 11.65) in chronic hepatitis D when patients with chronic HBV infection alone were used as the reference population [141], suggesting that HDV may indeed be a strong risk factor for HCC, at least in Europe. This seems confirmed by a large, nationwide retrospective study of 2175 US veterans who were tested for HDV, where the HDV-positive status was independently associated with HCC [142]. Interestingly, in this study, the association was also independent of the presence of cirrhosis, suggesting that HDV may be directly oncogenic, possibly via epigenetic changes, such as DNA methylation [143] or other interactions with the cell machinery (see below). On the other hand, the outcome of chronic hepatitis D may be less ominous in patients with HDV genotype-2, as suggested by a study from Taiwan [144]. More longitudinal studies from diverse parts of the world are needed to address these issues, including the role of cofactors, such as HDV and HBV genotypes and the metabolic syndrome.

Mechanisms of liver damage

In vivo, HDV replicates only in hepatocytes and thus pathologic changes are limited to the liver. At histology, hepatitis D is characterized by hepatocyte necrosis and inflammatory infiltrate that seems to correlate with the level of intrahepatic replication, a finding that is compatible with a preponderant immune-mediated liver damage [145], as confirmed by other studies [146]. Although some *in vitro* experiments have suggested a direct cytopathic effect of HDV [147, 148], this is unlikely a consequence of the expression of HDAg, since its expression in transgenic mice is not associated with any sign of liver damage [136]. An international study including 80 chronic hepatitis D patients at different stages of fibrosis failed to show any correlation between HDV replication levels and any histological feature [149], although a weak correlation was found between histological activity and serum HBsAg levels. Thus, it is unclear whether the cell death observed *in vitro* is due to the very high levels of HDV RNA in infected cells [150]. On the other hand, due to the frequent suppression of HBV replication, with the exception of patients in whom both viral agents replicate at high levels, the liver damage is more likely induced by HDV rather than HBV. However, the pattern of mutual inhibition may be multifaceted, especially in patients in whom multiple infections coexist [151].

HDV-specific adaptive immune responses have been reported by some studies. A specific T cell response to HDAg in the peripheral blood of patients with hepatitis D correlates with the decrease of HDV-induced liver disease activity [152]. A cytokine profiling study showed that interleukin (IL)-2, IFN- γ , IFN-inducible protein-10 and IL-10-responses were detectable in 53, 35, 65 and 6 percentage, respectively, of chronic hepatitis D patients, and that HDV-specific IFN- γ responses were more often observed in those with low HDV RNA levels [153]. In this study, moreover, the quality of the HDV-specific T cell response was able to predict the response to IFN- α -based therapy. In another, comparative study on 77 patients with chronic viral hepatitis of different origin, the highest frequencies of CD4⁺ cytotoxic T lymphocytes were found in patients with chronic hepatitis D ($P = 0.04$ vs. HBV and HCV patients) [154]. Even more interestingly, HDV-specific CD8⁺ T cells to peptides expressed by a HDV DNA vaccine administered to mice were also detected in two patients who had eradicated HDV, suggesting that appropriate T cell responses may lead to viral clearance [155].

In spite of these findings, the quantity and quality of the HDV-specific adaptive immune responses occurring in the vast majority of chronic hepatitis D patients seem to be insufficient to contain the

infection. This has prompted some authors to suggest that innate immune responses may play a more important role than previously suggested. The IFN signaling pathway have been shown to be activated *in vitro* [118, 156, 157] and in experimental mice models [158, 159]. For example, in a hepatoma cell line-based cotransfection model, the HDV large antigen was shown to inhibit HBV replication by trans-activating the IFN- α -inducible MxA gene [118]. However, even if the responses of cells expressing HDV RNA and antigen to IFN and dsRNA appear to be intact, IFN- α fails to inhibit HDV *in vitro* [157]. The misfolded HDV ribozyme sequence alone can induce protein kinase R [156], although it is unknown whether this occurs *in vivo*. HDV triggered an antiviral state of human hepatocytes in chimeric mice [158]. However, the nuclear accumulation of STAT1 was most pronounced in human hepatocytes lacking detectable amounts of HDAg, suggesting that intracellular inhibitory mechanisms may occur in the presence of elevated expression of the viral protein. This is in agreement with a previous *in vitro* study, where HDV replication was associated with the inhibition of the cell response to IFN- α , via a blockade of the tyrosine phosphorylation of the JAK kinase Tyk2, thereby impairing the activation and translocation of STAT1 and STAT2 to the hepatocyte nucleus and inhibiting the antiviral effect of IFN- α [160]. A recent work has provided an extensive analysis of natural killer (NK) cells in the peripheral blood of patients with chronic HDV infection [161]. This work compared frequency and phenotype of NK cells before and during treatment with IFN- α , and between persons with HDV or HBV infection, or healthy controls, and showed a distinct natural killer (NK) cell profile in patients responding to treatment. The number of NK cells was increased in chronic hepatitis D patients compared to healthy controls, but the phenotype was less activated. More work is however warranted to fully understand the role of NK cells in HDV pathogenesis.

Interactions between HDV and the cell machinery

HDV RNA and proteins have been shown to interact with several components of the cell machinery [162, 163], although the possible meaning of these interactions has been tested in only a fraction of them. Cell interactants of HDV proteins appear to be numerous. Although most of them are involved in the viral transcription and replication, some may affect also HDV pathogenesis. However, appropriate validation studies in humans are lacking. HDV may increase the cell survival potential. HDV replication increases histone H3 acetylation within the clusterin promoter, leading to an enhanced clusterin expression [164]. It is therefore conceivable that this epigenetic regulation by HDV contribute to the

carcinogenesis associated to HDV-related cirrhosis. Clusterin overexpression has indeed been reported in 89% of human HCC cases [165], but although 73% of clusterin-positive HCCs were HBsAg-positive, no mention was made about HDV coinfection. However, the observations concerning the relationship between HDV and cell proliferation are far from conclusive. HDV has been shown to impair cell proliferation [166], to induce cell cycle arrest [167] or even cell death [147]. On the other hand, the L-HDAg has been shown to activate the serum response factor-associated transcription [168], and may do so in synergism with the HBV X protein [169]. A detailed analysis of the cell proteome changes in human embryonic kidney HEK-293 cells identified 89 proteins (out of ~3000 quantified) differentially expressed in the presence of HDAg or HDV RNA replication [170]. Most of these proteins were associated with cell cycle regulation and pyruvate metabolism. Interestingly, p53 was down regulated, and the G2/M DNA damage checkpoint was among the most affected pathways, hinting at a possible mechanism involved in the promotion of HDV-associated HCC.

HDV may increase intrahepatic inflammation via the NF- κ B signaling. L- but not S-HDAg, activates the TNF- α -induced NF- κ B signaling *in vitro*, probably via a direct interaction with the TNF receptor-associated factor 2 (TRAF2), a protein involved in the early signal transduction events. This effect is independent of the L-HDAg farnesylation [171]. Alternatively, the NF- κ B activation may also proceed via an increased ER stress [172] or the production of reactive oxygen species [173].

Finally, HDV may directly affect fibrogenesis. A single *in vitro* study showed that L-, but not S-HDAg, increased the TGF- β - and the c-Jun-induced signaling activation, in a farnesylation-dependent manner [174]. This activation was carried out in synergism with the HBV X protein. Thus, HDV may also contribute to the progression of liver damage – and, indirectly, to HCC development – via a direct effect on fibrogenesis.

Conclusions

HDV remains a fascinating – and hitherto unique – example of a viral parasite with significant impact in terms of human disease worldwide. It is estimated that there are between 15 and 20 million carriers of HDV around the world [175], and all geographical regions are affected, although to a variable extent. Unfortunately, although HBV vaccination protects HBV-uninfected persons from both HBV and HDV infection, an HDV-specific vaccine aimed at protecting the large population of HBV chronic carriers has yet to be developed. Furthermore, treatment options for HDV carriers are unsatisfactory.

Therefore a better, in-depth knowledge of the HDV life cycle and the virus-host cell interactions is warranted for the development of more effective and well-tolerated antivirals. Although recent approaches targeting HDAg farnesylation or NTCP function at viral entry have been proposed [176, 177], the available results – albeit encouraging – are clearly suboptimal. On the other hand, the identification of NTCP as HBV/HDV receptor [90] should lead to the development human-NTCP transgenic mice susceptible to HDV, if not to HBV, paving the way to new drug testing. Future approaches may target the viral ribozyme, the HDV matrix domain in HBV envelope proteins or even the host immune response. The field of HDV research should also benefit from the recent progress made in growing HDV in tissue culture systems based of NTCP-expressing human hepatoma cell lines [178], and from the development of humanized liver mouse models [158].

Legend to the figures

Figure 1. Schematic representation of the HDV virion. The particle comprises two types of components: i) the viral envelope of HBV origin (in red) , including the HBV envelope proteins S-HBsAg, M-HBsAg and L-HBsAg, and ii) the ribonucleoprotein (RNP) (in blue) that comprises the circular genomic HDV RNA associated to multiple copies of the HDV-encoded S-HDAg and L-HDAg proteins. The diameters of the HDV virion and HDV RNP are indicated in nanometers (nm).

Figure 2. A) The different forms of HDV RNAs in HDV-infected cells. Genomic (g), antigenomic (ag) and messenger (mRNA) HDV RNAs are represented. The position of the tips of the rod-like structures is indicated. The HDAg ORF is indicated (thick blue line); the position of the ribozymes on genomic and antigenomic strands is indicated (closed circle). Arrows on the RNA strands indicate the 5' to 3' direction. B) The two forms of HDAg proteins are depicted. Functional domains such as coiled-coil dimerization domain (CCD), nuclear localization domain (NLS), nuclear export domain (NES), farnesylation signal, and assembly domain (including proline residues in blue) are indicated. The farnesyl group covalently linked to cysteine-211 is represented (thick blue line).

Figure 3. Intracellular replication of HDV RNA. Genomic HDV RNA (g) and messenger RNA (mRNA) are synthesized by cellular RNA polymerase II (Pol-II) in the nucleoplasm. Antigenomic (ag) RNA is synthesized by Pol-II - possibly Pol-I - in the nucleolus. HDV mRNAs are exported to the cytoplasm for synthesis of HDAg proteins. Genomic HDV RNA is also directed to the cytoplasm for assembly of ribonucleoprotein (RNP) by association with HDAg proteins.

Figure 4. Double rolling circle mechanism of HDV RNA in the cell nucleus. A) Cellular RNA polymerases (Pol-II and presumably Pol-I) are in charge of HDV RNA replication: in the infected cell nucleus the genomic HDV RNA (g RNA) is first used as a template for synthesis of HDAg mRNA by the RNA polymerase II (Pol-II), which also synthesizes antigenomic RNA (ag RNA) through a rolling-circle mechanism from genomic template. Greater than monomer linear form of de novo synthesized ag RNA is then cleaved into monomers by the ag ribozyme activity. The linear ag RNA monomer then self ligates to form a circular ag molecule, which in turn serves as a template for synthesis by Pol-II - eventually Pol-I - of g RNA using the same rolling circle mechanism. Genomic and antigenomic

ribozymes are indicated in thick lines. B) Host adenosine deaminase acting on RNA 1 (ADAR1), a key regulator of HDV replication cycle. ADAR1 converts adenosine to inosine by deamination on the antigenomic RNA, leading to the change of UAG amber codon for S-HDAg ORF to UGG tryptophan codon for L-HDAg ORF. ADAR1 controls the switch between early phase of HDV RNA amplification and late phase of virion assembly.

Figure 5. The HBV envelope proteins at the surface of HDV virions. Three determinants within the HBV envelope proteins are essential to the HDV replication cycle: the HDV matrix domain in the C-terminus of the small envelope proteins (S) for assembly of HDV virions (1), the antigenic loop (AGL) in S for interaction with cell surface heparan sulfate proteoglycan (HSPR) chains (2, and the pre-S1 domain (3) of the large envelope protein (L) for binding to the sodium taurocholate cotransporting polypeptide (NTCP) receptor. L and S HBV envelope proteins are the only 2 elements that HDV requires from its helper (in red). M-HBsAg (M) is dispensable. The HDV ribonucleoprotein (RNP) is indicated in blue. The carboxyl-terminus of the large HDAg protein (LHDAg-C-ter), including the farnesyl group inserted in the viral membrane is represented (thick blue line).

Figure 6. Schematic representation of the HBV envelope proteins functions in the HDV replication cycle. Intrinsic HDV functions (genome replication and RNP assembly) are indicated in blue. HBV helper functions (virion assembly, cell exit and entry) are indicated in red. HDV RNA can replicate in hepatocytes and form HDV RNPs without the assistance of HBV - this however leads to an abortive infection (0). The first step of HDV propagation is the RNP egress as HDV virions after envelopment with HBV envelope proteins in a HBV coinfecting cell (1). The HDV RNP is thereby protected and can travel in the extracellular space (2). HDV virions are then targeted to human hepatocytes - the HBV susceptible cells - for a new round of HDV RNA replication (3). HDV-infected hepatocytes are either non-infected with HBV leading to abortive infection (3), or already - or to become - infected with HBV, leading to a productive HDV infection (3 and 1).

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Figure-1

Figure-2

Figure-3

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B

Figure-4

Figure-5

Figure-6