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MINI-REVIEW

Unraveling the Enigma of Hepatitis B Virus cccDNA: Novel Insights and Therapeutic Prospects

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Abstract

Hepatitis B virus (HBV) is a significant global health concern, with its ., genome containing a small, partially double-stranded, relaxed circular (RC) DNA. The conversion of RC DNA to covalently closed circular (ccc) DNA serves as the essential template for all viral RNA transcription, making it a pivotal target for therapeutic intervention. Currently, antiviral drugs such as interferon-α (IFN-α)/pegylated IFN (PEG-IFN) and nucleos(t)ide analogues (NUC) represent the primary treatment options for patients with chronic hepatitis B (CHB). Nevertheless, the persistence of cccDNA poses a formidable challenge for further research and complete viral eradication. Understanding the intricate processes of cccDNA formation and decay in HBV-infected livers has been the focus of extensive investigation, although many mechanisms remain elusive. Both viral and host factors play crucial roles in governing HBV replication and gene expression. Notably, recent research has shed light on host factors influencing HBV cccDNA, opening new avenues for exploration.

Specifically, Interleukin-6 (IL6), transforming growth factor β (TGFβ), heme oxygenase-1 (HO-1), hepatocyte Nuclear Factor 1 (HNF1), and histones (HIS) have emerged as key regulators of HBV cccDNA dynamics. Epigenetic modifications, such as methylation of HBV DNA and ubiquitinylation of HBV proteins, play integral roles in the complex regulation of cccDNA during HBV infection. Looking towards the future, novel therapeutic strategies hold immense promise for combatting HBV. Among these, RNA interference (RNAi) techniques offer exciting potential as antiviral therapeutics, heralding a new era in HBV management.

This comprehensive review explores the enigmatic nature of HBV cccDNA, highlighting recent breakthroughs in understanding the pivotal host factors influencing its persistence. Moreover, we discuss the significance of epigenetic modifications in cccDNA regulation and underscore the potential of RNAi-based interventions as innovative anti-HBV therapeutics. By advancing our knowledge in these areas, we may pave the way for more effective and targeted treatments, ultimately aiming to reduce the global burden of chronic hepatitis B.

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Introduction

The Hepatitis B virus (HBV) possesses a small, partially double-stranded, relaxed circular (RC) DNA genome, which undergoes conversion to covalently closed circular (ccc) DNA. The cccDNA acts as the primary template for viral RNA transcription. Current treatment options for chronic hepatitis B (CHB) patients mainly involve antiviral drugs such as interferon-α (IFN-α)/pegylated IFN (PEG-IFN) and nucleos(t)ide analogues (NUC). However, the persistence of cccDNA remains a significant challenge for further research. Although investigations have been conducted on the formation and decay of cccDNA in HBV-infected liver, many of the underlying mechanisms remain largely unknown. Understanding the regulation of cccDNA could pave the way for the ultimate resolution of HBV infection in patients.

Significance of HBV cccDNA in clinical samples

Studies have shown varying implications of HBV cccDNA in clinical samples. Takkenberg et al. observed higher plasma cccDNA levels in HBeAg positive samples compared to HBeAg negative samples, independent of total HBV DNA levels and HBV genotype. Additionally, levels of alanine aminotransferase (ALT) and hepatic activity index (HAI)-score did not correlate with plasma cccDNA levels, indicating that increased hepatocyte degeneration may not be the primary cause. Coffin et al. detected HBV cccDNA in all liver samples, which is expected since HBV replicates in hepatocytes. However, the presence of cccDNA in circulation or peripheral blood mononuclear cells (PBMCs) remains unclear. Mazet-Wagner et al. developed a real-time PCR quantitation method for cccDNA in PBMCs, revealing that less than 1% of HBV DNA existed in the cccDNA form in the serum and PBMCs. Cabrerizo et al. detected HBV cccDNA in 47% of PBMC samples from CHB patients. Attempts to detect cccDNA after incubating PBMCs with HBV were unsuccessful, suggesting that previous observations could be attributed to adsorbed virus. The decline of liver cccDNA is used as a marker of response to antiviral therapy. Zhou et al. demonstrated the dependence of HBeAg production on cccDNA in HepAD38 cell cultures, indicating its potential as a surrogate marker for cccDNA in antiviral screening assays. Yasunaka et al. found a linear correlation between pre-orthotopic liver transplantation (OLT) serum HBV DNA and HBcrAg levels with post-OLT cccDNA levels. High levels of serum HBV DNA and HBcrAg at the time of OLT were associated with elevated post-OLT cccDNA levels. Faria et al. detected cccDNA more frequently in hepatocellular carcinoma (HCC), suggesting that HBV replication in tumor cells may contribute to HBV recurrence post-OLT. However, Hussain et al. found that the presence of intrahepatic cccDNA is not predictive of HBV recurrence after liver transplantation. Hosaka et al. discovered that HBcrAg is a predictor of post-treatment HCC recurrence during antiviral therapy, emphasizing the importance of suppressing serum HBcrAg and intrahepatic cccDNA through NUC treatment to prevent recurrence. Wong et al. demonstrated a positive correlation between HBcrAg concentration and cccDNA levels. Additionally, Chen et al. identified the presence of cccDNA in renal tissues of patients with HBV-related glomerulonephritis, indicating its involvement in the disease. Ceccarelli et al. emphasized the predictive value of cccDNA in HBV reactivation.

Viral and Host Factors Regulating Formation of HBV cccDNA

1. Viral Factors Regulating Formation of HBV cccDNA

The regulation of cccDNA formation in HBV involves various viral factors. Gao et al. investigated these factors by analyzing transient and stable DNA transfections of duck hepatitis B virus (DHBV) (Gao et al., 2007). They found that defects in the viral envelope protein led to increased cccDNA formation, particularly in the form of partially double-stranded relaxed circular DNA (PF-RC), suggesting a negative regulatory role of the envelope protein(s) in the early stages of cccDNA formation prior to deproteination. Lentz et al. demonstrated that the absence of envelope protein expression resulted in elevated cccDNA levels, while restoration of envelope protein expression reduced cccDNA levels, indicating the involvement of the envelope protein in cccDNA regulation (Lentz et al., 2011). They further showed that the presence of the L protein, alone or in combination with M and/or S proteins, decreased cccDNA levels, suggesting a role for the L protein in cccDNA regulation. Coexpression of L and M proteins had a greater regulatory effect compared to L alone or L and S together. Coexpression of all three envelope proteins limited the completion of plus-strand DNA synthesis, and this effect was correlated with protein levels and virion secretion.

HBV core protein (HBcAg) is a key component of the cccDNA minichromosome and also influences cccDNA formation. Guo et al. demonstrated that HBcAg binds to the CpG islands of cccDNA, promoting an epigenetically permissive state (Guo et al., 2011). Analysis of liver biopsy samples from CHB patients revealed that HBcAg preferentially binds to CpG island 2, an important region for HBV transcription regulation. The abundance of HBcAg binding to CpG island 2 positively correlated with the ratios of relaxed

circular (rc) DNA to cccDNA. Interestingly, HBcAg binding to CpG island 2 was associated with the binding of CREB binding protein (CBP) and hypomethylation in CpG island 2 of cccDNA minichromosomes. However, higher levels of HBcAg binding to CpG island 2 were accompanied by lower levels of HDAC1 binding. Multivariate analysis identified the abundances of HBcAg binding to CpG island 2 of cccDNA and positive HBeAg as independent factors associated with HBV replication. These findings indicate that HBcAg acts as a positive regulator of HBV transcription and replication, maintaining an epigenetically permissive state in the critical region of cccDNA minichromosomes.

Chou et al. investigated the transcriptional activity of cccDNA and the role of the HBxAg protein in transcriptional regulation using a modified 1.3-fold HBV genome (Chou et al., 2005). Their findings revealed that the HBxAg protein significantly regulated the transcriptional activity of cccDNA. Lucifora et al. observed that HBxAg-deficient HBV particles (HBV(x-)) did not result in productive HBV infection, in contrast to wild-type HBV particles (HBV(wt)) (Lucifora et al., 2008). Although nuclear cccDNA uptake and import were comparable between HBV(x-) and HBV(wt), active transcription was only observed from HBV(wt) genomes. Trans-complementation of HBxAg rescued transcription from the HBV(x-) genome and led to antigen and virion secretion, even weeks after infection. Continuous expression of HBxAg was necessary for HBV antigen expression and replication. HBxAg was not packaged into virions during assembly but was expressed within the new host cell after infection to exert epigenetic control over HBV transcription from cccDNA. These findings demonstrate that HBxAg is required for initiating and maintaining HBV replication and highlight its role as a key regulator during the natural infection process.

2. Host Factors Regulating Formation of HBV cccDNA

2.1 Interleukin-6

Interleukin-6 (IL-6), a pleiotropic cytokine, plays a crucial role in regulating biological responses in various target cells, including hepatocytes. Elevated serum levels of IL-6 have been reported in patients with CHB, liver cirrhosis, and HCC, making it a significant marker of HBV-related clinical progression. Kuo et al. demonstrated that IL-6 effectively suppressed HBV replication and prevented cccDNA accumulation in a human hepatoma cell line (Kuo et al., 2009). The suppression of HBV replication by IL-6 was associated with a moderate reduction in viral transcripts/core proteins and a marked decrease in viral genome-containing nucleocapsids. The stability of existing viral capsids indicated that IL-6 prevented the formation of genome-containing nucleocapsids, similar to the effect of IFNs. However, IFN-α/β and IFN-γ did not participate in the IL-6-induced suppression of HBV replication.

2.2 Epidermal Growth Factor

Schorr et al. observed that epidermal growth factor (EGF)-induced cell proliferation was associated with a slight increase in cccDNA synthesis and a decrease in viral transcription (Schorr et al., 2006). Conversely, transforming growth factor-beta (TGF-β) blocked cell cycle progression, diminished cccDNA synthesis, but increased viral transcription.

2.3 Heme Oxygenase-1

Heme oxygenase-1 (HO-1) induction has been shown to be beneficial in immune-mediated liver damage. Protzer et al. investigated the effects of HO-1 induction in HBV infection models (Protzer et al., 2007). In an acute hepatitis B model, HO-1 induction significantly reduced liver injury. Furthermore, HO-1 exhibited a pronounced antiviral effect, as demonstrated in hepatoma cells stably transfected with HBV and transgenic mice with persistent HBV replication. HO-1 induction directly repressed HBV replication in hepatocytes at a posttranscriptional step by reducing the stability of HBcAg, thereby inhibiting the replenishment of nuclear cccDNA. Small interfering RNA targeting HO-1 confirmed that this effect depended on the expression level of HO-1. In addition to its hepatoprotective effect, HO-1 showed a potent antiviral activity against HBV infection. Therefore, the induction of HO-1 may represent a novel therapeutic option for inflammatory flares of hepatitis B.

2.4 Histones

Nuclear cccDNA accumulates in hepatocyte nuclei as a stable minichromosome organized by histone and non-histone viral and cellular proteins. Levrero et al. emphasized the importance of studying the molecular mechanisms regulating cccDNA stability and its transcriptional activity at the RNA, DNA, and epigenetic levels to identify potential therapeutic targets for depleting the cccDNA reservoir (Levrero et al., 2009).

Belloni et al. demonstrated that cellular histone acetyltransferases CBP, p300, and PCAF/GCN5, as well as histone deacetylases HDAC1 and hSirt1, are recruited in vivo to cccDNA (Belloni et al., 2009). In cells replicating the HBxAg mutant, the recruitment of p300 was impaired, and cccDNA-bound histones were rapidly hypoacetylated, while the recruitment of hSirt1 and HDAC1 was increased and occurred earlier. These findings support the existence of a complex network of epigenetic events influencing cccDNA function and HBV replication, and they identify an epigenetic mechanism by which HBxAg controls HBV replication (preventing cccDNA deacetylation).

Gong et al. investigated the remodeling of HBV minichromosomes and the post-translational modifications of histone proteins during HBV replication (Gong et al., 2011). They found that cccDNA-bound H3 histones were phosphorylated, mono-methylated, and acetylated in HepG2 cells with replicating HBV.

The acetylation and methylation status of cccDNA-bound H3 histones paralleled HBV replication levels. These modifications were associated with the extent of minichromosome remodeling and represented potential targets for inhibiting HBV replication in the development of novel antiviral agents.

Belloni et al. demonstrated that interferon-alpha (IFN-α) repressed HBV replication by reducing the transcription of pregenomic RNA (pgRNA) and subgenomic RNA from cccDNA minichromosomes (Belloni et al., 2012). IFN-α treatment resulted in hypoacetylation of cccDNA-bound histones and the recruitment of transcriptional corepressors to cccDNA. The binding of STAT1 and STAT2 transcription factors to active cccDNA was also reduced by IFN-α. The inhibitory effect of IFN-α on HBV replication was linked to the IFN-stimulated response element (IRSE), as IRSE-mutant HBV exhibited reduced pgRNA transcription and was not repressed by IFN-α treatment. These findings elucidate a molecular mechanism by which IFN-α mediates the epigenetic repression of cccDNA transcriptional activity, providing insights for the development of effective therapeutics.

Pollicino et al. employed anti-acetyl-H4/-H3 antibodies to investigate the regulation of cccDNA stability and transcriptional activity by histone acetylation (Pollicino et al., 2011). In vitro and ex vivo analysis revealed that HBV replication is regulated by the acetylation status of cccDNA-bound H3/H4 histones. Inhibitors of class I histone deacetylases induced increased acetylation of cccDNA-bound H4 and enhanced HBV replication. Histone hypoacetylation and recruitment of histone deacetylase 1 onto cccDNA were observed in liver tissues with low HBV viremia in patients with hepatitis B. The development of a chromatin immunoprecipitation (ChIP)-based assay enabled the analysis of cccDNA minichromosome transcriptional regulation both in vitro and ex vivo. These findings shed light on the regulation of HBV replication and identify the enzymatic activities involved in histone acetylation of cccDNA-bound histones as potential therapeutic targets for anti-HBV drugs.

2.5 Hepatocyte Nuclear Factor 1

The role of hepatocyte nuclear factor 1α (HNF1α) in the regulation of HBV transcription and replication in vivo was investigated using an HNF1α-null HBV transgenic mouse model. While HBV transcription was not significantly affected by the absence of HNF1α, the intracellular accumulation of viral replication intermediates was increased in mice lacking functional HNF1α protein. This increase in encapsidated cytoplasmic replication intermediates was associated with the appearance of nonencapsidated nuclear cccDNA. In contrast, HNF1α-expressing HBV transgenic mice did not readily exhibit detectable viral cccDNA. Raney et al. suggested that the synthesis of nuclear cccDNA, the proposed transcriptional template found in natural infection, is regulated either by subtle alterations in viral transcript levels or by changes in the physiological state of hepatocytes in this in vivo model of HBV replication (Raney et al., 2001).

Methylation and Regulation of HBV cccDNA

CpG islands within HBV DNA have been found to undergo methylation in human tissues, indicating a potential role for methylation in regulating viral protein production. Sa-Nguanmoo et al. demonstrated that transfection of methylated HBV DNA resulted in reduced HBV mRNA levels, decreased expression of surface and core proteins, and reduced secretion of HBV viral proteins in HepG2 cells (Sa-Nguanmoo et al., 2011). These findings provide direct evidence of CpG island regulation of HBV gene transcription. Moreover, methylated cccDNA was detected in both tumor and nonneoplastic liver tissues. In vitro experiments with an equivalent of cccDNA showed that DNA methylation decreased viral protein production in HepG2 cells.

Guo et al. investigated the methylation status of CpG island 2 within intrahepatic cccDNA and its correlation with HBV replication capability (Guo et al., 2007). cccDNA was extracted from liver biopsies of CHB patients and analyzed for methylation status and quantity. Methylation of the two Hpa II recognition sequences (CCpGG) within CpG island 2 was observed in infected liver tissues. The positive ratios of cccDNA methylation were significantly higher in HBeAg-negative patients compared to HBeAg-positive patients. The percentage of methylated cccDNA relative to total cccDNA was also significantly higher in HBeAg-negative samples. Ratios of rcDNA to cccDNA molecules indicated that cccDNA methylation correlated with impaired virion productivity in HBeAg-positive individuals. Bisulfite DNA sequencing revealed higher methylation density in HBeAg-negative patients compared to HBeAg-positive patients, suggesting a potential association between cccDNA methylation and HBV replication capability.

Kim et al. found variable degrees of CpG methylation in cccDNA from patients with HBV-related liver cirrhosis (Kim et al., 2009). Increased cccDNA methylation was significantly associated with older age, lower serum HBV DNA levels, and reduced virion productivity. HepAD38 cells with higher cccDNA methylation levels also exhibited lower virion productivity. In vitro transcription assays demonstrated that increased cccDNA methylation suppressed the transcriptional activity of cccDNA.

Guo et al. examined the binding of HBcAg to cccDNA and its impact on the cccDNA epigenetic profile in liver biopsy samples from CHB patients (Guo et al., 2010). HBcAg preferentially bound to CpG island 2 of cccDNA, a crucial region for HBV

transcription regulation. The relative abundances of HBcAg binding to CpG island 2 positively correlated with the ratios of relaxed circular DNA to cccDNA and the levels of serum HBV DNA. Interestingly, the relative abundances of HBcAg binding to CpG island 2 were associated with the binding of CBP and hypomethylation in CpG island 2 of cccDNA minichromosomes. However, higher amounts of HBcAg binding to CpG island 2 were accompanied by lower amounts of HDAC1 binding. Multivariate analysis revealed that the abundances of HBcAg binding to CpG island 2 of cccDNA and the presence of HBeAg were independent factors associated with HBV replication. These findings indicate that HBcAg acts as a positive regulator of HBV transcription and replication, maintaining a permissive epigenetic state in the critical region of cccDNA minichromosomes.

RNA Interference and Regulation of HBV cccDNA

HBV replication involves RNA intermediates that can be targeted by RNA interference (RNAi), offering a potential anti-HBV strategy. Li et al. (2007) demonstrated that siRNA1 effectively inhibited cccDNA replication by targeting the nuclear localization signal (NLS). Targeting the HBV NLS through RNAi could be a novel approach to combat HBV infection, enhancing the efficacy of anti-HBV therapies and overcoming current limitations.

Kim et al. (2009) utilized lentiviral vectors to express two shRNA sequences, sh1580 and sh1685, targeting the HBV protein HBxAg. These shRNAs were efficiently delivered to HepAD38 cells using lentiviral vectors, resulting in a significant reduction in total intracellular HBV DNA content and suppression of HBV cccDNA. Lentivirus-mediated delivery of shRNAs against HBxAg effectively suppressed HBV replication and reduced cccDNA levels in cell culture systems.

Panjaworayan et al. (2010) conducted computational analysis of the PRE sequence and identified conserved regulatory elements that are predicted to form secondary structures, some of which are located within known regulatory regions. Deletion analysis revealed that different sub-elements of the PRE have distinct effects on reporter activity, suggesting the presence of multiple regulatory elements within the PRE. Predicted conserved siRNA targets at nucleotide positions nt 1317-1337 and nt 1329-1349 were identified. While the siRNA targeting nt 1329-1349 had no effect on reporter gene expression, the siRNA targeting nt 1317-1337 significantly decreased reporter protein expression. This siRNA also specifically reduced the level of cccDNA in transiently HBV-infected cells. These findings suggest that the HBV PRE likely contains multiple regulatory elements, with the conserved target at nt 1317-1337 being an effective siRNA target.

To simulate chronic HBV infection, Starkey et al. (2009) initiated HBV replication in HepG2 cells through transduction with HBV baculovirus. Subculturing of HBV-expressing HepG2 cells post-transduction established a system with ongoing HBV replication, where HBV expression primarily originated from cccDNA. This system served as a model for studying chronic HBV infection. HepG2 cells were transduced with baculovirus expressing short hairpin RNA (shRNA) either prior to initiating HBV replication or during chronic HBV replication. The levels of HBV RNA, HBsAg, replicative intermediates (RI), extracellular (EC) DNA, and cccDNA species were measured. Treatment with shRNA prior to or during chronic infection significantly reduced HBsAg, HBV RNA, and DNA levels. However, the effect on cccDNA species was observed only when shRNA was administered before initiation of infection. These results indicate that RNAi may have therapeutic potential for controlling HBV replication at the level of RI and EC DNA, as well as reducing cccDNA establishment during HBV infection. These findings support previous research demonstrating the stability of cccDNA following antiviral therapy. Additionally, this study introduced a novel HBV baculovirus subculture system for evaluating antiviral effects on chronic HBV replication.

Antivirals and Inhibition of HBV cccDNA

1. Inhibition of HBV cccDNA Formation by Interferon α and Pegylated Interferon

Both conventional IFNα and PEG-IFN play crucial roles in the treatment of chronic hepatitis B (CHB). A study by Tangkijvanich et al. (2010) evaluated the virological response to PEG-IFN therapy. The responders showed significantly lower levels of baseline HBsAg, HBeAg, cccDNA, and intrahepatic HBV DNA compared to non-responders. Baseline and reduced levels of HBsAg and HBeAg were strongly correlated with cccDNA. Another study by Sung et al. (2005) also identified cccDNA as a reliable predictor of virological response to PEG-IFN. At the end of treatment, HBV DNA levels showed a strong correlation with intrahepatic cccDNA and total HBV DNA levels. Patients with sustained virologic response had significantly lower levels of intrahepatic cccDNA and total DNA. Intrahepatic cccDNA and total HBV DNA levels at the end of therapy proved to be better indicators of sustained virologic response than serum HBV DNA. Similarly, Takkenberg et al. (2011) demonstrated that intrahepatic cccDNA and total intrahepatic HBV DNA were predictive of sustained virologic response in HBeAg positive patients at the end of treatment. Additionally, Wursthorn et al. (2006) utilized PEG-IFN and ADV for the treatment of HBsAg-positive CHB patients. Combination therapy resulted

in significant reductions in serum HBV DNA and intrahepatic cccDNA, with a strong correlation between cccDNA reduction and decreased HBsAg levels.

2. Inhibition of HBV cccDNA Formation by Nucleos(t)ide Analogs

Nucleos(t)ide analogs (NUCs) also play a crucial role in the treatment of CHB. While HBV DNA, HBeAg seroconversion, and HBsAg loss are commonly used as surrogate markers to evaluate antiviral efficacy, the impact of NUCs on cccDNA levels remains controversial. Future drug development efforts should focus on the eradication of cccDNA from liver tissue.

2.1 Lamivudine

Chong et al. (2011) conducted a study using a stably HBV-producing cell line, 1.3ES2, to examine the dynamic changes of cccDNA during different cellular growth stages. The results showed a sudden increase in cccDNA during the initial proliferation phase of cell growth, possibly due to its replenishment from intracellular nucleocapsids. Subsequently, cccDNA decreased dramatically during the cells' exponential proliferation, similar to the loss of extrachromosomal plasmid DNA during cell division. However, it gradually accumulated as the host cells reached confluency. The study also found that cccDNA was reduced in dividing cells and could be eliminated with long-term treatment of lamivudine (LAM). Yuen et al. (2005) tested the effect of LAM therapy on serum cccDNA levels in CHB patients and observed a significant reduction in cccDNA levels at week 24 and week 52 compared to baseline. Patients with YMDD mutations had a lesser reduction in cccDNA levels compared to those without mutations. However, Soemohardjo et al. (2003) argued that LAM does not affect the cccDNA of infected hepatocytes; it only inhibits the formation of new viruses.

2.2 Adefovir Dipivoxil

Delmas et al. (2002) investigated the ability of ADV to suppress viral cccDNA both in vitro and in vivo using the DHBV model. They observed dose-dependent inhibition of virion release in culture supernatants and synthesis of intracellular viral DNA. While ADV inhibited cccDNA amplification, it did not eliminate cccDNA or prevent the formation of cccDNA from incoming viral genomes. Werle-Lapostolle et al. (2004) demonstrated that 48 weeks of ADV therapy resulted in a significant decrease in cccDNA levels. Changes in cccDNA correlated with a similar reduction in serum HBsAg levels but not with a decrease in the number of HBV antigen-positive cells. Additionally, Cheng et al. (2011) compared cccDNA reduction with liver histology in NUC-naïve chronic hepatitis B patients receiving oral ADV monotherapy. They found only slight reductions in intrahepatic cccDNA levels, which were not statistically different from the placebo group. The level of intrahepatic cccDNA correlated with necroinflammation activity and fibrosis severity before treatment, and treatment-induced cccDNA reduction was associated with improved necroinflammation and fibrosis. However, one year of ADV treatment was insufficient for cccDNA eradication.

2.3 Entecavir

Entecavir (ETV), a nucleoside analogue, has been shown to reduce cccDNA to undetectable levels in woodchucks chronically infected with hepatitis virus. Wong et al. (2006) assessed the effect of ETV therapy on intrahepatic total HBV DNA and cccDNA levels. Entecavir reduced serum viral load, intrahepatic total HBV DNA, and cccDNA levels. Residual HBV DNA could still be detected after the resolution of acute HBV infection. A study on DHBV-infected ducks indicated that approximately 80% of residual DHBV DNA in the liver exists in cccDNA form, suggesting suppression of viral DNA synthesis. Furthermore, Reaiche et al. (2010) investigated whether the maintenance of residual DHBV cccDNA depends on ongoing viral DNA synthesis. Ducks that recovered from acute DHBV infection were treated with ETV or placebo. No major differences were observed in the stability or levels of residual cccDNA in liver biopsy tissues taken 95 days apart from ETV-treated and placebo control ducks. These findings suggest that residual DHBV cccDNA is highly stable and present in a cell population with a turnover rate similar to normal uninfected hepatocytes.

2.4 Clevudine

Anderson et al. (2009) demonstrated that clevudine (CLV) stands out from other oral agents due to its sustained suppression of HBV DNA for several months after therapy cessation. CLV also showed significant reduction of cccDNA in animal models. In treatment-naïve patients, a significant proportion achieved undetectable HBV DNA after 24 weeks of CLV treatment. However, Seignères et al. (2003) evaluated the antiviral activity of CLV in the DHBV model and failed to demonstrate a reduction in cccDNA. Although CLV effectively suppressed viremia and viral replication during a 4-week administration, viral clearance from the liver was not observed, suggesting that the antiviral effect of CLV was insufficient for cccDNA suppression and HBV eradication from infected cells.

New Agents Targeting HBV cccDNA Formation

Quintero et al. (2011) conducted a study to identify natural products capable of inhibiting HBV replication. They investigated hydro-alcoholic extracts from 31 species found in the Venezuelan Amazon rainforest using the HBV-producing cell line HepG2 2.2.15. Several species, including Euterpe

precatoria, Jacaranda copaia, Jacaranda obtusifolia, Senna silvestris, Warscewiczia coccinea, and Vochysia glaberrima, exhibited varying degrees of inhibition on HBV replication. Notably, the leaves of W. coccinea demonstrated significant antiviral activity, achieving 80% inhibition at 100 μg/ml extract concentration. This extract also exhibited inhibitory effects on cccDNA production. Fractionation experiments revealed that the ethanol fraction was primarily responsible for the observed antiviral activity. The study concluded that the extract from Warscewiczia coccinea showed promising inhibition of HBV replication, suggesting the presence of an antiviral compound that could be isolated through bioassay-guided purification.

Mycophenolate mofetil (MMF), the morpholinoethyl ester of mycophenolic acid (MPA), is an immunosuppressive agent used in kidney transplant recipients. MPA, the active compound produced upon hydrolysis of MMF, acts as a potent inhibitor of inosine monophosphate dehydrogenase (IMP-DH), depleting intracellular GTP and dGTP pools. Gong et al. (1999) investigated the inhibitory effect of MPA on HBV replication using primary human hepatocytes and HBV-transfected HepG2 2.2.15 cells. In vitro infection with HBV resulted in detectable cccDNA and HBV mRNA levels in human hepatocytes during the 10-day post-infection period, along with secretion of HBV DNA and HBsAg into the culture medium. However, the presence of 10μg/ml MPA in the culture medium, equivalent to therapeutic serum levels of MPA as an immunosuppressive agent, led to the disappearance of cccDNA and HBV mRNA within 5 days of treatment initiation. The secretion of HBV DNA and HBsAg into the medium was significantly reduced as well. No cytotoxic effects of MPA were observed during the experiments. The inhibitory effect of MPA on HBV replication was abolished in the presence of guanosine (50μg/ml). In HepG2 2.2.15 cells containing an integrated tandem dimer of the HBV genome, MPA treatment did not significantly inhibit the secretion of HBV DNA and HBsAg into the culture medium, nor did it affect HBV cccDNA and mRNA levels. These findings suggest that the observed effect of MPA on HBV replication in primary human hepatocytes may involve only episomal replication and may have clinical implications, particularly prior to the integration of HBV DNA into the host genome.

Nucleoside analogs can reduce virus production by inhibiting the viral polymerase; however, complete clearance is uncommon due to the persistence of the HBV episome. HBV DNA exists in the nucleus in the form of cccDNA, driving viral transcription and progeny virus production. cccDNA is not a direct target of antiviral nucleoside analogs and serves as the source of HBV reemergence when antiviral therapy is discontinued. To specifically target cccDNA, Zimmerman et al. (2008) designed six different zinc finger proteins (ZFPs) capable of binding to DNA sequences in the DHBV enhancer region. The binding kinetics of these ZFPs were assessed using electrophoretic mobility shift assays and surface plasmon resonance. Two candidates were selected for further investigation. The ZFPs were cloned into a eukaryotic expression vector and cotransfected with the plasmid pDHBV1.3, which replicates the DHBV life cycle, into longhorn male hepatoma cells. In the presence of each ZFP, viral RNA levels were significantly reduced, and protein levels were dramatically decreased. Consequently, intracellular viral particle production was also significantly diminished. In summary, the designed ZFPs successfully bound to the DHBV enhancer and disrupted viral transcription, resulting in decreased production of viral products and progeny virus genomes.

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