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Hong Tang *Editor*

Hepatitis B Virus Infection

Molecular Virology to Antiviral Drugs

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Preface

Hepatitis B virus (HBV) is a DNA virus, belonging to the *Hepadnaviridae* family. More than 50 years ago, the serendipitous discovery of “Australian antigen” as the HBsAg of HBV by Dr. Baruch S. Blumberg paved the way to the development of diagnosis, prevention, and treatment of HBV infection. HBV infection remains a global public health problem. The WHO estimates that globally, two billion people have been infected with HBV. In 2015, an estimated 257 million persons, or 3.5% of the world population, were living with chronic HBV infection. Uncontrolled chronic HBV infection can progress to life-threatening end-stage chronic liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC). In recent years, tremendous advances in the field of HBV basic and clinical research have been achieved. The deeper understanding of the key steps in the viral life cycle, the key regulators in the HBV transcription and replication, and the mechanisms of immunopathogenesis contribute greatly to the better clinical management of HBV infection. Current antiviral treatment with nucleos(t)ide analogs (NAs) or/and interferon enables full suppression of serum HBV DNA to undetectable levels in the majority of patients, thus leading to the improvement of liver function and the decreased incidence of cirrhosis and HCC. However, due to the difficulty in clearing the covalently closed circular DNA (cccDNA, the template for HBV transcription and replication), current anti-HBV agents and strategies can only achieve a clinical HBV cure in few patients. Thus, HBV infection remains a vital topic, and the advances in HBV basic and clinical research are crucial for achieving the ultimate goal of virological cure of HBV.

To review the achieved advances in the field of HBV study, we invite the world-leading experts in their respective fields to compile this book titled *Hepatitis B Virus Infection: From Molecular Virology to the Development of Antiviral Drugs*. This book aims to provide a state-of-the-art review of the current understanding of HBV genome and life cycle, the regulation of HBV transcription and replication, and the immunopathogenesis of HBV. It also provides an update of the cell and animal models employed in HBV basic and preclinical research. Those advances are the driving forces for the identification of new viral and host markers for guiding the clinical practice, as well as the identification of new therapeutic targets for the

development of novel antiviral agents. The last chapter of this book discusses the clinical management of chronic hepatitis B covering the standard and optimization antiviral therapies in treatment-naïve and treatment-experienced patients, as well as in the special populations. The up-to-date advances in the development of new anti-HBV drugs are also discussed. Future treatment option for achieving an HBV cure may be a combination of the current antiviral drugs and the newly developed antiviral agents targeting the different steps of the viral life cycle or the newly developed agents modulating the host immune responses.

I believe the content of this book provides a timely and comprehensive reference for medical students and young researches, as well as for experienced clinicians and researchers working in the field of HBV study. Hopefully, this book may also serve as a valuable source to enlighten the creative minds to develop new strategies or novel antiviral drugs to cure HBV infection in the foreseeable future. Finally, I would like to express my sincere appreciation to all the coauthors who provided great efforts and contributions to this book.

Chengdu, Sichuan, China

Hong Tang

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Chapter 1

Hepatitis B Virus Infection: Overview



Hong Li, Libo Yan, Ying Shi, Duoduo Lv, Jin Shang, Lang Bai,
and Hong Tang

Abstract Hepatitis B virus (HBV) is a DNA virus, belonging to the *Hepadnaviridae* family. It is a partially double-stranded DNA virus with a small viral genome (3.2 kb). Chronic HBV infection remains a global public health problem. If left untreated, chronic HBV infection can progress to end-stage liver disease, such as liver cirrhosis and hepatocellular carcinoma (HCC). In recent years, tremendous advances in the field of HBV basic and clinical research have been achieved, ranging from the HBV biological characteristics, immunopathogenesis, and animal models to the development of new therapeutic strategies and new drugs against HBV. In this overview, we begin with a brief history of HBV discovery and treatment milestones. We then briefly summarize the HBV research advances, which will be detailed in the following chapters.

1 Discovery of HBV

As early as the 1950s, clinicians observed the hepatitis that occurred after blood transfusion and proposed the concept of serum hepatitis [1]. In 1964, Blumberg and Alter collected blood samples from all over the world for the study of lipoprotein polymorphisms and serendipitously observed an unusual reaction between serum from a transfused hemophilic patient and an Australian aborigine, and the new antigen was designated as the “Australian antigen” [2, 3]. By 1967, accumulating evidences from Blumberg’s group and other research groups showed a strong correlation between the presence of the Australian antigen and serum hepatitis [4–8]. In 1970, David Dane used electron microscope to inspect the isolated virus particles from serum of patients with Australian antigen-associated hepatitis and demonstrated the famous “Dane particles” as the complete virus (42 nm in diameter) [9]. The outer

Hong Li and Libo Yan contributed equally to this chapter.

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surface protein of the Dane particle is the Australian antigen, which was later officially named by the World Health Organization (WHO) as the HBV surface antigen (HBsAg). In 1976, Blumberg was awarded the Nobel Prize in Physiology or Medicine for his scientific achievement in the discovery of HBV and his visionary concept in developing the first generation of plasma-derived HBsAg vaccine. In 1979, Galibert completed the whole genome sequence of HBV and demonstrated it as a partially double-stranded DNA virus with approximately 3200 base pairs of nucleotides in length [10], thus setting the stage for the second generation of HBV vaccine made from genetic engineering.

The development of HBV immunization began in 1971 by using HBV immunoglobulins to prevent HBV infection. In 1978, the efficacy of the first generation of HBV vaccine consisting of HBsAg particles made from the plasma of HBV carriers was successfully validated in clinical trials [11]. In 1979–1982, using modern molecular cloning methods, William Rutter cloned the HBsAg gene in *Escherichia coli* (*E. coli*) to express the HBsAg protein [12], thus opening the door for recombinant HBV vaccine [13]. Subsequently, the HBsAg gene was successfully transferred to yeast for the mass production of HBsAg as HBV vaccine [14]. In 1984, the yeast-expressed HBsAg vaccine prepared by Merck company was used to vaccinate chimpanzees, demonstrating that the vaccinated chimpanzees were totally protected from intravenous HBV challenge of human serum source [15]. Subsequently, the yeast-expressed HBsAg vaccine was approved by the US Food and Drug Administration (FDA) in 1986. In 1989, Dr. Roy Vagelos, on the behalf of Merck, provided all the technology needed to manufacture HBV vaccine to China with the largest burden of HBV infection. In 1991, HBV vaccine was incorporated into the neonatal immunization program by WHO, which significantly reduced the global HBV infection rate. Since 1992, HBV vaccination has been incorporated into the national routine immunization program by the Chinese government, which led to a markedly decline of HBV infection in China.

HBsAg is the most important marker for the diagnosis of HBV infection. Since the discovery of the “Australian antigen” by Blumberg, a variety of HBsAg qualitative detection methods have been developed, from the detection of HBsAg by immunoelectrophoresis in the 1960s to the enzyme-linked immunosorbent assay in the 1980s. Quantitative detection of HBsAg was introduced as early as in the 1990s, but fully automated and high-throughput quantitative assays have only been recently available. Serum HBsAg quantification not only serves as a useful test in clinical practice to define the specific immunological conditions of the single HBV carrier during the dynamic natural history of HBV infection but also a prediction marker of virological response to antiviral therapy and long-term prognosis [16, 17]. In addition to HBsAg, other commonly used HBV serum markers include anti-HBs, HBeAg and anti-HBe, and anti-HBc. HBV nucleic acid detection is mainly to detect HBV DNA level. HBV DNA testing has also undergone a process from qualitative to quantitative testing. The most widely used HBV DNA quantification method is the real-time polymerase chain reaction (PCR) method. Serum HBV DNA levels reflect the viral replication, and monitoring HBV DNA levels during treatment allows evaluation of antiviral therapy effect.

In the 1990s, interferon- α was approved by the US FDA for the treatment of HBV infection, marking the treatment of HBV infection into the era of antiviral therapy [18]. In the late 1990s, lamivudine became the first nucleos(t)ide analog (NA) approved for anti-HBV treatment, representing the treatment of HBV infection into the era of NAs [19]. At the beginning of the twenty-first century, the long-acting pegylated IFN (Peg-IFN) and the potent and low-resistant NAs entecavir (ETV) and tenofovir disoproxil fumarate (TDF) were approved for HBV treatment [18]. Long-term antiviral treatment with full suppression of serum HBV DNA to undetectable levels not only halts hepatic inflammation but also decreases the incidence of liver cirrhosis and HCC.

2 Epidemiology and Natural History of HBV Infection

HBV infection remains the most common chronic viral infection in the world. WHO estimates that globally, 2 billion people have been infected with HBV. In 2015, an estimated 257 million persons, or 3.5% of the world population, were living with chronic HBV infection [20]. About 887,000 people die each year from HBV-related liver disease with approximately half coming from China. In 2006, the epidemiology serosurvey of HBV in China revealed that the prevalence of HBsAg for population aged 1–59 years was 7.18% [21, 22]. Based on this calculation, there were about 93 million HBsAg carriers in China, of which approximately 20 million are chronic hepatitis B (CHB) patients [23]. The latest epidemiological data showed that the estimated national HBsAg prevalence in the general population was 6.1% in 2016 [24].

The natural history of HBV infection depends on the interaction among the virus replication and evolution, the host immune response, and the environment factors. The age at which HBV is infected is the most important factor affecting chronicity. The risks of progression from acute to chronic HBV infection are approximately 95% if the infection occurs during the perinatal period, 20%–30% in children aged 1–5 years, and less than 5% in adults. The natural history of chronic HBV infection can be characterized into four phases: (1) immune-tolerant phase, (2) HBeAg-positive immune-active phase, (3) inactive CHB phase, and (4) HBeAg-negative immune reactivation phase [25, 26]. In the immune-tolerant phase, the HBV-infected subjects are characterized by high levels of HBV DNA, positive HBeAg, but normal ALT levels and the absence of significant inflammation or fibrosis in the liver. HBV-infected subjects in this phase can be defined as HBV carriers. In the HBeAg-positive immune-active phase, the HBV-infected patients are characterized by elevated ALT and HBV DNA levels together with liver injury by liver histology, and therefore, those patients are defined as HBeAg-positive CHB patients [27]. The transition from the immune-active phase to the immune-inactive phase is reflected by the seroconversion from HBeAg to antibody to HBeAg (anti-HBe), the normal ALT levels, and the low or undetectable HBV DNA levels. However, around 10–30% of those patients who have HBeAg seroconversion continue to have

elevated ALT and high HBV DNA levels and, therefore, are defined as HBeAg-negative CHB patients [27]. Most of these patients have mutations in the pre-core or core promoter region. Patients with sustained liver injury and persistent HBV replication are prone to develop liver cirrhosis and HCC.

HBV infection not only leads to acute and chronic hepatitis but also is one of the most important etiological factors for liver cirrhosis and HCC. In the absence of antiviral treatment, the annual incidence of liver cirrhosis in CHB patients is 2–10% [28], the annual risk of progression from compensated to decompensated cirrhosis is 3–5%, and the 5-year survival rate in patients with decompensated cirrhosis is only 14%–35% [29]. The annual incidence of HCC in non-cirrhotic patients is about 0.5%–1.0% [29], whereas in cirrhotic patients, the annual incidence of HCC increases to 3–6% [28, 30, 31]. Liver cirrhosis has been classified by WHO as one of the top 10 causes of death in low-middle-income countries, and HCC as one of the top 10 causes of death in upper-middle-income countries [32].

3 Biological Characteristics and Immunopathogenesis of HBV

3.1 HBV Genome and Life Cycle

HBV particles are composed of the HBV genome, nucleocapsid, and the envelope proteins. The HBV genome is a partially double-stranded DNA with approximately 3200 base pairs. The longer-strand DNA is complementary to pregenomic RNA (pgRNA) and therefore is designated as minus (–) strand, whereas the shorter strand is designated as plus (+) strand. The (–) strand contains four overlapping open reading frames (ORFs) (PreC/C, P, PreS/S, and X). Under the joint regulation of the four promoters (the core promoter, the PreS1 promoter, the PreS2/S promoter, and the X promoter) and two enhancers (EnhI and EnhII), four distinct classes of HBV transcripts are transcribed: the 3.5 kb PreC/C mRNA, the 2.4 kb PreS1 mRNA, the 2.1 kb PreS2/S mRNA, and the 0.7 kb X mRNA. The PreC/C ORF is responsible for encoding HBeAg and HBcAg; the P ORF encodes the HBV DNA polymerase; the PreS/S ORF encodes the large (L), the middle (M), and the small (S) envelope proteins; and the X ORF is responsible for encoding the X protein (HBx).

The life cycle of HBV involves the viral entry into host cells; rcDNA's entry into the nucleus to form cccDNA; the expressions of viral RNAs and proteins; viral capsid assembly; reverse transcription and rcDNA formation; and, finally, viral packaging, maturation, and budding. Mediated by the antigenic loop (AGL) present in the S domain of HBsAg, HBV is initially attached to heparan sulfate proteoglycan (HSPG) on the surface of hepatocyte membrane [33]. Subsequently, through the preS1 region of the L protein, HBV is tightly bound to the sodium-taurocholate cotransporting polypeptide (NTCP) on the surface of hepatocytes [34, 35]. After HBV entry into the hepatocytes, the nucleocapsid is released, and the rcDNA of

HBV enters the nucleus to form cccDNA, which resides inside the hepatocyte as a microchromosome [36]. Current anti-HBV NAs have no direct effect on cccDNA, which explains why HBV infection is currently manageable but still incurable by current treatments. Under the action of host RNA polymerase II, cccDNA serves as the template for transcription of the abovementioned four HBV transcripts and the translation of seven viral proteins. Of note, in addition to encode the translation of HBcAg, HBeAg, and DNA polymerase, the 3.5 kb pgRNA also has an important function as a template for viral reverse transcription and replication. During viral replication, HBV DNA polymerase binds to the ϵ -stem-loop structure near the 5' end of the pgRNA, forming a specific pgRNA-polymerase ribonucleoprotein (RNP) complex, which is encapsulated by the core antigen polypeptide dimer to form an immature nucleocapsid [37]. Under the catalysis of HBV DNA polymerase in the nucleocapsid, the 3.5 kb pregenomic RNA serves as the template for reverse transcription of the (–) strand DNA, which subsequently serves as the template for the synthesis of the (+) strand DNA, thus forming the progeny rcDNA. The recycling of the de novo synthesized rcDNA into the nucleus makes more cccDNA, maintaining the cccDNA reservoir. Double-stranded linear (dsl) DNA may be generated due to erroneous viral DNA replication [38]. Mature nucleocapsid and envelope protein aggregate in the endoplasmic reticulum to complete the packaging, maturation, and viral budding [39]. The discovery of HSPG and NTCP and their crucial roles in HBV entry into hepatocytes and the immature nucleocapsid containing the pgRNA and the dslDNA has significantly advanced our understanding of the HBV life cycle.

3.2 *HBV Transcription and Translation*

Using the reporter gene system containing the HBV promoter, a series of *cis*-acting transcriptional regulatory sequence elements and *trans*-acting DNA-binding proteins have been discovered. The 3.5 kb pgRNA is primarily regulated by *cis*-acting regulatory sequence elements within the HBV genomic enhancer I (EnhI/Xp) and enhancer II (EnhII/Cp). HBV EnhI is located within the ORF P, between the ORF S and X, and overlaps with the X promoter, which enhances the transcription of C, SPI, SPII, and X promoters. The transcription factors reported bound to EnhI/Xp include C/EBP, P53, IRF, NF1, HNF3, HNF4, RXR, PPAR, COUPTF, RFX1, AP1, CREB, and ATF2 [40, 41]. EnhII is located to the upstream of Cp. The transcription factors bound to HBV EnhII/Cp include SP1, RFX1, C/EBP, FTF, HLF, E4BP4, HNF4, HNF3, RXR, PPAR, COUPTF1, and ARP1 [40, 41].

HNF4, RXR, PPAR, FXR, and LRH1 are liver-enriched transcription factors capable of supporting HBV replication in non-hepatoma cells [41, 42]. The identification of these liver-enriched transcription factors contributes significantly to the hepatocyte-specific tropism of HBV. In addition to the transcription factors, some transcriptional co-activators (including PGC1, CBP, SRC1, and PRMT1) and co-repressors (including SBP) are also involved in the regulation of HBV transcription

and replication [43–45]. In addition, other host proteins including APOBEC3B, PRMT5, and PRKAA/AMPK have also been reported to participate in the HBV transcription and replication [46–48].

The viral protein HBx can also act as a *trans*-acting factor possibly through interacting with certain host proteins to enhance HBV transcription and replication. It has been reported that HBx not only enhances HBV DNA replication but also activates the transcription of 3.5 kb HBV RNA. The augmentation of HBV transcription is always observed in parallel to HBV replication, strongly suggesting that the stimulation of HBV replication is mainly due to the enhanced transcription by HBx [49, 50]. It has also been suggested that HBx enhances HBV replication through posttranscriptional regulation mechanisms, such as the regulation of intracellular calcium signaling and activation of downstream Pyk2/FAK kinase [51]. Recent studies have found that HBx can promote the degradation of SMC5/6 to enhance HBV replication [52, 53].

3.3 Immunopathogenesis of HBV

The immunopathogenesis of CHB is complex and has not yet been fully elucidated. Numerous studies have shown that HBV is a non-cytopathic hepatotropic virus. Persistent liver inflammation plays an important role in the progression of CHB to cirrhosis and HCC. The innate immune response plays a role in the early stages of HBV infection and induces subsequent adaptive immune responses [54]. HBV inhibits the innate immune response by interfering with Toll-like receptors and retinoic acid receptors through the viral components such as HBeAg and HBx [55–59]. Although the innate immune response of CHB patients is impaired, antiviral cytokines of the innate immune pathway can still inhibit HBV. This is supported by the ability of IFN α or TLR agonists to induce innate immunity to inhibit HBV replication [60]. At present, many novel anti-HBV drugs are being developed to inhibit HBV replication through the upregulation of innate immunity.

Many CHB patients are characterized with impaired frequency and function of myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC) in their peripheral blood. Immature mDC and pDC with decreased capacity to produce IFN- α are associated with tolerogenic T-cell responses and HBV persistence [61]. HBV-specific immune response plays a major role in HBV clearance. The virus-specific effector CD8+ T-cell response is central to HBV pathogenesis. Major histocompatibility complex (MHC) class I-restricted CD8+ cytotoxic T lymphocytes can induce hepatocyte apoptosis and secrete IFN- γ , thus inhibiting HBV replication through non-cytolytic mechanisms. In chronic HBV infection, apoptosis-prone HBV-specific CD8+ T cells, the reduced cytokine function and proliferative capacity, and the T-cell exhaustion contribute to HBV persistence [60].

4 Current Antiviral Drugs Against HBV and New Antiviral Drugs Under Development

4.1 Current Antiviral Drugs Against HBV

Current antiviral drugs for HBV include IFNs and NAs. The first milestone in CHB treatment is the utilization of IFN- α (IFN- α -2a, IFN- α -2b, and IFN- α -1b) produced by recombinant DNA technology. Meta-analysis has shown that conventional IFN-treated HBeAg-positive CHB patients have higher HBeAg seroconversion and HBsAg clearance, but lower cirrhosis and HCC incidence than the patients without IFN treatment [62, 63]. However, due to its limited efficacy, low sustained viral response, and frequent injections, the conventional IFN has been largely replaced with the long-acting Peg-IFN, which was approved by the US FDA for HBV treatment in 2002. International multicenter randomized controlled clinical trials showed that HBeAg-positive CHB patients treated with Peg-IFN- α -2a for 48 weeks resulted in HBeAg seroconversion rate of 32% at 24 weeks posttreatment follow-up [64] and the HBeAg seroconversion reached 41% at 48 weeks posttreatment follow-up [65]. Similar HBV DNA inhibition and HBeAg seroconversion can also be achieved in HBeAg-positive CHB patients treated with Peg-IFN- α -2b [66].

The second milestone in CHB treatment is the use of the lamivudine (LAM), which revolutionized the treatment of CHB. LAM exhibits good antiviral effects in HBeAg-positive and HBeAg-negative CHB patients, even in CHB patients with advanced liver diseases. However, resistance to LAM can be easily developed. It has been reported that LAM resistance can reach up to 80% after 5 years of treatment [67]. Following LAM, adefovir dipivoxil (ADV) was the second antiviral drug approved for anti-HBV therapy. However, like LAM, ADV has low genetic barrier and drug resistance can be easily developed and another drawback for ADV is its nephrotoxicity [18]. Telbivudine (LdT) is another nucleoside analog for antiviral treatment of CHB. With a proven safety profile, LdT is a pregnancy category B medication and has been applied to prevent mother-to-child-transmission (MTCT) in mothers with HBV infection [68–70]. However, similar with LAM and ADV, long-term LdT treatment leads to high rate of drug resistance (34% after 3-year TBV therapy) [71].

The third milestone in CHB treatment is the clinical use of the potent and low-resistant NAs: ETV and TDF. Both ETV and TDF can strongly inhibit HBV replication and have high genetic barrier to drug resistance. It has been shown that the 3-year cumulative ETV resistance rate is 1.7–3.3% [72]. Resistance to TDF was not detected in CHB patients after 6 years of TDF monotherapy [73]. Most CHB patients with long-term use of ETV or TDF can achieve histological improvement and even the reversal of liver fibrosis. Tenofovir alafenamide (TAF) has been recently approved for treatment of CHB in adults. Compared to TDF, TAF has a better safety profile (lower rates of bone and renal abnormalities) and similar