Postexposure Prophylactic Effect of Hepatitis B Virus (HBV)-Active Antiretroviral Therapy against HBV Infection

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Retrospective study indicates that hepatitis B virus (HBV)-active nucleoside (nucleotide) analogues (NAs) used for antiretroviral therapy reduce the incidence of acute HBV infections in human immunodeficiency virus (HIV)-infected patients. Learning from HIV postexposure prophylaxis (PEP), we explored the possibility of using NAs in PEP following HBV exposure, if preexposure prophylaxis is feasible clinically. Using freshly isolated primary human hepatocytes cultured in vitro, we analyzed the effect of HBV-active tenofovir and lamivudine in primary HBV infection and also the effect of treatment with these NAs after HBV infection. HBV-active NAs applied from 24 h before inoculation could not prevent the secretion of hepatitis B surface antigen into the culture medium, and cessation of the NAs after inoculation allowed the cells to establish an apparent HBV infection. In contrast, hepatitis B immune globulin was able to prevent HBV infection completely. NA treatment before infection, however, can control the spread of HBV infection, as detected by immunohistochemistry. Practically, starting NA treatment within 2 days of primary HBV infection inhibited viral spread effectively, as well as preexposure treatment. We demonstrated that preexposure NA treatment was not able to prevent the acquisition of HBV infection but prevented viral spread by suppressing the production of mature progeny HBV virions. The effect of postexposure treatment within 2 days was similar to the effect of preexposure treatment, suggesting the possibility of HBV PEP using HBV-active NAs in HIV- and HBV-susceptible high-risk groups.

Patients infected with human immunodeficiency virus (HIV) are at high risk of hepatitis B virus (HBV) infection, and an estimated 10% of HIV-infected individuals worldwide have chronic hepatitis B (1). Because of the shared transmission routes of the two viruses, i.e., sexual intercourse and blood contact (2), HIV-infected individuals, particularly men who have sex with men (MSM) and intravenous drug users, are at high risk of acute HBV infection.

Two recent retrospective studies from Japan and the Netherlands indicate that HBV-active nucleoside (nucleotide) analogues (NAs) used for antiretroviral therapy (ART) reduced the incidence of acute HBV infections in HIV-infected patients (3, 4). The prophylactic effects of regimens containing NAs as reverse transcription (RT) inhibitors are well accepted against HIV infection (5, 6), because RT is an initial essential step following HIV entry into susceptible cells. However, considering the differences between the HIV and HBV replication cycles, this notion cannot be applied simply to HBV infection.

Unlike HIV, when HBV enters the hepatocyte, its genomic DNA is transported to the nucleus and converted to covalently closed circular DNA (cccDNA), which serves as the template for transcription. One transcript, the pregenomic RNA, is converted to genomic DNA by RT, which is the target of NAs.

Studies of occult HBV infections strongly suggest that HBV cccDNA remains in the hepatocyte nuclei for a long time after resolution of acute infection (7), often leading to reestablishment of infection (HBV reactivation) following immunosuppressive therapy, i.e., anticancer chemotherapy (8). The phenomenon of HBV reactivation suggests that cccDNA remaining in the hepatocytes can produce infectious virions, leading to very rapid viral spread. Therefore, establishment of cccDNA in the nuclei means HBV infection of susceptible cells.

Nonetheless, human clinical studies have shown that HBV-active ART protects against the occurrence of de novo HBV infection, indicating that NA-based strategies inhibit the serological changes of HBV markers (hepatitis B surface antigen [HBsAg], hepatitis B surface antibody [anti-HBs], and hepatitis B core antibody [anti-HBc]) that provide evidence of HBV infection and have a clinical prophylactic effect against incident HBV infection. Learning from HIV postexposure prophylaxis (PEP), we consider the possibility of NA-based PEP against HBV exposure, given that preexposure prophylaxis (PrEP) is feasible clinically. Therefore, using freshly isolated primary human hepatocytes (PHH) cultured in vitro, we analyzed the effect of HBV-active NAs on primary HBV infection and showed the possibility of prophylaxis by NAs not only as PrEP but also as PEP against acute HBV infection.

MATERIALS AND METHODS

Freshly isolated PHH. Freshly isolated primary human hepatocytes (PHH) from severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator, whose livers were repopulated with human hepatocytes, were purchased from Phoenix Bio Co., Ltd. (Higashihiroshima, Japan), without cryopreservation. The isolation and culture of PHH were described previously (9).
HBV infection experiments. Inoculation of PHH with 10 HBV genome equivalents per cell was carried out in culture medium without polyethylene glycol for 48 h at 37°C. PHH were washed with medium three times on days 2 and 3 to remove the inoculum. Supernatants were collected, and the culture media were replenished on days 3, 5, 7, 12, 17, and 22 postinfection. To specifically block HBV attachment to the PHH, the HBV inoculum (2 $\times$ 10^6 copies/ml) was preincubated with 1,000 mIU/ml of hepatitis B immunoglobulin (HBIG) for 2 h, and the mixture of HBIG and HBV was added to the PHH. Also, 2.5 mM lamivudine (LAM) or 100 μM tenofovir (TFV) was added to the culture medium, starting 24 h before inoculation. According to previous results, using the HBV-expressing cell lines (Hep2.B.15), the 50% effective concentration (EC50) value of LAM (10) is around 0.12 μM and that of TFV (11) is around 1.1 μM. Thus, the doses of LAM and TFV used in our study are 20,000 times and 100 times higher, respectively, than the EC50 for HBV inhibition and thus provide sufficient excess for the desired clinical effect. In addition, the high concentration of LAM and TFV could not cause any cytotoxicity on the cells.

Determination of HBsAg and HBV DNA levels. The levels of HBsAg were determined by chemiluminescent enzyme immunoassay as described previously (12). The detection limit of the HBsAg assay is 0.05 IU/ml. HBV DNA was quantified by quantitative PCR as described previously (13). The detection limit of HBV DNA was set to 2.0 $\times$ 10^3 copies/ml. cccDNA was measured by quantitative PCR with primers cccF2 and cccR4, described previously (14), and a fluorescent probe, cccP2 (5′-FAM-CTGTAGGCATAAAATTGGT-MGB-3′ [FAM is 6-carboxyfluorescein]). The detection limit of cccDNA was set to 1.0 $\times$ 10^2 copies/μg DNA.

Southern hybridization. Southern hybridization was performed with full-length probes for HBV as described previously (12, 15).

Immunofluorescence assay. Fluorescence staining of intracellular HBsAg was performed by standard methods using goat anti-HBs (Bioss, Inc., MA, USA) and donkey anti-goat IgG conjugated with Alexa Fluor 488 (Life Technologies, Maryland, USA).

RESULTS

Effects of HBV-active NAs against primary infection. Freshly isolated PHH were confirmed to be susceptible to HBV primary infection by using Southern blot analysis (Fig. 1A). We examined the effect of NAs on the establishment of HBV infection in the hepatocytes. PHH were treated with the HBV-active NAs, LAM and TFV, at extremely high concentrations (2.5 mM and 100 μM, respectively) starting 24 h before HBV inoculation to gain full efficacy of NAs. Figure 1B shows the kinetics of HBV DNA (top) and HBsAg (bottom) concentrations in the culture medium of HBV-infected PHH. After day 5, HBV DNA concentrations increased in the supernatants of nontreated cells, indicating HBV replication, i.e., accumulation of cccDNA in the cell and release of progeny virions into the culture medium. Because NAs inhibit RT activity, treatment with LAM or TFV results in a continuous decrease of HBV DNA in the supernatants. In contrast, HBIG, which inhibits viral entry by blocking receptor binding (16), effectively prevented HBV infection, and HBV DNA could not be detected for 22 days. As shown in the bottom panel of Fig. 1B, LAM and TFV treatment have a small effect on the HBsAg concentration in the

PHH were treated continuously with lamivudine (LAM), tenofovir (TFV), or hepatitis B immune globulin (HBIG) up to 22 days. Ongoing treatment with LAM or TFV resulted in a continuous decrease in HBV DNA in the supernatant. However, the production of HBsAg increased after day 5. Data are presented as means ± standard deviations (SD) ($n = 3$ experiments). The NAs were LAM and TFV. Mock, the HBV infection control experiment without treatment.
supernatant up to day 12; presumably, these NAs could not protect against the formation at high concentration but do inhibit the accumulation of cccDNA as the template for HBsAg. Through the experiments, we did not see evidence of cytotoxicity by 3-(4,5-di-

FIG 2 Preexposure NA treatment fails to prevent HBV infection. (A) PHH were inoculated with HBV for 48 h at 37°C and washed several times, and sampling of the supernatants was performed (as described in Fig. 1B). The concentration of HBV DNA increased gradually after day 12 when the LAM or TFV treatment was stopped on day 5, despite the complete cessation of hepatitis B immune globulin (HBIG) treatment. The production of HBsAg in the control experiment increased further after day 12, and HBsAg productions in cases in which NA treatment was stopped on day 5 (Off-Tx) gradually increased in the supernatant. Off-Tx, HBV-infected PHH were treated for only 5 days with lamivudine (LAM), tenofovir (TFV), or HBIG. Data are presented as means ± SD (n = 3 experiments). (B) Increasing production of HBsAg in the supernatants during the period from day 12 to day 22. In order to confirm the change of HBsAg production, the HBsAg titer on day 22 was divided by that on day 12. The increase of the rate in the nontreatment experiment was 6.8 from day 12 to day 22; otherwise, the rates of the cells treated continuously with NAs (On-Tx of both LAM and TFV) were maintained at steady levels (the ratio was approximately 1). In the cases with cessation of NA treatment on day 5, the increase of HBsAg production during the period from day 12 to day 22 was observed in the culture medium (Off-Tx of LAM, 2.3; Off-Tx of TFV, 2.6). Data are presented as means ± SD (n = 3 experiments). Mock, the HBV infection control experiment without treatment. Statistical analysis of the difference was conducted using the analysis of variance with a nonparametric Mann-Whitney U test. *, P < 0.05.

FIG 3 HBV-active NAs can inhibit viral spread after acquisition of HBV infection in freshly isolated PHH. (A) Immunohistochemical staining of HBV-infected PHH with various treatments. Viral spread occurred over 10 days from day 12 to day 22 in the nontreatment experiment (Mock); however, the numbers of cells staining for HBsAg did not change from day 12 to day 22 with continuous treatment by either LAM or TFV. HBIG, treatment with HBIG for 12 days; no HBV, PHH at day 22 without HBV inoculation; green, staining of HBsAg; blue, staining of nuclear DNA. (B) The level of cccDNA in HBV-infected PHH at day 22. Compared to the control experiment (Mock), the levels of cccDNA treated continuously with NAs (On-Tx of both LAM and TFV) were suppressed and that with LAM treatment for only 5 days (LAM Off-Tx) exhibited an increase following the cessation of LAM treatment. The asterisk indicates a value below the detection limit.
methyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays or by microscopic examination of cell morphology after treatment with these high concentrations (data not shown).

Next, to confirm the prophylactic effect of NAs, treatment was stopped at day 5 (Fig. 2A). As expected, HBIG continued to prevent HBV infection. When LAM or TFV treatment was stopped on day 5, however, the concentration of HBV DNA increased gradually after day 12, suggesting that these agents did not protect against the establishment of HBV infection (Fig. 2A). The production of HBsAg in the control experiment also increased after day 12, and HBsAg production in cases in which NA treatment had ceased on day 5 (Off-Tx) also slowly increased. Previously, it was thought that the rapid differentiation of PHH leads to a loss of susceptibility to HBV infection within a few days. However, the PHH used in this study were isolated from living chimeric mice without cryopreservation. We therefore confirmed the susceptibility to HBV infection over 10 days using the increase in HBsAg production as a surrogate marker for the accumulation of cccDNA and thus the increasing number of HBV-infected PHH.

Although HBsAg production increased markedly in the nontreatment experiment (the relative increase ratio was 6.8) during the period from day 12 to day 22 (Fig. 2B), there was no increase in the number of infected cells was observed following continuous LAM or TFV treatment. Although the level of cccDNA in cells treated continuously with NAs (LAM and TFV) was suppressed on day 22, compared to the control experiment, those treated with LAM for only 5 days (LAM Off-Tx) exhibited an increase following cessation of LAM treatment (Fig. 3B). Because NAs inhibit the production of infectious virions at the stage of conversion of pregenomic RNA to genomic DNA, the release of progeny virions is inhibited by NAs. This allows us to suggest that secondary infection of neighboring cells by newly produced HBV virions, viral spread, occurred in the in vitro system. From these results and basic mechanisms regarding the HBV replication cycle, we conclude that NAs do not prevent the establishment of cccDNA and acquisition of HBV infection but prevent viral spread by suppressing the production of progeny HBV virions.

**Feasibility of NA-based HBV postexposure prophylaxis.** Considering that HBV-active NAs act only on viral spread, we explored the possibility that the addition of LAM to the culture medium shortly after HBV inoculation might have an effect similar to that of preexposure LAM treatment, as shown in Fig. 1 and 3. Again, to confirm the susceptibility to HBV infection during the

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**FIG 4** HBIG treatment in freshly isolated PHH post-HBV inoculation inhibits viral spread, while delaying HBIG addition allows viral spread. (A) Schematic representation of the schedule for continuous treatment (On-Tx) with hepatitis B immune globulin (HBIG) after HBV inoculation. IHC, immunohistochemical staining for intracellular HBsAg. (B) IHC of PHH on day 22 with HBIG treatment started at 2, 3, 5, 7, 12, and 17 days after HBV inoculation, as indicated in panel A. The addition of HBIG after inoculation did not protect against primary HBV infection (On-Tx Day 2), and delaying the addition of HBIG allowed gradual viral spread (On-Tx Day 2 to Day 17), detected by fluorescence IHC at day 22 postinoculation.
follow-up periods, the addition of HBIG after inoculation was
explored for 22 days (Fig. 4A), because HBIG treatment protects
completely against new HBV infection (Fig. 1B, 2A, and 3B). The
addition of HBIG after inoculation was not able to protect against
primary HBV infection (On-Tx Day −1 compared to Day 2), and the delay in the addition of LAM resulted in viral spread at day 22 postinoculation. These results strongly indicate that delaying starting HBIG treatment after HBV inoculation allowed viral spread into the freshly isolated PHH system.

Next, the effect of the addition of LAM after inoculation was monitored and quantified, based on the HBsAg titer in the supernatant (Fig. 5A). The addition of LAM before and after inoculation did not protect against primary HBV infection (On-Tx Day −1 and Day 2), and delaying the addition of LAM (Fig. 5B, On-Tx Day 2 to Day 17 compared to None). These results strongly indicate that delaying starting HBIG treatment after HBV inoculation allowed viral spread into the freshly isolated PHH system. Next, the effect of the addition of LAM after inoculation was monitored and quantified, based on the HBsAg titer in the supernatant (Fig. 5A). Delaying the addition of LAM (Fig. 5A) allowed gradual viral spread, as observed in the HBIG treatment. However, the addition of LAM on day 2 after inoculation effectively inhibited the viral spread observed on day 22 (Fig. 5B, On-Tx Day 2). Furthermore, the HBsAg levels in the culture medium on day 22 in-

FIG 5 Effects of LAM on post-HBV inoculation in vitro. (A) Schematic representation of the schedule for continuous treatment (On-Tx) with lamivudine (LAM) after HBV inoculation. IHC, immunohistochemical staining for intracellular HBsAg. (B) IHC of PHH on day 22 with LAM treatment started at 2, 3, 5, 7, 12, and 17 days after HBV inoculation, as indicated in panel A. The addition of LAM before and after inoculation did not protect against primary HBV infection (On-Tx Day −1 and Day 2), and the delay in the addition of LAM resulted in viral spread at day 22 postinoculation. (C) The HBsAg concentrations in the culture medium collected on day 22 from the HBV-infected PHH with LAM treatment as shown in panel A. Data are presented as means ± SD (n = 3 experiments). ctrl, HBV-infected PHH without treatment.

DISCUSSION

Coinfection with HBV and HIV increases the morbidity and mor-
tality beyond those caused by either virus alone, and the burden of coinfection is greatest in developing countries, particularly in Southeast Asia and sub-Saharan Africa (1). Therefore, the assessment of HBV status is warranted for all HIV-positive persons, and hepatitis B vaccination should be offered to all susceptible individuals. Although immunization remains the mainstay of disease prevention, HIV-positive individuals mount poorer antibody responses to hepatitis B vaccine than healthy donors (17, 18). Moreover, as the course of hepatitis B immunization involves injections at 0, 1, and 6 months, a coordinated effort and period of time are required.
Because NA-based PEP has been used as an HIV prevention strategy for nearly 20 years, we explored the possibility of HBV PEP using NAs, with reference to clinical evidence that PrEP may be feasible for de novo HBV infection. In Japan, Gatanaga et al. explored NA-based PrEP using sequential serum samples from HIV-infected MSM who had no detectable HBsAg, anti-HBs, and anti-HBc at baseline (3). Evidence of HBV infection was detected in follow-up samples from 43 of the 354 men (12.1%) after a median time period of 1.6 years. The rate of incident infection was approximately 90% lower for patients taking LAM or TDF than for those receiving no ART or a non-LAM/TDF-containing regimen. Moreover, no new HBV infections occurred in patients taking TDF; however, the 7 men who developed HBV infection despite taking LAM were more likely to be infected with LAM-resistant strains (50.0%) than the 36 individuals not receiving HBV-active ART (7.1%). Another serological follow-up study from the Netherlands reported that LAM and TDF protected against the occurrence of de novo HBV infection among HIV-infected MSM over a median of 6.8 years and also demonstrated a superior protective effect on HBV seroconversion in the group taking TDF (4). These clinical data indicated that HBV-active NAs can prevent acute HBV infection, defined by the absence of the serological markers HBsAg, anti-HBs, and anti-HBc, and TDF-based PrEP has a significant effect on HBV primary infection. In the case of HBV infection, the prophylactic effects of NAs as RT inhibitors are well accepted, because these block the initial step following virus entry. However, during HBV infection, RT inhibitors act after cccDNA formation and do not prevent HBV infection completely, confirmed by our original HBV-infected PPH system. Our results clearly indicate that the effect of NAs on HBV primary infection was simply prevention or inhibition of viral spread.

It remains largely unknown how NAs act as prophylaxis. As clinical data showed, treatment with NAs protects against the acquisition of HBV without an antibody response. We speculated that acquired immunity, especially humoral immunity, could not be responsible for the clinical outcome of prophylaxis by NAs. Recent reports show that natural killer (NK) cells contribute to protection against acute HCV infection (19–21), and NK cells may be responsible for the clinical outcome of prophylaxis by NAs. Clinical data showed, treatment with NAs protects against the acquisition of de novo HBV infection among HIV-infected MSM over a median of 6.8 years and also demonstrated a superior protective effect on HBV seroconversion in the group taking TDF (4). These clinical data indicated that HBV-active NAs can prevent acute HBV infection, defined by the absence of the serological markers HBsAg, anti-HBs, and anti-HBc, and TDF-based PrEP has a significant effect on HBV primary infection. In the case of HBV infection, the prophylactic effects of NAs as RT inhibitors are well accepted, because these block the initial step following virus entry. However, during HBV infection, RT inhibitors act after cccDNA formation and do not prevent HBV infection completely, confirmed by our original HBV-infected PPH system. Our results clearly indicate that the effect of NAs on HBV primary infection was simply prevention or inhibition of viral spread.

Although the extent to which the data from in vitro studies can be extrapolated to clinical human studies is largely unknown, we postulate that treatment with NAs should be used for longer periods of HBV prophylaxis than of HIV prophylaxis, typically for 4 weeks in HIV PEP (5, 25), because of the evidence from our in vitro data regarding acquisition of HBV infection. Correspondingly, the use of NAs for preexposure and postexposure prophylaxis to prevent HBV transmission must also be studied in the context of HIV prevention in areas where there may be problems in obtaining and/or storing HBIG. Despite the fact that our results with higher concentrations of NAs than those in the clinical setting indicate the possibility of NA-based HBV PEP, as well as PrEP shown by retrospective human studies, further research and human clinical trials are needed to evaluate brief screening, drug selection, as well as dose, timing, and duration of NA-based PEP against de novo HBV infection in susceptible high-risk groups.

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