ANTICANCER RESEARCH *36*: 6059-6070 (2016)
doi:10.21873/anticanres.11195

Apoptosis of Hepatitis B Virus-expressing Liver Tumor Cells Induced by a High Concentration of Nucleos(t)ide Analogue

EUNYOUNG TAK[1], SHIN HWANG[2], HAN CHU LEE[3], GI-YOUNG KO[4], CHUL-SOO AHN[2], YOUNG-IN YOON[2], YOUNG-SUK LIM[3], DAE-YOUNG JUN[1], KI-HUN KIM[2], GI-WON SONG[2], DEOG-BOK MOON[2], BAEK-YEOL RYOO[5], NAYOUNG KIM[1] and SUNG-GYU LEE[2]

[1]*Asan Institute for Life Sciences, and Departments of* [2]*Surgery,* [3]*Gastroenterology,* [4]*Diagnostic Radiology and* [5]*Oncology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea*

Abstract. *Background/Aim: We investigated the expression of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) and HBV X protein (HBx) in human hepatocellular carcinoma (HCC) and evaluated the effect of high-concentration nucleos(t)ide analogs (NUCs) on liver tumor cell lines. Materials and Methods: This study consisted of three parts: part I used human blood and non-tumor liver tissues; part II used human HCC and adjacent liver tissues; and part III used an HBV-expressing liver tumor cell line. Results: There were close correlations among blood and liver HBV DNA and liver cccDNA. HBV cccDNA and HBx were highly up-regulated in HCC compared to adjacent liver tissues despite NUC therapy. HBV cccDNA and HBx were highly up-regulated in the cccDNA-expressing HepG2.2.15 cell line. Their expression was down-regulated and apoptosis was induced by a very high concentration of NUCs in dose- and time-dependent manner. Conclusion: Very high concentrations of NUCs may have a novel potential to kill replicating HBV-expressing liver tumor cells.*

The close association between hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC) development is well recognized. Due to a high prevalence of HBV infection in the general population in many Asian countries, including Korea, chronic hepatitis B (CHB)-associated HCC has become one of the most common causes of patient death. Inhibition of HBV replication through administration of nucleos(t)ide analogs (NUCs) is known to effectively prevent *de novo* HCC development and HCC recurrence in addition to blockage of progression of fibrosis (1-5).

Because NUCs with a high genetic barrier to resistance (hgbNUCs), such as entecavir and tenofovir, can effectively suppress replication of HBV for a long time, the sustained virological suppression will improve liver histology (6, 7). However, it is generally accepted that these agents do not control the covalently closed circular DNA (cccDNA) or integrated DNA of HBV in the liver (8). HBV antiviral therapy should be maintained throughout life because cccDNA is integrated into the hepatocyte nucleus. Thus, it is difficult to eliminate cccDNA from the infected reservoir of hepatocytes. Because cccDNA is the transcriptional template for production of HBV viral RNA and functions as a key intermediate in the HBV life cycle, it appears to be the factor preventing complete eradication of HBV infection in patients with CHB (9, 10). HBV cccDNA has been detected in the context of HCC recurrence in liver transplantation (LT) recipients who developed recurrence of both HBV and HCC (11). HBV X protein (HBx) is a major regulator encoded by the HBV genome that plays an important role in HBV-associated hepatocarcinogenesis (12).

It is well known that HBV cccDNA and *HBx* is up-regulated in HBV-associated HCC cells, thus we searched for a method to induce their down-regulation because this may be associated with induction of antitumor effect. In the present study, we investigated the effect of NUCs on the viral load of HBV DNA and cccDNA in the blood, liver tissue and HCC, and then evaluated the antitumor effect of high-concentration hgbNUCs on a cccDNA-expressing liver tumor cell line.

Materials and Methods

Study design. This study actually comprised of three consecutive studies: two clinical studies, one study using human blood and non-tumor liver tissue samples (part I), another using paired human HCC and non-tumor liver tissue samples (part II), and a laboratory study using liver tumor cell lines associated with and not associated with HBV (part III).

Correspondence to: Shin Hwang, MD, Ph.D., Department of Surgery, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Korea. Tel: +82 230103930, Fax: +82 230106701, e-mail: shwang@amc.seoul.kr

Key Words: Tenofovir, entecavir, covalently closed circular DNA, HBV X protein, transcatheter arterial chemoembolization.

In part I, we investigated the association between HBV DNA and cccDNA in the peripheral blood and non-tumor liver tissue samples obtained from patients with HCC undergoing LT (n=27). In part II, we analyzed the expression status of HBV cccDNA and *HBx* in paired tumor and non-tumor liver tissues obtained from patients with HCC undergoing surgical resection (n=13). Finally, in part III, we evaluated the influence of hgbNUCs (entecavir and tenofovir) on HBV cccDNA and HBx in liver tumor cell lines.

The two clinical studies using human blood and tissue samples were performed after the study protocols were approved by the Institutional Review Board of Asan Medical Center (2010-0359 for part I and 2014-0465 for part II). Written informed consent was obtained from all patients who were enrolled for the part I and II studies.

Blood and liver tissues of LT recipients (Part I). The peripheral blood was sampled just before laparotomy and non-tumor liver tissues were obtained immediately after recipient liver removal. These samples were obtained from 27 recipients who underwent LT in 2010. Liver tissue and serum were stored at –74˚C.

All patients were hepatitis B surface antigen (HBsAg)-positive and NUCs were being administered to 16 patients at the time of LT (entecavir in 11, lamivudine in two, and lamivudine with adefovir in three). Their mean age was 50.3±6.3 years (range=37-65 years) and 24 patients were male. The Model for End-stage Liver Disease (MELD) score was 16.5±10.2 (range=6-37), and the Milan criteria were met in 20 patients. The HCC tumor stage according to the Seventh American Joint Committee on Cancer staging system was I in 8, II in 14, and III in five study subjects.

Paired HCC tissues (Part II). The human liver tissues were randomly obtained from 13 patients with CHB who underwent surgical resection of HCC between February 2015 and May 2015. Paired wedge resection was performed for both HCC and adjacent non-tumor liver tissues immediately after liver specimen delivery from the abdomen and the tissues were then stored at –74˚C.

All patients were HBsAg-positive and at the time, NUCs were being administered to nine patients (entecavir in five, tenofovir in two, both entecavir and tenofovir in one, and adefovir in one). Their mean age was 56.1±6.2 years (range=31-62 years) and all of these patients were male. The HCC tumor stage according to the Seventh American Joint Committee on Cancer staging system was I in five, II in six, and III in two patients.

In addition, paired wedge resection was also performed for both HCC and adjacent non-tumor liver tissues in seven patients with HCC without viral hepatitis.

Liver tumor cell lines (Part III). The human HCC-derived HepaRG cell line (HPRGC10), which was used as a control liver tumor cell line without HBV association, was purchased from Life Technology (Grand Island, NY, USA).

Since primary cell culture with HBV-infected human HCC tissues (*e.g.* fresh surgical specimens obtained during Part II study) was very difficult, as well as not reliable regarding HBV replication, we searched for HBV-infected HCC cell lines but no human HCC cell line producing intact HBV with viral infectivity was available at that time. Thus we chose the HepG2.2.15 cell line, which is derived from the human hepatoblastoma cell line HepG2 with HBV transfection and is characterized by having stable HBV expression (each cell contains ~10 copies of cccDNA) (13, 14). This cccDNA-expressing HepG (HepG2.2.15) cell line (1×10^6/ml) was obtained from the Korean Advanced Institute of Science and Technology.

Both liver tumor cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, both purchased from Gibco-BRL (Grand Island, NY, USA).

The effect of the hgbNUCs entecavir and tenofovir and hepatitis B immunoglobulin (HBIg) was evaluated by using these liver tumor cell lines. The add-on *in vitro* NUC concentration was determined after repeated titration with consideration of the *in vivo* therapeutic ranges in patients with CHB. The steady-state maximal and 24-hour trough plasma concentrations are reported to be 4.2 ng/ml and 0.3 ng/ml for entecavir (Baraclude 0.5 mg; Bristol-Myers Squibb, New York, NY, USA) and 0.3 μg/ml and 0.07 μg/ml for tenofovir (tenofovir disoproxil fumarate: VIREAD 300 mg; Gilead Sciences, Foster City, CA, USA), respectively. Thus, we set the *in vitro* baseline concentration as 5 ng/ml for entecavir and 0.3 μg/ml for tenofovir. As a preliminary test, each NUC of 100 μmol (molecular weight: 277.3 g/mol for entecavir and tenofovir 287.2 g/mol) was added to the culture medium of 2 ml, thus making each NUC concentration around 50 μmol/ml or 14 μg/ml. This concentration is 2,800-fold higher than the baseline entecavir concentration and 47-fold higher than the baseline tenofovir concentration. At this concentration, down-regulation of HBV cccDNA and *HBx* was clearly observed. Thereafter, each NUC concentration was increased to 3-fold, as well as gradually reduced close to the baseline concentration.

Two types of HBIg were used: one was a blood product containing polyvalent anti-hepatitis B surface antibodies (anti-HBs) (Hepabig; Green Cross, Seoul, Korea); the second was a recombinant monoclonal antibody (Hepabig-Gene; Green Cross; under clinical trial only). The add-on *in vitro* HBIg concentration was determined after repeated titration with consideration of the *in vivo* peak concentration (500-1,000 mIU/ml for Hepabig 10,000 U per 1-2 months) in adult LT patients. We used *in vitro* HBIg concentration >800 mIU/ml with Hepabig and >5,000 mIU/ml with Hepabig-Gene.

Serum and liver tissue HBV DNA and cccDNA measurement. In part I, the pre-transplant serum HBV DNA levels were initially checked at our clinical hospital laboratory using the Abbott Realtime HBV system (sensitivity of 15 IU/ml; Abbot Laboratories, Abbot Park, IL, USA). These absolute DNA values were matched with the relative DNA values obtained from our research laboratory because of different polymerase chain reaction (PCR) cycles and specimen preparations.

On the day of LT, patient blood was collected for the measurement of relaxed circular DNA (rcDNA) and cccDNA. A 200-mg liver tissue sample was minced with a sterilized surgical knife, crushed, and then mixed with 2 ml of normal saline. The mixture was centrifuged for 1 minute at 1,300 × *g* and the supernatant was used for the detection of rcDNA and cccDNA.

The HBV rcDNA levels of the blood and liver tissues were measured by nested PCR using a primer set located within the S gene. First-round PCR was performed using the following primers: *S1*, 5'-ACTCGTGGTGGACTTCTCTC-3' (nucleotides 252–271) and *S2*, 5'-GAACCACTGAACAAATGGCA-3' (nucleotides 703-684). The second-round primers were: *S3*, 5'-GTCTGCGGCGTTT TATCATA-3' (nucleotides 381-399) and *S4*, 5'-GGATGGGAATA CAGGTGCAA-3' (nucleotides 611-592). To detect cccDNA, real-time PCR was performed by using the following primers: forward,

5'-CTCCCCGTCTGTGCCTTCT-3' (nucleotides 1548-1566); reverse, 5'-GCCCCAAAGCCACCCAAG-3' (nucleotides 1903-1886). The sequence of the cccDNA probe was 5'-FAM-AC GTCG CATGGARACCACCGTGAACGCC-TAMRA-3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control gene with the following primers: forward, 5'-CACAT GGCCTCCAAGGAGTAA-3'; reverse, 5'-GAGGGTCTCTCTCTT CCTCTTGT-3'; and probe, 5'-FAM-CTGGACCACCAGCCCC AGCAAG-TAMRA-3'. When no DNA was detected after 55 PCR cycles, the sample was regarded as DNA not detected. Details of these PCR methods are described elsewhere (15, 16). DNA was quantified using the values obtained after 55 PCR cycles.

Antibodies for apoptosis-related proteins and HBx. Antibodies against poly (ADP ribose) polymerase (PARP) (B-10, sc-74470) and cleaved PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against actin (AC-15, A3854) were purchased from Sigma-Aldrich (Poole, Dorset, UK); antibodies against cleaved caspase-3 (Asp175, cat no. 9664) were obtained from Cell Signaling (Danvers, MA, USA); and antibodies against HBx (ALX-804-278-C100) were purchased from Enzo Life Sciences (Farmingdale, NY, USA).

RNA Isolation and cDNA synthesis. For analysis of cccDNA and *HBx* mRNA, total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA purity was estimated by spectrophotometry at 260, 270, and 280 nm. RNA integrity was assessed by running 1 μl of every sample in an RNA NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized from 1 μg of total RNA using a cDNA synthesis kit in accordance with the manufacturer's specifications (Bio-Rad, Hercules, CA, USA).

Reverse transcription-PCR. The following primer sets were used: *GAPDH* control (Hs_GAPDH_1_SG) purchased from Abcam (Cambridge, UK); *HBx* forward, 5'-TGCCAACTGGATCCTTCG CGGGACGTCCTT-3'; and *HBx* reverse, 5'-GTTCACGGTGGTC TCCATG-3'. Relative transcription levels were measured as previously described (16). The data are expressed as the fold change in the *HBx* level from the treatment groups to the control group. *GAPDH* was amplified as a control for the real-time reverse transcription-PCR.

Western blot. Cell extracts were separated by polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with primary antibody against PARP, actin, cleaved caspase-3, and HBx followed by incubation with a secondary antibody. Proteins in samples were detected with a Supersignal pico-enhanced chemiluminescence kit purchased from Pierce (Rockford, IL, USA). Unless specified, cell lysates containing 20 μg of protein were analyzed.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assays were performed to quantify cell viability using 12-well plates. Optical density was assessed at 550 nm using a microplate reader (Bio-Rad). Cell survival was expressed as the percentage of absorbance of NUC-treated cells relative to that of untreated cells. MTT was purchased from Duchefa (Haarlem, the Netherlands).

Statistical analysis. Continuous variables are presented as the mean±SD or median. The Student *t*-test was used for comparisons with a value of $p<0.05$ being considered statistically significant. Simple linear regression analysis is presented with a regression equation, correlation coefficient (r), and coefficient of determination (r^2). Statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad, La Jolla, CA, USA), SPSS (version 20; IBM, New York, NY, USA), and Statistica (version 6.0; StatSoft, Tulsa, OK, USA) software.

Results

Quantitative correlation of serum HBV DNA, tissue HBV DNA and cccDNA in non-tumorous liver tissues. Among the 27 patients who underwent LT, in our current series serum HBV DNA was detected in 20 cases in the clinical hospital laboratory setting with a sensitivity of 15 IU/ml (or 84 copies/ml). HBV DNA was detected in 10 out of 16 patients who were administered NUCs (median=275 IU/ml) and in 10 out of 11 patients who were not administered NUCs (median=1,110 IU/ml).

In contrast, serum HBV rcDNA was detected in all 27 patients in the research laboratory setting with 55 PCR cycles. The correlation between the serum HBV DNA amounts obtained from the clinical and research laboratory settings is depicted in Figure 1A (r=0.875, r^2=0.766; y=6.7 + 6.8×10^{-6} × x). The values of serum HBV DNA (unit: IU/ml) in the clinical setting were 1.5×10^5-fold greater than the research setting DNA values (no unit). Serum HBV cccDNA was detected in only five patients, who had a median serum HBV DNA level of 7.1×10^6 IU/ml (Figure 1A). Both HBV rcDNA and cccDNA were detected in all liver tissue samples.

There was a close positive correlation between the serum HBV rcDNA and liver rcHBV DNA loads (r=0.886; r^2=0.785; $p<0.0001$), as well as between the liver HBV rcDNA and liver cccDNA loads (r=0.871, r^2=0.758; $p<0.0001$) (Figure 1B). These data suggest that there are high-grade linear correlations among blood HBV DNA, liver rcDNA, and liver cccDNA. NUCs reduced DNA load in the blood and liver, but were unable to eradicate HBV from infected hepatocytes.

HBV cccDNA and HBx are up-regulated in HCC tissues. In 13 of our study patients undergoing surgical resection, serum HBV DNA >15 IU/ml was detected in three out of nine cases who were administered NUCs (range=16-3,600 IU/ml) and three out of four patients who were not administered NUCs (range=1,100-49,000 IU/ml). HBV cccDNA transcription was barely detected in the non-tumor liver tissues, but was significantly up-regulated in HCC tissues of all 13 patients (mean=2,158-fold, $p=0.008$) (Figure 2A). *HBx* transcript was also barely detected in the non-tumor liver tissues (n=13) and non-HBV HCC samples (n=7), but was markedly up-

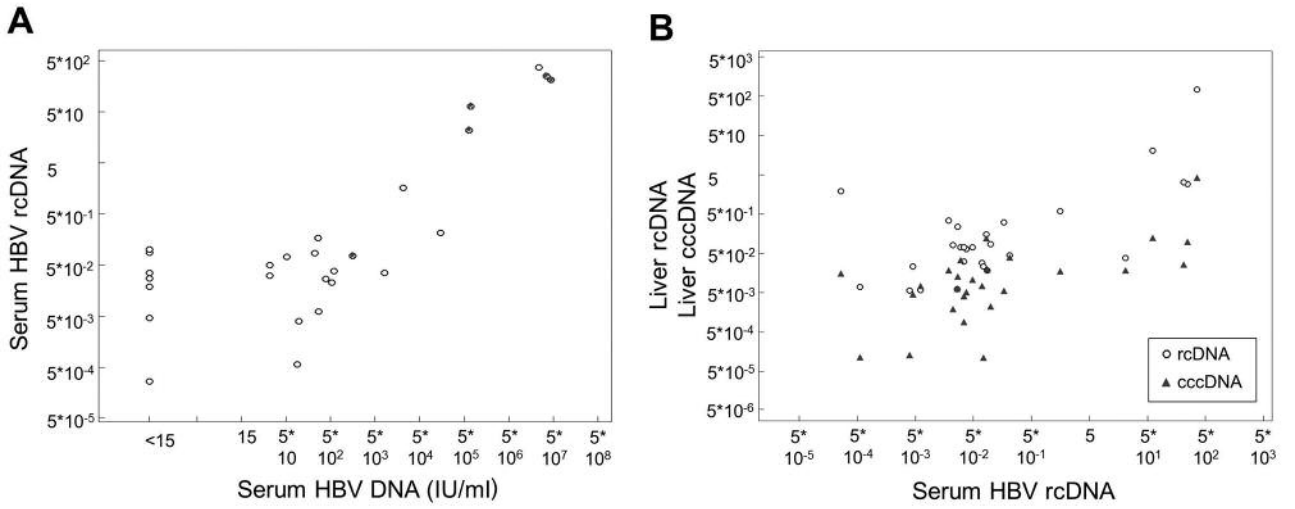

Figure 1. *A: Correlation of hepatitis B virus (HBV) tissue relaxed circular DNA (rcDNA) and HBV DNA in serum obtained from the clinical (x-axis) and research laboratory (y-axis) settings. Correlation between the serum HBV DNA amounts. Closed symbols denote patients with detectable serum HBV cccDNA. B: Correlation of liver HBV rcDNA load and liver cccDNA loads with serum HBV rcDNA. No units are provided for rcDNA and cccDNA due to a lack of reference samples for determining these concentrations.*

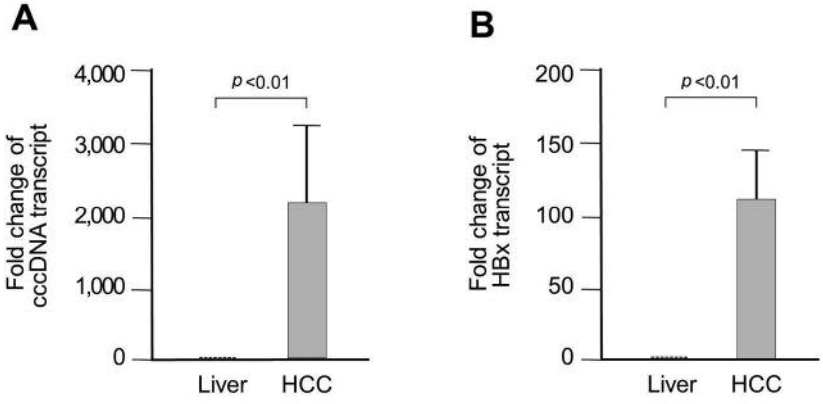

Figure 2. *Comparison of the transcript measurements of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) (A) and HBV protein X (HBx) (B) between hepatocellular carcinoma (HCC) and adjacent non-tumor liver tissue (Liver). The height of bars in the adjacent non-tumor liver tissue group was set at 1.*

regulated in HBV-associated HCC tissues (mean=109-fold, p=0.007) (Figure 2B). These data indicate that the HBV viral load within the replicating HCC cells was dramatically increased, even when the HBV viral load in the surrounding liver tissue was fully suppressed with long-term use of hgbNUCs.

Expression of HBV cccDNA and HBx in the HepG2.2.15 cell line. HBV cccDNA transcript was not detected in the HepaRG cell line but was found to be markedly up-regulated in the HepG2.2.15 cell line (mean=1,447-fold, p=0.002) (Figure 3A). *HBx* transcription was practically undetected in the HepaRG cell line and markedly up-regulated in the HepG2.2.15 cell line (mean=204,744-fold, p=0.0002) (Figure 3B). Western blot densitometric analysis using ImageJ software showed marked up-regulation of *HBx* in the HepG2.2.15 cell line (mean=9-fold, p=0.025) (Figure 3C).

NUC-induced apoptosis in the HepG2.2.15 cell line. In the HepaRG cell line, *HBx* transcript was not influenced by administration of entecavir nor of tenofovir for 24 h [entecavir and tenofovir concentrations ranging from 0 to

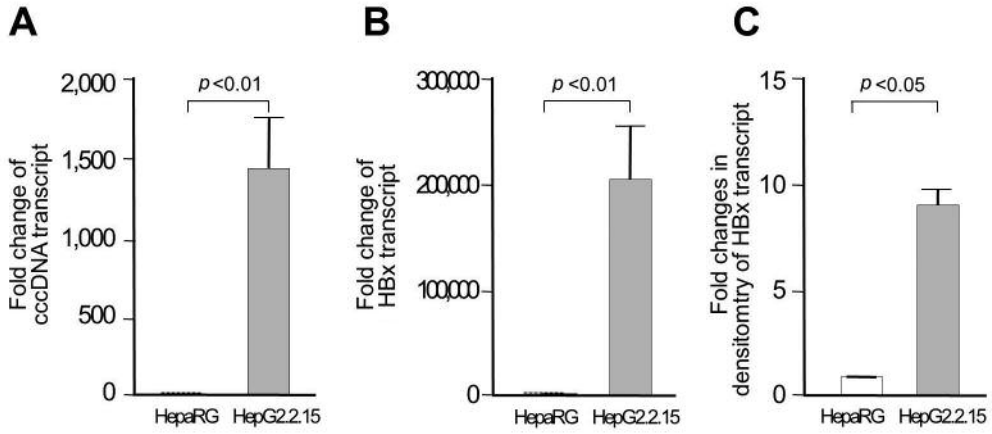

Figure 3. *Comparison of the transcription of covalently closed circular DNA (cccDNA) cccDNA (A) and hepatitis B virus protein X (HBx) (B) between the HepaRG and HepG2.2.15 hepatitis B virus-expressing cell line. HBx expression was also analyzed by western blot and measured by densitometry (C). The height of bars in the HepaRG group was set at 1.*

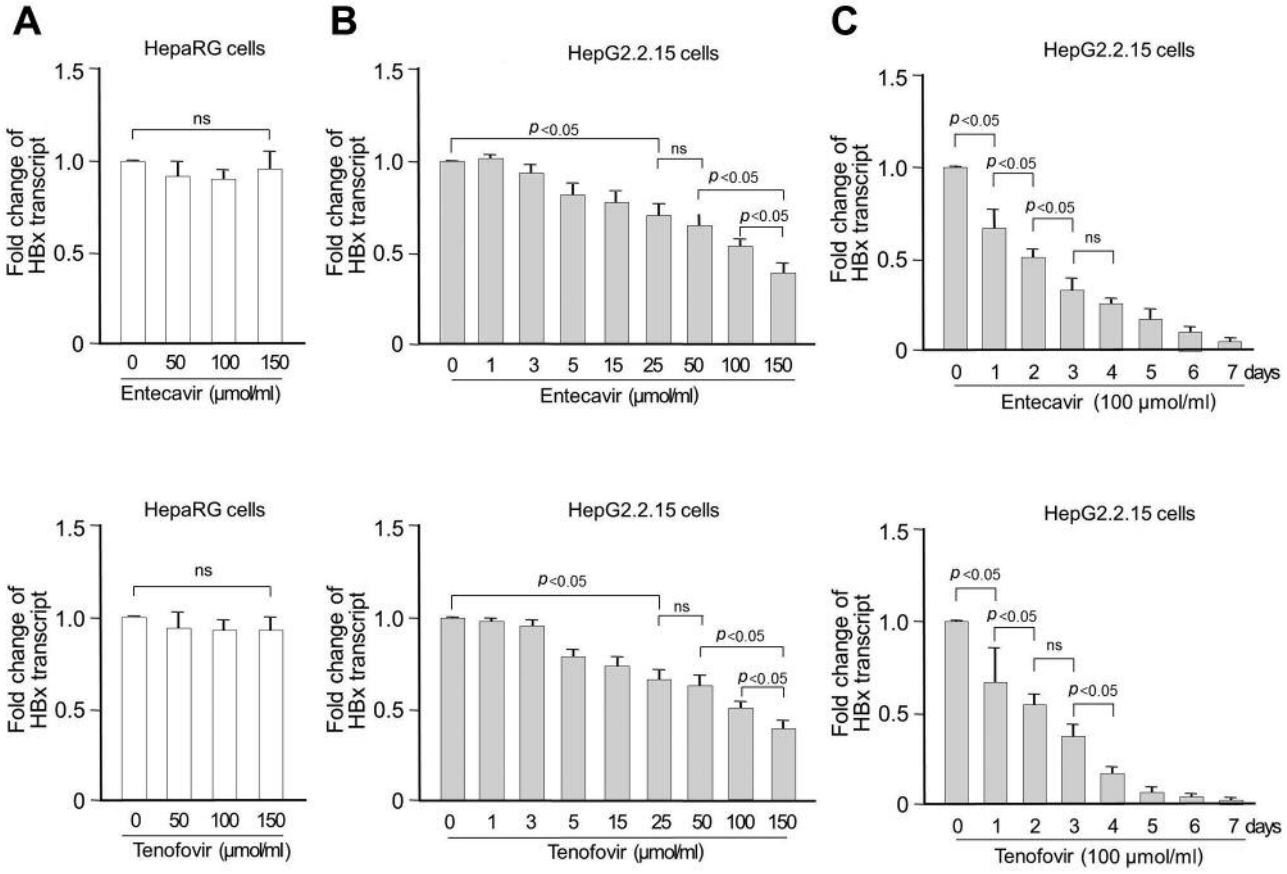

Figure 4. *Expression of hepatitis B virus protein X (HBx) after administration of nucleos(t)ide analogs. Either entecavir or tenofovir was administered for 24 h at 0-150 µmol/ml to the HepaRG (A) and HepG2.2.15 hepatitis B virus-expressing (B) cell lines. Additionally, either entecavir or tenofovir at 100 µmol/ml was administered for 7 days to the HepG2.2.15 cell line (C). ns: Not significant.*

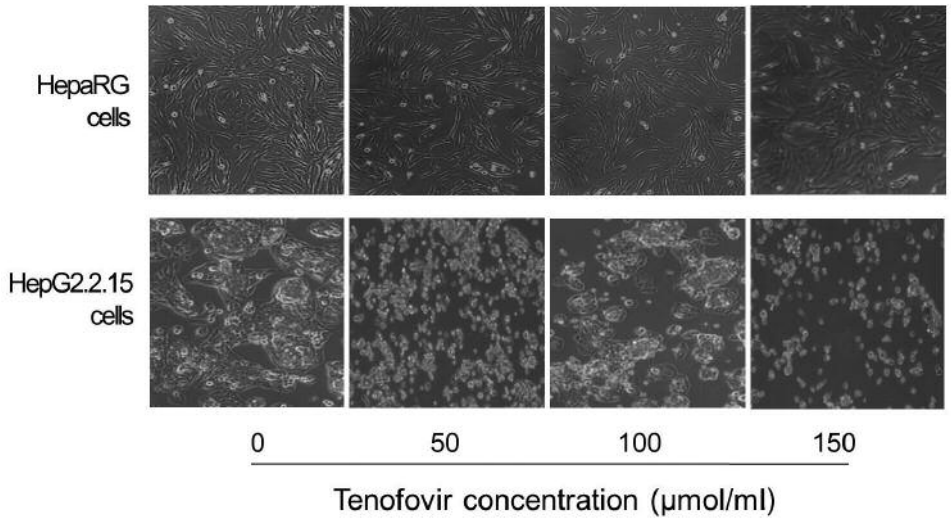

Figure 5. *Comparison of microscopic cell morphology of the HepaRG (upper) and HepG2.2.15 hepatitis B virus-expressing (lower) cell lines after administration of tenofovir at 0-150 μmol/ml for 24 h.*

150 μmol/ml (0.28-42 μg/ml); all $p>0.8$) (Figure 4A). In contrast, addition of either entecavir or tenofovir to the HepG2.2.15 cell line led to noticeable concentration-dependent down-regulation of *HBx* expression at dug concentrations ≥5 μmol/ml (1.4 μg/ml) for 24 h, but there were no changes at concentrations <5 μmol/ml (Figure 4B).

Daily measurement of *HBx* transcript levels showed that addition of either entecavir or tenofovir at a concentration of 100 μmol/ml (28 μg/ml) resulted in a time-dependent down-regulation of *HBx* transcription (all $p<0.05$) (Figure 4C). The patterns of down-regulation of entecavir and tenofovir (regarding the NUC concentration and treatment duration) were very similar.

Microscopic assessment of cell morphology revealed abundant apoptosis in the HepG2.2.15 cell line after addition of tenofovir (concentration ranging from 0 to 150 μmol/ml) for 24 h, but few changes were observed in the HepaRG cell line (Figure 5). The MTT assay for cell survival assessment showed a concentration-dependent decrease of cell survival [NUC concentration ≥50 μmol/ml (14 μg/ml)] was observed in the HepG2.2.15 cell line but not in the HepaRG cell line after addition of either entecavir or tenofovir for 24 hours (all $p<0.05$) (Figure 6A and B). Daily measurement of MTT assays showed that addition of either entecavir or tenofovir at a concentration of 100 μmol/ml (28 μg/ml) resulted in a time-dependent decrease of HepG2.2.15 cell survival (all $p<0.05$) (Figure 6A), showing no noticeable differences in the effect between entecavir and tenofovir, although the *in vivo* therapeutic ranges of these two drugs were quite different.

Apoptosis was assessed by staining for total PARP, cleaved PARP, and cleaved caspase-3 on treatment of cells with a range of NUC concentration ≥50 μmol/ml (14 μg/ml). These experiments showed that concentration-dependent up-regulation of apoptosis was consistently observed in the HepG2.2.15 cell line after addition of either entecavir or tenofovir for 24 h, but no noticeable changes were observed in the HepaRG cell line (Figure 7).

Effect of HBIg on the HepG2.2.15 cell line. In the HepaRG cell line, *HBx* transcript was not influenced by the administration of a high concentration of HBIgs for 24 h (polyvalent anti-HBs at 200, 400 and >800 mIU/ml and monoclonal anti-HBs at 2,000, 4,000, and >5,000 mIU/ml; all $p>0.7$). Addition of a high concentration of HBIgs to the HepG2.2.15 cell line also did not noticeably down-regulate *HBx* transcription (polyvalent anti-HBs at 200, 400, and >800 mIU/ml and monoclonal anti-HBs at 2,000, 4,000, and >5,000 mIU/ml; all $p>0.8$) in both HepaRG and HepG2.2.15 cell lines. Microscopic cell morphological analysis showed no noticeable induction of apoptosis. MTT assays also did not show any noticeable decrease in cell survival ($p>0.7$).

Discussion

The results of our three consecutive studies suggest that several cascade mechanisms are involved in the effects of NUCs. In part I of this study, NUC administration in the clinical setting effectively reduced the HBV DNA load in both blood and liver tissues. HBV cccDNA load in the liver tissue also decreased along with HBV DNA load in the liver tissue, but HBV eradication was not achieved. In part II of this study, HBV-associated HCC cells presented definitely

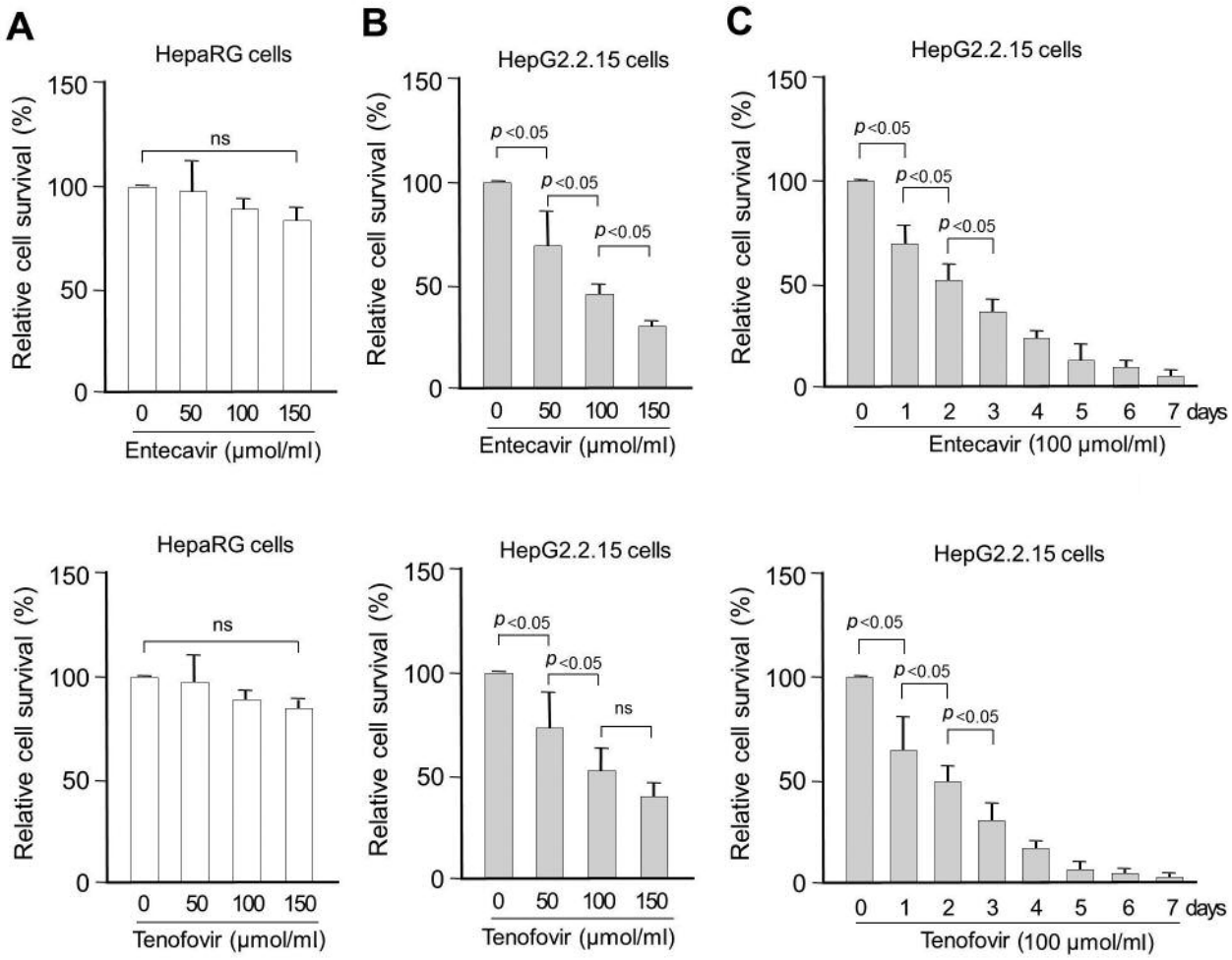

Figure 6. *Assessment of cell survival assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after administration of nucleos(t)ide analogs. Either entecavir or tenofovir was administered for 24 h at 0-150 μmol/ml to the HepaRG (A) and HepG2.2.15 HBV-expressing (B) cell lines. Additionally, either entecavir or tenofovir at 100 μmol/ml was administered for 7 days to the HepG2.2.15 cell line (C). ns: Not significant.*

higher expression of HBV cccDNA and *HBx* than the adjacent non-tumor liver tissues, suggesting that such large increases are closely associated with hepatocarcinogenesis. In part III of this study, a very high concentration of NUCs induced the down-regulation of *HBx* transcript and apoptosis in the cccDNA-expressing liver tumor cell line, which was dependent on NUC concentration and treatment duration. Briefly, NUCs at *in vivo* therapeutic concentrations reduces viral loads in both liver tissues and blood, but only a very high concentration of NUCs appears to reduce the HBV cccDNA load and HBx production in the liver tumor cells through induction of tumor cell apoptosis. It is well-known that standard NUC therapy can reduce the HBV viral load and thereby reduce the risk of hepatocarcinogenesis (1-8), but its direct antitumor effect on pre-existing or replicating cccDNA-expressing liver tumor cells themselves has not been reported yet, probably because the *in vivo* therapeutic concentration of NUCs was too low to induce such a unique antitumor effect.

The results of part I of this study showed that there are linear correlations among serum HBV DNA, liver tissue HBV DNA, and liver tissue HBV cccDNA. Even if the serum HBV DNA load becomes lower than the clinical detection limit after long-term NUC therapy, the liver tissues still carry a low HBV DNA load, as well as persistent HBV cccDNA, although these viral loads are markedly decreased. These findings are consistent with previous studies indicating that prolonged NUC therapy cannot eradicate cccDNA in hepatocytes (9,10). The sustained NUC-induced virological response is beneficial for preventing deterioration of liver function and progression of liver fibrosis in patients with CHB. Potent suppression of HBV replication with NUC

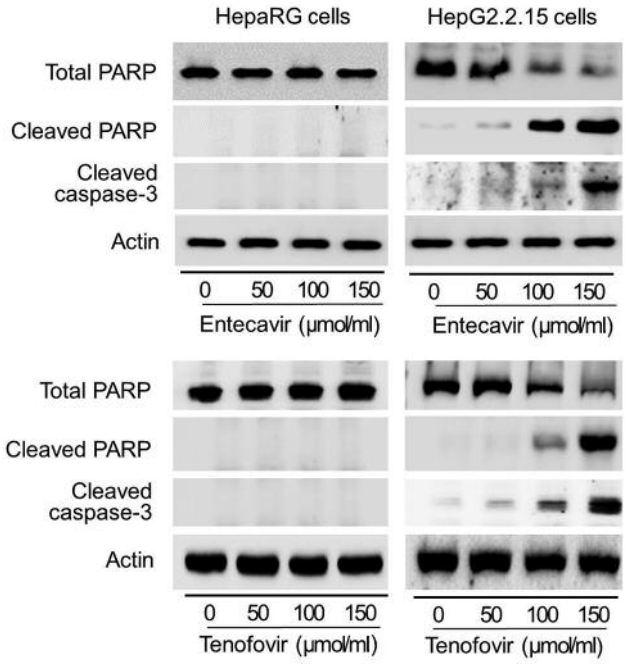

Figure 7. *Apoptosis in the HepaRG (left) and HepG2.2.15 hepatitis B virus-expressing (right) cell lines assessed by staining for poly-ADP-ribose polymerase (PARP), cleaved PARP, and cleaved caspase-3 after administration of entecavir and tenofovir for 24 h.*

therapy also effectively prevents HCC recurrence and *de novo* occurrence (1-8). However, the incidence of HCC is still relatively high in patients with CHB with NUC therapy-induced HBV DNA-negative seroconversion and even after HBsAg seroclearance (17-19). Based on our previous report of 5,374 patients with CHB treated with NUCs, *de novo* HCC developed in 9.8% during follow-up of up to 6 years (20). Although it cannot block hepatocarcinogenesis completely, hgbNUC administration is highly recommended from the viewpoints of both oncological and hepatic function (21).

The results of part II of this study support the contention that HBV within HCC tumors replicates in conjunction with tumor growth in patients with CHB or after LT (11, 22). After our random selection of 13 HCC resection samples without consideration of the blood HBV DNA load, there were universal high increases in HBV cccDNA (thousand-fold) and HBx (hundred-fold) in the HCC tissues compared with the paired surrounding non-tumor liver tissues. These results were even seen in the six patients with undetectable serum levels of HBV DNA in the clinical hospital laboratory setting after long-term NUC therapy or as a natural course. Thus, HBV-associated carcinogenesis is closely associated with marked up-regulation of *HBx* and HBV cccDNA (23,24).

The results of part III of this study strongly suggested that only therapy with a very high concentration of NUC can down-regulate *HBx* and apoptosis in HBV-expressing liver tumor cells, although the underlying mechanisms were not clearly elucidated. Although NUC therapy has been frequently performed in patients with CHB who undergo HCC treatment, such a direct NUC-associated antitumor effect has not been reported before probably due to a too low NUC concentration. We speculate that a very strong antiviral effect from very high concentration NUC therapy greatly contributes to this unique antitumor effect. This finding indicates that a high concentration of NUCs may have an unknown potential to kill replicating HCC cells expressing HBV or cccDNA. The results of this study revealed that such high-concentration NUC-induced antitumor activity is evident because only a small proportion of liver tumor cells survived after such therapy for 7 days in our research laboratory setting. Therefore, we presume that a very high concentration of NUCs has an antiviral activity-associated antitumor potential.

In part III of this study, we initially tested intravenous HBIg, which contains highly purified polyvalent anti-HBIg. HBIg combines with the 'a' determinant region of the major hydrophilic region of HBV DNA to induce antibody-dependent cell-mediated cytotoxicity (25). It is generally accepted that HBIg has no antiviral effect on HBV cccDNA in the hepatocyte nucleus. As expected, we did not observe any antitumor effect of HBIg on the cccDNA-expressing liver tumor cell line. We subsequently used the newly developed recombinant monoclonal Hepabig-Gene to increase the anti-HBs concentration by more than 10-fold but no significant effects were found. Thus, we confirmed that high-concentration HBIg, similarly to standard-dose NUC, has no antitumor effects.

Active replication of HBV in HCC cells acting as a viral reservoir has been well recognized in the clinical sequence of HCC recurrence and treatment after LT (11). In one patient who presented with both HBV and HCC recurrences, HBsAg disappeared after resection of adrenal metastasis but reappeared after the second extrahepatic tumor recurrence. In this patient, HBV DNA was detected in the adrenal metastasis but not in the liver graft. It is generally accepted that HCC recurrence is a risk factor for post-transplant HBV recurrence (11, 22, 26). We previously reported that 24.2% of LT recipients with post-transplant HCC recurrence were also HBsAg-positivite despite vigorous combination therapy with high-dose HBIg and NUC (27). Thus, potent HBV prophylactic therapy with HBIg and hgbNUC is strongly recommended in LT recipients with a high risk of HCC recurrence (22, 28).

In contrast, unlike in LT, the incidence of HBV DNA-positive seroconversion at the time of HCC recurrence after surgical resection was lower than expected in CHB patients

who showed sustained virological response to NUC therapy. In our previous study of the resection of solitary HCC in 2,558 patients (29), more than 90% of 2,117 of patients with HBV-associated HCC became HBV DNA-negative during postoperative follow-up following vigorous NUC therapy. After excluding the patients who exhibited a sustained post-resection virological response for more than 6 months, HCC recurrence after post-resection for 6 months and use of hgbNUC for more than 6 months, finally 576 patients were selected. Of these cases, only 28 patients (4.9%) exhibited serum HBV DNA positivity at the time of first HCC recurrence and 50% exhibited re-disappearance of HBV DNA positivity after treatment for HCC recurrence. These findings support the following two hypotheses: firstly, that hgbNUCs are sufficiently potent to make blood HBV DNA undetectable in most patients with CHB, even at HCC recurrence; and secondly, that NUCs damage HBV-infected tumor cells, causing them to lose their ability to propagate. NUC-induced apoptosis of HBV-infected liver tumor cells appears to be unusual considering the natural history of HBV-infected hepatocytes, but it was also reported that HBV particles released from apoptotic hepatocytes are immature, non-enveloped and not infectious, thus preventing HBV propagation (30).

However, our recent study revealed that hgbNUC therapy cannot suppress the viral replication effectively during rapid HCC progression (31). Ten patients with advanced HCCs with detectable blood HBV DNA were administered tenofovir up to 5-fold of the recommended dosage for 4-8 weeks. Three patients did not tolerate the side-effects of high-dose tenofovir and another seven patients persistently had detectable HBV DNA in their blood and tumor progression despite high-dose tenofovir therapy. Because of these negative results, the clinical study was terminated early.

The antitumor effect induced by a very high concentration of NUC must be a novel finding, but it is not possible to achieve such high concentrations in human peripheral blood because the required drug dosage is more than 1000-fold for entecavir and 50-fold for tenofovir. The majority of our patients barely tolerated even the 5-fold dosage of tenofovir (31). It is very unique that the therapeutic range of concentration for entecavir and tenofovir are quite different, but their *in vitro* concentration-dependent antitumor effects were very similar. From the viewpoint of patient safety, daily ingestion of 1000-fold dosage of entecavir could never be considered for clinical application. Of course, ingestion of 50 tablets of 300 mg tenofovir is also not possible in practice. In contrast, local NUC delivery into HCC tumor is feasible through a transcatheter arterial approach. For example, if the tumor diameter is 5 cm (37 ml in volume), only 0.1 mg of tenofovir is theoretically necessary to achieve an intratumoral concentration of 42 μg/ml. Considering a daily intake of 300 mg of tenofovir, such a conventional amount may not induce adverse side-effects regardless of administration route.

However, prolonged maintenance of a very high concentration of NUC in HCC tumors is very difficult. At first we thought that the intravenous form of tenofovir is mixed with embolic material during transcatheter arterial chemoembolization (TACE), by which NUC is directly delivered into the HCC tumor. We simulated this method with the clinical backgrounds learned from TACE, in which we recognized that it may also be difficult to maintain the high concentration of tenofovir within the tumor for a few days because a majority of infused tenofovir will be washed out from the tumor within several hours. Next to conventional TACE, we simulated the use of TACE with drug-eluting beads (DEB), in which the negatively charged acrylate of microsphere beads interacts with positively charged doxorubicin during he loading process and the loaded doxorubicin is eluted by a reversible ionic exchange mechanism. According to a controlled study comparing conventional TACE and DEB-TACE (32), pharmacokinetic assays revealed that the peak drug concentration of doxorubicin was reached within 5 min after injection in all cases, but it was 12-fold higher in patients treated by conventional TACE than in patients in the DEB-TACE group. The blood concentration area under the curve during the first 7 days was 2.3-fold higher in the conventional TACE group than in the DEB-TACE group. Thus DEB-TACE appears to be a potential candidate for delivery high-concentration NUC into HCC tumors. However, at this time, we do not know whether the intravenous form of tenofovir is positively charged, hence we also do not know whether NUCs interact well with negatively charged beads.

There is another type of bead commercially available, superabsorbent polymer microspheres, which absorb fluid and swell when exposed to aqueous media (33). These particles differ from the usual DEBs in that they are soft and deformable and conform to the lumen of embolized vessels. In addition, they can absorb several chemotherapeutic drugs that can be released from the microsphere, by which many drugs can be used through diffusion regardless of electrical charge. In a rabbit model with hepatic VX2 tumors, intra-arterial administration of superabsorbent polymer microspheres loaded with doxorubicin showed that intratumoral doxorubicin fluorescence was detected at all time-points up to 14 days, but only on the first day after treatment in the conventional TACE group (34). Therefore, we presume that superabsorbent polymer microspheres are more suitable than DEBs because they may enable the sustained release of a high concentration of NUC to HCC tumors.

We feel that our concept of high-concentration NUC therapy through sustained release of NUC into HCC tumors is worthy of performing further *in vivo* studies with animal models, which may be similar to the abovementioned study

using a rabbit model (34). From the viewpoint of safety regarding potential adverse side-effects, we believe tenofovir may be superior to entecavir. We expect that synergistic antitumor effects of the chemotherapeutic agent and high-concentration NUC will develop. If safety and antitumor effect are shown in future animal studies, it will bring us closer to performance of clinical trials.

There were several limitations to this study. Firstly, the analyses consisted of three small separate consecutive studies, thus consistent clinical outcomes were not provided. Secondly, the high-concentration NUC-induced antitumor effect was demonstrated only in a cccDNA-expressing liver tumor cell line, not in actual HCC cells obtained from patients. Thirdly, most of our patients with HCC had already been administered hgbNUCs, thus the number of patients showing the natural sequence of HBV infection was small.

In conclusion, high-concentration hgbNUCs induced apoptosis of HBV-expressing liver tumor cells, supporting the possibility that high-concentration NUCs, in accordance with their potent HBV suppression capacity, may have a novel potential to kill replicating HCC cells. Further studies are necessary to validate the potential oncological benefit of using high-concentration NUCs against HCC cells.

Conflicts of Interest

The Authors declare no competing interests with respect to this study.

Funding

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (grant no. 2015R1A2A2A04007141 to S. Hwang and 2015K1A4A3046807 to G. W. Song) and by research funding from the Asan Institute for Life Sciences (2011-474 to C.S. Ahn).

References

1 Wu CY, Lin JT, Ho HJ, Su CW, Lee TY, Wang SY, Wu C and Wu JC: Association of nucleos(t)ide analogue therapy with reduced risk of hepatocellular carcinoma in patients with chronic hepatitis B-A nationwide cohort study. Gastroenterology *147*: 143-151.e5, 2014.

2 Sung JJ, Tsoi KK, Wong VW, Li KC and Chan HL: Meta-analysis: Treatment of hepatitis B infection reduces risk of hepatocellular carcinoma. Aliment Pharmacol Ther *28*: 1067-1077, 2008.

3 Kumada T, Toyoda H, Tada T, Kiriyama S, Tanikawa M, Hisanaga Y, Kanamori A, Niinomi T, Yasuda S, Andou Y, Yamamoto K and Tanaka J: Effect of nucleos(t)ide analogue therapy on hepatocarcinogenesis in chronic hepatitis B patients: a propensity score analysis. J Hepatol *58*: 427-433, 2013.

4 Hosaka T, Suzuki F, Kobayashi M, Seko Y, Kawamura Y, Sezaki H, Akuta N, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Kobayashi M and Kumada H: Long-term entecavir treatment reduces hepatocellular carcinoma incidence in patients with hepatitis B virus infection. Hepatology *58*: 98-107, 2013.

5 Wu CY, Chen YJ, Ho HJ, Hsu YC, Kuo KN, Wu MS and Lin JT: Association between nucleoside analogues and risk of hepatitis B virus–related hepatocellular carcinoma recurrence following liver resection. JAMA *308*: 1906-1914, 2012.

6 Marcellin P, Gane E, Buti M, Afdhal N, Sievert W, Jacobson IM, Washington MK, Germanidis G, Flaherty JF, Aguilar Schall R, Bornstein JD, Kitrinos KM, Subramanian GM, McHutchison JG and Heathcote EJ: Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. Lancet *381*: 468-475, 2013.

7 Buti M, Fung S, Gane E, Afdhal NH, Flisiak R, Gurel S, Flaherty JF, Martins EB, Yee LJ, Dinh P, Bornstein JD, Mani Subramanian G, Janssen HL, George J and Marcellin P: Long-term clinical outcomes in cirrhotic chronic hepatitis B patients treated with tenofovir disoproxil fumarate for up to 5 years. Hepatol Int *9*: 243-250, 2015.

8 Baran B: Nucleos(t)ide analogs in the prevention of hepatitis B virus related hepatocellular carcinoma. World J Hepatol *7*: 1742-1754, 2015.

9 Guo JT and Guo H: Metabolism and function of hepatitis B virus cccDNA: Implications for the development of cccDNA-targeting antiviral therapeutics. Antiviral Res *122*: 91-100, 2015.

10 Thursz M: Basis of HBV persistence and new treatment options. Hepatol Int *8(Suppl 2)*: 486-491. 2014.

11 Faria LC, Gigou M, Roque-Afonso AM, Sebagh M, Roche B, Fallot G, Ferrari TC, Guettier C, Dussaix E, Castaing D, Brechot C and Samuel D: Hepatocellular carcinoma is associated with an increased risk of hepatitis B virus recurrence after liver transplantation. Gastroenterology *134*: 1890-1899, 2008.

12 Tarocchi M, Polvani S, Marroncini G and Galli A: Molecular mechanism of hepatitis B virus-induced hepatocarcinogenesis. World J Gastroenterol *20*: 11630-11640, 2014.

13 Sells MA, Chen ML and Acs G: Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci USA *84*: 1005-1009, 1987.

14 Bloom K, Ely A, Mussolino C, Cathomen T and Arbuthnot P: Inactivation of hepatitis B virus replication in cultured cells and *in vivo* with engineered transcription activator-like effector nucleases. Mol Ther *21*: 1889-1897, 2013.

15 Takkenberg RB, Zaaijer HL, Menting S, Weegink CJ, Terpstra V, Cornelissen M, Dijkgraaf MG, Jansen PL, Reesink HW and Beld MG: Detection of hepatitis B virus covalently closed circular DNA in paraffin-embedded and cryo-preserved liver biopsies of chronic hepatitis B patients. Eur J Gastroenterol Hepatol *22*: 952-960, 2010.

16 Song GW, Ahn CS, Lee SG, Hwang S, Kim KH, Moon DB, Ha TY, Jung DH, Park GC1, Kang SH, Jung BH and Kim N: Correlation between risk of hepatitis B virus recurrence and tissue expression of covalently closed circular DNA in living donor liver transplant recipients treated with high-dose hepatitis B immunoglobulin. Transplant Proc *46*: 3548-3553, 2014.

17 Cho JY, Paik YH, Sohn W, Cho HC, Gwak GY, Choi MS, Lee JH, Koh KC, Paik SW and Yoo BC: Patients with chronic hepatitis B treated with oral antiviral therapy retain a higher risk for HCC compared with patients with inactive stage disease. Gut *63*: 1943-1950, 2014.

18 Young AL, Adair R, Prasad KR, Toogood GJ and Lodge JP: Hepatocellular carcinoma within a noncirrhotic, nonfibrotic, seronegative liver: surgical approaches and outcomes. J Am Coll Surg *214*: 174-183, 2012.
19 Kim GA, Lee HC, Kim MJ, Ha Y, Park EJ, An J, Lee D, Shim JH, Kim KM and Lim YS: Incidence of hepatocellular carcinoma after HBsAg seroclearance in chronic hepatitis B patients: a need for surveillance. J Hepatol *62*: 1092-1099, 2015.
20 Lim YS, Han S, Heo NY, Shim JH, Lee HC and Suh DJ: Mortality, liver transplantation, and hepatocellular carcinoma among patients with chronic hepatitis B treated with entecavir vs lamivudine. Gastroenterology *147*: 152-161, 2014.
21 Kim GA, Lim YS, An J, Lee D, Shim JH, Kim KM, Lee HC, Chung YH, Lee YS and Suh DJ: HBsAg seroclearance after nucleoside analogue therapy in patients with chronic hepatitis B: clinical outcomes and durability. Gut *63*: 1325-1332, 2014.
22 Hwang S, Ahn CS, Song GW, Kim KH, Moon DB, Oh HB, Lim YS, Lee HC, Ha TY, Jung DH, Chung YH and Lee SG: Posttransplantation prophylaxis with primary high-dose hepatitis B immunoglobulin monotherapy and complementary preemptive antiviral add-on. Liver Transpl *17*: 456-465, 2011.
23 Seeger C and Mason WS: Molecular biology of hepatitis B virus infection. Virology *479-480*: 672-686, 2015.
24 Tian Y, Yang W, Song J, Wu Y and Ni B: Hepatitis B virus X protein-induced aberrant epigenetic modifications contributing to human hepatocellular carcinoma pathogenesis. Mol Cell Biol *33*: 2810-2816, 2013.
25 Park GC, Hwang S, Ahn CS, Kim KH, Moon DB, Ha TY, Song GW, Jung DH, Shin YW, Kim SH, Chang KH, Namgoong JM, Park CS, Park HW, Park YH, Kang SH, Jung BH and Lee SG: Analysis of S gene mutation of the hepatitis B virus in adult liver transplant recipients showing resistance to hepatitis B immunoglobulin therapy. Transplant Proc *45*: 3047-3051, 2013.
26 Chun J, Kim W, Kim BG, Lee KL, Suh KS, Yi NJ, Park KU, Kim YJ, Yoon JH and Lee HS: High viremia, prolonged Lamivudine therapy and recurrent hepatocellular carcinoma predict posttransplant hepatitis B recurrence. Am J Transplant *10*: 1649-1659, 2010.
27 Hwang S, Lee SG, Ahn CS, Kim KH, Moon DB, Ha TY, Song GW, Jung DH, Park JI, Ryu JH, Lee HJ, Suh DJ and Lim YS: Prevention of hepatitis B recurrence after living donor liver transplantation: primary high-dose hepatitis B immunoglobulin monotherapy and rescue antiviral therapy. Liver Transpl *14*: 770-778, 2008.
28 Gish R, Jia JD, Locarnini S and Zoulim F: Selection of chronic hepatitis B therapy with high barrier to resistance. Lancet Infect Dis *12*: 341-353, 2012.
29 Hwang S, Lee YJ, Kim KH, Ahn CS, Moon DB, Ha TY, Song GW, Jung DH, Lee SG: The impact of tumor size on long-term survival outcomes after resection of solitary hepatocellular carcinoma: single-institution experience with 2558 patients. J Gastrointest Surg *19*: 1281-1290, 2015.
30 Arzberger S, Hösel M and Protzer U: Apoptosis of hepatitis B virus-infected hepatocytes prevents release of infectious virus. J Virol *84*: 11994-12001, 2010.
31 Hwang S, Song GW, Jung DH, Yoon YI and Tak E: High-dose tenofovir is not effective to suppress hepatitis B virus replication in patients with hepatocellular carcinoma progression: a preliminary result. Korean J Hepatobiliary Pancreat Surg *20*: 8-11, 2015.
32 Varela M, Real MI, Burrel M, Forner A, Sala M, Brunet M, Ayuso C, Castells L, Montañá X, Llovet JM and Bruix J: Chemoembolization of hepatocellular carcinoma with drug eluting beads: efficacy and doxorubicin pharmacokinetics. J Hepatol *46*: 474-481, 2007.
33 Khankan AA, Osuga K, Hori S, Morii E, Murakami T and Nakamura H: Embolic effects of superabsorbent polymer microspheres in rabbit renal model: comparison with tris-acryl gelatin microspheres and polyvinyl alcohol. Radiat Med *22*: 384-390, 2004.
34 Gupta S, Wright KC, Ensor J, Van Pelt CS, Dixon KA and Kundra V: Hepatic arterial embolization with doxorubicin-loaded superabsorbent polymer microspheres in a rabbit liver tumor model. Cardiovasc Intervent Radiol *34*: 1021-1030, 2011.

Received March 17, 2016
Revised April 24, 2016
Accepted April 26, 2016