Construction and Characterization of an Anti-Hepatitis B Virus preS1 Humanized Antibody that Binds to the Essential Receptor Binding Site

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Hepatitis B virus (HBV) is a major cause of liver cirrhosis and hepatocellular carcinoma. With recent identification of HBV receptor, inhibition of virus entry has become a promising concept in the development of new antiviral drugs. To date, 10 HBV genotypes (A–J) have been defined. We previously generated two murine anti-preS1 monoclonal antibodies (mAbs), KR359 and KR127, that recognize amino acids (aa) 19–26 and 37–45, respectively, in the receptor binding site (aa 13–58, genotype C). Each mAb exhibited virus neutralizing activity in vitro, and a humanized version of KR127 effectively neutralized HBV infection in chimpanzees. In the present study, we constructed a humanized version (HzKR359-1) of KR359 whose antigen binding activity is 4.4-fold higher than that of KR359, as assessed by competitive ELISA, and produced recombinant preS1 antigens (aa 1–60) of different genotypes to investigate the binding capacities of HzKR359-1 and a humanized version (HzKR127-3.2) of KR127 to the 10 HBV genotypes. The results indicate that HzKR359-1 can bind to five genotypes (A, B, C, H, and J), and HzKR127-3.2 can also bind to five genotypes (A, C, D, G, and I). The combination of these two antibodies can bind to eight genotypes (A–D, G–J), and to genotype C additively. Considering that genotypes A–D are common, whereas genotypes E and F are occasionally represented in small patient population, the combination of these two antibodies might block the entry of most virus genotypes and thus broadly neutralize HBV infection.

Keywords: Hepatitis B virus, preS1, virus entry, humanized antibody, genotypes, binding activity

Introduction

Hepatitis B virus (HBV) is a small, enveloped, partially double-stranded DNA virus with strict tropism to human hepatocytes [1, 2]. Hepatitis B viral infection attacks the liver and can cause both acute and chronic diseases [2]. An estimated 240 million people are chronically infected with hepatitis, and over one million people die every year owing to complications of hepatitis B, including cirrhosis and liver cancer [3, 4]. Hepatitis B prevalence is highest in sub-Saharan Africa and East Asia, where between 5% and 10% of the adult population is chronically infected [5, 6].

The genotypes of HBV are defined by an intergroup divergence of more than 8% in the entire genome sequence [7, 8]. To date, 10 distinct genotypes (A–J), which show a distinct geographic distribution, have been described [9-12]. Genotypes A and D are ubiquitous but most prevalent in Europe and Africa, whereas genotypes B and C are confined to Asia and Oceania. Genotypes E, F, G, and H are also occasionally observed in Asia. Though rare, genotype I is found in Laos, Vietnam, India, and China, and Genotype J has been described in Japan. Genotypes A–D are common and constitute 97% of the total patient population, whereas genotypes E–G represent less than 2% [13, 14].
The hepatotropism of HBV is partially attributed to the entry step mediated by viral envelope proteins. The HBV envelope contains three membrane glycoproteins, namely the large, middle, and small surface proteins, which are translated from a single open reading frame via different in-frame start codons. The small surface protein is the major component of the viral envelope. The middle protein has an extra N-terminal preS2 region, whereas the large protein, on the basis of the middle protein, harbors an additional preS1 region of 108 aa (genotypes D and J) or 119 aa (the other subtypes) [15, 16]. Cell entry of HBV presumably begins with reversible, low-affinity attachment of HBV to the cell membrane via the interaction between viral envelope proteins and heparin sulfate proteoglycan, followed by high-affinity binding of viral envelope proteins to a specific receptor, the bile-acid pump sodium-taurocholate cotransporting polypeptide (NTCP) [17–20]. NTCP is an integral membrane protein that is exclusively expressed on the basolateral membrane of hepatocytes, which explains the tropism of HBV for the liver. Multiple lines of evidence have indicated that preS1, in particular, the N-terminal half (aa 2–47, genotype D) and the myristoyl moiety at its amino terminus, is critical for the binding of viral particle to NTCP, whereas aa 9–18 of the preS1 component (genotype D) are essential for HBV attachment and subsequent infection [21, 22]. Therefore, inhibition of virus entry that can neutralize the activities of viral surface proteins or target the cellular receptors has become a major concept in the development of new antiviral drugs.

Monoclonal antibodies (mAbs) have great potential for both diagnostic and therapeutic applications [23]. Murine mAbs are easy to produce, but their therapeutic use in humans is limited because of the human anti-mouse antibody response during treatment [24]. To obviate this problem, humanized antibodies have been constructed by grafting the complementarity determining regions (CDRs) of murine mAbs, which form an antigen-binding pocket, onto homologous human antibody variable domains, while retaining some murine residues in framework regions (FRs) that are predicted to influence the conformation of CDRs. This humanization technique is referred to as CDR grafting [25, 26].

We previously generated two murine mAbs, KR359 and KR127, which recognize aa 19–26 and 37–45 of preS1 (genotype C), respectively [27], and demonstrated that they exhibit neutralizing activity in infection of human hepatocyte primary culture by HBV in vitro [28]. Subsequently, we constructed a humanized version (HzKR127) of KR127 by CDR grafting and provided evidence that this humanized antibody can neutralize HBV infection in chimpanzees [29]. In addition, we developed an improved version (HzKR127-3.2) of HzKR127 through further humanization and affinity maturation [30]. In the present study, we constructed a humanized version (HzKR359-1) of KR359 via the CDR-grafting method and characterized its binding capacities to the preS1 region of HBV genotypes (A–J). In addition, we analyzed the binding capacities of HzKR127-3.2 and combined binding activities of HzKR359-1 and HzKR127-3.2 to the preS1 region of HBV genotypes. The results indicate that the combination of these two humanized antibodies can bind to most HBV genotypes and thus may broadly neutralize HBV infection.

### Materials and Methods

#### Cell Culture

Murine hybridoma KR359 [27] cells were routinely cultured at 5% CO₂, 37°C in IMDM (Gibco: Thermo Fisher Scientific, Inc., USA) supplemented with 10% (v/v) fetal bovine serum (Atlas, USA). Suspension-adapted HEK293F cells (Invitrogen; Thermo Fisher Scientific) were cultured in FreeStyle 293 Expression medium (Gibco) in 125 ml Erlenmeyer flasks (Corning, USA) at 125 rpm on a shaker (N Biotek, Korea) in a humidified 37°C and 8% CO₂ atmosphere.

#### Construction of Humanized Antibody HzKR359-1

For the cloning of the cDNAs encoding the mouse heavy chain variable region (V_{H}) or κ light chain variable region (V_{κ}) of KR359 antibody (IgG1, κ), total RNA was isolated from the KR359 hybridoma, and the first-strand cDNA was synthesized from the total RNA using reverse transcriptase (Superscript II, Thermo Fisher Scientific) and the 5′-primers specific to the γ1 heavy chain (C_{H},1) or κ light chain (C_{κ}) constant region of KR359. The cDNA templates were subjected to PCR using the 5′-primer specific to the N-terminal sequence of V_{H} or V_{κ} of KR359 and the 3′-primer specific to the mouse C_{H},1 or C_{κ}. The resulting PCR products were cloned into the pBluescript vector to determine their nucleotide sequences.

For the construction of a humanized version of KR359, the amino acid sequences of the mouse V_{H} and V_{κ} were compared with those of human immunoglobulin germline segments in IMGT (http://www.imgt.org), and the most homologous V_{H} (VH4-59) and V_{κ} (VK1-39) germline segments were selected. To construct a humanized V_{H}, the CDRs (HCDR1–HCDR3) of the mouse V_{H} were grafted onto the human VH4-59 germline segment and JH4, and 13 mouse FR residues that were predicted to influence the conformation of HCDRs were also grafted. To construct a humanized V_{κ}, the CDRs (LCDR1–LCDR3) of the mouse V_{κ} were grafted onto the human VK1-39 germline gene segment and JK4. The resulting humanized V_{H} or V_{κ} sequence was synthesized and
subcloned into the EcoRI-ApaI or HindIII-BsiWI sites, respectively, of pCMV-dhfrC [29] containing human C1 or Cκ sequences, respectively, to construct heavy or light chain expression plasmids, respectively.

Expression and Purification of Recombinant Human Antigen

The heavy and light chain expression plasmid DNAs were mixed with polyethyleneimine (PEI, linear 25 kDa; Polysciences, USA) at a ratio of 1:4 (60 μg:240 μg) and incubated at room temperature for 25 min, as described previously [31]. The mixture was added to 30 ml of HEK293F cells (1.0 × 10⁶ cells/ml) and cultured for 7 days. The culture supernatant was recovered by centrifugation at 2,300 × g for 30 min at 4°C and subjected to indirect ELISA and quantitative ELISA to determine its antigen-binding activity. For purification of the humanized antibody, the culture supernatant was subjected to affinity chromatography on Protein A-agarose beads (Amiconic, Inc., Korea). The antibody was eluted from the column with 0.1 M sodium citrate buffer (pH 3.2) and neutralized with 1.0 M Tris-HCl buffer. Finally, the buffer was changed to storage buffer (25 mM sodium citrate, 150 mM NaCl, pH 6.4) by using Vivaspin (MWCO 30,000; Sartorius, Germany), as described previously [32]. Antibody concentration was determined with a NANO-DROP 2000 spectrophotometer, and the integrity of the purified protein was assessed by SDS-PAGE and western blot analysis.

Construction, Expression, and Purification of Recombinant PreS1 Antigens

For the production of recombinant preS1 antigens of six HBV genotypes (B–G), the preS1 sequences (aa 1–60) of the HBV genotypes were individually synthesized and fused to the C-terminus of the Ig1-Ig5 domains of human L1 cell adhesion molecule (LICAM), whereas a Streptactin Superflow high capacity column (Iba, Germany), according to the protocol suggested by the supplier. Briefly, the column was equilibrated with buffer W (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) and the bound protein was eluted with 5 column volumes of buffer W supplemented with 2.5 mM dithiorebiotin. Finally, the buffer was changed to PBS. The protein concentration was determined using the NANO-DROP 2000 spectrophotometer, and the integrity of the purified protein was assessed by western blot analysis.

Western Blot Analysis

The purified HzKR359-1 antibody was analyzed by 10% SDS-PAGE under reducing conditions and 6% SDS-PAGE under non-reducing conditions, followed by Coomassie Brilliant Blue G-250 staining. The protein bands were transferred to a nitrocellulose membrane (GE Healthcare, France), and then the membrane was incubated with anti-human IgG(H+L)-HRP conjugate (1:10,000; Thermo Fisher Scientific). Finally, the bands were visualized using a chemiluminescent substrate (WEST-ZOL plus; iNtRON BioTechnology, Korea).

For the recombinant preS1 antigens, the purified proteins were subjected to 10% SDS-PAGE and western blot analysis with a murine mAb A10-A3 (0.5 μg/ml) that binds to the Ig1 domain of human LICAM [35], followed by anti-mouse IgG (Fc-specific)-HRP conjugate (1:6,000 (v/v); Thermo Fisher Scientific).

Enzyme-Linked Immunosorbent Assays

All the incubations were performed at 37°C for 1 h. For the quantitative ELISA of humanized antibody, antibody was serially diluted in PBST (PBS with 0.05% Tween 20) and incubated with goat anti-human IgG (Fc-specific) antibody (200 ng) and 50 mM NaHCO₃, pH 9.6; Thermo Fisher Scientific). The preS1 antigen was detected using anti-Strep-tag II antibody-HRP (1:10,000; Iba). For indirect ELISA of humanized antibody, antibody A10-A3 (200 ng) coated on each well of the 96-well Maxisorp plate (Nunc, Denmark). After washing, the bound humanized antibody was incubated with anti-human IgG (Fc-specific)-HRP conjugate (1:10,000; Thermo Fisher Scientific). For the quantitative ELISA of the recombinant preS1 antigen, the preS1 antigen was serially diluted and incubated with anti-LICAM antibody A10-A3 (200 ng) coated on each well, and then bound preS1 antigen was detected using anti-Strep-tag II antibody-HRP (1:8,000; Iba). For indirect ELISA of humanized antibody, serially diluted humanized antibody was incubated with the recombinant preS1 antigen (200 ng) coated on each well, and then the bound humanized antibody was incubated with anti-human IgG (Fc-specific)-HRP conjugate (1:10,000; Thermo Fisher Scientific). Finally, TMB substrate reagent (BD Biosciences, USA) was added and the reaction was stopped with 2.5 M H₂SO₄. The optical density was read at 450 nm in a microplate reader (Molecular Devices, USA).
mouse antibody was detected using anti-mouse IgG(Fc-specific)-HRP (1:6,000; Thermo Fisher Scientific). Herceptin was used as an irrelevant antibody control in this assay.

Analysis of Humanized Antibody Using Octet Red

The anti-human Fc-coated biosensor (Fortebio, Inc., USA) was activated in 0.1% PBA (PBS containing 0.1% BSA) for 20 min by agitating at 1,000 rpm. Antibody (1 µg/ml) was captured for 10 min and then washed with 0.1% PBA for 2 min. For affinity determination, the sensor-loaded antibody was incubated with the preS1 antigen (100, 50, 25, 12.5, or 6.25 nM in 0.1% PBA) for 10 min for the association step. Then, the dissociation step was carried out in 0.1% PBA for 30 min. The sensor without loading antibody was used as a reference well. The whole experiment process was performed at 30°C and by agitating at 1,000 rpm. Data were analyzed using a 1:1 interaction model with a ForteBio data analysis software ver. 7.1.

For the analysis of additive binding of two mAbs to the preS1 antigen, the sensor-loaded humanized antibody was incubated with the preS1 antigen (100, 50, or 25 nM in 0.1% PBA) for 10 min, and then incubated with mouse antibody binding to a different epitope. Baselines were established before and after the loading step.

Results and Discussion

Construction, Expression, and Purification of Humanized Antibody HzKR359-1

The cDNAs encoding the V\(^{\text{H}}\) and V\(^{\text{\kappa}}\) of KR359 were cloned from the hybridoma and their nucleotide sequences were determined, as described in Materials and Methods. Comparison of the amino acid sequences of the mouse V\(^{\text{H}}\) and V\(^{\text{\kappa}}\) with those of human immunoglobulin germline gene segments in IMGT showed that the mouse V\(^{\text{H}}\) and V\(^{\text{\kappa}}\) were the most homologous to human VH4-59 and VK1-39 germline genes, respectively.

To construct a humanized V\(^{\text{H}}\), the CDRs (HCDR1–HCDR3) of the mouse V\(^{\text{H}}\) were grafted onto the human VH4-59 and JH4, and 13 mouse FR residues that were predicted to influence the conformation of HCDRs were also grafted. Similarly, to construct a humanized V\(^{\text{\kappa}}\), the CDRs (LCDR1–LCDR3) of the mouse V\(^{\text{\kappa}}\) were grafted onto the human VK1-39 and JK4. The resulting humanized V\(^{\text{H}}\) and V\(^{\text{\kappa}}\) sequences were codon-optimized and synthesized, and subsequently separately subcloned into an antibody expression cassette vector pCMV-dhfrC containing human C\(^{\gamma}\)1 or C\(^{\kappa}\), respectively, to construct humanized heavy or light chain expression plasmids, respectively.

The expression plasmids were cotransfected into HEK293F cells, and the resulting humanized antibody (HzKR359-1) was transiently expressed and purified from the culture supernatant by affinity chromatography on a Protein A column. The purity and integrity of the purified antibody were confirmed by SDS-PAGE (Fig. 1A) and western blot analysis using anti-human IgG-HRP (Fig. 1B) under reducing and non-reducing conditions.

Construction, Expression, and Purification of Recombinant PreS1 Antigens of Six Different HBV Genotypes

To produce the recombinant preS1 antigens, the Ig1–Ig5 domains of human L1CAM were used as a carrier protein because these domains were found to be stably expressed in mammalian cells and secreted well in our previous study [35], and a Strep tag was fused to the C-terminus of

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![Fig. 1. SDS-PAGE and western blot analysis of purified HzKR359-1 antibody.](image-url)

(A) 10% SDS-PAGE under non-reducing (NR) or reducing (R) conditions of purified antibody. Bands that correspond to a heavy chain (~50 kDa), light chain (~25 kDa), and whole antibody (150 kDa) are shown. (B) Western blot analysis of purified antibody under non-reducing (NR, 6%) or reducing (R, 10%) conditions with anti-human IgG (H+L)-HRP.
preS1 for the detection and purification of the recombinant preS1 antigen (Fig. 2A). The amino acid sequences of the preS1 region (aa 1–60) of 10 HBV genotypes (A–J), including the essential NTCP-binding site (aa 20–29) and the epitopes of HzKR359-1 (aa 19–26) and HzKR127-3.2 (aa 37–45), are shown in Fig. 2B. Comparison of the epitope sequences among the genotypes indicated that five genotypes (A, B, C, H, and J) have the same epitope sequence of HzKR359-1, whereas three genotypes (A, C, and I) have the same epitope sequence of HzKR127-3.2. Therefore, the preS1 antigens of six HBV genotypes (B–G) were produced to examine the antigen-binding activity of HzKR359-1 or HzKR127-3.2 to all of the HBV genotypes (A–J).

For the production of the preS1 antigens, the preS1 sequences of the six genotypes with Strep tags were synthesized and individually fused to the C-terminus of the Ig1-Ig5 domains. The recombinant preS1 fusion proteins were transiently expressed in HEK293F cells, and the culture supernatants were subjected to quantitative ELISA using a murine anti-L1CAM mAb (A10-A3) as a coating antigen and anti-Strep Tag-HRP to measure their expression levels. As a result, the preS1 antigens of the genotypes B–D (2.4–4.2 µg/ml) were expressed at higher levels than those of genotypes E–G (0.7–0.9 µg/ml; data not shown). The preS1 antigens were purified from the culture supernatants using a Strep tag and subjected to western blot analysis using A10-A3 antibody. The result showed that the preS1 antigens were stably expressed and purified (Fig. 2C).

Analyses of Antigen-Binding Activity and Affinity of HzKR359-1

The antigen-binding activity of the purified HzKR359-1 antibody was analyzed by indirect ELISA using the purified preS1 antigen (genotype C) as a coating antigen. HzKR359-1 bound to the preS1 antigen in a dose-dependent manner, and its antigen-binding activity was slightly lower than that of HzKR127-3.2, which was developed through affinity maturation of a humanized KR127 antibody constructed by CDR grafting (Fig. 3A). The data indicate that the HzKR359-1 antibody was successfully constructed.

To compare the antigen-binding activity of HzKR359-1 with that of KR359, competitive ELISAs were performed. HzKR359-1 and increasing concentrations of KR359 as a competing antibody were incubated with the preS1 antigen (genotype C) coated on the well, and the bound humanized antibody was detected using anti-human IgG (Fc-specific)-HRP, whereas anti-HER2 humanized antibody (Herceptin) was used as an irrelevant antibody control (Fig. 3B). Similarly, KR359-1 and increasing concentrations of HzKR359-1 were incubated with the preS1 antigen, and the bound mouse antibody was detected using anti-mouse IgG-HRP (Fig. 3C). The results indicated that HzKR359-1 and KR359 competed...
for the same epitope, indicating that HzKR359-1 retained the same epitope specificity as that of KR359. The IC$_{50}$ of HzKR359-1 was calculated to be 0.37 µg/ml (Fig. 3C), whereas that of KR359 was 1.62 µg/ml (Fig. 3B), indicating that the antigen-binding activity of HzKR359-1 is higher than that of KR359.

To precisely compare the affinity of HzKR359-1 with that of HzKR127-3.2, the affinities of these antibodies for the preS1 antigen (genotype C) were measured using Octet Red. As shown in Fig. 4, the affinity (3.28 × 10$^{-9}$ M) of HzKR359-1 was 5.8-fold lower than that (5.69 × 10$^{-10}$ M) of HzKR127-3.2.

Analyses of the Antigen-Binding Activities of HzKR359-1 or HzKR127-3.2 to Different HBV Genotypes

Having analyzed the antigen-binding activities of HzKR359-1 and HzKR127-3.2 to the preS1 of genotype C, we further extended the analysis to other genotypes. Because the epitope sequences of HzKR359-1 and HzKR127-3.2 in genotype A are identical to those in genotype C, we...
exempted the expression of the preS1 antigen of genotype A. HzKR359-1 or HzKR127-3.2 was incubated with each of the purified recombinant preS1 antigens of genotypes (B–G), bound by anti-LICAM antibody (A10-A3) coated on the well, to perform indirect ELISA. The results showed that HzKR359-1 bound to the preS1 of genotypes B and C (Fig. 5A), whereas HzKR127-3.2 bound to genotypes C, D, and G (Fig. 5B). Accordingly, HzKR359-1 plus HzKR127-3.2 bound to genotypes B, C, D, and G (Fig. 5C). Considering that the epitope sequences of HzKR359-1 and HzKR127-3.2 in genotype A are identical to those in genotype C, HzKR359-1 can bind to the preS1 of three genotypes (A–C), whereas HzKR127-3.2 can bind to four genotypes (A, C, D, and G).

Given that the epitope sequences of HzKR359-1 in genotypes H and J are identical to those in genotypes A–C, HzKR359-1 is expected to bind to five genotypes (A–C, H, and J). Likewise, because the epitope sequence of HzKR127-3.2 in genotype I is identical to that in genotypes A and C, HzKR127-3.2 is predicted to bind to five genotypes (A, C, D, G, and I). Collectively, the combination of HzKR359-1 and HzKR127-3.2 is expected to bind to eight genotypes (A–D and G–J), except genotypes E and F. Considering that genotypes E and F are occasionally represented in small patient populations, the combination of HzKR359-1 and HzKR127-3.2 may bind to HBV particles of most genotypes.

**Additive Binding of HzKR359-1 and HzKR127-3.2 to Genotype C**

Because the epitope of HzKR359-1 is only 11 aa distant from that of HzKR127-3.2, we examined whether these two antibodies compete in binding to the preS1 antigen of genotype C, using competitive binding assay. As shown in Fig. 6A, HzKR359-1 or Herceptin as a negative control did not inhibit the binding between KR127 and the preS1 antigen, whereas HzKR127-3.2 efficiently inhibited binding, suggesting that HzKR359-1 and HzKR127-3.2 can bind to genotype C simultaneously. To demonstrate this, HzKR359-1 or HzKR127-3.2 was incubated with different concentrations of the preS1 antigen followed by KR127 or KR359, respectively, using Octet Red (Figs. 6B and 6C). Indeed, HzKR359-1 and KR127 (or HzKR127-3.2 and KR359) bound to the preS1 antigen additively and, thus, showed enhanced binding activity to genotype C compared with either antibody alone.

In conclusion, we successfully constructed a humanized antibody (HzKR359-1) that binds to the essential receptor binding site and demonstrated that the affinity of HzKR359-1 is higher than that of the original murine mAb. HzKR359-1 can bind to five genotypes (A–C, H, and J). HzKR127-3.2 binds to six genotypes. The combination of HzKR359-1 and HzKR127-3.2 can bind to most HBV genotypes, with the exception of genotypes E and F, and showed enhanced binding to genotype C compared with that of either antibody alone. Given that HzKR127 exhibited

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**Fig. 5.** Analyses of the antigen-binding activities of HzKR359-1 (A), HzKR127-3.2 (B), and HzKR359-1 plus HzKR127-3.2 (C) to the preS1 of different genotypes.
neutralizing activity to genotype C in chimpanzees and that the epitopes of HzKR359-1 and HzKR127-3.2 are located in the receptor-binding site, these two humanized antibodies might effectively block the entry of most virus genotypes and thus broadly neutralizing HBV infection.

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