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Original Article

Hepatitis B immunoglobulin inhibits the secretion of HBV via antigen-antibody precipitation in the multivesicular body

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Abstract: Background and Aims: Although the main action of human hepatitis B immunoglobulin (HBIG) is to neutralize hepatitis B virus surface antigen (HBsAg) in serum, HBIG is known to be localized in the cell. However, the effect of intracellularly located HBIG is poorly understood because of the low purity of conventional plasma-derived HBIG (cHBIG). We attempted to elucidate the mechanism of action of internalized HBIG using recombinant HBIG (lenvervimab). Methods: We used HBsAg producing cell lines, non-HBsAg cell lines and human HBsAg-producing hepatocytes. The autophagosome lysis pathway-related proteins Rab5, calnexin, giantin, and Rab7 were used to localize HBsAg and anti-HBs-IgG in the cytoplasm using Western blotting and confocal microscopy. Results: Intracellular anti-HBs-IgG (lenvervimab and cHBIG) transported via Fc receptor-mediated endocytosis increased the number of autophagosomes. However, there was no change in autolysis. HBsAg and anti-HBs-IgG co-localized in the multivesicular body and precipitated in the cytoplasm. HBsAg secretion into culture medium decreased after lenvervimab treatment. Simultaneously, the amount of cellular HBsAg increased in the cell lines but decreased in human hepatocytes. Furthermore, intracellular lenvervimab is not easily removed from HBsAg cell lines. Conclusions: Lenvervimab decreases HBsAg secretion, and HBsAg antibody precipitation in the multivesicular body may play an important role.

Keywords: Autophagosome, lenvervimab, anti-human hepatitis B virus immunoglobulin, inhibition of secretion of HBsAg

Introduction

Viral hepatitis is a major global health concern. Human hepatitis B virus (HBV) infection is the most common infectious liver disease, affecting an estimated 257-290 million people, or 3.5% of the population [1, 2]. According to the 2016 WHO report, >0.7 million people die yearly due to HBV infection complications. Vaccination against HBV during the perinatal period provides effective prophylaxis and is essential for eradicating HBV. However, the vaccination rate is extremely low in Central Africa, the Eastern Mediterranean region, and Southeast Asia. The incidence of hepatitis B virus surface antigen (HBsAg) positivity remains high in Central and Southeast Asia (5.3%) and Middle Africa (5.6%) [1]. Considering the high incidence of HBV-related chronic liver diseases, HBV infection remains a clinical burden.

Hepatitis B immunoglobulin (HBIG) monotherapy was previously considered an effective prophylactic protocol after liver transplantation in earlier times; however, it might lead to inefficacy in the G145R mutant and is also expensive [3-5]. Additional administration and titer monitoring are necessary to maintain adequate serum levels [3]. With the development of antiviral nucleos(t)ide analogs (NA), the combination of HBIG and NA has the greatest potential. It showed the most outstanding results in real-world practice and reported data better than NA monotherapy [6, 7]. NA monotherapy also showed excellent long-term survival of 85% at 9 years and an undetectable HBV DNA rate of 100% at 8 years [8]. However, NA causes 9-14% HBsAg loss of efficacy [8, 9] and nephrotoxicity, especially in patients with ongoing acute kidney injury before liver transplantation [10].

Potent and relatively low-cost HBIG could be a good alternative for overcoming the HBsAg loss of efficacy drawbacks of recent regimens. A recombinant monoclonal HBIG (lenvervimab) developed by GC Pharma (Yongin, South Korea) has consistent avidity to cloned S antigens, including the immune escape mutant G145R. Moreover, it did not interfere with antibody binding in HBV with mutations in the S gene sequence, which caused resistance to NA [5]. Furthermore, the neutralization of circulating HBV particles and inhibition of viral re-entry by binding to HBsAg have been reported [11, 12], however, the action of its intracellular component is not completely understood.

Therefore, we investigated the action of intracellularly located lenvervimab in HBV-infected cells.

Materials and methods

Cell lines and cell culture

We used five human hepatoma cell lines obtained from the Korean Cell Line Bank (KCLB) and Merck KGaA. Huh7 (KCLB No. 60104) and HepG2 (KCLB No. 88065) cells were HBV negative, while PLC/PRF/5 (KCLB No. 28024), HepG2.2.15 (Merck, No. SCC249), and Hep3B (KCLB No. 88064) cells express HBsAg. The cell lines were cultured in a 37°C and 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, Thermo Scientific Hyclone) and 1× Antibiotic-Antimyotic (Anti-Anti).

Human hepatocyte isolation

Human hepatocytes from patients with high titers of HBsAg and HBV DNA were used. Approximately 50-70 g of liver tissue was obtained from a patient infected with hepatitis B virus and positive HBsAg. After connecting two or three catheters filled with William's media to a large vessel exposed to a cross-section of the liver tissue, perfusion solution A [Hanks' balanced salt solution (HBSS) 250 mL + hydroxyethyl piperazine-ethane-sulfonic acid (HEPES) 2.5 mL + antibiotics 2.5 mL + EDTA 250 μL] was perfused through a peristaltic pump at a flow rate of 30 mL per minute per catheter. After approximately 10 min, solution B (HBSS 250 mL + HEPES 2.5 mL + antibiotics 2.5 mL + collagenase 0.125 g) was used for perfusion for another 10 min. After removing the catheter from the liver tissue that had turned yellow due to decomposition, the liver tissue was transferred to another bowl, and a cold William's medium was added. A knife was used to obtain enzymatically degraded tissue cells.

Immunoglobulins

Three immunoglobulins [control human IgG (Sigma, Dorset, UK), conventional plasma-derived hepatitis B immunoglobulin (cHBIG, GC Pharma, Yongin, Korea), and recombinant hepatitis B immunoglobulin along with its Fab portion (lenvervimab, GC Pharma, Yongin, Korea)] were diluted with Dulbecco's Phosphate Buffered saline (DPBS) or media corresponding to the cell line and used at various concentrations from 0.25 mg/mL to 10 mg/mL. For immunofluorescence and western blotting, the cell lines were treated for 15 min, 48 h, and 96 h, respectively, in a 37°C and 5% CO_2 incubator.

Immunofluorescence

Each cell line was cultured in an 8-well chamber slide (Nunc™ Lab-Tek™) at a density of 0.3 × 10^5 cells/well, or on a cover glass placed in a 24-well cell culture plate and seeded at 0.5 × 10^5 cells/well. After 24 h, control human IgG, cHBIG, or lenvervimab were treated in a 5% CO_2 incubator at 37°C for 15 min to 48 h. The cells were fixed with 3.7% paraformaldehyde for 15 min, and then incubated for 15 min using 0.2% Triton X-100. Blocking was performed with 1% bovine serum albumin (BSA) for an hour. The primary antibodies used were mouse anti-HBsAg, mouse anti-Rab5 (early endosome marker), rabbit anti-LC3 (autophagy marker), mouse anti-calnexin (ER marker), mouse anti-giantin (Golgi marker), and Rab7 (multivesicular body marker) at a ratio of 1:100. The corresponding secondary antibodies conjugated with fluorescence, goat anti-mouse IgG (488), goat anti-human IgG (568), and horse anti-rabbit IgG (568) were used at a ratio of 1:200. Wheat germ agglutinin (633) and Alexa Fluor-phalloidin 488 were used at a ratio of 1:500. Immunofluorescence (IF) samples were observed under a Leica TCS SP8 confocal microscope.

Western blot

Each cell line was seeded in a 6-well cell culture plate, treated with the necessary IgG or

reagents, and then the cells in each well were scraped and lysed on ice for 30 min after treatment with radioimmunoprecipitation assay lysis buffer and a proteinase inhibitor. The lysed cells were centrifuged at 4°C for 30 min to obtain the supernatant. A 5× sample buffer was added, boiled for 5 min, and cooled on ice.

Western blotting was performed for 55 min at 130 V and 300 mA using a 4-20% precast gel and then transferred to a polyvinylidene fluoride membrane at 80 V and 300 mA for 2 h at 4°C in a cold room. The membrane was blocked with 5% BSA and washed with Tris-buffered saline. The primary antibody was incubated in a cold room at 4°C overnight, and the secondary antibody was incubated at room temperature for 1 h. The results were measured and analyzed using a LAS 4000 instrument.

As described in the figure, each protein was loaded onto a gel and transferred to a sheet of membrane. The proteins were detected using each antibody after cutting the membrane to a size suitable for the protein of interest. For HBsAg, we used the entire membrane to detect various sizes of HBsAg proteins, including small, medium, and large (Supplementary Figure 1).

Cell culture medium protein assay

Western blot after polyethylene glycol (PEG): To characterize the proteins in the cell culture medium by Western blotting, the culture medium was collected separately and centrifuged at 8000 rpm. The supernatant (2 mL of supernatant was mixed with 1 mL of 40% PEG, 400 µL of 4 M NaCl, and DMEM to adjust to a total volume of 4 mL). This was mixed with a rotator at 4°C overnight, and the pellet was centrifuged at 4850 rpm at 4°C and used for Western blotting.

Enzyme-linked immunosorbent assay: PLC/PRF/5 cells were seeded in a 6-well plate. After 24 h, FBS-free DMEM was added to each plate. cHBIG or lenvervimab, and the Fab portion of lenvervimab were added. After 48 h, the supernatant was replaced with FBS-free Dulbecco's modified Eagle's medium (DMEM). The supernatant was obtained every 12, 24, and 48 h, centrifuged at 10,000 rpm for 20 min at 4°C, and used for enzyme-linked immunosorbent assay (ELISA).

A human HBsAg ELISA kit was used. Blank samples were added to each well and incubated at 37°C for 90 min. After removing the liquids from the wells and treating the supernatant with 100 µL of biotinylated detection antibody, it was gently tapped to mix well and incubated again at 37°C for 1 h. After washing each well thrice with wash buffer, 100 µL of Horseradish peroxidase conjugate working solution was added and incubated at 37°C for 30 min. After washing each well five times with wash buffer, 90 µL of the substrate was treated and incubated at 37°C for 15 min. After adding 50 µL of the stop solution, the optical density was measured at 450 nm using a microplate reader.

Ethics

This study followed the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of Seoul National University Hospital (nos. 1401-081-550 and 1701-004-819). In this study, we used human hepatocytes from patient samples after resection and obtained informed consent with IRB approval (IRB number is described above). A dataset from qualified researchers trained in human-subject confidentiality protocols was sent to the Seoul National University Hospital Institutional Review Board at cris@bri.snuh.org (https://cris.snuh.org).

Results

Intracellular location of lenvervimab and cHBIG in Hur7 and PLC/PRF/5 via endocytosis

Anti-HBs-IgG (lenvervimab and cHBIG) was added to Hur7 cells for 24 h, and anti-HBs-IgG (lenvervimab and cHBIG) and control human IgG were added to PLC/PRF/5 cells for 24 h. **Figure 1** shows the IF results. Lenvervimab and Rab5 (early endosome markers) were co-localized in the cytoplasm, and cHBIG showed a co-localized pattern in the Hur7 cell line (**Figure 1A**). However, the Fab portion, except for the Fc portion, and Rab5 were not co-localized and showed a diffuse distribution pattern. In PLC/PRF/5 cells, anti-HBs-IgG (lenvervimab and cHBIG) and Rab5 (an early endosome marker) were co-localized in the cytoplasm. Control human IgG was located in the cytoplasm with Rab5; however, anti-HBs-IgG was located more in the cytoplasm than control IgG

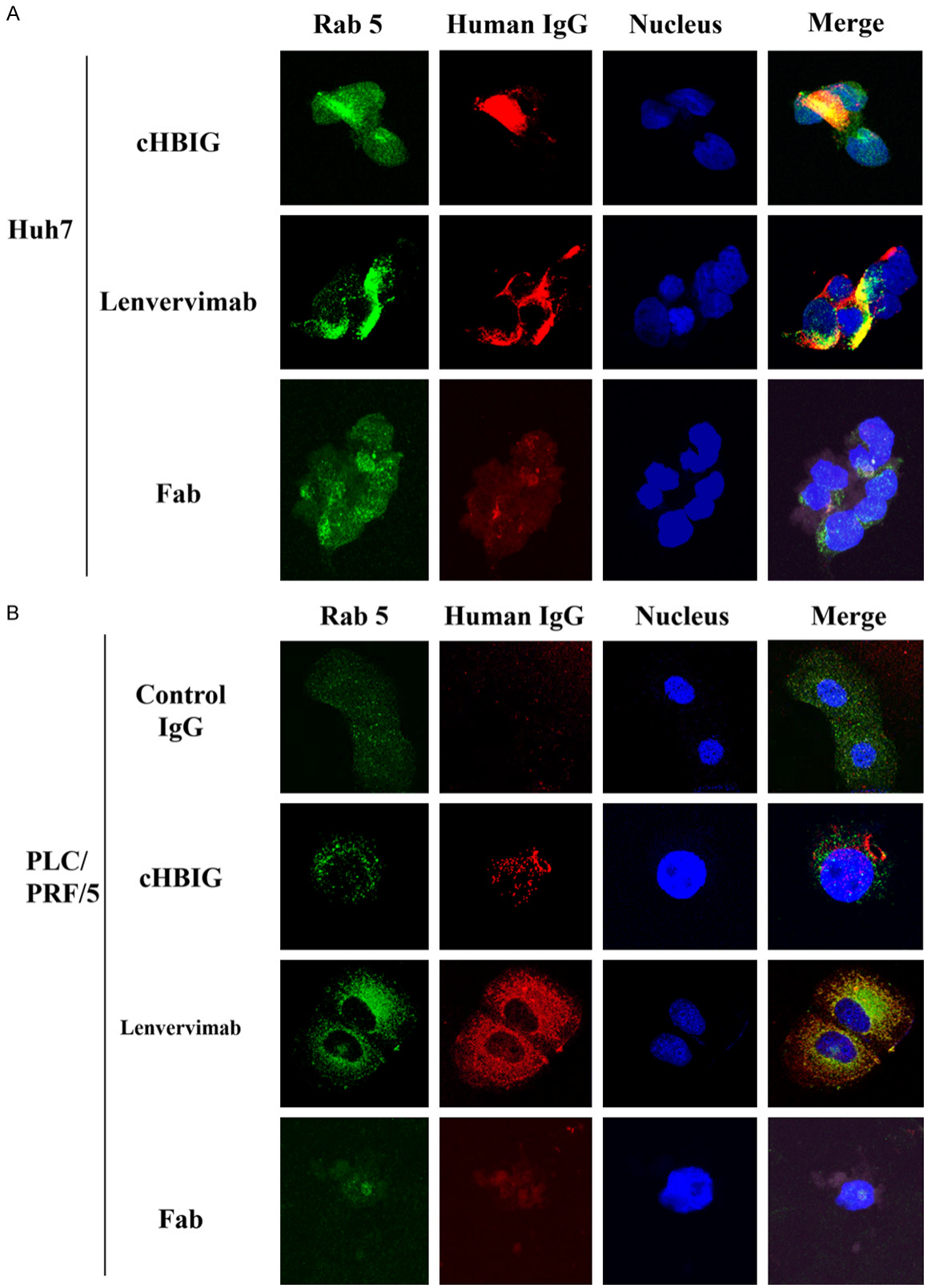

Figure 1. Immunofluorescence images of immunoglobulin marker and Rab5 (early endosome marker) in Huh7 and PLC/PRF/5 cell lines after treatment of anti-HBs-Ig (lenvervimab and cHBIG) for 24 hours (800× magnification). A. Rab5 and anti-HBs-IgG were co-localized (shown with yellow color). However, Fab portion (except Fc portion) and Rab5 were not co-localized with a diffuse distributed pattern in Huh7. B. Co-localizations of Rab5 and immunoglobu-

lin (control IgG anti-HBs-IgG) in PLC/PRF/5 cell line were shown; however, the Fab portion (except the Fc portion) and Rab5 were not co localized. Anti-HBs-IgG is located in cytoplasm more than control IgG, and the dot like patterns were more prominent in the anti-HBs-IgG group.

and appeared in prominent dot-like patterns (**Figure 1B**).

Formation of autophagosomes after internalization of anti-HBs-IgG (lenvervimab and cHBIG) in HepG2.2.15

Figure 2A shows the prominent dot-like patterns of LC3 and anti-HBsIgG (lenvervimab and cHBIG). However, the intensity graph showed that LC3 and anti-HBsIgG were not co-localized (**Figure 2B**). The Western blot analysis showed increased intracellular localization of anti-HBs-IgG and HBsAg in the anti-HBs-IgG group. The ratio of LC3 I to LC3 II was significantly increased in lenvervimab group, similar to that in ammonium chloride treatment group (positive control for autophagosomes), compared to the cHBIG and control groups. However, p62 (an autolysis marker) did not decrease in the anti-HBs-IgG (lenvervimab and cHBIG) groups compared with rapamycin group (positive control for autolysis) (**Figure 2C**).

Co-localized precipitation of HBsAg and anti-HBs-IgG (lenvervimab and cHBIG) in PLC/PRF/5 and HBsAg-producing human hepatocyte

IF was performed to detect anti-HBsAg-IgG (lenvervimab and cHBIG) and HBsAg markers in PLC/PRF/5 cells and HBsAg-producing human hepatocytes (**Figure 3**). Contrary to the treatment with control IgG, co-localization of HBsAg and anti-HBs-IgG (lenvervimab and cHBIG) was observed in the anti-HBs-IgG treatment group. Moreover, prominent dot-like staining of HBsAg and IgG was observed only in the anti-HBs-IgG group, compared to the control IgG and Fab groups. The dot-like staining was more prominent with lenvervimab than with cHBIG. In human hepatocytes (after lenvervimab treatment), co-localized HBsAg and lenvervimab and dot-like patterns were prominent, similar to those in the PLC/PRF/5 cell line (**Figure 3**).

Intracellular accumulation of HBsAg and reduced secretion of HBsAg in PLC/PRF/5 by lenvervimab

Western blot analysis of intracellular HBsAg and IgG and the culture supernatant was performed according to the treatment duration (48, 72, and 96 h after lenvervimab treatment). The bands of intracellular HBsAg (especially small HBsAg) and lenvervimab were prominent at 96 h after treatment than those of the control IgG (**Figure 4A**). All HBsAg band areas were faint in the culture supernatant compared to the control IgG. The lenvervimab band was less prominent than that of the control IgG in the supernatant. It appears that continuous treatment with lenvervimab resulted in a supernatant effect (**Figure 4B**). The cells were washed several times while preparing intracellular proteins; in contrast to the control IgG, HBsAg, and IgG remained within the cells in the lenvervimab-treated group of the PLC/PRF/5 cell line.

HBsAg and lenvervimab in multivesicular body with precipitation in PLC/PFR/5

Figure 5 shows the IF image and intensity graph of HBsAg, endoplasmic reticulum (calnexin), Golgi apparatus (giantin), and multivesicular body (Rab7) markers after treatment with anti-HBs-IgG in PLC/PRF/5 cell line. Some HBsAg and calnexin were co-localized in the control IgG and anti-HBs-IgG groups. However, dot-like patterns of HBsAg were observed only in the anti-HBs-IgG group, and the dot-like patterns of HBsAg and calnexin were not co-localized (**Figure 5A**, **5B**). Similarly, some HBsAg and giantin were co-localized in the control IgG and anti-HBs-IgG groups, whereas dot-like patterns of HBsAg and giantin were not co-localized (**Figure 5C**, **5D**). Rab7, HBsAg, and anti-HBs-IgG were co-localized; lenvervimab cells showed prominent dot-like patterns of HBsAg and lenvervimab staining, and the intensity graph showed the co-localization of lenvervimab and HBsAg (**Figure 5E**, **5F**).

Figure 6 shows a schematic representation of the life cycle of HBV and the mechanism by which HBV release is blocked. As shown in **Figure 5**, the main location of the antigen-antibody reactions was the multivesicular body (MVB), where the main HBsAg assembled organelles after the packaging process. Eventually, filament-type (containing medium and large HBsAg) virus-like particles and HBV could not be released by lenvervimab.

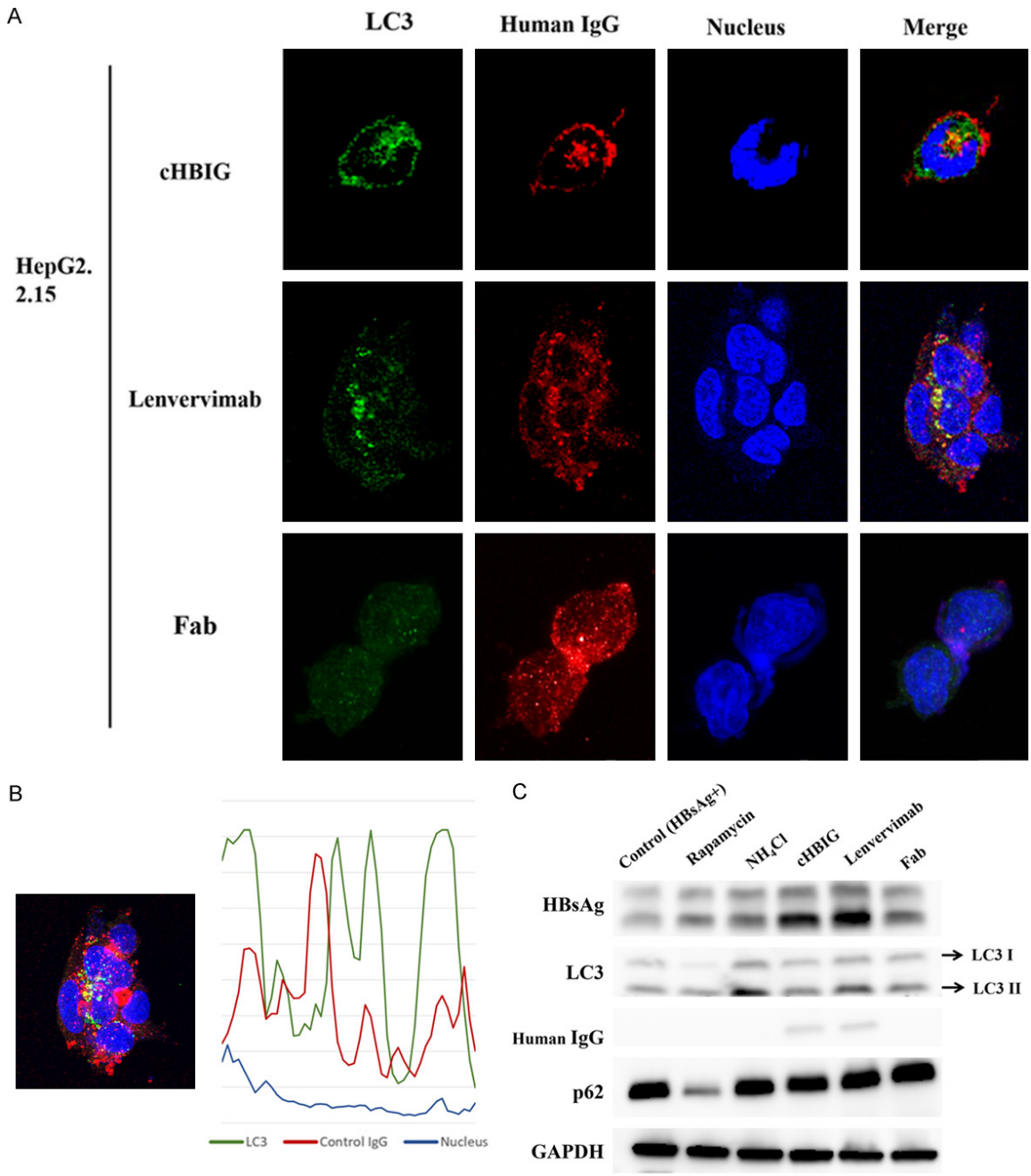

Figure 2. Autophagosome formation but autolysis was not observed. (Each protein was loaded on a gel and transferred to one sheet of membrane. The proteins were detected using each antibody after cutting the membrane suitable size of the interested protein. The supplementary file serves entire membranes) (600× magnification). A. LC3 and anti-HBs-Ig (lenvervimab and cHBIG) were prominent with dot like patterns. B. The intensity graph showed that LC3 and anti-HBs-Ig were not co-localized. C. Western blot analysis of the human Ig and HBsAg, LC3, P62. The intracellular location of anti-HBs-IgG and HBsAg and the increased ratio of LC3 I to LC3 II were prominent in lenvervimab group similar to ammonium chloride group (positive control for autophagosome). However, the P62 was not decreased in anti-HBs-Ig group compared to the rapamycin group (positive control for autolysis).

Discussion

While NA showed tremendous survival and HBV DNA clearance, anti-HBs IgG still had an additional effect in controlling HBV. One of the two main categories of prophylaxis after LT, the combination therapy (HBIG and NA), yielded better results than NA alone. NA treatment has

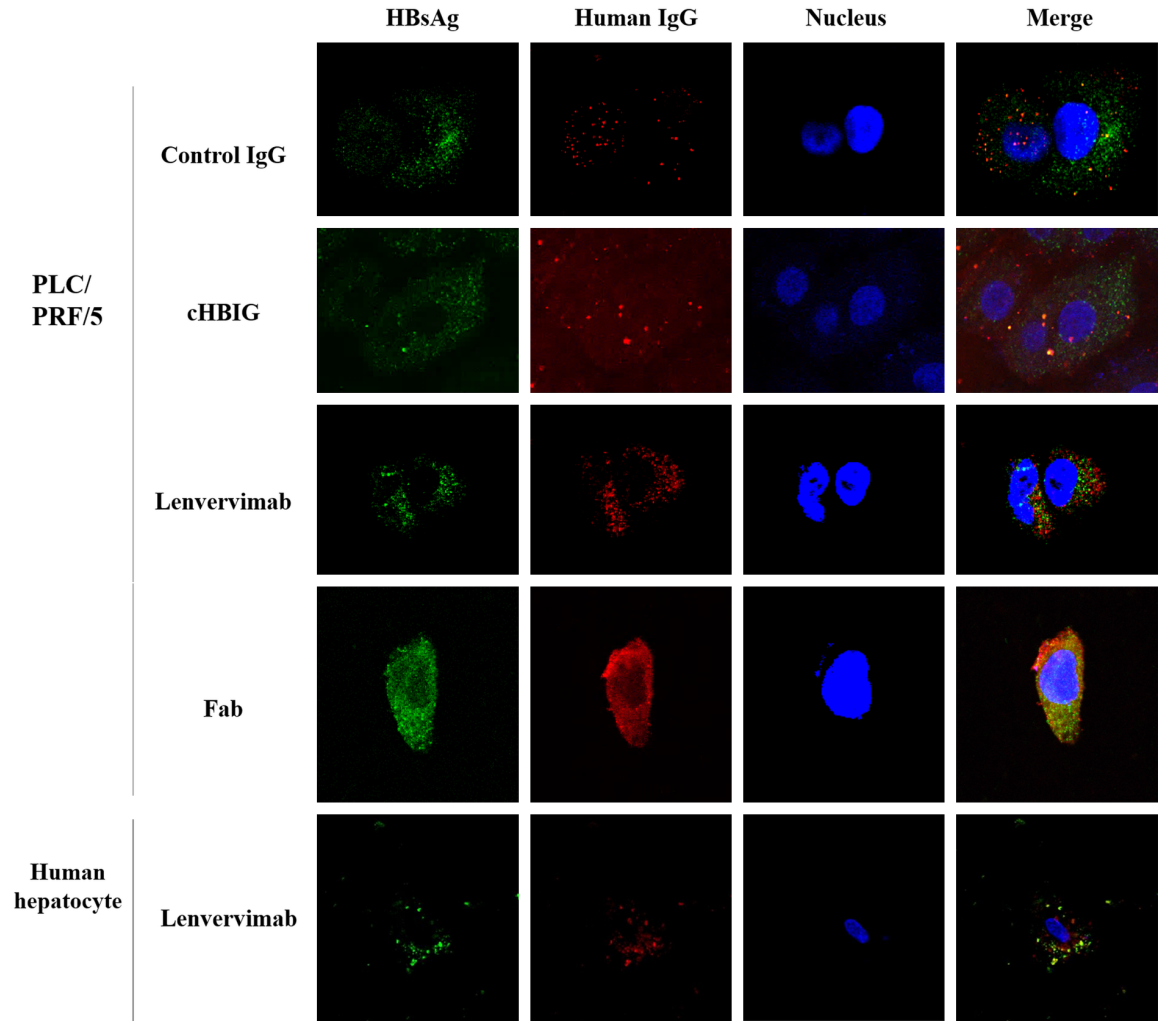

Figure 3. Immunofluorescence images of immunoglobulin marker and HBsAg in PLC/PRF/5 cellline and human hepatocytes isolated from the patient with HBsAg and DNA in the blood. Co-localization of HBsAg and anti-HBs-IgG (lenvervimab and cHBIG) was observed, and dot like pattern is prominent in the anti-HBs-IgG group. Lenvervimab formed more dot like patterns than cHBIG. For Fab portion except Fc portion immunoglobulin, dot like pattern was not prominent without co-localization with HBsAg. Co-localization of HBsAg and lenvervimab and dot like patterns were also observed in HBsAg producing human hepatocytes and dot like pattern was prominent and similar to the anti-HBs-Ab treating group in PLC/PRF/5 cell line (600× magnification).

several advantages in controlling HBV, including different mechanisms of suppression of DNA synthesis, reduced risk of HBIG-related HBsAg mutations, relatively high compliance, and low cost compared to HBIG. However, drug-related toxicity, especially in the kidneys, and a 22.1% chance of immune-escape mutation of HBsAg still exist in patients with NA exposure [13]. HBsAg is essential in maintaining HBV immune tolerance and suppressing HBV DNA via NA [14, 15]. HBsAg seroclearance is an important requirement for the discontinuation of NA by inducing spontaneous immune control [16]. This might be related to the poorer outcomes of the HBIG-free regimen in some patients undergoing transplantation and those with CHB [17, 18]. However, human plasma-fractioned HBIG has drawbacks such as low specific activity, which might lead to a loss of efficacy against the G145R mutant, and is expensive [3-5]. Lenvervimab is a recombinant IgG1-type and anti-HBs-IgG derived from the immunoglobulin genes of the vaccinated persons transferred to Chinese hamster ovary cells, and it has more potent activities in drug-resistant variants and all virus genotypes, par-

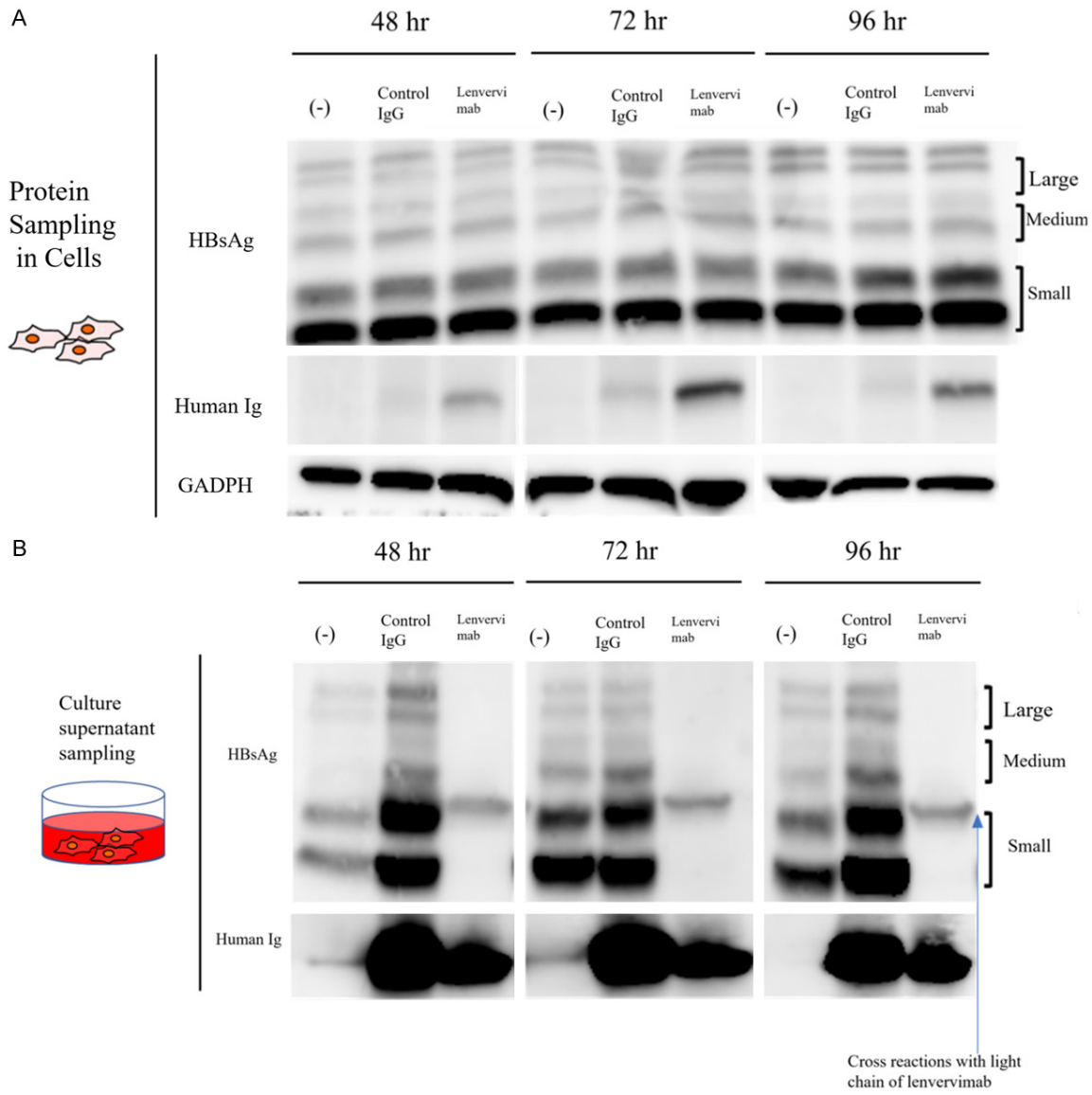

Figure 4. Western blot analysis of HBsAg and immunoglobulin in PLC/PRF/5 cell line after incubation with control human Ig and lenvervimab. Control immunoglobulin and lenvervimab were treated for 96 hours in PLC/PRF/5 cell line (Each protein was loaded on a gel and transferred to one sheet of membrane. The proteins were detected using each antibody after cutting the membrane suitable size for the interested protein. In the case of HBsAg, we used the entire membrane to detect the various size of proteins consisting of HBsAg as small, medium and large supplementary files that serve entire membranes) (800× magnification). A. Intracellular accumulation of HBsAg and lenvervimab was prominent in lenvervimab treated cells compared with to treated with control Ig. B. Reduced HBsAg in the supernatant after lenvervimab treatment and band of Ig in lenvervimab were less prominent than that after control Ig treatment.

ticularly in the antigenic 'a' determinants [5]. The mechanism of action of lenvervimab involves neutralization of circulating HBV particles and inhibition of viral re-entry by binding [11, 12]. We investigated the mechanism of action of lenvervimab in HBV-positive cell lines, focusing on the intracellular portion and the differences between lenvervimab and cHBIG.

The entry mechanism of lenvervimab and cHBIG is endocytosis, similar to that of typical immunoglobulins. **Figure 1** shows that most intracellular anti-HBs-IgG co-localized with the early endosome marker Rab5 in both PLC/PRF/5 and Huh7 cells, as in a previous study [19]. However, this effect was not observed in the Fab-treated group. Interestingly, the dot-

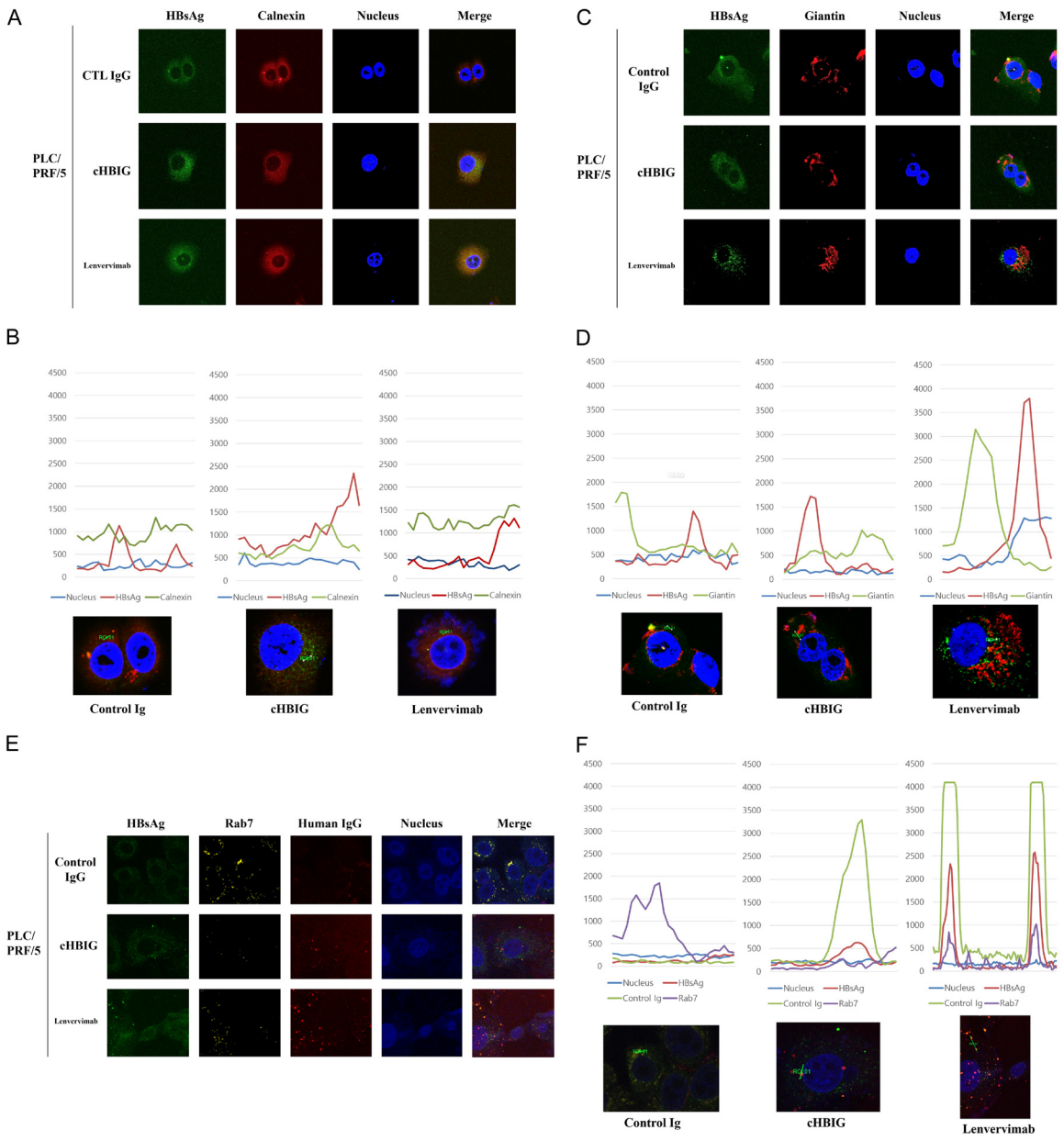

Figure 5. Immunoflorescence image of HBsAg and endoplasmic reticulum (calnexin), golgi apparatus (giantin), multivesicular body (Rab7) markers after treating with anti-HBs-Ig in PLC/PRF/5 cell line (800× magnification). A, B. Some parts of HBsAg and calnexin were co-localized in control Ig and anti-HBs-Ig groups. The dot like patterns of HBsAg was seen only in anti-HBs-IgG group and the dot like patterns of HBsAg and calnexin were not co-localized. C, D. Some parts of HBsAg and giantin were co-localized in the control Ig and anti-HBs-IgG groups and the dot like patterns of HBsAg and giantin were not co-localized. E, F. Rab7 and HBsAg, anti-HBs-IgG were co-localized and treating cells with lenvervimab showed more prominent dot like patterns of HBsAg and lenvervimab and the intensity graph also showed co-localization of lenvervimab and HBsAg, Rab7.

like pattern of anti-HBs-Ig was more prominent in PLC/PRF/5 cells than in Huh7 cells, suggesting that antigen-antibody reactions are related to these findings.

We attempted to elucidate this process after the internalization of anti-HBs-IgG, focusing on autophagy. We hypothesized that an HBsAg-specific antibody (anti-HBs-Ig) could form an HBsAg-antibody complex in the form of autophagosomes and undergo autolysis after endocytosis. Autophagosome formation was more prominent in the lenvervimab group than in the control IgG and cHBIG groups; however,

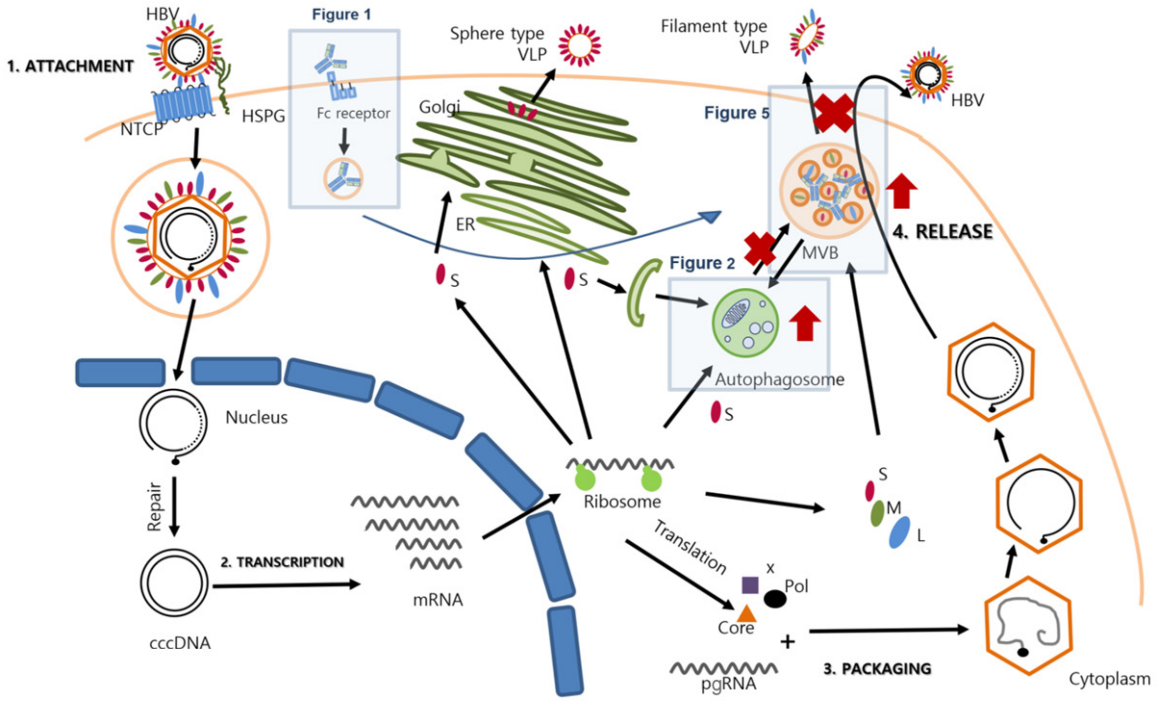

Figure 6. Schemes of the HBV life cycle and related figures. The main location of the antigen-antibody reactions was the multivesicular body (MVB), where HBsAg was assembled into organelles after the packaging process. Eventually, filament-type (containing medium and large HBsAg) virus-like particles and HBV could not be released by lenvervimab. cccDNA, covalently closed circular DNA; NTCP, sodium/taurocholate co-transporting polypeptide; HSPG, heparan sulfate proteoglycan; MVB, multivesicular body; pgRNA, pre-genomic RNA; VLP, virus-like particle.

sequential autolysis was not observed (**Figure 2C**). Rapamycin, a known autolysis-inducing agent, was added after lenvervimab treatment; however, autolysis after autophagosome formation was not observed (data not shown). Furthermore, LC3 (an autophagosome marker) did not colocalize with anti-HBs-IgG (**Figure 2B**). In general, autophagosome formation is induced by ER stress via phagopore-initiating processes [20, 21]. In this study, antigen-antibody reactions did not occur inside the autophagosome; however, the formation of autophagosomes increased. A possible mechanism underlying the increase in autophagosomes is the ER stress induced by lenvervimab in an HBsAg-producing cell line. Therefore, antigen-antibody reactions increase ER stress and induce autophagosome formation through the phagopore-initiating process [22]. Interference with the interactions between MVB and autophagosomes may be a possible reason. MVB is related to autophagosomes, forming amphisomes that can fuse with lysosomes, which are known to generate autophagolysosomes [23]. The accumulation and altered function of MVB after antigen-antibody reactions may be related to increased autophagosome formation in the absence of autolysis.

Dot-like patterns of co-localized HBsAg and anti-HBs-IgG were observed only in HBsAg-producing cell lines. Dot-like patterns are not observed when only the Fab portion is used (**Figure 3**). According to this result and previous studies, the Fc portion is necessary to stabilize the antigen-antibody reactions after endocytosis, and regulate lysosomal activity, and the endosomal sorting complex required for transport (ESCRT) [19, 24]. Therefore, the dot-like patterns are related to the Fc portion after antigen-antibody reactions, and the Fc portion seems to play an important role in maintaining a strong binding affinity for HBsAg or adjacent Ig.

In the cHBIG-and lenvervimab-treated groups, accumulation of HBsAg in the intracellular portion and reduced HBsAg secretion were observed in the supernatant. Elimination of the Fc portion results in HBsAg secretion in PLC/

PRF/5 cell line [19]. Even after washing cell culture plates, some HBsAg and lenvervimab remained intracellular (**Figure 4**). Inhibition of HBsAg by lenvervimab was observed in human hepatocytes; however, intracellular HBsAg levels were slightly decreased. A previous study has reported similar findings. Further studies are necessary to clarify the differences between HBsAg-producing cell lines and infected hepatocytes.

Eventually, these antigen-antibody complexes co-localized with Rab7 (MVB marker). This result is meaningful in terms of the inhibition of HBV infectivity, considering the pathways of subviral HBV particles. Small HBsAgs with sphere (without infectivity) secretion usually occur via the ER-Golgi network, are released by the general secretory pathway, do not generally accumulate, and do not significantly inhibit the production and secretion of small HBsAgs (spheres) in an inhibited MVB biogenesis cell line [25]. However, virions containing medium or large HBsAgs, which have infectivity, require ESCRT via the MVB for secretion [25]. HBV secretion is regulated through the activation of endocytic and autophagic compartments mediated by Rab7 stimulation. MVB has been known to participate in the final stage of HBV maturation and release [25, 26]. Silencing either Rab5 or Rab7 inhibits HBV infection, according to these findings [27]. Accumulated lesions of virions and antigen-antibody complexes occur in the MVB, which manifests as Rab7, as shown in this study. Accumulation of HBsAg in the MVB is not only unable to secrete the HBV particles themselves but also has no infectious ability because medium and large HBsAg bind with heparin sulfate proteoglycans, which means that only secretion of HBV particles could not have the infectious ability [28]. In chronic HBV infection, high levels of virus-like particles consisting of medium or large HBsAg particles are major obstacles to triggering effective immune responses and subsequent virus clearance [29]. Our findings suggest that antigen-antibody reactions in MVB compromise the HBsAg secretory pathway of virion-or filament-type VLPs (**Figure 6**). Monoclonal antibodies with strong multisite affinities, such as lenvervimab, showed stronger effects than cHBIG.

We focused on the mechanism of action of lenvervimab and HBsAg, which was confirmed in the Rab7 marker-positive organelles. It appears that not only suppressing the HBV release of anti-HBs-IgG but also the main location of the antigen-antibody reaction is the MVB, where the main HBV assembly organelles have not yet been reported.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

BSA, bovine serum albumin; cHBIG, conventional plasma-derived hepatitis B immunoglobulin; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's Phosphate Buffered saline; ESCRT, endosomal sorting complex required for transport; HBV, human hepatitis B virus; HBIG, Hepatitis B immunoglobulin; HBSS, Hanks' balanced salt solution; HEPES, hydroxyethyl piperazine-ethane-sulfonic acid; IF, immunofluorescence; KCLB, Korean Cell Line Bank; MVB, multivesicular body; NA, nucleos(t)ide analogs.

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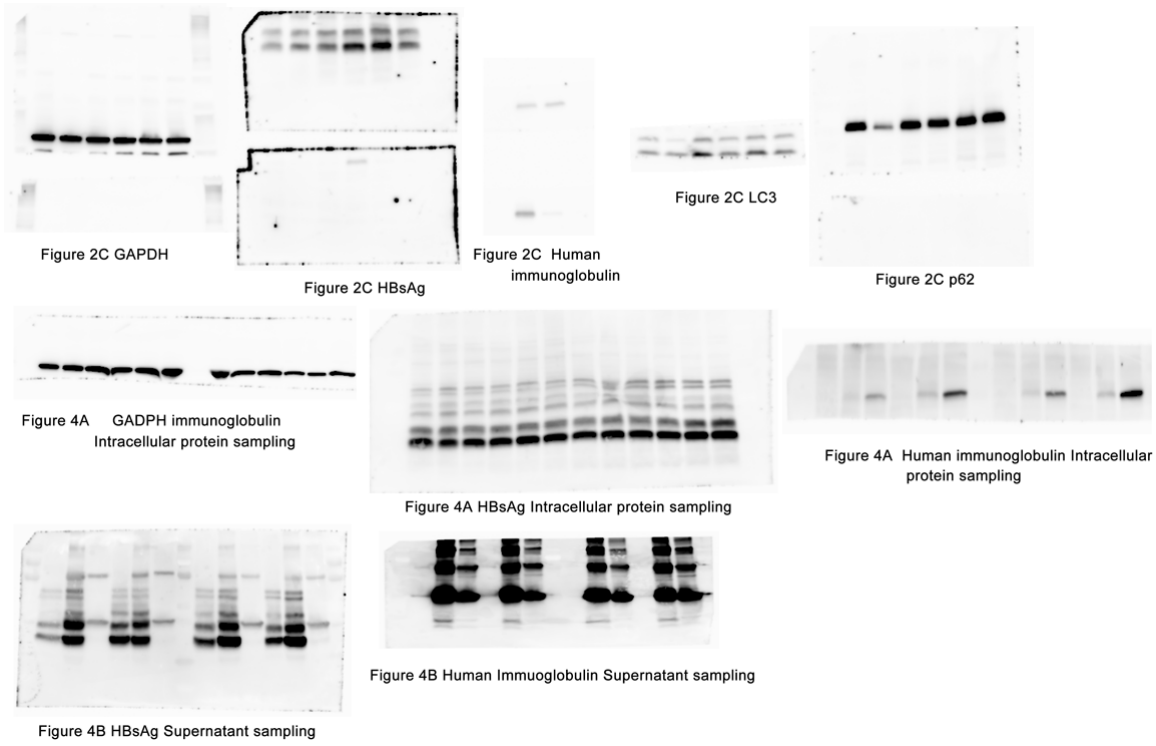

Supplementary Figure 1. Blot image.