performed. Especially, further investigation is needed to clarify the relationship between the change in hepatitis B surface antigen levels during treatment and HCC incidence in patients with HBV infection.

In conclusion, in the consecutive surveillance for HCC after the initiation of ETV treatment, monitoring the change in AFP levels at 24 weeks is essential, especially among patients of advanced age or with cirrhosis.

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Successful Generation of Hepatitis B virus (HBV) Pseudotype Particle; a versatile tool for Identification of the HBV Receptor and Investigation of HBV infectivity

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Abstract

It is near a half century since hepatitis B virus (HBV) was identified. HBV receptor molecules and the entry mechanism of HBV into hepatocytes have not been elucidated completely, though there are some reports on infection systems and on the receptor molecules. Thus, we still have not reached finding a real HBV receptor and there have been no useful and convenient infection system in vitro and in vivo for HBV, which makes it impossible for us to understand a precise HBV life cycle and HBV involved related diseases. An HBV infection system is really needed to explore ways and means of treatment of HBV related diseases based on evidence as well. Here, we designed and tried to generate an HBV pseudotype, which has a viral particle containing a retrovirus capsid and a genome inside surrounded by HBV membrane proteins. We proved successful generation of this pseudotype by immunoprecipitation with anti-HBVs antibodies and by CsCl density gradient ultracentrifugation, followed by RT-PCR targeting a retroviral gene, an *EGFP* gene in this case, respectively. Though our established system is constructed on growth dependent integration of retroviral

genomes and thus was very hard to observe its infection in a primary human hepatocytes culture system, successful generation of the HBV pseudotype will make it possible for us to perform a biological assay to clone an HBV receptor based on infectivity and will facilitate its separation and identification.

Key words; HBV, pseudotype, infection

Introduction

It has been near half a century, since hepatitis B virus (HBV) was identified by Blumberg [1]. Though treatment of chronic hepatitis B with interferons has been continued and nucleotide analogs, most of which were explored as anti-HIV agents, and prevention against HBV infection by vaccination has been developed, HBV infected patients yet reach three hundreds and fifty million people worldwide [2]. Thus, HBV infection has not been controlled and one of the biggest viral infections in the world.

There are several animal models; avian hepatitis B viruses such as duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHBV), rodent hepatitis B viruses such as ground squirrel hepatitis virus (GSHV) and woodchuck hepatitis virus (WHV) [2; 3]. HBV shows highly species specific spectrum for its infection and can infect only primates and amplify mainly in parenchymal hepatocytes, but never infects the other animals including mice [2; 4]. Furthermore, even human hepatocyte-originated hepatocellular carcinoma cell lines such as HepG2, HuH7, HuH6, Hep3B and so on never permit HBV infection, or extremely limited even if possible,

though there are some reports about in vitro infection of HBV to HepG2 [5; 6].

As HBV infection systems, human normal hepatocytes and a human hepatocellular carcinoma originated cell line have been available on the commercial base [7; 8] and uPA/SCID mice with human hepatocytes have been developed [9; 10; 11]. These systems are very useful for HBV and hepatitis C virus (HCV) research indeed but they are very expensive and inconvenient for daily HBV research. Thus, there is still a high hurdle to expand knowledge about HBV.

Actually, many studies about HBV and its related diseases such as acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma have been performed so far [12; 13; 14; 15]. Such studies, however, were very limited because of absence of a useful and convenient *in vitro* infection system for HBV as mentioned. In order to overcome such a big problem, we need very useful, convenient and inexpensive systems for HBV infection to understand a true HBV life cycle and pathogenesis of HBV related diseases, and then to develop specific drugs and means to treat such diseases.

Identification of an HBV receptor is indispensable to establish *in vitro* and *in vivo* or a convenient animal infection model. Information on the HBV receptor has

been reported, including non-human hepadnaviruses such as duck hepatitis B virus [16; 17; 18] and such seems not to be a real and complete HBV receptor, and has not reached establishing an HBV infection system in culture system. HBV can infect primary hepatocytes for sure whose preparation is still troublesome, and although there are several human hepatocytes originated hepatocellular carcinoma cell lines as noted, we do not know why such cell lines becomes insusceptible to HBV infection, which might mean that differentiation status of hepatocyte could be critical for the expression and/or the activity of an HBV receptor [19; 20].

It should be very difficult to identify an HBV receptor and thus we must design a revolutionary method for the identification. Pseudotype viruses could be very useful not only to transfer and express some genes exogenously but also to monitor their infectivity [21]. VSV-G based pseudotype containing retroviral vectors and lentiviral VSV-G pseudotype vectors have been proved to be extremely useful to transduce heterologous genes into cells and in case of the latter [22; 23], even into resting cells such as well-differentiated neuronal cells [24]. On the other hand, specific viral enveloped pseudotype viruses could be helpful to test what kinds of cells and how such

viruses can infect. Though HCV pseudotype particles (HCVpp), in which lentiviral capsids are enveloped by HCV membrane proteins, has been successfully generated and utilized without any knowledge of viral particle maturation and egress [25]. Thus, the bottleneck is dependent on whether such pseudotype particles really can be generated or not.

Here, we challenged to develop HBV pseudotype particles (HBVpp), which consist of HBV membrane particles outside and murine leukemia virus (MLV) originated capsids and genomes insides. Therein, we prepared a packaging cell system, where the cells produce MLV gag-pol fusion polypeptides and a retroviral genome containing an *EGFP* gene and a hygromycin resistant gene (hyg^R) in addition to murine leukemia virus packaging signal ψ. Pantropic retroviruses with vesicular stomatitis virus G proteins were successfully generated in our generated packaging system and infected the other cell lines, meaning that the packaging cell lines were practicable. Introducing an HBV membrane protein expression vector into the packaging cells, which expresses three HBV membrane proteins, large S (LS), middle S (MS) and small S (SS) enabled release of particles enveloped by HBV membrane proteins.

Immunoprecipitation with anti-S antibodies followed by RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) targeted the *EGFP* gene revealed the designed retroviral genome inside. Physico-chemical studies using CsCl-density gradient ultracentrifugation followed by RNA extraction and RT-PCR targeted the *EGFP* gene also revealed that the particles focused around 1.22~1.25 g/ml density zone, which was consistent with the HBV particle density, and the retroviral genomes were inside.

Successful formation of an HBVpp seems to be very useful and practical for identification of an HBV receptor since we are able to establish an assay system based on the viral infectivity by using versatile cell culture systems, though our system is an MLV based packaging system, which depends on cell growth for their integration.

Materials and Methods

Cells.

HEK293, a human embryonic kidney cell line, and its derivatives, GP2-293 (Clontech-Takara Bio), in which an MMLV gag-pol gene was stably integrated for its expression, were maintained in Dulbecco's modified essential medium (DMEM) (nakalai tesquec) supplemented with 10 IU/ml penicillin G, 10 μg/ml streptomycin and 10 % fetal bovine serum (FBS), and BC3, a human primary effusion lymphoma [5] cell line in RPMI1640 (nakalai tesque) supplemented with the same antibiotics and 20 % fetal bovine serum. All cells were cultured in 5 % CO2 atmosphere. For the selection, hygromycin B (Wako Chemicals Japan) was added into each medium at 0.5 mg/ml.

Plasmids.

A retroviral expression vector, pHyTc-GFP was consisted of 5' and 3' LTR, a ψ packaging signal, a hygromycin resistant gene (hyg^R) at the gag-pol position, the cytomegalovirus (CMV) enhancer-promoter region followed by an *EGFP* gene from pEGFP C1 (Clontech) at the env position (see Fig. 1 A). pCEP4 LS-S was an HBV membrane protein expression vector and contained BstEII to EcoRV fragment of the cloned HBV DNA subtype adw2 (Accession No. X02763.1, GI: 59418) at a PvuII site of pCEP4 vector (Invitrogen).

Transfection and establishment of packaging cell lines.

pHyTc-GFP was transfected to GP2-293 cells (Clontech-Takara Bio) with a transfection reagent, TransIT LT1 (Takara Bio) according to manufacturer's direction. Two days after transfection, the cells were selected in 0. 5 mg/ml hygromycin B (Wako Chemicals, Japan) containing DMEM with supplements mentioned above. Several colonies with GFP-positive signals were isolated with a penicillin ring and propagated. To check their packaging activity, a VSV-G expression vector (Clontech) was

150 transfected to the isolated clones and the culture medium was harvested three days
151 post-transfection. The medium was passed through a 0.22 μm filter unit (Millex®,
152 Millipore) and stored at 4° C until use. 2×10^5 BC3 cells or HEK293 were prepared in
153 a 6-well plate and the virus containing medium was contacted with them for 4 hours.
154 The medium was withdrawn and incubated for two days in the DMEM for HEK293 and
155 RPMI1640 for BC3 supplemented with antibiotics and FBS as mentioned above.
156 Then, the medium changed to hygromycin B containing one as above and GFP-positive
157 colonies were counted and the best clone was maintained for further experiment. The
158 established packaging cell lines were termed gfp/GP2.

159 To generate HBV pseudotype particles (HBVpp), the established packaging
160 cell line, gfp/GP2 was transfected with the pCEP4 LS-S expression vector with TransIT
161 LT1 (Takara Bio) as mentioned. Two days after transfection, the supernatant was
162 harvested and passed through a 0.22 μm filter as mentioned.

163

164 **Immunoprecipitation and Western blotting analysis.**

165

Supernatant from pCEP4 LS-S transfected gfp/293GP2 cells was adjusted at 0.3M NaCl and incubated with goat polyclonal anti-HBs antibodies (Austral Biologicals) at 4 °C overnight. Then the supernatant was added 20 μl protein G Sepharose® (GE Healthcare) and incubated for 20 min at RT. The complex was pelleted and washed with sodium phosphate buffer (50mM Na-phosphate pH 7.8, 0.3M NaCl) three times. Finally, half of the pellet was suspended in sample buffer (5mM 2-mercaptoethanol, 5% SDS, 0.3% bromophenol blue, 10% glycerol) and an aliquot was subjected to SDS-PAGE followed by Western blotting analysis using a mouse monoclonal antibody against HBsAg (HBs mAb #7-2).

CsCl density gradient ultracentrifugation and Enzyme-linked immunosorbent assay (ELISA) for HBs and preS1.

The culture medium of pCEP4 LS-S transfeted gfp/GP2 cells was harvested as above and was added 30% polyethyleneglycol (PEG6000, nakalai tesque)-3M NaCl at 5% PEG and 0.5M NaCl of final concentration. This solution was stood at 4°C

more than half an hour and then centrifuged at 10,000 × g for 30 min at 4°C. The precipitate was suspended in 500μl 10mM Tris-HCl, pH 7.6-100mM NaCl-1mM EDTA (TNE) solution and clarified insoluble materials. The soluble fraction was loaded at the top of CsCl gradient solution (39F 350 μl, 35F 2500μl, 31F 250μl, 27F 250μl, 23F 250μl, 19F 250μl and 15F 150μl from the bottom, F; CsCl percentage in weight) and centrifuged at 50, 000 rpm at 10°C for 30 hours using a Beckman Ti55 rotor and Optima® TLX ultracentrifuge (Beckman). After the centrifugation, eighteen fractions were taken from the top by the 120 μl. An aliquot (about 10 μl) from each fraction was diluted with 90μl PBS and subjected to ELISA using HBs Antigen (HBsAg) Quantitative ELISA Kit Rapid II® for HBs and HBV PreS1 Antigen Quatitative Kit® (Beakle Inc., Japan) for preS1, respectively, according to the manufacturer's direction. Forty microliter of the aliquot was subjected to RNA extraction followed by RT-PCR targeting an *EGFP* gene (see below).

Detection of a retrovirus gene in the particle by RT-PCR.

198 The supernatant from pCEP4 LS-S transfected gfp/GP2 cells was adjusted at
199 0.3M NaCl was incubated either with goat polyclonal anti-HBs antibodies (1μg/ml)
200 (Austral Biologicals) or rabbit anti-VSV-G antibodies (1μg/ml) (MBL) at 4°C overnight.
201 This mixture then incubated with 20 μl protein-G Sepharose® (GE Healthcare) for 20
202 min at RT. The complex was pelleted and washed with sodium phosphate buffer
203 (50mM Na-phosphate pH 7.8, 0.3M NaCl) three times. The final pellet and 40 μl from
204 each fraction of CsCl density gradient ultracentrifugation as mentioned above was
205 added 200μl or 160 μl TNE, respectively, and 2 μl 10 % SDS and incubated with 20 μg
206 Protainase K (Roche) at 56 °C overnight, respectively. The solution was extracted
207 with phenol-chloroform-isoamylalcohol (24 : 24 : 1) and precipitated with ethanol with
208 10 μg yeast tRNA by centrifugation at 10, 000 × g at 4 °C for 10 min. The precipitate
209 was rinsed with 70 % ethanol and dried and suspended 20 μl TE (10 mM Tris-HCl pH
210 7.6, 1 mM EDTA).
211 5 μl of the preparation was subjected to reverse transcription reaction by using
212 first strand synthesis kit (Transcriptor First Stand cDNA Synthesis Kit®, Roche)
213 according to the manufacturer's direction. This reaction was stopped with

phenol-chloroform-isoamylalcohol (24: 24: 1) extraction followed by ethanol precipitation with 10 μg yeast tRNA as mentioned above. Finally, the dried pellet was suspended in 20 μl TE. Either 5 μl out of 20 μl cDNA sample or 5 μl of RNA preparation sample was constituted in PCR using an EGFP 5' primer; 5'-ACGGCAACATCCTGGGGCACAAGC-3', and an EGFP 1327RV primer; 5'-TGTACAGCTCGTCCATGCCGAGAG-3' and Ex Taq® DNA polymerase (Takara Bio) according to the manufacturer's direction. The PCR condition was 96°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min with 35 cycles. One forth of the reaction (5 μl) was run in the 2 % agarose gel electrophoresis stained with ethidium bromide. A digital image of the DNA was obtained using a fluorescent imager (FX-800®, BioRad).

Electron microscopy.

Cells were cultured on a polystyrene cover slip, Cell Desk (Sumitomo Bakelite Co., Ltd., Japan), fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium-cacodylate buffer (pH7.4) and washed for 5 min three times in the same buffer.

Cells were post-fixed for 1hr with 1% osmium tetroxide and 0.5% potassium ferrocyanide in 0.1 M sodium-cacodylate buffer (pH7.4), dehydrated in graded series of ethanol and embedded in Epon812® (TAAB Co. Ltd., UK). 80 nm ultra thin sections were stained with saturated uranyl acetate and lead citrate solution. Electron micrographs were obtained with a JEM-1011® transmission electron microscope (JEOL, Japan).

237 **Results**

238

239 **Establishment of HBV psuedotype packaging cells.**

240

241 We utilized a murine leukemia virus (MLV) packaging system since

242 considering *in vitro* use for biological cloning of an HBV receptor in cultured cell lines

243 based on infectivity and for checking infectivity of HBV with ease afterwards, even

244 though mature hepatocytes have a limited growth activity in vitro. Retroviral genomes

245 are integrated into host genome after reverse-transcribed only in growing cells except

246 those of lentiviruses, which were utilized in a previous report [24]. Thus, the

247 packaging design was a commonly used retrovirus packaging system consisting of

248 5'-LTR-packaging signal (Ψ)-hyg^{R}-CMV IE promoter-GFP-LTR-3' (Fig. 1A). This

249 construct was integrated into a retrovirus gag-pol expressing GP2-293 cell line

250 (Clontech), the cell line of which was termed gfp/GP2 (Fig. 1B). Thus, the transcribed

251 RNA genome was packaged into MLV capsids. We attempted that upon envelope

252 expression and processing, the capsids would be incorporated into enveloped particles

253 and secreted. If HBV membrane proteins could envelope the retroviral capsid, HBV
254 pseudotype particles (HBVpp) would be formed (Fig. 1C).
255 In order to confirm whether the established packaging cell lines were
256 available or not, we transfected to the cells with a VSV-G expression vector (Takara
257 Bio Clontech) to form a VSV-G pseudotype (in case, this pseudotype is called a
258 pantropic retrovirus) and its infectivity. If the packaging cell lines were available,
259 so-called pseudotype retroviruses wrapped by VSV-G envelope would be generated and
260 secreted into the culture medium. Thus, the supernatant of the VSV-G expressing
261 packaging cells was contacted with BC3, a primary effusion lymphoma cell line or with
262 the other cell lines. Two days after the contact, the infected BC3 cells were selected
263 with a hygromycin B containing medium and we observed that hygromycin B resistant
264 cells were growing and such cells showed GFP positive signals (Data not shown).
265 Several cell lines including HeG2, HuH7, and HEK293 cells were effectively infected
266 with the VSV-G pseudotype retrovirus. These results showed that the packaging
267 system was functional and termed gfp/GP2.
268

Expression of HBV envelope genes.

There are three envelope genes termed large S (LS), middle S (MS) and small S (SS, also called simply HBs) (Fig. 2A) [2; 26]. Each gene is independently transcribed but the coding regions are overlapped. We constructed an HBV membrane protein expression vector termed pCEP4 LS-S under CMV immediate early enhancer and promoter (CMV IEp) in the pCEP4 expression vector (Invitrogen) (Fig. 2B). We were afraid that such strong enhancer and promoter just upstream the LS gene preferentially might drive mainly the LS gene expression, because a balance among HBV envelope proteins affected secretion activity of the virus particles [27]. Transfected cells with the pCEP4 LS-S, however, expressed generally all kinds of HBV envelope genes as reported (Fig. 2C) [28].

Formation and secretion of HBV pseudotype particles into medium.