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Baculovirus-mediated production of HDV-like particles in BHK cells using a novel oscillating bioreactor

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Abstract

We have recently demonstrated the assembly of hepatitis delta virus-like particles (HDV VLP) by co-transducing hepatoma cells using two recombinant baculoviruses, one encoding hepatitis B surface antigen (HBsAg), and one encoding large delta antigen (L-HDAg). In this study, we further demonstrated the assembly and secretion of VLP in other mammalian cells. The assembly efficiency varied depending on cell lines, the baculovirus constructs and the relative dosage of both recombinant viruses. The co-transduction of BHK cells led to the formation of VLPs resembling authentic virions in size and appearance. The production process was transferred to a novel oscillating packed bed bioreactor, BelloCell, in which the transduction efficiency was up to ≈90% for a high cell density of 1.5×10^7 cells/cm^3 bed and a total yield of 427 μg, based on HBsAg in the VLP (harvested from 940 ml medium) was obtained. The particle yield corresponded to an average volumetric yield of 454 ng ml^{-1} and a specific yield of 285 μg/10^9 cells, and is significantly superior to that can be obtained by the commonly employed transfection method. The combination of baculovirus transduction and BelloCell reactor, thus, may represent a simple and efficient approach for the production of HDV VLP and viral vectors.

Keywords: Hepatitis delta virus; Virus-like particle; Baculovirus; BelloCell; Mammalian cell; Virus transduction

1. Introduction

Hepatitis delta virus (HDV) is a satellite virus of hepatitis B virus (HBV) and requires the supply of HBV surface antigen (HBsAg) for replication and transmission (Rizzetto et al., 1977). HDV superinfection of HBV carriers generally cause fulminant hepatitis and liver cirrhosis (Rizzetto et al., 1980a); consequently, HDV imposes a huge threat to the health of approximately 350 million HBV chronic carriers worldwide. However, effective vaccines or diagnostic assays remain unavailable.

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The HDV virion is coated with an outer envelope composed of lipids and HBsAg (He et al., 1989). The envelope surrounds an inner ribonucleoprotein complex comprising a circular RNA genome, as well as large and small forms of hepatitis delta antigens (L-HDAg and S-HDAg). HBsAg exists as large (L)-, middle (M)- and small (S)-forms in the HDV virion. These three isoforms are encoded in a single open reading frame and translated from different in-frame initiation codons, thus, sharing a common C-terminal domain. M-HBsAg carries a preS2 domain at the N terminus of S-HBsAg, while L-HBsAg carries a preS1 domain at the N terminus of M-HBsAg. Each of the three HBsAg exists in two forms with different degrees of glycosylation. The S-HDAg and L-HDAg are identical in sequence, except that L-HDAg contains a C-terminal extension that allows for the isoprenylation of L-HDAg, which is crucial for HDV assembly and inhibition of HDV replication (Chang et al., 1991; Kuo et al., 1989; Taylor et al., 1991). It has been shown that L-HDAg along with S-HBsAg is necessary and sufficient for the assembly of HDV-like particles (HDV VLP) (Ryu et al., 1992; Wang et al., 1991).

VLPs (virus-like particles) are devoid of viral nucleic acids, thus, they are non-infectious. However, VLPs can generally induce broad and strong immune responses, thanks to the preservation of many essential epitopes (Lenz et al., 2001); therefore, VLPs have gained increasing attention as potential vaccine candidates (Roy, 1996; Schiller and Lowy, 2001) or diagnostic reagents (Xiang et al., 2002). Although, numerous studies have focused on the HDV VLP assembly (Chang et al., 1991; Gudima et al., 2002; Jenna and Sureau, 1999; Wang et al., 1996), the possibility of using HDV VLP as a vaccine candidate or a diagnostic reagent has not been assessed. One impediment to this lies in the difficulty in producing sufficient amounts of HDV VLP as transfection of HDV cDNA into cultured hepatoma cells or animal tissues remains the dominant approach to study the assembly (Lai, 1995). Plasmid transfection of hepatoma cells, however, suffers from notoriously low efficiency. Consequently, the VLP yield is generally low and obtaining sufficient VLP for immunological evaluation becomes difficult.

To address this problem, we have recently constructed two recombinant baculoviruses, Bac-GD expressing L-HDAg and Bac-GB expressing HBsAg, to transduce mammalian cells. The co-transduction resulted in efficient self-assembly and secretion of HDV VLP from Huh-7 cells (Wang et al., in press). Comparing to plasmid transfection, baculovirus transduction was simpler and the particle yield was approximately 2-fold higher; therefore, baculovirus transduction may be an attractive approach alternative to plasmid transfection for HDV VLP synthesis. Despite the efficient VLP synthesis in baculovirus-transduced Huh-7 cells, the production was performed in cells cultured in 10-cm dishes. However, subsequent analysis and immunological study require more VLPs, and thus, a production process of larger scale is desired.

In this study, we exploited the advantage of BelloCell bioreactor for the HDV VLP production. BelloCell (Cesco Bioengineering Co., Hsinchu, Taiwan) is a novel oscillating bioreactor that is composed of a packed bed in the upper chamber and a compressible "bellow" in the lower chamber. We have previously shown that BelloCell enables high-density insect cell culture and efficient baculovirus production (Hu et al., 2003a), yet whether BelloCell is suitable for baculovirus transduction of mammalian cells and HDV VLP production, has not been evaluated. Therefore, the major objective of the present study is aimed at increasing the HDV VLP yield by combining the merits of baculovirus transduction and BelloCell bioreactor.

2. Materials and methods

2.1. Cells and recombinant baculoviruses

Mammalian cells Huh-7, CHO, 293, HeLa and BHK were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10 or 5% fetal bovine serum (FBS, Gibco). The recombinant baculoviruses, Bac-GD encoding L-HDAg under the control of cytomegalovirus immediate early (CMV) promoter and Bac-GB encoding the HBV PreS2/S/enhncer domains, were constructed earlier (Wang et al., in press). Both gene fragments were cloned into multiple cloning site II (MCS II) of pFastBac DUAL plasmid (Invitrogen). The reporter gene *egfp* under the control of CMV promoter was subcloned into MCS I (Fig. 1). The resultant plasmids were used for the generation of recombinant baculoviruses using Bac-to-Bac® system (Invitrogen) according to the manufacturer's instructions.

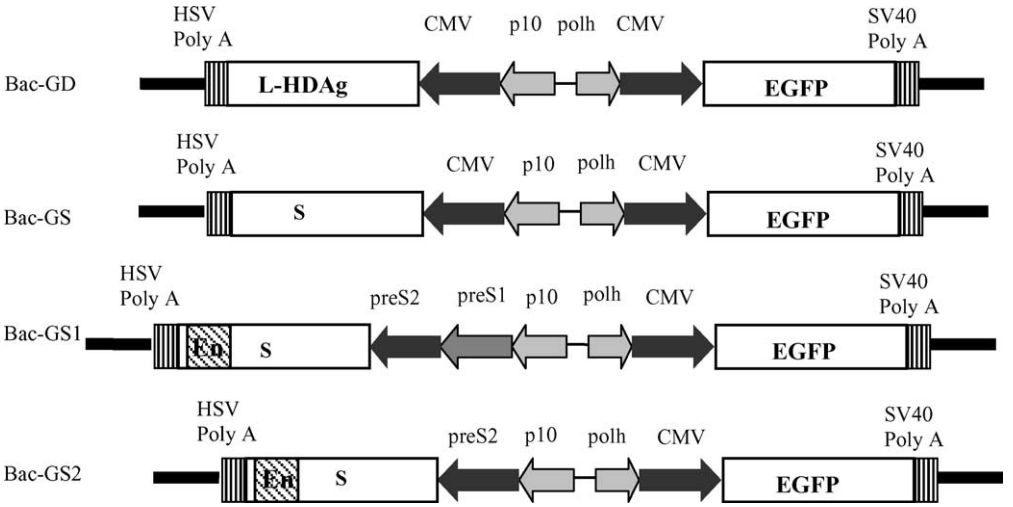

Fig. 1. Schematic illustration of the baculovirus vectors used in this study. Polyhedrin (polh) and p10 are endogenous baculovirus promoters in the plasmid but they are not functional in mammalian cells. HSV and SV40 Poly A are polyadenylation signals provided in the plasmid. Bac-GS2 was previously designated Bac-GB (Wang et al., in press).

Additionally, two more viruses were constructed in a way similar to Bac-GB construction, except that PreS1/PreS2/S/enhancer or CMV-S regions were cloned into MCS II (Fig. 1). The PreS1/PreS2/S/enhancer fragment consisted of PreS1, PreS2, S and enhancer regions, plus the PreS1 promoter upstream of the PreS1 coding region. The CMV-S region was generated by cloning the S coding sequence into pcDNA3.1 plasmid (Invitrogen) under the control of CMV promoter, then the whole region was PCR-amplified and subcloned into pFastBac DUAL plasmid. The resultant viruses were named Bac-GS1 and Bac-GS, respectively. To make the terminology more consistent, Bac-GB was re-designated Bac-GS2 here. The viruses were amplified by infecting insect cells cultured in TNM-FH medium and titered by end-point dilution assay using insect cells as the host (O'Reilly et al., 1992). The titers of all viruses were 1×10^8 plaque forming units (pfu)/ml. The viruses were not concentrated by ultracentrifugation.

2.2. Carrier for cell immobilization

The carrier used for cell immobilization, BioNOC II (Cesco Bioengineering Co.), was non-woven fabric strips (width ≈5 mm, length ≈10 mm) made of 100% polyethyleneterephthalate (PET). Each BelloCell was pre-packed with ≈860 pieces (bed volume ≈ 100 cm^3) of BioNOC II carriers, and then pre-sterilized by γ-irradiation.

2.3. BelloCell configuration and operation

The working liquid volume of BelloCell bioreactor used in this study was 500 ml. The upper chamber of BelloCell contained two carrier support screens to accommodate BioNOC II carriers, while the compressible lower chamber (i.e., the "bellow") contained the medium (Fig. 2). The "bellow" could be alternately compressed and released by BelloStage® (Cesco Bioengineering Co.) whose configuration was described previously (Hu et al., 2003a). The compression raised the medium level to submerge the carriers (Fig. 2), thus allowing for nutrient transfer. On the other hand, the relaxation dropped the medium to the lower bellow, thus exposing the carriers to air for oxygen transfer (Fig. 1).

For cell culture in BelloCell, 5×10^7 BHK cells, suspended in 30 ml DMEM, were added to BelloCell that was pre-filled with 470 ml DMEM (supplemented with 5% FBS), then the reactor was mounted onto BelloStage® in a 37 °C incubator. The linear moving rate was set at 2 mm s^{-1} for 2 h until cells attached, and then was lowered to 1.5 mm s^{-1} throughout the

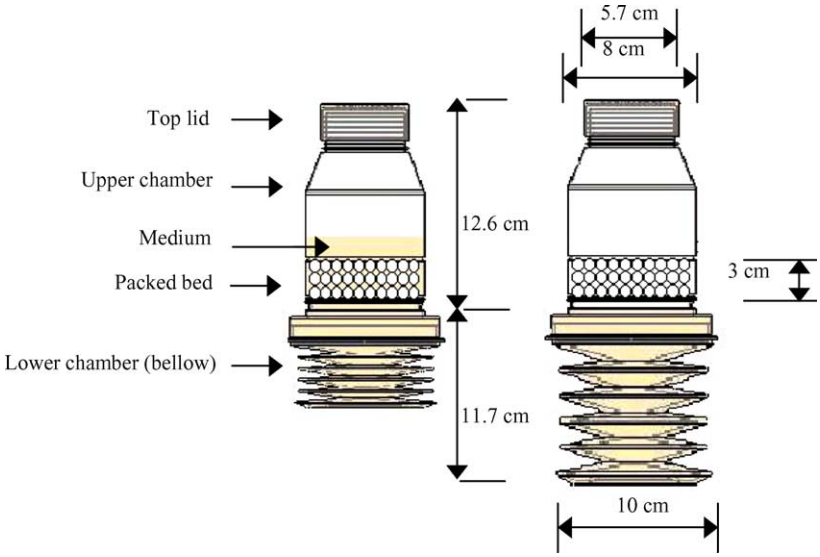

Fig. 2. Schematic illustration of the BelloCell bioreactor. The reactor consisted of an upper chamber containing the packed bed for cell immobilization and a compressible bellow that can be alternately compressed and released by BelloStage®. The photograph of BelloStage® was shown previously (Hu et al., 2003a). The working volume was 500 ml and the dimensions of the reactor are shown. The top lid was equipped with a 0.22 μm filter for gas exchange and can be opened for medium replacement.

culture period. When the pH fell to 6.8, or when the glucose concentration as determined by YSI 2700 analyzer (Yellow Spring Instruments), fell to 1–1.5 g l^{-1}, the spent medium was completely replaced by fresh medium.

During the culture, six BioNOC II carrier strips were taken daily from the top, middle and bottom layers of the bed and evenly distributed to three microfuge tubes. Each tube was filled with 1 ml lysis solution (0.1% crystal violet in 0.1 M citric acid), and incubated for 1 h at 37 °C. The nuclei were released by vortexing and counted by a hemacytometer. The cell numbers calculated from the three tubes were averaged and converted to the total cell number in the reactor.

2.4. Virus transduction and VLP production

One feature of the transduction protocol was that volumes of unconcentrated virus, rather than multiplicity of infection (MOI), were used to indicate the virus dosage (Hsu et al., 2004). Another feature of this protocol was that Dulbecco's phosphate-buffered saline (PBS) was employed as the surrounding solution, and a volumetric ratio (PBS to virus solution) of 3–4 was optimal for BHK cells with regard to enhancing the transduction efficiency and transgene expression (Wang et al., in press). In light of our previous findings, the cells (1×10^7) cultured on 10-cm dishes were transduced by incubating the cells with unconcentrated virus solution for 6 h at 27 °C using 3.2 ml PBS as the surrounding solution (Wang et al., in press). The volumes of the virus solutions varied, depending on the experiment design (see Section 3).

To transduce BHK cells cultured in BelloCell, the spent medium was poured and the immobilized cells were washed with 400 ml PBS for 5 min. After washing, PBS was discarded and the virus transduction was initiated by the addition of 100 ml Bac-GS2 and 25 ml Bac-GD. The reactor was swirled several times to allow uniform contact between cells and viruses, and then filled with 375 ml PBS. This gave a volumetric ratio (PBS to virus solution) of 3. The transduction continued for 6 h at 27 °C by mounting the reactor onto BelloStage® and setting the linear moving rate at 1.5 mm s^{-1}. At the end of the transduction period, the virus solution was removed and 500 ml fresh medium containing 2.5 mM sodium butyrate (Sigma) was added. The reactor was mounted onto BelloStage® in a 37 °C incubator, then the VLP production phase commenced (BelloStage® settings were identical to

those in the culture phase). During the production phase, 30 ml medium was sampled at 24 and 72 h post-transduction (hpt) while ≈470 ml medium was harvested at 48 and 96 hpt. After the harvest at 48 hpt, the reactor was replenished with 500 ml fresh medium containing no sodium butyrate.

For the collected medium samples, 20 ml was ultracentrifuged through 20% sucrose cushion at 38,000 rpm (P40ST rotor, Hitachi) for 5 h for Western blot analysis and 1 ml was used for enzyme immunoassay (EIA). Besides, nine BioNOC II carrier strips were taken daily from different layers and locations in the bed, and evenly distributed into three microfuge tubes. The cells were detached three times by trypsinization, resuspended in PBS, and analyzed for the percentage of GFP+ cells and mean fluorescence intensity (FI) by flow cytometry (FACSCalibur, Becton Dickinson), as described previously (Ho et al., 2004). The data of three tubes were averaged.

2.5. Enzyme immunoassay (EIA)

The amounts of secreted HBsAg in the medium samples were quantified using the EIA kits (AxSYM/HBsAg Confirmatory Assay, Abbott Laboratories), and the data are reported as S/N (sample versus negative controls) ratios. The medium samples were diluted 50-fold and measured by EIA again, so that the S/N values of the samples fell within the range of known amounts of HBsAg. The S/N ratios were converted to the HBsAg yield by interpolation.

2.6. Purification of VLP

The VLPs from the cells cultured in 10-cm dishes were purified as described earlier (Wang et al., in press). For the VLPs produced in BelloCell, 200 ml medium was concentrated by Stirred Cell (Millipore) to 50 ml. The retenate was ultracentrifuged through 20% sucrose cushion (38,000 rpm, 5 h), and the VLPs were purified by 10–40% CsCl gradient ultracentrifugation (38,000 rpm, 24 h). The aliquots of CsCl gradient were fractionated from bottom to top and ultracentrifuged (38,000 rpm, 5 h) again. The pellets were resuspended in 50 μl deionized water for Western blot and transmission electron microscopy (TEM) analyses.

2.7. Western blot

The Western blots were performed as described previously (Wang et al., in press). The membranes were developed with BCIP/NBT color developing reagent (Sigma).

2.8. TEM examination

The diameter and morphology of the purified particles were examined by TEM (Hitachi, H7500), as described previously (Hu et al., 1999). At least three representative micrographs were used to measure the particle size for histogram analysis.

3. Results

3.1. Efficiency of HDV-like particle (HDV VLP) secretion using different cells and viruses

We have previously demonstrated the expression and secretion of HDV VLP from Huh-7 cells that were co-transduced by Bac-GS2 (previously designated Bac-GB) and Bac-GD (Wang et al., in press). Despite the high yield (≈1.5 μg HBsAg per 10-cm dish), Huh-7 cells cultured in BelloCell exhibited longer lag phases (≈48 h) and lower final cell yields (≈2×10^9 cells) comparing to other mammalian cells (unpublished data). This would impose practical difficulties upon process scale-up, and prompted us to explore whether an alternative cell line could be used for VLP production. To this end, BHK, HeLa, CHO, 293 and Huh-7 cells were co-transduced by Bac-GS2 and Bac-GD (800 μl each) on 10-cm dishes. Fig. 3a depicts that the secretion of both S-HBsAg (glycosylated, gp27, and unglycosylated, p24) and L-HDAg from BHK cells was slightly lower than from Huh-7 cells, but was significantly higher than those from CHO and 293 cells. HeLa cells secreted abundant S-HBsAg, but considerably lower amount of L-HDAg. M-HBsAg expression was barely detectable (but was evident using chemiluminescence enhancement, data not shown) because S-HBsAg accounts for ≈95% of the envelope proteins (Bonino et al., 1986). In light of these data, BHK was chosen for subsequent VLP production.

To determine whether HBsAg expression mediated by different virus constructs could improve the

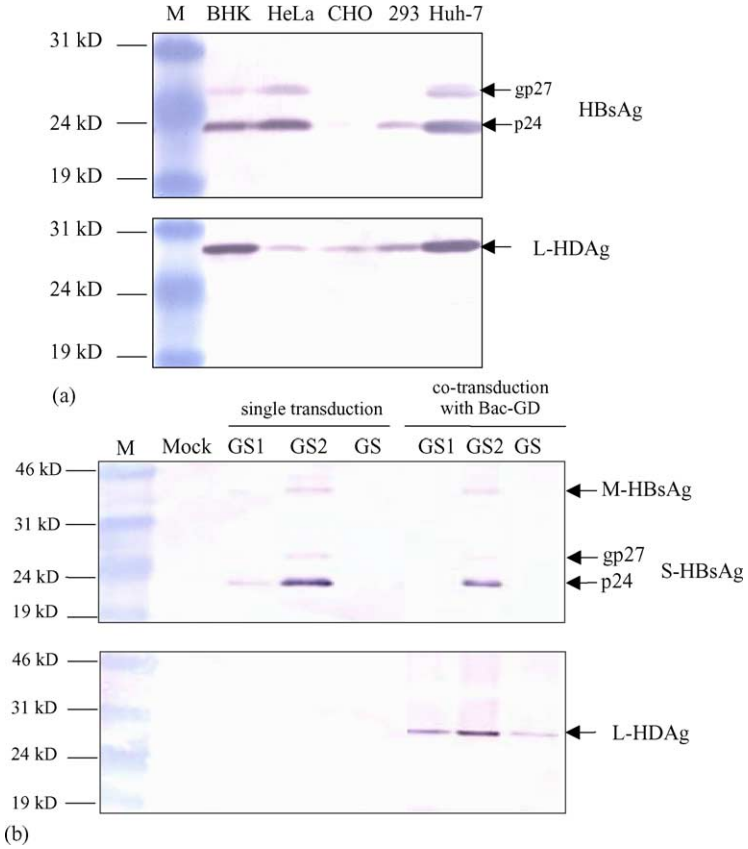

Fig. 3. (a) Western blot analysis of the VLP secreted into the medium of BHK, HeLa, CHO, 293 and Huh-7 cells that were co-transduced by Bac-GS2 and Bac-GD (800 μl each). (b) Western blot analysis of the VLP secreted into the medium of BHK cells that were either singly transduced by one of the three virus constructs (800 μl Bac-GS, Bac-GS or Bac-GS2), or additional co-transduced with Bac-GD (800 μl). The cells (1×10^7 cells) were cultured in 10-cm dishes and transduced for 6 h at 27 °C using 3.2 ml PBS as the surrounding solution. The medium was collected at 2 dpt and the particles were pelleted by ultracentrifugation (38,000 rpm, 5 h). M, protein marker; Mock, mock-transduction.

VLP secretion, Bac-GS1 and Bac-GS were constructed. BHK cells were either singly transduced with one of these three viruses or additionally co-transduced with Bac-GD. As shown in Fig. 3b, the single transduction by Bac-GS2 resulted in the highest expression levels of S-HBsAg. Also, some M-HBsAg proteins were detected. In contrast, Bac-GS1 yielded lower S-HBsAg expression and Bac-GS yielded nearly undetectable expression. Accordingly, upon co-transduction with Bac-GD, Bac-GS2 led to a significantly higher level of VLP secretion in comparison with Bac-GS and Bac-GS1, hence, Bac-GS2 was chosen for subsequent co-transduction experiments.

3.2. Effect of relative virus dosage on VLP secretion

In all aforementioned experiments, the cells were co-transduced with equal virus volumes. Since, the relative virus dosage, and hence, the relative expression levels of structural proteins could affect the particle assembly (Hu and Bentley, 2001), the effect of relative virus dosage on VLP secretion was examined by co-transducing BHK cells at varying volumetric ratios. As shown in Fig. 4a, when the Bac-GS2 dosage was fixed at 800 μl (MOI 8), 200 μl Bac-GD (MOI 2) appeared to give the highest S-HBsAg and L-HDAg secretion. Increasing the Bac-GD dosage to 400 and

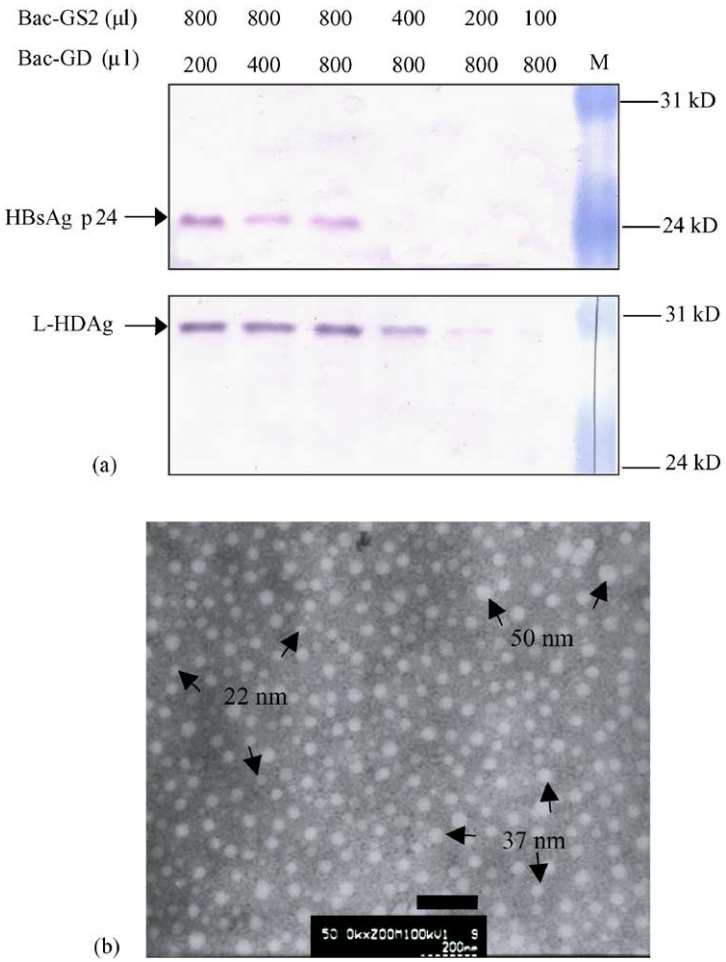

Fig. 4. Effect of relative virus dosage on VLP assembly and secretion. BHK (1×10^7) cells in 10-cm dishes were co-transduced by Bac-GS2 (1×10^8 pfu ml^{-1}) and Bac-GD (1×10^8 pfu ml^{-1}) with varying volumetric ratios. The co-transduction, medium collection, sample centrifugation and Western blot were performed, as in Fig. 3b, except that different volumes of virus solutions were added. Western blot analysis of the pelleted VLP is shown in (a). The electron micrograph of the CsCl-purified particles from the cells co-transduced at a volumetric ratio of 4 is shown in (b). The arrows indicate particles of different sizes. Bar, 200 nm; magnification, 50,000×; M, protein marker.

800 μl, surprisingly, did not increase L-HDAg secretion, but instead decreased the amount of S-HBsAg slightly. Likewise, when the Bac-GD dosage was fixed at 800 μl, the particle secretion efficiency decreased dramatically with decreasing Bac-GS2 dosage. These data obviously indicated the importance of relative virus dosage for particle secretion, and a volumetric ratio of 4 (800 μl Bac-GS2 to 200 μl Bac-GD) appeared optimal for particle secretion. Besides, a ratio of 4, comparing to a ratio of 1, decreased the Bac-GD dosage required for transduction.

The average diameter of the HDV virion was reported to be 35–37 nm (Bonino et al., 1986; Rizzetto et al., 1980b). However, the higher amount of S-HBsAg at the ratio of 4 may arise as a result of excess HBsAg empty particles, since S-HBsAg alone could assemble into 22 nm particles and be secreted into the medium (Dane et al., 1970). To investigate this hypothesis, the particles secreted from BHK cells were purified by CsCl gradient ultracentrifugation (Wang et al., in press). The TEM examination revealed that the co-transduction by a volumetric ratio of 4 (Fig. 4b) resulted

in the formation of 36–37 nm particles that resembled the VLP expressed by Huh-7 cells (Wu et al., 1997) in morphology and size. Note, however, that a distribution of particle size (22–50 nm) existed. The histogram analysis estimated that the 36–37 nm particles accounted for ≈75% of the total particles (data not shown), while 22 nm particles accounted for only ≈15%. The TEM examination, thus confirmed that HBsAg particles were not excessively formed when using a volumetric ratio of 4 and justified its use.

3.3. Culture and transduction of BHK cells in BelloCell

After selecting the suitable cell line, virus construct and relative virus dosage for VLP synthesis, the production process was transferred to BelloCell. BHK cells were seeded at 5×10^7 cells and the culture proceeded in a fed-batch mode with intermittent medium exchange. As shown in Fig. 5a, the cells experienced a short lag phase, and then, the exponential growth phase rapidly commenced. Without baculovirus transduction, a final cell yield of $\approx 5.5 \times 10^9$ cells was reached in 192 h. This corresponded to a density of $\approx 1.1 \times 10^7$ cells/ml medium and proved the excellent culture characteristics of BHK cells in BelloCell.

In parallel, cells were cultured under identical conditions but were co-transduced at 96 h (when the cell number reached $\approx 1.5 \times 10^9$ cells) with 100 ml Bac-GS2 (MOI ≈ 6.7) and 25 ml Bac-GD (MOI ≈ 1.7), which corresponded to a volumetric ratio of 4, as determined above. Fig. 5a shows that the transduced BHK cells continued to grow but at a relatively lower rate compared to the untransduced cells, probably due to the overexpression of EGFP under the control of CMV promoter, which could be toxic or cause extra metabolic burden for some cells (Detrait et al., 2002; Wang et al., 2000). Fig. 5b shows the transduction efficiency and transgene expression profiles of three independent reactor runs, as measured by flow cytometry, and demonstrates that our transduction protocol resulted in very high percentages of GFP+ cells (≈90%) and strong mean FI (>2000 a.u.) throughout the production phase. This transduction efficiency was only slightly lower than that in 2-D cultures (95%; data not shown), proving that the packed bed and high cell density (1.5×10^7 cells/cm^3 bed) did not hamper the virus attachment to cells and subsequent virus uptake. The

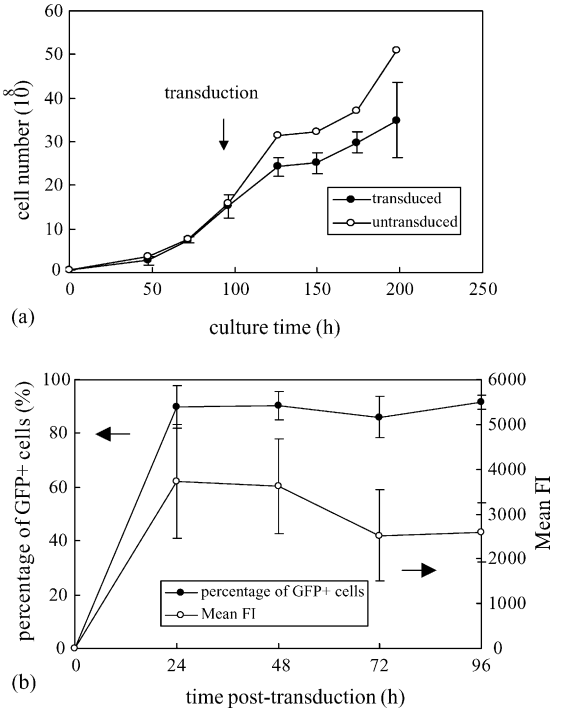

Fig. 5. (a) The growth curves of BHK cells cultured in BelloCell. (b) Time-course profiles of the percentages of GFP+ cells and mean FI after transduction. For the untransduced experimental group, the culture continued until 192 h. For the transduced experimental group, the cells were co-transduced at 96 h (cell number $\approx 1.5 \times 10^9$) with 100 ml Bac-GS2 and 25 ml Bac-GD for 6 h at 27 °C. In the course of culture, six BioNOC II carriers were taken every 24 h for cell count. After transduction, nine more BioNOC II carriers were taken daily and the cells were detached to analyze the percentage of GFP+ cells and mean FI by flow cytometry. The mean FI are expressed in arbitrary units (a.u.). The data represent the mean ± standard deviations (S.D.) of three independent bioreactor runs.

high-transduction efficiency was partly attributed to the oscillating movement of medium caused by the compression and relaxation, which theoretically enhanced the probability of contact between viruses and cells. Furthermore, the strong mean FI throughout the production phase suggested that the cells were actively producing recombinant proteins.

3.4. Production of HDV VLP in BelloCell

Since, the molar ratio of HBsAg and L-HDAg in the VLP was unknown, and a proper method to quantify L-HDAg was unavailable, the production kinetics of HDV

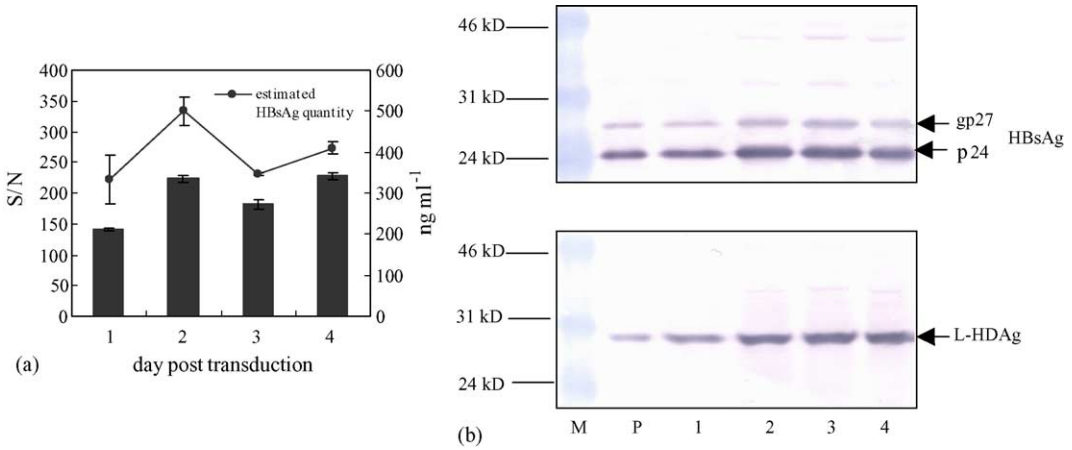

Fig. 6. Time-course profiles of VLP production in BelloCell as monitored by EIA (a) and Western blot (b). The BHK cells were cultured and transduced in BelloCell as described in Fig. 5. The medium samples were assayed by EIA and the S/N values (bars) represent the mean ± S.D. of three independent bioreactor runs. The medium samples were diluted 50-fold for another EIA assay and the S/N values were used to estimate the VLP yield (●) based on the HBsAg concentrations. The VLPs were pelleted as in Fig. 3 for Western blot. Lanes 1–4, samples collected at 1–4 dpt. P, positive control (VLP from Huh-7 cells).

VLP was monitored by the HBsAg yield in the particle. Fig. 6a shows the EIA results of three independent reactor operations, and depicts that the S/N values (bars) increased sharply at 2 dpt, but dropped slightly at 3 dpt because of the medium change at 2 dpt. Despite this, the S/N ratio rapidly increased at 4 dpt again, suggesting a very active VLP production throughout the 4-day period. Note the product yield precipitously decreased thereafter (data not shown). The corresponding HBsAg concentrations in the medium were calculated to be 500, 350 and 410 ng ml^{-1} at 2, 3, and 4 dpt, respectively. The identities of the secreted proteins were examined by Western blot. Fig. 6b confirmed that the particles contained both S-HBsAg (glycosylated and

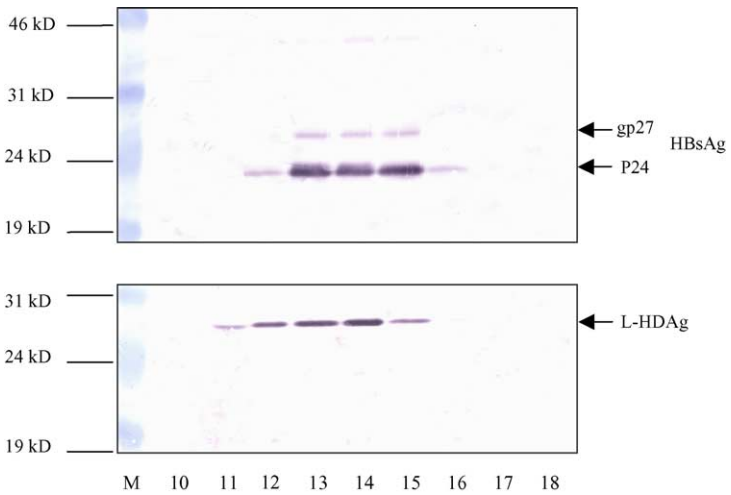

Fig. 7. Western blot analysis of VLP purified by CsCl gradient ultracentrifugation. The medium (200 ml) harvested at 4 dpt was concentrated to 50 ml by Stirred Cell and the VLPs were pelleted by sucrose-cushioned ultracentrifugation. The VLPs were then purified by CsCl gradient ultracentrifugation (38,000 rpm, 24 h). Fractions 10–18 were subjected to Western blot analysis.

unglycosylated forms) and L-HDAg, and the expression kinetics agreed well with the S/N values. Namely, the VLPs were actively expressed and secreted as early as 1 dpt, and the high-level expression continued for 4 days, confirming that BelloCell was suitable for the production of HDV VLP.

3.5. Purification of HDV VLP

Since, the total volume of the medium, harvested from the bioreactor, was large (≈940 ml), Stirred Cell was implemented to concentrate the samples prior to CsCl gradient ultracentrifugation. Membranes with 300 kDa molecular mass cut-off were chosen because the average diameter of HDV VLP was 36–37 nm. The Western blot revealed no S-HBsAg or L-HDAg in the filtrate (data not shown), indicating the successful retention of VLP in the retenate. Further, Western blot analysis of the gradient-purified samples revealed the presence of S-HBsAg and L-HDAg in fractions 13–15 (Fig. 7).

4. Discussion

We have recently reported on the efficient formation and secretion of HDV VLP from Huh-7 cells by baculovirus co-transduction. In this study, we further demonstrate that HBsAg and L-HDAg, upon co-transduction, could be expressed and secreted from other mammalian cell lines. Since, L-HDAg alone is located within the nucleus (Wu et al., 1992), the co-secretion indicates the VLP assembly and secretion (Sheu et al., 1996; Wang et al., 1996; Wu et al., 1991). The VLP yield varied among cell lines and followed the order: Huh-7 > BHK > HeLa > 293 > CHO. In general, the baculovirus transduction efficiency of BHK is greater than 90% (Condreay et al., 1999; Hu et al., 2003b), and the efficiencies of HeLa and 293 range between 50 and 85% (Condreay et al., 1999; Hu et al., 2003b; Kronschnabl et al., 2002; Sarkis et al., 2000), whereas, the efficiency of CHO is generally between 30 and 75% (Cheng et al., 2004; Condreay et al., 1999; Hu et al., 2003b). The cell-type dependence of VLP secretion, thus correlated well with that of transduction efficiency. Since, Huh-7 was more difficult to be cultured in BelloCell, BHK was chosen for subsequent VLP production. One additional advantage of using BHK is that it is one of the few cell lines approved by FDA for pharmaceutical protein production; thus, future process validation may be easier.

Besides, additional viruses Bac-GS and Bac-GS1 were constructed to evaluate whether higher VLP yield was possible. Disappointingly, these two viruses expressed S-HBsAg less efficiently, and thus, decreased the VLP yield. Nonetheless, we found that the volumetric ratio of Bac-GS2 to Bac-GD profoundly affected the particle assembly and secretion, and a relative dosage of 4 was found to be optimal in maximizing VLP secretion. This was likely due to the fact that L-HDAg suppressed the expression of HBsAg (Wu et al., 1991), thus, leading to a higher requirement for Bac-GS2. Another possibility was that each HDV VLP contained a larger amount of HBsAg than L-HDAg, leading to a higher requirement for HBsAg expression; yet, this hypothesis could not be confirmed at present because the molar ratio of HBsAg to L-HDAg in the HDV VLP remained unknown. Note, the HDV VLP encompasses lipids derived from host cell membranes and a size distribution has been observed for particles produced by yeast and Huh-7 cells (Wu et al., 1997). In this study, a volumetric ratio of 4 resulted in the formation of VLP with morphology and size distribution, similar to those of HDV virions, found in infected chimpanzees (Bonino et al., 1986; Rizzetto et al., 1980b), and the 36–37-nm particles were predominant (Fig. 4). In addition, few 22 nm HBsAg particles were observed, indicating that S-HBsAg was not expressed in excess using the current protocol. More importantly, isoprenylation of L-HDAg is crucial for virion formation (Lee et al., 1994; Ryu et al., 1992); thus, the assembly of HDV VLP implied that L-HDAg was also properly isoprenylated in BHK cells. Additionally, HBsAg in the HDV VLP produced from BHK cells exhibited identical glycosylation patterns, as in Huh-7 cells (Fig. 6b). Comparing to the VLP produced in yeast, in which most HBsAg molecules were not glycosylated (Wu et al., 1997), the VLP produced in this system may resemble the authentic particles more closely. Therefore, the VLP produced from BHK cells using BelloCell potentially possessed immunological properties similar, if not identical, to those of the VLPs produced in Huh-7 cells and authentic HDV. These attributes substantiate the feasibility of using the VLP as a vaccine candidate or a diagnostic reagent.

The transduction parameters characterized in 2D culture studies were further applied to the bioreactor

operation. With our protocol, the transduction efficiency was up to 90%, although, the corresponding MOI for Bac-GS2 and Bac-GD were only 6.7 and 1.7. Thanks to the highly efficient gene transfer, abundant VLPs were expressed and secreted into the medium in BelloCell. The EIA and Western blots (Fig. 6) indicated that the cells actively synthesized and secreted the VLP in the entire production phase. Using a standard provided in the EIA kits, the total particle yield for the entire reactor operation was estimated to be ≈427 μg (for 940 ml medium) based on the HBsAg content, which corresponded to an average volumetric yield of 454 ng ml^{-1}, and a specific yield of 285 μg/10^9 cells (divided by the cell number at transduction 1.5×10^9). The volumetric yield was approximately 1.5–2-fold and 5-fold higher compared to that (≈150 ng ml^{-1}) obtained from Huh-7 cells cultured in 10-cm dishes (Wang et al., in press) and that (80 ng ml^{-1}) obtained in the plasmid transfection system (Sureau et al., 2003). The high yield indicated that the efficient oxygen and nutrient transfer characteristics of BelloCell reactor (Hu et al., 2003a) provided an environment, not only suitable for BHK cell culture, but also for protein expression and VLP assembly.

Although, numerous groups have reported on the assembly of HDV VLP, follow-up studies of the chemical compositions, immunogenic properties and the potentials as a vaccine or a diagnostic reagent have been absent due to the low VLP yield. One alternative to resolve the problem is the use of baculovirus/insect cell system; however successful formation of HDV VLP in insect cells has not been reported, probably because L-HDAg expressed in insect cells could induce cell cycle arrest (Hwang and Park, 1999) and could be degraded rapidly (Hwang et al., 1992). Stable mammalian cell expression system may offer another alternative, but it takes months to generate the stable clones and lacks the flexibility to conduct necessary mutations in order to study the assembly. Besides, the relative expression levels of HBsAg and L-HDAg can not be readily manipulated. In contrast, baculovirus can be constructed in a few weeks, thus conferring flexibility in genetic manipulation-like plasmid systems; the protein expression levels can be manipulated by varying virus dosage, and the particle characterization can be readily performed thanks to the efficient transduction and high-level expression. The development of the production process, using the baculovirus/mammalian cell system in BelloCell, enables us to rapidly elevate the particle yield to more than 400 μg, a satisfactory quantity for laboratory research purposes. The yield may be further improved via process optimization. Currently, the bottleneck to subsequent analysis and immunologic evaluation of the VLP resides in the downstream purification. In this study, we successfully concentrated the medium samples by employing the Stirred Cell without appreciable loss of the VLP, but less than 1% of the VLP was recovered after CsCl gradient ultracentrifugation. Further development of a more efficient chromatographic purification scheme is ongoing to enhance the recovery yield.

Another potential application of this production process is the generation of viral vectors for gene therapy. Currently, the majority of retroviral or lentiviral vectors are produced via transfection of producer cell lines (Andreadis et al., 1999; Merten, 2004). However, transfection requires a huge amount of purified plasmids and the transfection process is difficult to scale-up. Since, HDV VLP can self-assemble in the baculovirus-transduced cells, similarly, the gene elements essential for retroviral vector assembly can be cloned into baculovirus and delivered into the producer cells. Since, baculovirus can confer highly efficient gene transfer and our protocol eliminates the need for virus purification, the combination of baculovirus transduction and BelloCell reactor may represent a simple and efficient approach for viral vector production.

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