Cancer Gene Therapy (2006), 1–8
 0929-1903/06 $30.00
www.nature.com/cgt

ORIGINAL ARTICLE

Gene therapy of liver tumors with human liver-specific nanoparticles

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The development of safe and efficient liver-specific gene delivery approaches offers new perspectives for the treatment of liver disease, in particular, liver cancer. We evaluated the therapeutic potential of hepatotropic nanoparticles for gene therapy of liver tumor. These nanoparticles do not contain a viral genome and display the hepatitis B virus L antigen, which is essential to confer hepatic specificity. It has not been shown whether a therapeutic effect could be obtained using L nanoparticles in a human liver tumor xenograft model. Rats bearing human hepatic (NuE) and non-hepatic tumors were injected with L nanoparticles containing a green fluorescent protein (GFP) expression plasmid. GFP expression was observed only in NuE-derived tumors but not in the non-hepatic tumor. The potential for treatment of liver tumors was analyzed using L nanoparticles containing the herpes simplex virus thymidine kinase gene, in conjunction with ganciclovir pro-drug administration. The growth of NuE-derived tumors in L particle-injected rats was significantly suppressed, but not of the non-hepatic tumor control. In summary, this is the first demonstration that nanoparticles could be used for delivery of therapeutic genes with anti-tumor activity into human liver tumors. This intravenous delivery system may be one of the major advantages as compared to many other viral vector systems.
Cancer Gene Therapy advance online publication, 22 September 2006; doi:10.1038/sj.cgt.7700990

Keywords: HBsAg L nanoparticle; gene therapy; liver tumor; HSV-*tk*/GCV system

Introduction

Gene therapy is recognized as one of the most promising approaches for treatment of serious diseases, including monogenic diseases, infectious diseases and cancer.[1,2] About 60% of the clinical trial protocols for gene therapy have addressed the treatment of cancer, and a number of preclinical studies are ongoing *in vitro* and *in vivo*. Many types of therapeutic genes have been investigated, and some of them have shown anti-tumor effects. Suicide genes are one such class of therapeutic genes.[3,4] Tumor cells expressing suicide genes become sensitive to pro-drugs, and prodrug treatment can be used to kill the tumor cells specifically. Herpes simplex virus thymidine kinase (HSV-*tk*) gene and ganciclovir (GCV) form one of the more important suicide gene/prodrug combinations. The use of various vectors carrying HSV-*tk* has been reported in treatment of malignant glioma,[4,5] head and neck cancer,[6] colorectal cancer[7] and prostate cancer.[8]

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Received 19 December 2005; revised 18 June 2006; accepted 19 August 2006

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy with a rising incidence worldwide.[9,10] Unresectable HCC lack effective therapy[9,10] and new therapeutic modalities are urgently needed. The transfer of therapeutic genes to liver tumors or to the peritumoral tissue is particularly attractive for the potential treatment of liver cancer and metastasis.[11,12] HCC is also a target of the HSV-*tk*/GCV system.[13–16] However, non-specific expression of suicide genes in non-target cells may cause undesirable adverse effects, such as bone marrow suppression,[17] and for such therapeutic genes to exert their function in a target-specific manner, it is important to specifically deliver them into the target cells or tissues while obviating inadvertent gene transfer into non-target cells.

Various viral vectors, including retroviral, lentiviral, adeno-associated viral (AAV), herpes viral and adenoviral vectors are currently available for gene therapy and are being explored for (liver) cancer gene therapy in particular.[13–16] These vectors do not have target selectivity which reflects the broad tropism of the viruses from which they are derived. Consequently, many vectors wind up transducing non-target cells which reduces the overall efficacy of the gene therapy approach.[18] Moreover, this lack of targeting specificity may cause unexpected adverse

effects, such as bone marrow suppression, due to the inadvertent transgene expression in non-target cells.[17] Although the current vector technologies are being improved further, the development of alternative novel vector systems with improved targeting specificity, higher transduction efficiencies and improved safety is warranted.

We have recently described a novel gene delivery approach based on hepatotropic nanoparticles.[19] These nanoparticles are engineered to display the hepatitis B virus surface L antigen (HBsAg) on their surface and are devoid of viral genomes,[20] which obviates potential safety concerns related to the emergence of replication-competent viruses. These nanoparticles possess high gene transfer efficiency and show high specificity to human liver cells.[19,21] We have succeeded in loading several different genes into the particles, and then transferring these genes into human liver cells *in vitro* and *in vivo*. In addition, L nanoparticles are easily produced by recombinant yeast cells and large-scale production of the particles has already been established,[20,22] whereas large-scale manufacturing of clinical-grade viral vectors remains a significant obstacle that hampers its clinical implementation.[23,24]

It is not known whether nanoparticles could be used successfully for targeted delivery of therapeutic genes with anti-tumor activity into human liver tumors. As a proof-of-concept and as a logical extension of our prior work,[19] we have therefore investigated the distribution of L nanoparticles in a human liver tumor xenograft rat model, using a green fluorescent protein (GFP) expression plasmid as a reporter, and analyzed their potential for treatment of liver tumors using the HSV-*tk*/GCV system.

Materials and methods

Purification and assays for the L protein from the yeast

HBsAg L nanoparticles were prepared from the yeast cells, *Saccharomyces cerevisiae* AH22R$^-$, carrying HBsAg L protein expression plasmid pGLDLIIP39-RcT.[20] The whole cell extract of the recombinant yeast was fractionated with PEG 6000, and separated twice by CsCl isopycnic ultracentrifugation and once by sucrose density gradient ultracentrifugation, as described previously.[20,22] The level of the HBsAg L proteins in the yeast cell extracts were determined by silver-stained SDS-PAGE. The S-antigenic activity in the cell extracts was determined by the IMx system (Abbott Laboratories, Abbott Park, IL, USA) in conjunction with a microparticle enzyme immunoassay (MEIA) in accordance with the manufacturer's instructions.

Cell cultures

Two cancer cell lines were used for the *in vivo* studies. A spontaneously immortalized cell line derived from a fetal liver cell, NuE,[25] was kindly provided by Professor T Tadakuma (National Defense Medical College, Japan). Hepatocyte specific antigen, keratins and α-fetoprotein (AFP) were examined immunohistochemically in this cell line. Hepatocyte specific antigen and keratins were positive, but AFP was negative in NuE (data not shown). We confirmed the growth of tumor when we implanted these cells into mice or rats. We used this transformed hepatocyte cell line as a human liver tumor cell line. The human colon cancer cell line, WiDr, was obtained from American Type Culture Collection (Rockville, MD, USA). NuE cells were maintained in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA). WiDr cells were maintained in Dulbecco's modified eagle's medium (DMEM; Sigma) supplemented with 10% FBS. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Preparation of L nanoparticles

Mammalian GFP expression plasmid (pTB701-hGFP)[26] (20 μg) under the control of the SV40 promoter or a plasmid expressing HSV-*tk* (pGT65-hINF-alpha) (InvivoGen, San Diego, CA, USA) (20 μg) under the control of the CMV promoter was mixed with purified L nanoparticles (100 μg of protein: 500 μl of 200 μg/ml) in phosphate-buffered saline (PBS; pH 7.2), and then packaging of these genes into the L nanoparticles was performed by electroporation (Gene Pulser II System; Bio-Rad Laboratories, Hercules, CA, USA), as described previously.[19] All experiments were carried out using these gene-containing nanoparticles.

Experimental animals

For the tumor gene therapy study using L nanoparticles, BALB/c nude mice (*nu/nu*, 5 weeks of age, male) and F344/N nude rats (*rnu/rnu*, 5 weeks of age, male) were purchased from CLEA JAPAN. All experimental procedures in this study were conducted according to the guideline of the Animal Care Committee of Keio University.

Morphological analysis

Subcutaneous tumor volume was estimated according to Carlsson's formula,[27] Hence, the largest (a) and smallest (b) superficial diameters of the tumor were measured 0, 5, 7, 9 and 12 days after transplantation of the tumors, and then the volume (V) of the tumor was calculated ($V = a \times b \times b/2$). Statistical analysis was performed using Student's *t* test. All values were expressed as means ± s.d.

Histological analysis

The rats that received L nanoparticles were killed 7 days after injection and tumors, brains, hearts, lungs, livers, spleens, kidneys, adrenal glands, intestines and skeletal muscles were isolated. These tissues were fixed in 4% neutralized formaldehyde, according to the method of Kamovsky,[28] and embedded using a Technovit 7100 kit (Heraeus Kulzer GmbH, Wehrheim, Germany). The blocks were sectioned at 5-μm thickness, and then GFP fluorescence in each section was observed with fluorescent microscopy (Nikon ECLIPSE E1000 with a fluorescence system) and with a laser scanning confocal microscope.

L nanoparticles transfer and prodrug treatment of rats

GCV (InvivoGen, San Diego, CA, USA) as a prodrug for HSV-*tk* was dissolved in sterile water, according to the manufacturer's instruction. Continuous GCV administration (50 mg/kg/day) was performed using an osmotic pump (DURECT Co., Cupertino, CA, USA) on the back of the tumor-bearing rats ($n = 5$), starting from the first day after transplantation. L nanoparticles for the expression of HSV-*tk* were injected on day 5 after transplantation. The effect of GCV/HSV-*tk* was estimated by measurement of tumor volume.

Results

Expression and characterization of L nanoparticles

L nanoparticles were overexpressed in *S. cerevisiae* AH22R$^-$ carrying L protein expression plasmid pGLDLIIP39-RcT.[20] After fractionation of the lyses by CsCl and sucrose gradient ultracentrifugation, L nanoparticles in the various fractions were separated by SDS-PAGE which was stained with silver reagent, as described previously.[20,22] The S-antigenic activity in the cell extracts was determined by the IMx system.

Distribution of L nanoparticles in the rat model

In pre-clinical experiments, it is important to assess the biodistribution and safety of gene transfer vectors *in vivo*. We adopted a xenograft rat model bearing hepatic (NuE) and non-hepatic (WiDr) cell-derived tumors. About 1×10^7 tumor cells (both NuE and WiDr) were subcutaneously injected into the rear flank of the mice. To normalize the size of the tumors, tumor-bearing mice were killed, and then both NuE- and WiDr-derived tumors (5 mm in diameter) were subcutaneously transplanted into the rear flank of the same nude rat. Rats bearing large enough tumors (NuE tumor: $434.1 \pm 134.8\,cm^3$, WiDr tumor: $400.2 \pm 146.0\,cm^3$) were injected with 100 μg (500 μl) of L nanoparticles carrying 20 μg of expression plasmids, via the tail vein ($n = 5$). As a negative control, rats were injected with PBS. The rats were killed 7 days after L nanoparticle or PBS injection. The tumors (NuE and WiDr-derived), livers, spleens and kidneys were extracted, and the morphological differences and the weights of these tissues were compared between the L nanoparticle and PBS control groups. There were no obvious differences in growth rates or tumor sizes of the tumors of rats injected with L nanoparticles containing GFP expression plasmids versus PBS controls (Figure 1a and b, Table 1) indicating that the *in vivo* transfection *per se* does not affect tumor growth. In addition, histological examination revealed no apparent histologic abnormalities in liver, lung, brain, kidney, heart, adrenal gland, skeletal muscle, spleen and intestine of tumor-bearing rats injected with L nanoparticles *versus* PBS controls (Figure 2, Table 1). Moreover, intravenous delivery of L nanoparticles in the tumor-bearing animals did not significantly alter body weight or the weight of the

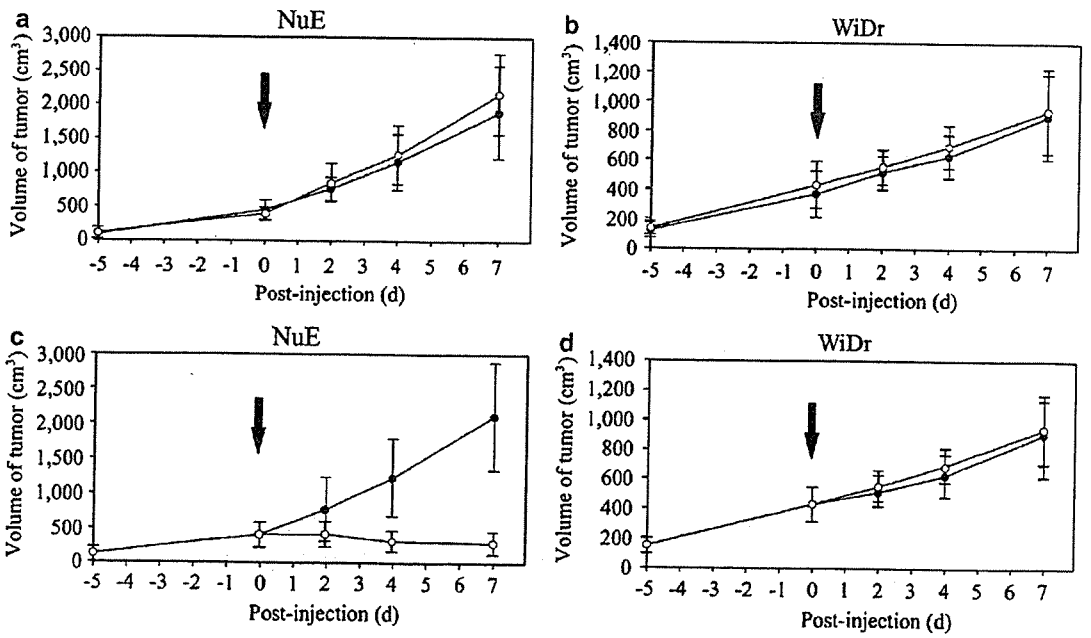

Figure 1 Analysis of anti-tumor effects of L nanoparticles in rat xenograft tumor model. The time course in days of the change in volume of subcutaneous NuE and WiDr tumors from the transplantation day (day −5) to the euthanasia day (day 7). After 5 days from the transplantation (day 0), rats were injected with L nanoparticles carrying expression plasmid. PBS or plasmid alone was injected as the control group. Experiments were repeated with five animals. (**a**, **b**) Effect of L/GFP particles on the growth of the transplanted tumors. NuE-bearing rats (**a**) or WiDr-bearing rats (**b**) were injected with 100 μg of L particles carrying 20 μg GFP expression plasmid (○) via the tail vein or injected with 500 μl of PBS (●). (**c**, **d**) Effect of L/ HSV-*tk* particles with GCV on the growth of the transplanted tumors. NuE-bearing rats (**c**) or WiDr-bearing rats (**d**) were injected with 100 μg of L particles carrying 20 μg HSV-*tk* expression plasmid (○) via the tail vein or injected with 20 μg HSV-*tk* plasmid alone (●). Continuous GCV administration (50 mg/kg/day) was performed on day −5.

individual organs (Table 1), nor was there any detectable change in blood platelet count and aminotransferase level in blood as markers of hepatic acute toxicity (data not shown). Collectively, these data indicate that there was no detectable toxicity of the L nanoparticles in this rat model based on histologic and macroscopic analysis, liver transaminase levels and hematologic parameters, which further corroborates their relative safety.

Table 1 Effects of intravenous injection of L nanoparticles into rats

	L (+)	*L (−)*	P
Body weight (g)	179.3±9.6	180.3±15.8	NS
Tumor (NuE) (g)	12.2±1.7	11.8±1.8	NS
Tumor (WiDr) (g)	7.0±1.0	7.1±1.0	NS
Liver (g)	10.7±0.7	10.3±0.5	NS
Spleen (g)	1.0±0.1	1.0±0.1	NS
Kidney (lt) (g)	1.1±0.1	1.1±0.1	NS

Abbreviation: NS, nonsignificant.

GFP fluorescence was measured to investigate the distribution of L nanoparticles, after killing the rats 7 days post-injection. Following injection of the GFP nanoparticles, fluorescence was observed in the hepatic NuE-derived tumors only but not in the non-hepatic WiDr-derived tumors (Figures 3, 4a and b). This underscores the hepatic specificity of the nanoparticles. No significant fluorescence was detectable in the NuE- or WiDr-derived tumors from rats injected with PBS control (Figure 4c and d). Furthermore, there was no detectable fluorescence in rat liver (Figures 2 and 3), indicating that the nanoparticles were specific for human liver tumor cells, whereas rat hepatocytes were refractory to L nanoparticle-mediated gene transfer. In addition, there was no evidence of transfection into non-hepatic tissues since only aspecific background fluorescence was

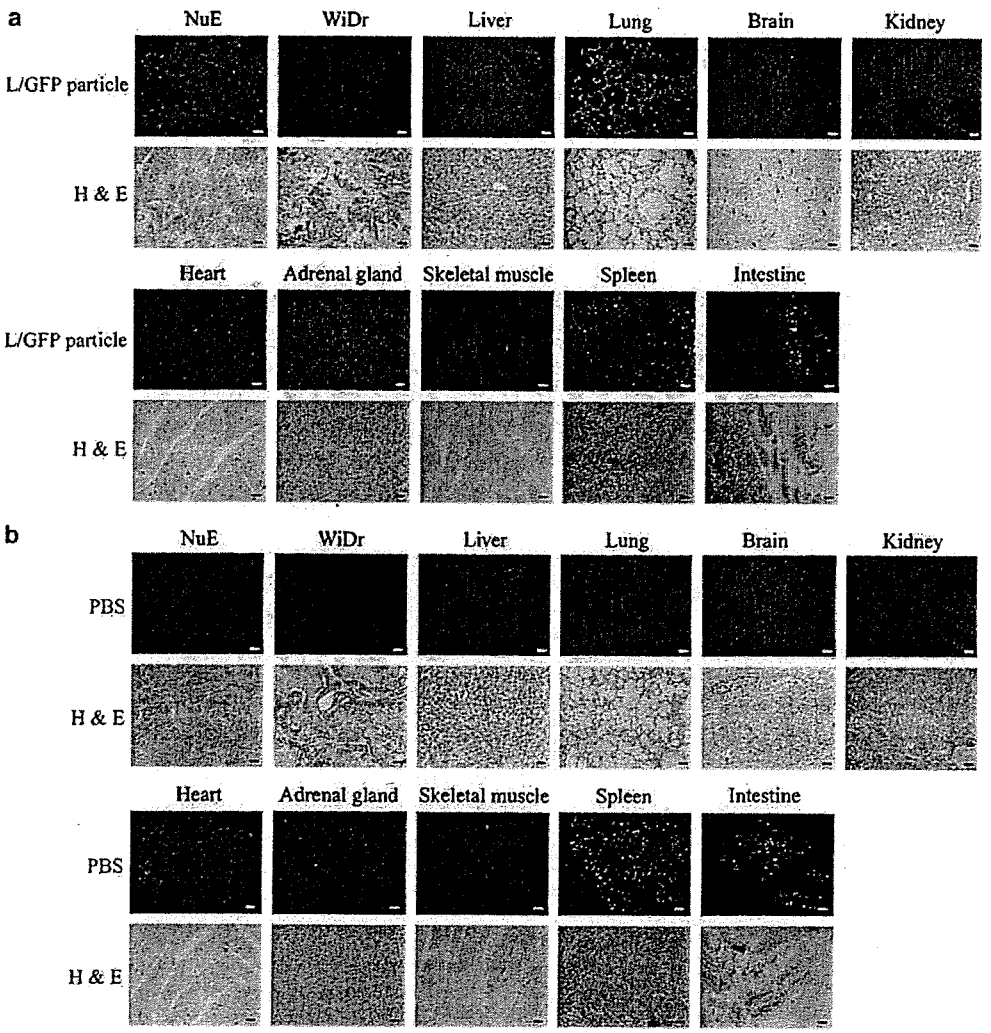

Figure 2 Biodistribution analysis in the rat xenograft model. 100 μg L nanoparticles containing 20 μg GFP expression plasmid (**a**) or PBS as the negative control (**b**) were injected. Fluorescence images and the histology (H & E staining) of NuE-derived tumors, WiDr-derived tumors, liver, lung, brain, kidney, heart, adrenal gland, skeletal muscle, spleen and intestine on day 7 after injection. Scale bars: (NuE, WiDr and kidney) 100 μm; (liver, lung, brain, heart, adrenal gland, skeletal muscle, spleen and intestine) 20 μm.

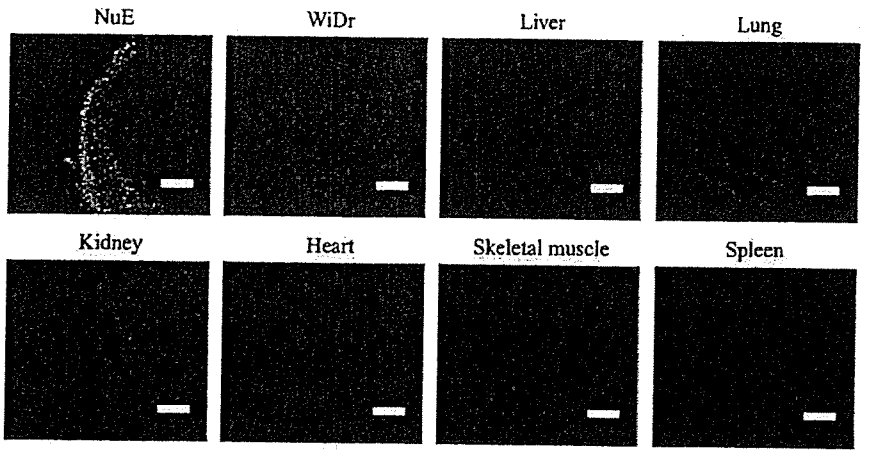

Figure 3 Fluorescence images with a laser scanning confocal microscope. 100 μg L nanoparticles containing 20 μg GFP expression plasmid were injected. Fluorescence images of NuE-derived tumors, WiDr-derived tumors, liver, lung, kidney, heart, skeletal muscle spleen on day 7 after injection. Scale bars: 500 μm.

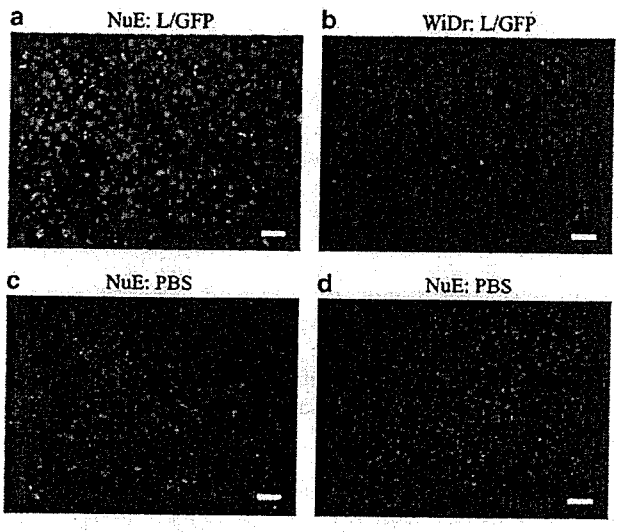

Figure 4 *In vivo* GFP gene delivery in the rat xenograft model. 100 μg L nanoparticles containing 20 μg GFP expression plasmid were injected in NuE-bearing rats (**a**) or WiDr-bearing rats (**b**). 500 μl PBS was injected in NuE-bearing rats (**c**) or WiDr-bearing rats (**d**). Scale bars: 20 μm.

apparent in brain, lung, kidney, heart, spleen, adrenal gland, intestine and skeletal muscle (Figure 2). This fluorescence, which could be observed through both GFP and Cy3 filters, was inherent to the examined tissue and undistinguishable from PBS controls.

Suicide gene therapy with L nanoparticles

The biodistribution studies based on GFP reporter gene expression indicate that the L nanoparticles are well suited to achieve targeted gene delivery and cell-specific expression in liver tumor. To determine whether L nanoparticles could be used for liver tumor-specific gene therapy, it was important to demonstrate that a therapeutic effect could be obtained by targeted delivery of therapeutic genes into the liver tumors. The therapeutic potential of the nanoparticles was determined by loading the particles with HSV-*tk* gene and by assessing the anti-tumor effect following GCV pro-drug administration in the rat xenograft tumor model.

Continuous GCV administration (50 mg/kg/day) was performed in rats ($n=5$) bearing NuE hepatic tumors, starting from the first day after transplantation. L nanoparticles containing 20 μg HSV-*tk* expression plasmids were injected intravenously into tumor-bearing rats on day 5 after transplantation. As a control, injection of 20 μg HSV-*tk* plasmids alone was used. The growth of NuE-derived hepatic tumors in L nanoparticle-injected rats was significantly ($P<0.05$) suppressed during the course of the experiment ($456.2\pm124.5\,cm^3$), whereas NuE-derived tumor growth progressed in the control group that only received the HSV-*tk* genes without L nanoparticles ($2120.0\pm778.5\,cm^3$) (Figure 1c). In contrast, there was no tumor regression following nanoparticle/HSV-*tk* and GCV administration in WiDr-derived non-hepatic tumor-bearing rats, compared to the WiDr tumor control group that received only HSV-*tk*/GCV (Figure 1d). Moreover, there was no anti-tumor effect in the NuE and WiDr controls injected with L nanoparticles/GFP (Figure 1a and b). There were no apparent side-effects associated with the nanoparticle transfection, nor was there a significant difference in body or organ weight between the experimental and control groups (data not shown). Hence, these results demonstrate for the first time that L nanoparticles could be used successfully and safely for delivery of therapeutic genes with anti-tumor activity into human liver tumors with unprecedented targeting specificity, resulting in liver tumor-selective expression of the therapeutic gene.

Discussion

In the present study, we investigated the effects of L nanoparticles *in vivo* in a rat xenograft tumor model. The

biodistribution of L nanoparticles was determined by analyzing GFP expression in hepatic and non-hepatic tumors and in various tissues. The fluorescence and histologic analysis indicate that GFP expression is restricted to and specific for the transplanted liver tumors (NuE) (Figures 2–4), indicating that nanoparticles are well suited for targeted gene delivery and expression for human liver tumor. In the current study, a detailed histologic analysis was undertaken and the results are consistent with and extend our previous observations regarding L nanoparticle-mediated delivery of GFP or fluorescent compounds in a mouse model.[19] This tropism of the L nanoparticles for human liver tumor reflects the natural tropism of the human hepatitis B virus (HBV) which is primarily determined by the pre-S1 targeting peptide located at the N-terminal end of the L antigen.[29] The lack of GFP expression in the rat liver following L nanoparticle transfection is consistent with the resistance of rodent hepatocytes to HBV infection.[30] The hepatotropic specificity of HBV and consequently also the L nanoparticles, is determined by the interaction of the HBsAg with an elusive cellular receptor, although several candidate receptors or co-receptors have been proposed, including annexin V[31] and squamous cell carcinoma antigen-1 homolog (SCCA-1).[32] L nanoparticles are well tolerated and do not appear to induce serious adverse effects (Table 1, Figure 2). Furthermore, L nanoparticles by themselves have no effect on tumor suppression or progression (Figure 1).

The lack of viral genomes in the L nanoparticles obviates concerns regarding the possible generation of replication-competent viruses that may arise by homologous recombination, as in the case of early-generation adenoviral vectors and retroviral vectors.[33] Moreover, the safety profile of the L nanoparticles compares favorably compared to the safety of several viral vectors.

The immunogenicity of the L nanoparticles is controversial. The ability for repetitive administration is of paramount importance in the clinical context. Most of the world's population has antibodies against hepatitis B because of prior infection or due to immunization. Hence, the application of L particles would be limited to the patients who have no antibodies against the virus. However, HBsAg immune escape variants exist in chronic hepatitis B patients and eliminating/modifying the immunogenic epitopes from L particles could minimize these limitations.

The HSV-*tk*/GCV paradigm is an attractive anti-cancer gene therapy approach, because it generally provides for a potent anti-tumor response, by virtue of the bystander effect.[5,34] However, the expression of HSV-*tk* in non-target cells may result in unexpected adverse effects, because all cells transduced with HSV-*tk* genes become sensitive to GCV. In studies of anti-HCC therapy, an AFP promoter or enhancer has been employed to drive HSV-*tk* specifically into HCC cells.[35,36] Although use of an AFP promoter or enhancer induces HCC-selective expression, the transgenes rarely work in non- or low-AFP-producing HCC cells. In this study, we attempted to express HSV-*tk* genes in NuE cells, which are low AFP-producing liver tumor cells.[37] L nanoparticles carrying HSV-*tk* genes were injected into tumor-bearing rats intravenously, and growth of NuE-derived hepatic tumors in the experimental group was suppressed in conjunction with GCV administration (Figure 1c), whereas WiDr-derived non-hepatic tumors showed progression during the course of the experiment (Figure 1d). The suppression of tumor-growth was strictly depending on the L nanoparticles since injection of HSV-*tk*/GCV without nanoparticles had no effect on tumor growth (Figure 1c). These results are consistent with the GFP biodistribution profile (Figures 2 and 3) and indicate that L nanoparticles can specifically deliver therapeutic transgenes into human liver cells. Nanoparticle-mediated targeted gene delivery enable liver tumor-selective expression while obviating the use of an AFP promoter or enhancer, which may not be ideally suited to target expression in HCC, especially in those cells in which AFP expression is either absent or very low. Nevertheless, it is possible that selective transgene expression in human hepatic tumor cells following nanoparticle-mediated gene transfer could be improved further when promoters are chosen that are expressed at higher levels in cancerous versus normal hepatic tissue, as identified by transcriptional profiling.[38,39] Although it is known that the L nanoparticles also transfect normal human hepatocytes but not murine hepatocytes,[19] it has been shown that only dividing cells are susceptible to phosphorylated GCV.[40] Thus, expression of HSV-*tk* with L particles in quiescent normal hepatocytes followed by GCV treatment might not cause damage to normal hepatocytes but would be restricted to dividing liver tumor cells instead. This would be consistent with recent observations showing strong antitumoral efficacy in the absence of normal liver toxicity following direct intratumoral injection of a lentiviral vector containing the HSV-*tk* gene and GCV treatment.[16] However, this study and our current observations are in contrast with the severe liver dysfunction and mortality that arose following systemically administered hepatotropic adenoviral vectors expressing HSV-*tk* and GCV treatment.[41] These data further confirm that the L nanoparticles may have an improved biosafety profile compared to adenoviral vectors for hepatic gene delivery. Interestingly, intravenous injection of L nanoparticles appeared to show similar efficacy to the intratumoral injection of adenoviral vectors into large tumor nodules.[42]

The current study underscores the potential of hepatotropic nanoparticles for gene therapy of liver tumor, which may pave the way towards a more efficacious and safer hepatic gene delivery approach. Future studies will require safety and efficacy analysis of the L nanoparticles in mouse models with 'humanized' liver[43] in conjunction with potent liver-tumor specific promoters to further refine the selectivity of expression.

Acknowledgements

We thank Takuji Tadakuma (the Department of Parasitology, National Defense Medical College) for the gift of

the NuE cell line, and the HBV nanobioparticle team for technical support. This study was supported by the Grants-in-Aid from Ministry of Education, Culture, Sports, Science and Technology, Japan (the 21st century Center of Excellence program and Scientific Research). Thierry VandenDriessche and Marinee KL Chuah are supported by an FWO grant (G.0341.05) and a grant from the Flemish Government (VIB Dotation).

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Characterization of bio-nanocapsule as a transfer vector targeting human hepatocyte carcinoma by disulfide linkage modification

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Received 5 September 2006; accepted 12 December 2006
Available online 28 December 2006

Abstract

The bio-nanocapsules (BNCs) composed of the recombinant envelope L-protein of hepatitis B virus constitute efficient delivery vectors specifically targeting human hepatocytes. Here, we have tried to enhance the stability of the BNCs because the L-proteins in the BNCs were aggregated due to random disulfide bridging when stored for a long period at 4 °C. The envelope protein contains fourteen cysteine residues in the S domain. Aggregation of the envelope proteins might be avoided if unessential cysteine residues are replaced or removed because the irreversible alkylation of the free sulfhydryl group protects against the aggregation and enhances the efficiency of encapsulation. In this study, the possibility of reducing the number of cysteine residues in the S domain to enhance the stability of the BNCs was assessed. The replacement of each cysteine residue by site-directed mutation showed that nine of fourteen cysteine residues were not essential to obtaining BNCs secreted into the culture media. Furthermore, upon evaluating the combination of these mutations, it was found that eight residues of replacement were acceptable. The mutant BNCs with replaced eight cysteine residues were not only more resistant against trypsin, but also more effective in transducing genes into human hepatoma-derived HepG2 cells than the original type BNC. Thus, we demonstrated that the minimized number of cysteine residues in the S domain could enhance the stability of the BNCs.

Keywords: Bio-nanocapsule; HBV surface antigen; Delivery vector; Hepatocyte specific targeting; Enhanced stability

1. Introduction

A molecular targeting method that transfers drugs efficiently and safely in a cell type specific manner has been a subject of great attention in the development of a novel drug delivery system (DDS). In general, recent DDS technologies are based on carriers that are categorized as two independent methods, viral and non-viral. Each has its advantages and disadvantages. Viral DDS systems, which exploit viruses, such as retroviruses, adenovirus, adeno-associated virus and lentivirus, have been developed as gene transfer procedures for efficient transduction. However, the patients

Abbreviations: DDS, Drug delivery system; BNC, Bio-nanocapsule; DMEM, Dulbecco's modified Eagle medium; HBV, Hepatitis B virus; GFP, Green fluorescent protein; EIA, Enzyme immunoassay; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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doi:10.1016/j.jconrel.2006.12.020

would have to face the risks of not only having the viral genome integrated into their own genomes, but also of having genes transferred to unexpected cells or tissues in a non-specific manner, resulting in serious diseases, such as cancer [1,2]. A non-viral system, represented by cationic liposome or polyethylene glycol, is considered to be rather safer than a viral system. However, liposome as a transfer vector still needs improvement in order to confer as much efficiency as viral vectors, and specificity on the target cells or tissues [3]. The development of novel DDS vectors with the advantages of both vectors is eagerly awaited [4–6].

Over the last decade, recombinant envelope proteins have been developed as vaccines for hepatitis B virus (HBV) [7]. The envelope of HBV is composed mainly of three closely related surface proteins that are known as the large (L), the middle (M) and the small (S) proteins. They are encoded in one open reading frame of HBV genome translated from three different in-frame initiation sites. When these proteins were expressed, each S-, M- and L-protein was found to form hollow virus-like nanoparticles. The particle composed of L-protein showed, in particular, a specific affinity to human hepatocytes due to the hepatocyte recognition site localized in the amino terminus [8–12]. For the purpose of developing safe and efficient DDS vectors having a targeting potential for specific cells or tissues, we exploited the nanoparticles composed of recombinant envelope protein as bio-nanocapsule (BNC) prepared from mammalian cells or yeast cells. The BNC composed of L-protein (L-BNC) is a sphere that has an average diameter of 80 nm, which has been observed under atomic force microscope of about 110 molecules of L-protein [13]. This nanoparticle formation is also confirmed under thin section electron microscope [14]. We found that the L-BNC was extremely useful as a vector to deliver genes and pharmaceutical drugs to the human liver in vivo cells, as well as in vitro [15].

However, we experienced aggregation of envelope proteins in the BNCs during long-term storage. We report here that the aggregation is due to the false disulfide bridges and that replacement of cysteine residues in the L-protein enhances the stability of the BNCs.

2. Materials and methods

2.1. Cell culture

COS7 cells were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 0.1% sodium bicarbonate. HepG2 cells and WiDr cells were maintained at the same conditions, but with 10% FBS.

2.2. Preparation of the L-BNCs in yeast cells

The L-BNCs were produced in *Saccharomyces cerevisiae* AH22$^-$ transformed with the L-protein expression plasmid pGLDLIIP39-RcT and purified as previously described [16].

2.3. Alkylation of L-BNCs with iodomonoacetic acid (IAA)

During purification of L-BNC, IAA was added to the yeast extracts to modify free SH-groups, thereby preventing them from forming false disulfide bridges. IAA was added to 10 mM 15 min before the precipitation of L-BNC with PEG6000 so that IAA could be removed after reaction.

2.4. Comparison of calcein encapsulation into L-BNC with/without IAA modification

Calcein (Dojindo, Japan) was mixed at 1 mM with 240 μg of L-BNCs with or without IAA modification in 500 μl of PBS. This was followed by electroporation with a Gene Pulser II electroporation system (Bio-Rad, VA) in a 4-mm cuvette, at 50 V and 750 μF. After removal of free calcein by gel filtration, calcein incorporated into BNCs was measured by a fluorescence image analyzer LAS-1000 (Fujifilm, Japan).

2.5. Expression of the mutant L-BNCs

The DNA fragment coding of the HBV L-protein that was fused to the secretion signal of chicken lysozyme was inserted downstream of SR alpha promoter to construct the expression vector pBO441. To replace the cysteine residues with serine or alanine residues, the gene for the L-protein was subjected to site-directed mutagenesis. Each resulting plasmid was introduced into COS7 cells by a Gene Pulser II. After 15 h, the medium was replaced by CHO-SFM II (Invitrogen, CA). Four days after transfection, the conditioned medium was collected and concentrated by a Vivaspin 20 (MWCO 1000 kDa, Vivascience, Germany). The BNCs and the derivatives in the conditioned media were immunologically detected and quantified with the Abbott IMx HBsAg assay system (Abbott Laboratories, IL) in the series of two-fold dilution. All the tests were performed and interpreted in accordance with the manufacturer's recommendation. Standard curve of EIA was evaluated with the positive control of HBsAg supplied with the system and antigen in the range of 10–100 ng/ml was estimated. Each sample was measured in triplicates and standard deviation was calculated.

3. Detection of the L-protein and its derivatives by Western blotting

The BNCs in the conditioned media were immunoprecipitated with anti-S antibody conjugated to microparticles, a component of the Abbott IMx HBsAg assay system, and then subjected to SDS-PAGE and Western blotting. The blots were probed with anti-S goat IgG conjugated to biotin and anti-biotin rabbit IgG conjugated to alkaline phosphatase. Immunoreactive bands were detected with the Phototope-Star Chemiluminescent Detection Kit (New England Biolabs, MA). The bands detected were densitometorically analyzed using NIH Image.

3.1. Protease protection assay

The BNCs were immunoprecipitated by anti-S antibody, and treated with various concentration of trypsin for an hour at 37 °C. The digestion fragments were detected using Western blotting, as described above.

3.2. Evaluation of the effects of reducing reagent, and oxidation on the L-BNCs

L-BNCs were treated with various concentration of reducing reagent dithiothreitol (DTT) for 10 min at 25 °C. The L-proteins were analyzed by SDS-PAGE and silver staining. L-BNCs were oxidized, on the other hand, by vigorous agitation at 25 °C. The L-proteins were then analyzed by SDS-PAGE in the presence of 1 mM DTT. Density of the protein bands were measured by Image J<http://rsb.info.nih.gov/ij/> and the time course changes of density were plotted.

3.3. Transduction of DNA into HepG2 cells with BNCs

Prior to transfection, HepG2 cells were seeded into the 8-well chamber slide (Nalge-Nunc international). On the following day, green fluorescent protein (GFP) expression plasmid pEGFP-N1 (Clontech Labs., CA) was introduced into the BNCs by electroporation at 50 V and 750 μF and added into the culture media of HepG2 cells. The final culture volume was adjusted to 500 μl/well. Three days after transfection, the transduction of the plasmid DNA was evaluated by the GFP fluorescence in HepG2 cells under confocal laser scanning microscopy (Carl Zeiss, Germany). The infectious efficiency was evaluated by mean of green color intensity of confocal microscopic images using RGB histogram in Image J.

4. Results

4.1. Stability of L-BNC

Our L-BNCs are efficient transfer vectors with which to target human hepatocytes. However, the L-proteins, the major component of an L-BNC, were difficult to observe, when analyzed on SDS-PAGE without DTT following the long-term storage of the L-BNCs at 4 °C (Fig. 1A lane 1 and Fig. 1B lane 3). In this experiment, we compared freshly prepared L-BNC and purified L-BNCs, which had been stored at 4 °C in PBS for periods of two, four, and nine months. When the L-BNCs were treated with 10 mM DTT for 5 min at 98 °C prior to the electrophoresis, all preparations showed bands at a molecular mass of 47 kDa (Fig. 1A, lanes 8 to 10). However, the lower concentrations of DTT, such as 0.5 or 1 mM, were ineffective on the L-BNCs that were kept for a long term (Fig. 1A, lanes 2 to 7). The longer the storage period became, the tighter appeared to be the interaction of L-protein via disulfide bridges.

To prevent cysteine residues in L-protein from false or random disulfide bonds, we modified the free sulfhydryl group of L-protein with IAA at the first step of preparation from the cell extracts. The SDS-PAGE assessment with DTT described above implies freshly prepared BNC should be free from unessential disulfide bridges. IAA reaction in this stage will not allow further formation of disulfide bridges in L-proteins. They should be kept as many as those just produced in the cells even after the whole purification procedures. When the BNCs modified with IAA were analyzed by SDS-PAGE without DTT, L-protein was shown as monomeric and dimeric forms,

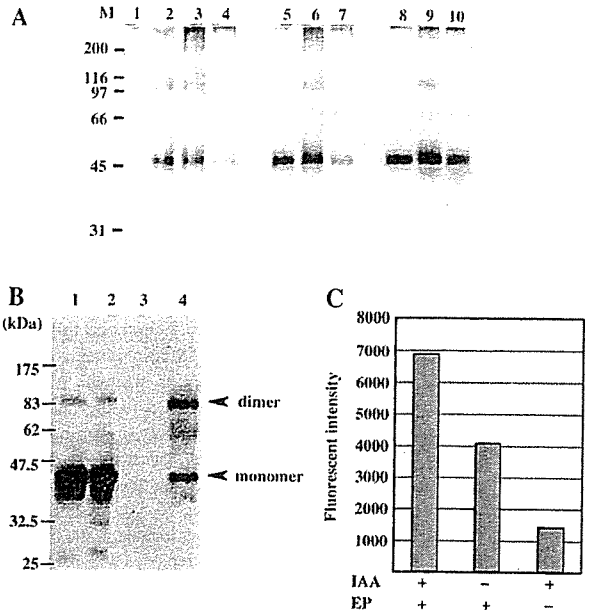

Fig. 1. The sensitivity of L-BNCs to reducing reagent. (A) The L-BNCs from yeast were kept at 4 °C in the refrigerator for two months (lanes 1, 2, 5 and 8), four months (lanes 3, 6 and 9) and nine months (4, 7 and 10). Prior to electrophoresis, these BNCs were treated with 0.5 mM DTT followed by incubation at 25 °C for 10 min (lanes 2, 3 and 4), 1 mM DTT followed by incubation at 25 °C for 10 min (lanes 5, 6 and 7), or 10 mM DTT followed by incubation at 100 °C for 10 min (lanes 8, 9 and 10). These L-BNCs were electrophoresed with or without DTT treatment (lane 1) and the presence of each protein was detected by silver staining. The monomeric form of L-protein was shown as the main band at about 47 kDa, and multimeric form of L-protein appeared at a higher molecular weight or at the top of the gel. (B) The wild type L-BNC (lanes 1 and 3) and the L-BNC modified with IAA (lanes 2 and 4) were analyzed by Western blotting in the presence of, or absence of, 10 mM DTT (lanes 1 and 2, and lanes 3 and 4 respectively). (C) The calcein was encapsulated into the L-BNC and the L-BNC modified with IAA by electroporation. The residual calcein was removed by gel filtration and the fluorescence from the fraction containing 240 μg of L-BNC was measured by LAS-1000.

while intact L-protein in BNCs was not (Fig. 1B). Furthermore, it was observed that the loading of calcein into the BNCs by electroprotation was more efficient when using BNC modified with IAA, rather than with the unmodified BNC (Fig. 1C).

4.2. Essential cys residues for secretion of L-BNC

Consequently, we hypothesized that dispensable inter-/intra-molecular S–S cross-linking would occur in L-protein randomly, with the result that the stability and ability to function as a carrier of BNCs would be destroyed. The unwanted S–S bridges made the formation of the BNCs so tight that the loading efficiency of substances was reduced. It should be important to keep the L-BNC flexible in order to maintain the optimal L-BNC encapsulation efficiency. Hence, we thought that as many as possible unessential cysteine residues should be replaced. Each cysteine residue in the hydrophobic region was replaced with alanine, whereas others in the hydrophilic region were replaced with serine (Fig. 2 and Table 1). The mutant plasmids were transiently expressed in COS7 cells and the amount of the mutant BNCs secreted into the media were

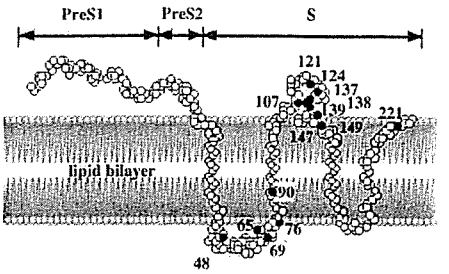

Fig. 2. Speculative secondary-structure model for HBsAg L-protein in the membrane. The L-protein consists of Pre-S1 and Pre-S2 domains followed by S domain. Pre-S1 contains the sequence for the specific binding to human hepatocytes. Pre-S2 is responsible for the association with the polymerized albumin-mediated interaction and for the integration into the membrane. S domain is indispensable for an L-protein to be spun in the lipid bilayer membrane (gray line). The cysteine residues in the S domain are depicted as black circles with the numbers of their positions in the S domain. Then cysteines' residue located in the hydrophobic region, 76, 90, 107, 147, 149 and 221, was replaced with alanine and that of the others located in the hydrophilic region, 48, 65, 69, 121, 124, 137, 138 and 139, was replaced with serine by site-directed mutation.

quantified by EIA system and qualified by immunoprecipitation and Western blotting. This assay system, IMx HBsAg, is based on highly sensitive sandwich EIA aiming at the same epitope localized in the surface region of S domain in L-protein so that it is important for one antigen to have multiple epitopes to be detected. Namely, this one antigen should be a form of virus like particle, which is soluble in aqueous phase. In this case the virus like particle is L-BNC. Since L-protein is a typical transmembrane type protein (Fig. 2), random aggregation of this protein would make insoluble precipitates in denatured state, which could not be detected in EIA. Taking this into consideration, we considered the mutant L-proteins prepared in this study as nanocapsules if they are detected by EIA in aqueous phase.

Each secreted amount of BNC with a single mutation was represented by the percentage relative to that of wild type BNC in Table 1. Each mutation of the cysteine residues at 76, 139, 147, 149 and 221 facilitated the secretion of the BNC remarkably without affecting the essential disulfide bonds, and the mutation of cysteine residue at 90 did not change the level of secretion. Still, more than 50% of the secreted wild type of BNC was detected in the mutations of cysteine residues at 107, 137 and 138. The mutation of cysteine residues at 48, 65, 69, 121 and 124 did not show detectable secretions of BNC. From the results, cysteine residues at 76, 90, 147, 149 and 221 were found dispensable for the secretion. On the other hand, cysteine residues at 48, 65, 69, 121 and 124 were found to be essential for the secretion of BNCs.

Western blot analysis exhibited the characteristic patterns of wild type and derivative mutant L-proteins (Fig. 3). A number of immunoreactive bands were detected in wild type BNCs due to *N*-glycosylation in PreS1, PreS2 and S domain, and also due to alternative translations for M- and S-proteins. The five main bands in wild type L-BNC are recognized to be: (1) the 44 kDa glycosylated form (gpL 44) (2) the 47 kDa glycosylated form (gpL 47) of L-protein (3) the 36 kDa glycosylated form of M-protein (gpM 36) (4) the 28 kDa glycosylated form of S-protein (gpS 28) and (5) the nonglycosylated forms of M and S, which were undetectable. (Fig. 3, lane 1 and 9) [17]. All proteins with mutations at each single cysteine residue displayed characteristic patterns different from that of wild type. In the mutation of cysteine residues at 48, 65 and 69, the bands of L-protein from secreted BNCs were hardly detected (Fig. 3, lanes 2 to 4), and a small number of bands were detected in the mutation of cysteine residues at 121, 124 and 139 (Fig. 3, lanes 8, 10, 13). As for the other mutations of cysteine residues at 76, 90, 107, 137, 138, 147, 149 and 221, the bands of gpL44 and gpL47 were detected almost at the same level as those in the wild type. The results of the Western blot analysis are almost consistent with those of EIA analysis, except for mutations of cysteine residues at 107 and 139.

The results of EIA should indicate that BNC has been detected, rather than the Western blotting, which detects denatured proteins on the membrane filter. For this reason, we concluded that cysteine residue 139 was dispensable, but have withheld any conclusion about cysteine residue at 107. As a consequence, the experiments demonstrated completely that cysteine residues at 76, 90, 137, 138, 139, 147, 149 and 221 were dispensable for the assembly and secretion of the BNCs.

In order to design the most suitable BNCs for the transfer vector, we further assessed the combinations of these nine mutations. As summarized in Table 2, various combinations of the mutation were investigated for the ability to form BNCs. The amounts of the secreted BNCs were estimated by EIA and shown as the percentage relative to that of wild type BNC. In these multiple mutation experiments, cysteine residues at 48, 65, 69, 107, 121 and 124 were not included since these mutations had decreased the apparent production of BNC in the single mutation experiment described above. Because the cysteine residue at 90 was considered to be located in the trans-membrane and the mutation of this residue did not change

Table 1
Production of BNCs with single mutation

Positions of cysteine residues replaced[a]	Immunoreactivity as HBsAg (%)[b]
Cys/48/Ser	21.0±3.0
Cys/65/Ser	21.3±9.0
Cys/69/Ser	22.0±8.6
Cys/76/ Ala	241.0±94.0
Cys/90/ Ala	93.5±4.5
Cys/107/ Ala	56.0±8.5
Cys/121/Ser	14.0±8.5
Cys/124/Ser	13.7±8.7
Cys/137/Ser	65.7±10.7
Cys/138/Ser	58.0±31.9
Cys/139/Ser	186.5±46.3
Cys/147/ Ala	236.3±64.5
Cys/149/ Ala	167.0±66.3
Cys/221/ Ala	142.5±39.1

[a] The name of each mutant BNC indicates the position of the cysteine residue that was replaced by the amino acid, Ser or Ala. The amino acid number is its position in the S region.

[b] The amount of wild type L- and mutant BNCs secreted into the culture media of COS7 cells was estimated by enzyme immunoassay. The averages of the EIA values of each BNC were calculated as a percentage of that of the wild type L-BNC. The standard deviation (S.D.) in the right column was obtained from triplicates of independent experiments.

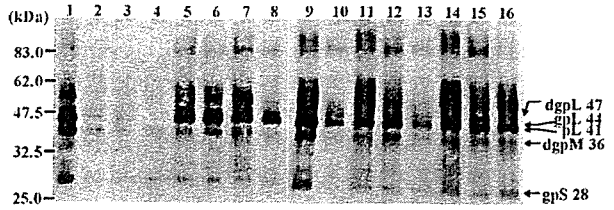

Fig. 3. Analysis of the L-proteins with single substitution at cysteine residues. Wild type (lanes 1 and 9) and mutants (lane 2, C/48/S; lane 3, C/65/S; lane 4, C/69/S; lane 5, C/76/A; lane 6, C/90/A; lane 7, C/107/A; lane 8, C/121/S; lane 10, C/124/S; lane 11, C/137/S; lane 12, C/138/S; lane 13, C/139/S; lane 14, C/147/A; lane 15, C/149/A; lane 16, C/221/A) of BNCs expressed in COS7 cells were immunoprecipitated with anti-S monoclonal antibody conjugated with agarose beads and detected by anti-S polyclonal antibody in Western blotting. SDS polyacrylamide gel electrophoresis was carried out in reducing conditions. The arrows indicate the position of glycosylated (gpS 28) S-protein, diglycosylated (dpM 36) M-protein and glycosylated (gpL 44) and diglycosylated (dpL 47) L-protein. The migration of marker protein (molecular masses in kDa) is shown at the left.

Table 2
Production of BNCs with multiple mutations

Positions of cysteine residues simultaneously replaced[a]	Immunoreactivity as HBsAg (%)[b]
76/90	167.7±30.3
90/139	222.0±71.9
90/147	326.7±71.6
90/149	154.7±86.6
90/221	108.7±25.0
76/90/221	157.0±12.8
76/90/149/221	207.3±62.8
76/90/139/149/221	114.0±22.6
76/90/147/149/221	112.0±40.3
76/90/139/147/149/221	86.3±21.3
76/90/138/139/147/149/221	67.3±7.8
76/90/137/139/147/149/221	97.0±34.4
76/90/137/138/139/147/149/221	80.0±32.3
76/90/107/137/138/139/147/149/221	45.0±18.5

[a] The numbers in the left column represent the position of the cysteine residues, which were substituted as shown in Table 1.
[b] The immunoreactivity of mutant BNCs were measured as described in Table 1.

the productivity of the BNC, this mutation was added to the mutation combination of the cysteine residues at 76, 139, 147, 149 and 221. Each of the latter resulted in greater productivity than that afforded by use of the wild type. All of the double mutants demonstrated more efficient production of BNCs than did the wild type. In fact, the two double mutants, 76/90 and 90/221, gave such good productivity that production of only the triple-mutant 76/90/221 was assessed. As a result, the triple mutant showed an expression level of 157% better than that of wild type. On this basis, we then designed further mutations of quadruple mutations at cysteine residues of 76/90/149/221, which showed a good expression level of 207%. The expression level of quintuple mutations at either cysteine residues at 76/90/139/149/221 or 76/90/147/149/221 was as much as that of the wild type. The sextuple mutations at all of the cysteine residues at 76/90/139/147/149/221 gave an almost equivalent level of expression as that of wild type. Then, we added two other cysteine residues at 137 and 138 to be assessed for the mutation. As a result, the two septet mutations at either cysteines 76/90/138/139/147/149/221 or 76/90/137/139/147/149/221 and the octet mutations at cysteine residues 76/90/137/138/ 139/147/149/221 did not seriously decrease the expression level when compared with the wild type. However, the nine mutations at cysteine residues 76/90/107/137/138/139/147149/221, which include the additional mutation at 107, showed decreased levels of the secreted BNCs that were less than 50% of the wild type by EIA. These results suggest that the eight cysteine residues are probably not essential for the formation and secretion of BNCs.

In Fig. 4, all of the L-BNCs that have multiple mutations were subjected to Western blotting analysis under reducing conditions. The pattern of the derivative L-proteins did not change in the double to octet mutants (Fig. 4, lanes 2 to 10). The band of gpL50 appeared, in contrast to the disappearing gpL44, due to the mutation of cysteine residues 147 and/or 149. These results indicate that substitution of the eight cysteine residues should be effective in producing BNCs, which will lead to avoiding unwanted disulfide bridging.

To compare the sensitivity to DTT of wild type L-BNC and the octet mutant 76/90/137/138/139/147/149/221 (Lm8-) BNC, both BNCs were treated with various concentrations of DTT at 25 °C, and analyzed by SDS-PAGE. The Lm8-protein was about 10 times more sensitive to DTT than was the L-protein (Fig. 5A). To assess the effect of oxidation on both BNCs, L-BNCs and Lm8-BNCs were oxidized by vigorous agitation for various periods and analyzed on SDS-PAGE in the presence of 1 mM DTT (Fig. 5B). The Lm8-protein resisted oxidation for up to 1 min, whereas the L-protein showed little resistance to oxidation (Fig. 5C). The time for the 50% decrease of protein by oxidation was about 10 s in the case of L-BNC while it was around 40 s for Lm8-BNC. These differences in sensitivity to reduction and oxidation between the BNCs might be explained by the number of resultant disulfide bridges due to the replacement of cysteine residues.

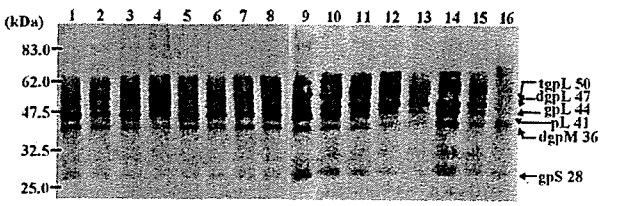

Fig. 4. Analysis of the L-proteins with multiple substitutions at cysteine residues. The wild type and multiple mutant BNCs (Lanes 1 and 9, wild type; lane 2, C/76,90/A; lane 3, C/90/A, C/139/S; lane 4, C/90,147/A; lane 5, C/90,149/A; lane 6, C/90,221/A; lane 7, C/76,90,221/A; lane 8, C/76,90,149,221/A; lane 10, C/76,90,149,221/A, C/139/S; lane 11, C/76,90,147,149,221/A; lane 12, C/76,90,147,149,221/A, C/139/S; lane 13, C/76,90,147,149,221/A, C/138,139/S; lane 14, C/76,90,147,149,221/A, C/137,139/S; lane 15, C/76,90,147,149,221/A, C/137,138,139,S; lane 16, C/76,90,107,147,149,221/A, C/137,138,139/S) were transiently expressed in COS7 cells and were immunoprecipitated and analyzed by Western blotting as described in Fig. 2. The arrows indicate the position of glycosylated (gpS 28) S-protein, diglycosylated (dgpM 36) M-protein, glycosylated (gpL 44), diglycosylated (dgpL 47), and triglycosylated (tgpL50) L-protein.

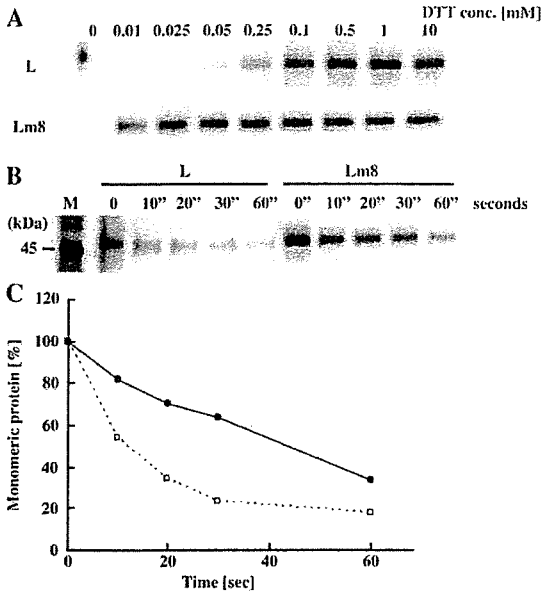

Fig. 5. Sensitivity to redox conditions of wild type and Lm8 BNCs. (A), the BNCs purified from yeast cells were treated with various concentrations of DTT for 10 min at 25 °C and analyzed on SDS-PAGE. For the reference of total amount, the L-protein treated with 10 mM DTT was boiled prior to SDS-PAGE. (B), the effect of oxidation on the BNCs was assessed. The wild type L-and Lm8-protein were analyzed in the presence of 1 mM DTT by SDS-PAGE. The protein bands were made visible by silver staining. Lane M, ovalbumin (molecular weight marker at 45 kDa). (C), resistance against oxidization. The bands of L-and Lm8-protein in (B) were densitometorically analyzed by Image J. Based on the density, the time course change of the amount of monomeric proteins was plotted. The amount at time 0 is taken as 100% for each protein. Solid circle depicts Lm8-BNC and open square depicts L-BNC.

4.3. Conformation of Lm8-protein compared with L-protein

Since the primary sequence of envelope protein contains several potential trypsin cleavage sites, consisting of Arg and Lys in the surface region of the BNCs, fragments cleaved with trypsin could possibly differ if there is a significant difference in the topology between these proteins. To determine if there is a difference of topological structures between L- and Lm8-proteins, the same amount of L- and Lm8-BNCs were digested with various concentrations of trypsin and analyzed by Western blotting (Fig. 6A). As a result, the three major fragments of Trs, Trl, and gTr at 24, 25, and 27 kDa respectively, were observed in both BNCs. It was noted that there was little significant structural difference between L- and Lm8-protein in the BNC due to the mutation. However, Lm8-BNC was resistant to trypsin at the concentration of 1.5 ng/ml, while the wild type L-BNC was digested at 0.75 ng/ml. Furthermore, digestion of Lm8-BNC showed that the major tryptic fragment was gTr, while Trl and Trs were apparent in the digestion of wild type L-BNCs. When treated

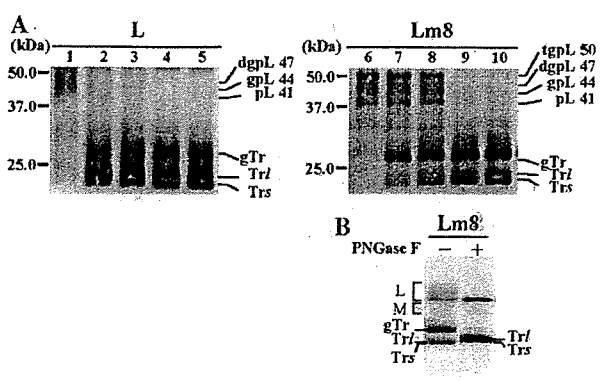

Fig. 6. Comparison of the topology of wild type L-and Lm8-BNCs by trypsin. After each BNC of the same EIA value was immunoprecipitated (A) the BNCs of wild type (lane 1to 5) and Lm8 (lane 6 to 10) were incubated with trypsin (lanes 2 to 5 and 7 to 10) or without trypsin (lanes 1 and 6) for an hour at 37 °C. The digested fragments were detected by Western blotting using anti-S antibodies. These BNCs were digested with various concentration of trypsin at 0.75 ng/ml (lane 2, 7), 1.5 ng/ml (lane 3, 8), 3 ng/ml (lane 4, 9), 6 ng/ml (lane 5, 10). (B) The BNCs of Lm8 were incubated with trypsin and treated with (left) or without (right) glycosidase F (PNGase F). The degraded tryptic fragments were observed to be glycosylated fragments (gTr), non-glycosylated short fragments (Trs) and large fragments (Trl).

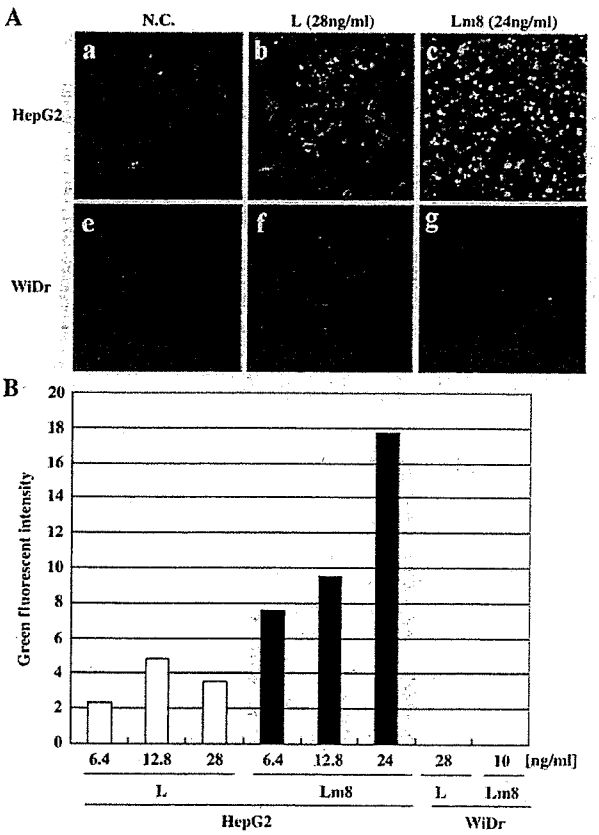

Fig. 7. (A) Gene transduction with wild type L-and Lm8-BNCs. The BNCs incorporating GFP expression vectors were infected into the human hepatocarcinoma-derived HepG2 cells (a, b, c d) and human colon adenocarcinoma-derived WiDr cells (e, f, g, h). GFP expression vector (500 ng) mixed with either wild type BNCs (28 ng/ml) or Lm8-BNCs (24 ng/ml) was subjected to electroporation and added to the culture media of each cell line (b, f and c, g, respectively). As a control for background, only GFP expression vector (500 ng) without any BNCs was added to the media (a, b). Cells were observed for fluorescence on day 3 following addition under a confocal laser scanning microscope. The magnification is ×20. (B) Comparison of infectious efficiency between L-BNC and Lm8-BNC. The images of cells expressing GFP obtained from the confocal laser scanning microscope were analyzed for green color intensity by RGB histogram in Image J. Cells were equally distributed in each field of microscopic observation.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with *N*-glycosidase F after digestion of both BNCs by trypsin, gTr was found to be degraded to Trs or Trl, so that gTr was implied as the *N*-glycosylated form of Trs or Trl (Fig. 6B) [18]. Lm8-protein should cause the glycosylation attachment site to be more accessible than the wild type. These results suggest that the structure of Lm8-BNC should differ in critical aspects from that of L-BNC.

4.4. Lm8-BNC targeting to HepG2 cells

The potential of Lm8-BNC as a vector specific to human liver cells was evaluated by comparison with L-BNC. An infection experiment was carried out on both human hepatoma-derived HepG2 cells and human colon adenocarcinoma-derived WiDr cells. The L- and Lm8-BNCs incorporated GFP expression plasmid were added into the culture media of these cells. Three days after infection, the cells were observed for GFP fluorescence. Both L-and Lm8-BNCs showed fluorescence, specifically in HepG2 cells, whereas no expression of GFP was observed in WiDr cells incubated with either BNC (Fig. 7). Furthermore, Lm8-BNCs appeared to show more expression of GFP in HepG2 cells than L-BNC of the same amount. The analysis of the images obtained by confocal microscope showed the increase of green fluorescence in a dose dependent manner. The greater the amount of Lm8-BNC that was used, the more expression of GFP in HepG2 cells was shown.

5. Discussion

The significance of the fourteen cysteine residues of L-protein has been extensively studied [19–21]. However, the essential residues for the assembly of HBV particles still remained to be studied. In this study, we explored how to prepare BNCs with enhanced stability by optimizing the number of cysteine residues. The ultimate objective is the development of a far more efficient vector for drug delivery systems.

When each cysteine residue at 48, 65 and 69 was replaced, the secretion of the BNC was not detectable at all. These cysteine residues, which are only conserved among all hepadnaviruses [22,23], appear indispensable for the secretion of vial particles. As schematically drawn in Fig. 2, these three residues are located inside of the BNC and present in the cytoplasm when L-protein is integrated into the membrane. They appear to hardly form a disulfide bridging due to the reducing conditions of the cytoplasm. They might be involved in some significant function such as palmitoylation, often found in the juxtamembranous cysteine residues in viral glycoproteins. Replacement of the cysteine residues present at the cytoplasmic side in the envelope has been reported to prevent the budding and infection of sindbis virus and human immunodeficiency virus [24]. This might be also the case with HBV.

Mutation of cysteine residue at either 121 or 124 significantly decreased the secretion of the BNC down to the same level as those of the mutations at the three internal residues described above. Since these two cysteine residues are located in the outer surface hydrophilic region of the S domain, they are possibly involved in the essential disulfide bridging [19,25]. Cys121 is considered to be involved in the intra-molecular disulfide bond, not only with Cys124, but also with Cys147 [21]. On the other hand, Cys124 is reported to bind with Cys137 [26].

The mutations at 76, 90, 139, 147, 149 and 221 did not show drastic decreases in the level of secretion. The mutations at 107, 137 and 138 showed slight decreases, but not less than 50% of the secretion of wild type BNCs, although the mutation of one or two of these cysteine residues has been described to be essential for secretion [27]. Furthermore, cysteine residue at 221 is not conserved in HBV subtype adw, showing that cysteine residues at this position might not be essential [28]. Taking these results into consideration, we assumed that the cysteine residues at 76, 90, 139, 147, 149 and 221 are not essential to form a BNC, leaving the possibility that those at 107, 137 and 138 are involved in some disulfide bridging in the formation of BNC.

To design more stable BNCs, multiple replacements of cysteine residue were evaluated. Even in the BNCs with multiple replacement from double up to sextuple, the secreted amount of the BNCs was almost equivalent to that of the wild type L-BNC. The sextuple mutant was subjected to further replacement of cysteine residues at each position of 107, 137 and 138. Interestingly, the replacement of the seventh cysteine residues at both 137 and 138 did not change the secretion level, so that the secreted level of the octet mutation, including both of these two, was 80% of wild type L-BNC. However, the ninth mutation at 107 showed a significant decrease of secretion, which was lower than 50% of wild type L-BNC. In contrast, it was previously reported that the simultaneous replacement of cysteine residues at 107, 137 and 138 show an inhibitory effect on the secretion of subviral particles [27]. Taking this into consideration, the cysteine residue at 107 should be essential for the secretion of BNCs. Finally, we obtained Lm8-BNCs, which have the eight cysteine residues replaced. The Lm8-BNCs were more sensitive to reducing reagent and less sensitive to oxidation than L-BNCs. This implied that Lm8-BNCs should be free from false disulfide linking. When the BNCs were expressed in COS7 cells, the glycosylation site appeared to be more accessible in Lm8-protein than in wild type L-protein. This suggests that the site is open without unessential cross-linking between the cysteine residues. This open structure should be created by the replacement of two cysteine residues at 147 and 149, thereby enhancing the accessibility of the enzyme responsible for the glycosylation at Asn146 [18,29,30]. Although the roles of glycosylation in HBV are not yet clear, there have been several reports that describe *N*-linked glycosylation as necessary for virion secretion [31], for reduction of the viral antigenicity and for resistance against proteolytic degradation [32]. This glycosylation might be associated with the resistance to trypsin that was acquired in Lm8-BNCs when compared to L-BNCs. The glycosaccharide moiety might make the proteases inaccessible to its recognition site. It is also conceivable that Lm8-protein might acquire stability with less false disulfide bridging, which would result in the denatured state accessible for proteases.

When Lm8-BNC was assessed as a gene transduction vector, the cell type specific GFP expression was successfully observed

in a dose-dependent manner of the BNC (Fig. 7). The gene transduction efficiency by Lm8-BNC was more than two-fold when compared with that by L-BNC. L-BNC itself did not show any cytotoxic activity [15] so that the replacement of cysteine residues should not be related with the decrease of toxic events. The increased efficiency of gene transduction may be due to the enhanced stability of the L-BNC caused by the removal of unessential disulfide bridging. A reason for this is that proteases on the cell surface may degrade the BNC at the infection [33,34]. Degradation of the sequence including hepatocyte recognition site present in PreS1 would seriously impairs the attachment of BNC to hepatocytes [10,17,35] and result in low efficiency of internalization. There might also be an advantage to Lm8-BNC to have pores opened to incorporate substances into the BNCs with less disulfide bridging. One of the biggest hurdles in gene therapy is finding a safe and effective delivery vehicle. Several viral delivery mechanisms are efficient at gene transfer, but pose manufacturing and safety problems. Non-viral vectors are easier to manufacture and often safer but are not as effective. BNC offers tissue specificity and efficiency of a viral approach without the harmful side effects [36]. In this paper, BNC is successfully sophisticated as Lm8-BNC with impaired stability.

6. Conclusion

In this study, we successfully engineered Lm8-BNCs, eliminating unessential disulfide linkages to confer the stability necessary for a delivery vector with enhanced efficiency of gene transduction. This stability is explained by the hyper-glycosylation and by the decreased opportunity of false disulfide links, both of which would prevent the accessibility of proteases. Due to the stability the efficiency of transduction should be enhanced simultaneously. The minimized number of disulfide links, which would not allow the unnecessary intermolecular bonds between L-proteins, will confer the mobility of the components of BNCs showing the enhanced efficiency of gene encapsulation by electroporation. This design of Lm8-BNC should be further applicable to other engineered BNCs, which are expected to appear in the future, such as those devised for protein delivery vector described by Yu et al [37].

Acknowledgments

The authors thank Mr. Nobuhiro Nagatomo, Mr. Tomonori Yabuki and Ms. Kumiko Soga for their excellent technical assistance. Fukuda, T. was supported by the fellowship from Katayama Chemicals Ind. Co. Ltd. This project is partly supported by the Grants-in-Aid from the Japan Science and Technology Corporation (Research Fund for Patenting) and the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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中空バイオナノ粒子を用いたDDSの開発とその産業化

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Development of DDS using hollow bio-nanoparticles and their commercialization

We succeeded overproduction of the HBV envelope L particles with an approximate average particle size of 80 nm in yeast cells. Because the L particle is an empty bionanoparticles containing no viral DNA, it can be used as a safe and efficient carrier for human liver-specific delivery (pinpoint delivery) of drug and gene. In addition, genetically engineered L particles that are able to target to various organs were constructed by deleting the hepatocyte binding domain of L protein (pre-S region) and displaying targeting peptide or protein ligands. Therefore, bionanoparticles are a novel nano-carrier applicable to the broad range of pinpoint DDS.

筆者らは，B型肝炎ウイルス(HBV)の外殻L蛋白質から形成され約80 nmの平均径を持つL粒子を酵母で量産することに成功した．この粒子はウイルスゲノムを含まない中空のバイオナノ粒子であることから，薬剤や遺伝子などを肝細胞特異的に送達(ピンポイントDDS)する安全かつ高効率なナノキャリアとし利用できる．さらに，L蛋白質の肝細胞認識部位(pre-S領域)を各種のターゲティングペプチドやリガンドに置き換えることで，任意の組織・臓器に再標的化された粒子も構築できるため，広範なピンポイントDDSへの応用が期待されている．

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key words : nanoparticle, hepatitis B virus, pinpoint drug delivery, gene therapy, targeting

近年の創薬技術の目覚ましい進展により，有望な医薬品候補を効率よく探索することが可能になってきている．また，薬物としては，主流である低分子化合物に加えて，蛋白質や核酸も使用されるようになってきている．さらに，近年のライフサイエンスの進展や分子レベルでの発症機構の解明は，新しい治療法としての遺伝子治療を可能にしつつある．遺伝子治療は，遺伝子を目的の細胞に送達・導入して，治療効果を持つ蛋白質をつくり出そうとするものである．

しかしながら，依然として薬物は全身投与が主流であるため，生体吸収率や目的部位へ送達率が低い場合も多い．また，高い治療効果を持つものでも，患部以外で生じる副作用のためにドロップアウトする医薬品候補が多く，副作用を抑えるうえで投薬量を下げた場合は，限定的な効果になる場合もある．

このような現状から，遺伝子などを含めた広い意味での薬物を，薬効を保ったまま，安全かつ効果的に投与を行う技術として，ドラッグデリバリーシステム(DDS)への関心が高まっている．DDSの中核技術として，標的化(ターゲティング)があげられる．これは生体内において薬物を運搬体(キャリア)を用いて細胞や組織特異的に送達すること(ピンポイントDDS)で，副作用を最小限に抑えて，本来の効能を発揮させようとする試みである．特に血液中には低濃度でも，積極的に患部に薬剤や遺伝子を集中できる能動的なピンポイントDDS法が開発できれば，薬物治療効果を飛躍的に高めることが出来る．また，がん治療を考えても，周辺・遠隔臓器に転移したものも含めて，ターゲティングにより多臓器への転移がんを網羅的に治療することが可能になれば，波及効果は大きい．

能動的なターゲティングを行ううえでは，標的部

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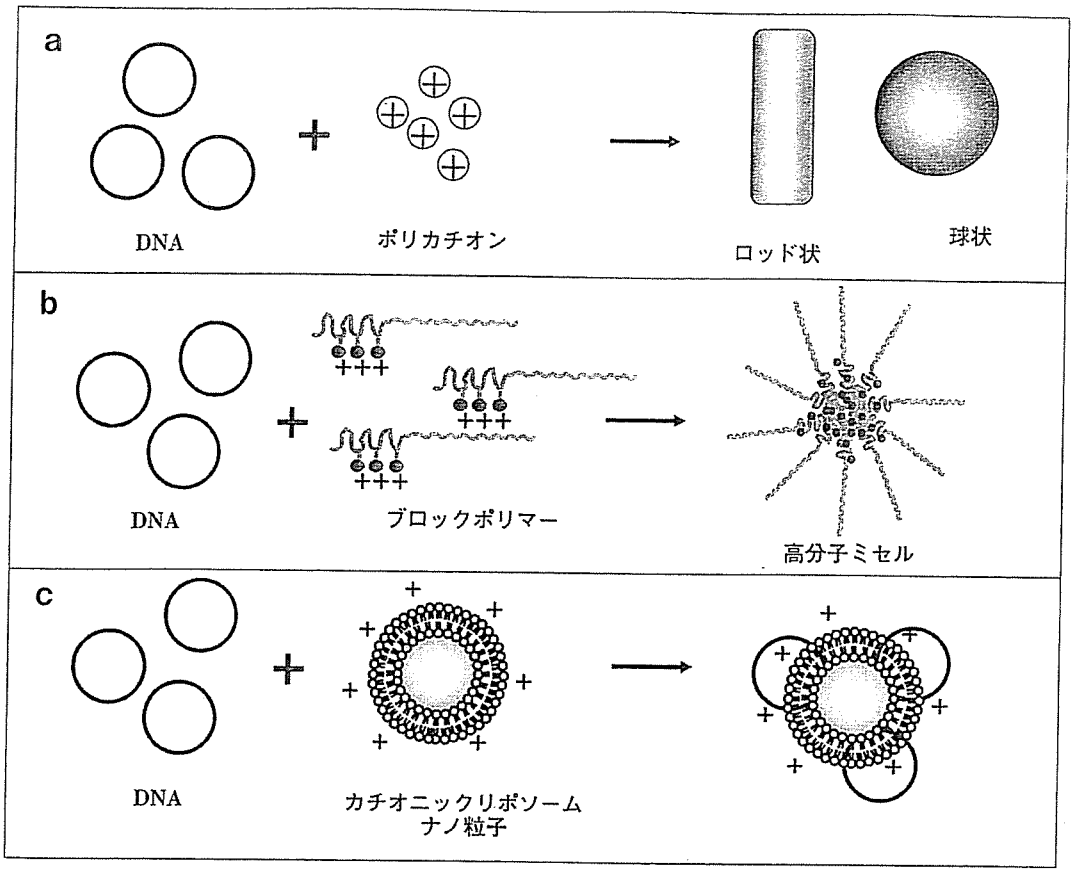

図1　人工DNAナノ粒子のアセンブル法

位になんらかの親和性を持つ物質をキャリアとして用いる．このキャリアは目的部位に到達したのち，分解されるか，薬物を効率よく放出することが重要である．

筆者らは，まったく新しいタイプの薬剤や遺伝子などのDDS用ナノキャリアとして，ウイルス由来の蛋白質を利用した中空バイオナノ粒子を考案した．ウイルスのなかでも，B型肝炎ウイルス(hapatitis B virus：HBV)が肝臓のみに特異的かつ効率よく感染する能力を有することに着目し，HBV表面抗原からなる蛋白質のナノ粒子(中空バイオナノ粒子)を目的臓器への能動的なピンポイントDDSを可能とするナノキャリアとして利用しようというものである．

本稿では，中空バイオナノ粒子の概念，その製造や物性と遺伝子治療をはじめとする広い意味でのDDSへの応用について紹介する．

遺伝子治療

1990年に臨床研究がはじまった遺伝子治療は，成功例がいくつも報告され，研究が活発になった．遺伝子治療においてはピンポイントな送達が不可欠である．これは，生体内で分解を受けやすい遺伝子を目的臓器に効率よく送達できなければ，充分な治療効果を出すことが難しく，逆に遺伝子が他の部位に導入されると思わぬ副作用が生じる可能性があるためである．

現在までに，実験的に行われている遺伝子治療の多くは，アデノウイルス，レトロウイルス，アデノ随伴ウイルスなどのウイルスゲノムに目的の遺伝子を組み込んだ感染性ウイルスを利用したものである[1]．これらのウイルスは高い遺伝子導入効率を持つ大きな利点があるが，組織・細胞に対して非特異的に感染してしまう．このため，目的臓器に送達するために手術などにより患部に直接投与する必要が

あり，患者への負担が大きい．また，ウイルスを利用すると，そのゲノムを患者の染色体に導入する可能性があり，予測不可能な副作用の危険性がある．実際，アメリカやフランスで臨床試験中の事故が報告されるなど，ウイルスベクターの安全性への懸念が広がっている[2,3]．さらに，その製造においても安全性の確保が大きな問題となる．

こうしたことから，高機能で安全な非ウイルス性の人工的なキャリアの開発が強く求められている．人工キャリア(あるいはナノ粒子化法)として検討されてきた代表的なものを図1に示す[4]．ポリカチオンあるいはポリカチオン＋ターゲティング用のリガンドによってDNAをロッド状や球状に凝縮する方法(図1a)，ブロックコポリマーによりポリマーミセルを形成させる方法(図1b)，正電荷を持ったリポソームや微粒子に結合させる方法(図1c)などが考えられている．この場合，遺伝子の導入効率をいかに高められるかが大きなポイントである．

近年，ヒトゲノムの概要が明らかにされており，遺伝子治療に利用可能な遺伝子群が多数見いだされると予想され，安全で有効な遺伝子治療法を早急に確立する必要がある．そのために，ピンポイントDDSを可能とするナノキャリアが必須である．

中空バイオナノ粒子

まず，中空バイオナノ粒子のもとになるHBVの構造について述べる．

HBVは，直径約42 nmの球状ウイルスである．図2aに示すように，HBVは，そのゲノムが，蛋白質(ウイルス表面抗原蛋白質という：図では楕円形で示す)と脂質からできた外殻(エンベロープ：蛋白質間を宿主由来の脂質の膜が埋める形で形成されたカプセルで厚さは7 nm)とコアで二重に包まれた構造をとっている．コアの中には，ウイルス本体の不完全二本鎖のHBV DNA(約3,200塩基)や遺伝子を複製するのに必要なDNAポリメラーゼが存在している．

HBVは，L蛋白質の標的細胞への結合と，引きつづいての標的細胞との膜融合によって感染する．HBVは，微量のB型肝炎患者血液を指に刺しただけでも感染することでもわかるように，極少量のウイルス粒子でもヒト肝臓に到達して選択的に感染して複製するすぐれた能力を持つ．

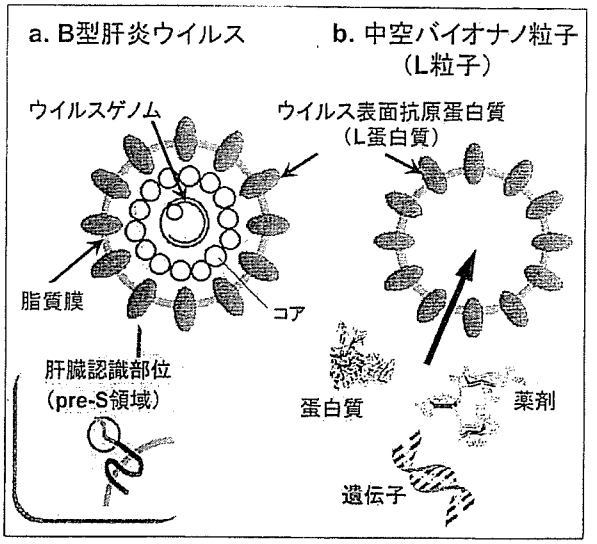

図2　B型肝炎ウイルス(a)と中空バイオナノ粒子(b)

外殻をつくるL蛋白質は，226アミノ酸残基のS蛋白質のN末端側にpre-S領域(55アミノ酸残基のpre-S2領域と108または119アミノ酸残基のpre-S1領域からなる)が付加した形になっており，分子量約52kDaの糖蛋白質である．pre-S1については，肝細胞と特異的に結合する部位を含んでおり，HBVが感染する際に中心的な役割を担っていることが明らかになっている[5,6]．また，pre-S1領域の作用により，HBVのトロピズム(感染する宿主域，細胞指向性)はチンパンジーとヒトの肝細胞に厳格に限定されている[7]．ウイルスは，その構造や感染から複製といった機能をみるとき，蛋白質，脂質，DNAといったバイオ分子群のパーツから組み上げられたきわめて精巧なナノマシンを連想させる．

中空バイオナノ粒子とは，HBVのL蛋白質と脂質からなる外殻のみを，人工的かつ安全な形で生産したナノカプセルである(図2b：L粒子とよばれる)．L蛋白質は肝臓を分子認識するpre-S1領域を持つため，この粒子はウイルスが本来持つ肝臓への高い感染力を保持すると考えられる．また，HBVの表面抗原蛋白質が形成する中空バイオナノ粒子は，脂質膜と糖蛋白質からなる柔軟な構造を有して