widely used in chemical processes involving copper and nickel catalysts as well as in fermentation processes carried out in steel tanks.

To construct novel yeast cells for practical use as bioadsorbent, suspended cells must normally be separated from the media prior to further processing. For complete separation, the cell culture is commonly centrifuged, which, however, requires use of a device and is expensive. By contrast, aggregation is a cheap and simple procedure, since the cells spontaneously from the culture medium. Cell aggregation is observed in *S. cerevisiae* and utilized in the production of pharmaceutical and food products. Overexpression of *GTS1*, a gene affecting the timing of budding, cell size, heat tolerance, and cell aggregation, has been reported to result in constitutive aggregation (Bossier et al. 1997; Mitsui et al. 1994; Yaguchi et al. 2000). Kuroda et al. (2002) endowed the hexa-His-displaying strain of *S. cerevisiae* with the ability to self-aggregate in response to environmental copper ion, thus allowing easy removal of the bioadsorbent from treated water.

Endocrine disruptor binding protein

One current focus in the field of endocrine disruption is to determine whether estrogenic compounds can mimic the effects or modify the activity of steroid hormones. To evaluate the ability of chemical compounds acting as endocrine disruptors to bind to steroid hormone receptors, various in vivo and in vitro assay systems have been developed and used to determine estrogenicity (Nishikawa et al. 1999; Routledge et al. 1997; Sohoni et al. 1998). Of concern is whether exposure to chemicals with steroid-like activity can disrupt normal endocrine function, leading to altered reproductive capacity, infertility, endometriosis, and cancers of the breast, uterus, and prostate (Jensen et al. 1995; Sharpe 1998).

A novel arming yeast displaying the ligand-binding domain of the rat estrogen receptor (ERLBD) on its cell surface (Yasui et al. 2002) heralded the possibility of a new assay system based on strains of *S. cerevisiae* displaying steroid hormone receptors. The binding activity of ERLBD, displayed on the yeast cell surface, to fluorescent 17β–estradiol (17–FE), an analogue of the natural ligand of the estrogen receptor, was comparable to that of the native receptor. The number of 17–FE molecules entrapped on the cell surface demonstrates that a system based on this new arming yeast will be useful not only in screening hormone-like compounds through competitive experiments but also in removing them from the environment.

Cellulose-binding protein

Cellulose is hydrolyzed by the cellulolytic enzyme system as described above. Like many other cellulases, two cellobiohydrolases (CBHI and CBHII) of the filamentous fungus *T. reesei* have a cellulose-binding domain (CBD) linked by a linker peptide to the catalytic core domain. The CBDs, the domains of CBHI and CBHII, display two distinct faces, one flat form and the other rough. The flat face is the binding surface to cellulose and contains several aromatic amino acids. The CBDs of CBHI (CBD1) and CBHII (CBD2) have similar binding properties, but the former binds reversibly and the latter irreversibly. A basic analysis of the binding ability of CBD to cellulose might contribute not only to understanding its binding mechanism but also to a wider development of the applications of yeast immobilization, for instance, in whole-cell biosensors and as bioadsorbents (Nam et al. 2002).

Genes encoding the CBD of CBHI and CBHII from *T. reesei* have been expressed on the cell surface of *S. cerevisiae* by cell-surface engineering (Nam et al. 2002), with tandemly aligned CBHI (CBD1) and CBHII (CBD2) fusion genes also constructed to display the two CBDs simultaneously on the cell surface. The binding affinity of the CBD-displaying yeast cells to a cellulose substrate is similar between CBD1-displaying and CBD2-displaying cells, and cells displaying the fusion protein of CBD1 and CBD2 show much higher binding affinity to cellulose than either of the single CBD-displaying yeast cells.

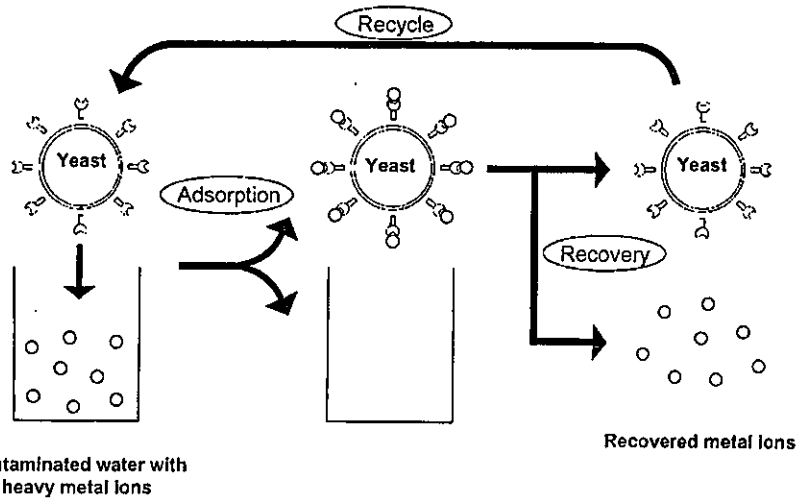

Fig. 4 Recovery of heavy-metal ion adsorbed by yeast strain displaying hexa-His

No studies have ever tried to bind yeast cells to cellulose through yeast cell-surface display of a fungal CBD, although the binding of recombinant *Staphylococcus carnosus* or *E. coli* to cellulose by CBD display on the cell surface has been reported recently (Lehtiö et al. 2001; Wang et al. 2001). The binding ratio of yeast cells is dependent on the number of CBD molecules displayed on the cell surface. In future studies, CBD- and mutated-CBD-displaying yeast cells will be used to analyze the binding mechanism of CBDs to cellulose, while yeast cells engineered to co-display CBDs and target enzymes will allow the construction of a bioreactor system with sequential and/or multistep enzyme reactions on the cellulose filter.

Fluorescent protein

A visible reporter located on the cell surface, which is an extremely important interface between the cell and its environment, represents a more powerful and vital marker for gene transcription, membrane protein localization, and ligand binding in molecular and biological analysis than an enzyme-type reporter expressed within the cell, as monitoring can be carried out without cell disintegration and at the level of a single cell. A green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used in the molecular design ofa novel visible type of reporter to target the cell surface of *S. cerevisiae* (Ye et al. 2000). Quantification of the number of enahnced GFP (EGFP) molecules displayed on the yeast-cell surface by the α-agglutinin fusion protein under the control of the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter produces results similar to those obtained by fluorometry and by confocal laser-scanning microscopy, indicating the accuracy of the count (Shibasaki et al. 2001a). These counts indicate that 10^4–10^5 molecules of α-agglutinin-fused molecules are expressed on each cell and that the amount of fluorescent protein expressed on cells harboring a multicopy plasmid is three to four times greater than on cells with the gene integrated in the genome.

A combination of reporters and promoters to sense intra- and extracellular conditions should allow the construction of a novel self-sensing yeast cell. The *UPR-ICL* promoter from *Candida tropicalis*, which has been found to be functional in *S. cerevisiae* (Kanai et al. 1996), is suitable for sensing glucose exhaustion, and the GFP variant BFP (blue fluorescent protein) is utilizable as the second reporter for this purpose. Shibasaki et al. (2001b) placed the BFP-encoding gene on a vector for integration into the yeast genome under the control of the *UPR-ICL* promoter and evaluated the relationship between intra- and extracellular glucose concentrations and the intensity of fluorescence from the surface-engineered cells. They also used the *PHO5* promoter and the *MEP2* promoter derived from *S. cerevisiae* to monitor nutrient concentration (Shibasaki et al. 2001c). The former is the upstream sequence of the gene (*PHO5*) for a secreted acid phosphatase (Harashima et al. 1995; Meyhack et al. 1982; Rudolph and Hinnen 1987) and its transcription is known to be regulated by the extracellular concentration of inorganic phosphate, that is, repressed in high-phosphate medium and derepressed in low-phosphate medium. The latter is the upstream sequence of the gene (*MEP2*) for an ammonium-ion transporter protein (Lorenz and Heitman 1998; Marini et al. 1994, 1997). In the presence of the nitrogen sources, glutamine, asparagine, and ammonium, all three MEP genes are repressed, whereas under poor nitrogen supply *MEP2* expression is much greater than

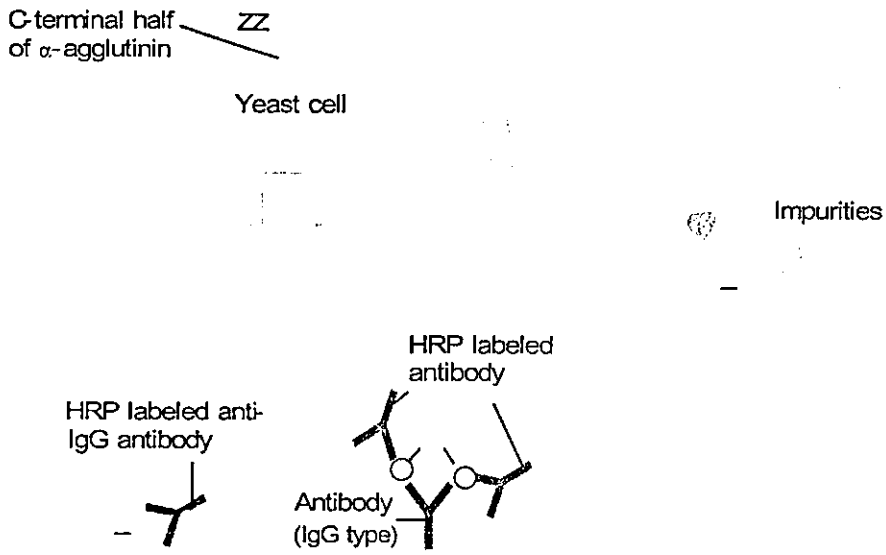

Fig. 5 ELISA to detect rabbit IgG and sandwich ELISA to detect antigen, and separation of IgG-type antibodies using yeast cells displaying ZZ

MEP1 and MEP3 expression. The GFP variants ECFP (enhanced cyan blue fluorescent protein) and EYFP (enhanced yellow fluorescent protein) have been used as reporters, and flow cytometric studies demonstrated that fluorescence emission from ECFP and EYFP displayed on the yeast cell surface reflected the concentration of phosphate ion or ammonium ion. This novel cell-surface-engineered system for non-invasively identifying environmental changes using various promoters allows the creation of intelligent cells able to independently sense extracellular environmental conditions and intracellular states without the need to disintegrate the cell. Novel yeast strains will also facilitate the computerized control of various bioprocesses through measurement of the intensity of emitted fluorescence.

Protein A derivative

Nakamura et al. (2001; Shimojo et al. 2004) employed cell-surface engineering using the C-terminal half of α-agglutinin to construct a yeast strain displaying the ZZ domain derived from *Staphylococcus aureus* (SPA), which binds to the Fc part of immunoglobulin G (IgG) (Nilsson et al. 1987). The ZZ protein has a structure repetitive of the small Z domain (58 amino acid residues), which consists of two α-helical domains. ZZ interacts with IgG from various species, including human (IgG1, IgG2 and IgG4) and rabbit (all of the IgG class), and has been used as an affinity tag to purify recombinant proteins and in immunoassay. *S. cerevisiae* cells displaying ZZ could therefore be widely used as immunoadsorbents in immunoassay and affinity purification (Fig. 5). Enzyme-linked immunosorbent assay (ELISA) and sandwich ELISA using *S. cerevisiae* cells displaying ZZ can both detect IgG and antigen (e.g. human serum albumin) down to a concentration of 1–10 ng/ml. The detection range covered by these assay systems is broad and can be varied by adjusting the amount of cells and the duration of reaction with the enzyme (horseradish peroxidase) substrate. Yeast cells displaying ZZ have also been successfully used for repeated affinity purification of IgG from serum. These results suggest that *S. cerevisiae* displaying ZZ may constitute a novel and genetically renewable whole-cell immunoadsorbent widely applicable for immunoassay and affinity purification.

Single-chain antibody and single-chain T-cell receptor

In the use of antibodies as therapeutics, rapid and cost-effective identification along with the development of antibodies with high specificity and high affinity are critical. Yeast cell-surface display systems have been found to be effective for the display of single-chain antibody (scFv) and the development of antibodies with enhanced affinity and stability (Boder and Wittrup 1997; Boder et al. 2000; Feldhaus et al. 2003). In the studies cited, functional scFv was successfully expressed on the yeast cell surface by fusion to the C-terminus of Aga2p. The Aga2p–scFv fusion protein is linked to Aga1p by two disulfide bonds. When the scFv fragment of antifluorescein antibody was displayed on the cell surface, it bound fluorescein-conjugated dextran, demonstrating that the antibody-binding site is accessible to very large macromolecules (Boder and Wittrup 1997). Yeast cell-surface display systems also offer many advantages for screening large combinatorial scFv libraries, and together with fluorescence-activated cell sorting (FACS) allow rapid quantitative isolation of rare clones with the desired characteristics (Boder and Wittrup 1997; Boder et al. 2000; Feldhaus et al. 2003). In addition, solid-phase capturing methods, such as magnetic separation, are efficient and convenient tools to isolate target cells from large cell-surface display libraries without using expensive equipment (Furukawa et al. 2003). Several successful instances of isolation of scFv with high antigen-binding affinity have been reported (Boder and Wittrup 1997; Boder et al. 2000; Feldhaus et al. 2003)

There is significant clinical interest in the T-cell receptor (TCR), which specifically recognizes peptide–major histocompatibility complex antigens in cell-mediated immunity. Although antibodies and the extracellular domains of TCR have similar structures, they have evolved to recognize different classes of ligands. It is therefore very important to clarify the difference in the V region of antibodies and TCRs in terms of molecular recognition. Single-chain T-cell receptor (scTCR) was constructed by connecting the $V_\beta$and V_α regions from TCR with a linker peptide and has been successfully displayed on the cell surface through fusion with Aga2p (Keike et al. 1999; Shusta et al. 2000). Thus, the scTCR yeast cell surface-display system is effective for T-cell receptor engineering.

Antibody Fab fragment

Functional hetero-oligomeric proteins, such as antibodies, have been successfully displayed on the yeast cell surface (Lin et al. 2003). This is probably attributable to the fact that yeast possesses typical eukaryote-specific, post-translational modification mechanisms for functions including proteolytic processing, folding, glycosylation, and efficient disulfide isomerization, and a secretory machinery homologous to that of mammalian cells. One model system uses the antibody 6D9, prepared by immunization of the transition-state analog haptenic phosphonate. A hetero-oligomeric Fab fragment of the catalytic antibody 6D9 efficiently hydrolyzed a non-bioactive chloramphenicol monoester derivative to produce chloramphenicol (Miyashita et al. 1997). The gene encoding the light chain of the Fab fragment was expressed with the tandemly linked C-terminal half of α-agglutinin, and the gene encoding the Fd fragment of the heavy chain of the Fab fragment as a secretion protein. The Fab fragments associated together on the yeast cell surface had an intermolecular disulfide linkage between the light and heavy chains and showed high stability and reactivity in

antibody-catalyzed reactions. The combination of the yeast cell-surface display system and the system for secretion from cells makes it possible to display functional hetero-oligomeric proteins of Fab fragments. The successful display of a functional hetero-oligomeric catalytic antibody allows a larger protein library to be constructed and clones of interest to be selected easily from a mutagenized protein pool using a fluorescently labeled hapten.

Combinatorial protein library

In vitro selection from molecular libraries has rapidly come of age as a protein-engineering tool. In the absence of quantitative and computational structure-function relationships for proteins, rational approaches to mutagenesis have limited potential to successfully and rapidly alter protein molecular properties to meet predefined criteria. As an alternative strategy, the construction and selection of randomly mutated combinatorial libraries has yielded numerous successes.

A combinatorial random protein library can be constructed from random DNA fragments generated by what is termed DNA random priming, an improved method of random-priming recombination (RPR) (Zou et al. 2001). Combining this method with cell-surface display techniques results in some "unusual fragments" or "unusual proteins" different from the natural genes and amino-acid compositions. In order to express this library on the yeast cell surface, a yeast multicopy cassette vector is constructed.

As an example, an *n*-nonane-tolerant yeast strain screened out from transformants displaying this combinatorial library was found to grow very well in nonane-overlaid culture medium. In both prokaryotic and eukaryotic organisms, organic-solvent tolerance has been considered as a cell-surface-related function and/or a function of the lipid composition (Miura et al. 2000). While there has been no report on the mechanism of this tolerance in yeast, it is highly likely that the yeast cell surface is intimately connected with organic-solvent tolerance. If this was the case, a protein library displayed on the yeast-cell surface would be very helpful in screening for proteins responsible for organic-solvent tolerance. When a combinatorial protein library constructed through displaying about 4×10^4 independent colonies on yeast cells was screened against the pressure of *n*-nonane, only one clone retained nonane tolerance after several passages of cultivation in the presence of the solvent (Zou et al. 2002). The tolerance was confirmed to be due to the random protein displayed on the yeast cell surface. Genetic analysis showed that a fragment of 274 bp was inserted in-frame into the multicopy cassette vector. The inserted protein fragment was relatively hydrophilic, which seemed to confer the clone with surface characteristics similar to those of the isooctane-tolerant mutant. This similarity suggests that the organic-solvent tolerance of yeast cells may be closely related to the structures and functions of the cell surface, although further investigation is required to confirm this and to identify the mechanism in detail. This strain, which is the first genetically constructed recombinant yeast strain able to tolerate the organic solvent *n*-nonane, will provide important information on the organic-solvent tolerance of eukaryotes, which may lead to a much wider application of yeast in industrial bioprocesses in general. At the same time, the strain may act as a model for screening of other new phenotypes and novel functional proteins from combinatorial protein libraries.

Future directions

The *S. cerevisiae* strains reviewed here are the first examples of surface-engineered yeasts in which active enzymes and functional proteins targeted to the cell surface endow cells with new beneficial properties. Yeast-based systems allow successful display of large protein molecules, not only of single-subunit proteins but also hetero-oligomeric multi-subunits. In addition, several different proteins can be displayed simultaneously. These surface-engineered yeast strains have been referred to as "arming yeasts" (Anonymous 1997).

The displayed proteins are self-immobilized on the cell surface, with this feature being passed on to daughter cells as long as the genes are retained by the cells.

The ability to display enzymes should further enhance the status of *S. cerevisiae* as a novel and attractive microorganism able to act as a whole-cell biocatalyst. This is particularly important when target substrates cannot be taken up by the cells. Furthermore, the system allows the production of renewable biocatalysts. In previous studies, surface-displayed enzymes were mainly used as simple catalysts. However, cellular engineering studies have resulted in the ability to change or improve intracellular metabolic capabilities by the addition or deletion of certain enzymes. In cell-surface engineering, cell-surface display is combined with the endowment of yeast cells with additional metabolic functions. The resulting yeast strains can thus be used as "cell factories" suitable for a wide variety of industrial applications.

The various types of protein libraries displayed on the yeast cell surface promise to supply novel protein molecules with significantly elevated binding affinity and stability, and endowed with new functions. The combination of efficient screening methods (e.g. high throughput flow cytometry and solid-phase capture methods such as magnetic separation) with cell-surface display will significantly accelerate the development of protein engineering. In particular, efficient and simple screening methods such as magnetic separation (Furukawa et al. 2003) are critical for the broader application of cell-surface display systems.

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Novel Tissue and Cell Type-specific Gene/Drug Delivery System Using Surface Engineered Hepatitis B Virus Nano-particles

Tadanori Yamada[1], Masakazu Ueda[2,5], Masaharu Seno[3,5], Akihiko Kondo[4,5], Katsuyuki Tanizawa[1] and Shun'ichi Kuroda[1,*]

[1]*Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan,* [2]*Keio University, School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan,* [3]*Graduate School of Natural Science and Technology, Okayama University, 3-1-1 Tsushimanaka, Okayama 700-8530, Japan,* [4]*Faculty of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe, Hyogo 657-8501, Japan,* [5]*Beacle Inc., 314 Okayama Research Incubation Center, 5303 Haga, Okayama 701-1221, Japan*

Abstract: The hepatitis B virus (HBV) surface antigen (HBsAg) L particle is a hollow nano-scale particle. HBsAg L particles have many properties that make them useful for *in vivo* gene transfer vectors and drug delivery systems. Gene therapy so far has required the *in vivo* pinpoint delivery of genetic materials into the target organs and cells. Gene transfer by HBsAg L particles might be an attractive method, since their tropism is the same as that of HBV. The HBsAg L particles are able to deliver therapeutic payloads with high specificity to human hepatocytes. In addition, the specificity of L particle can be altered by displaying various cell-binding molecules on the surface. Our results indicate that the L particle is suitable for a cell- and tissue-specific gene/drug transfer vector. In this review, we discuss HBsAg L particles as a gene/drug transfer vector and its potential for the treatment of infectious diseases.

Key Word: HBsAg L particle, gene delivery, drug system, *in vivo* system.

INTRODUCTION

Gene therapy is recognized as a promising approach for treating serious diseases, including monogenic diseases, infectious diseases and cancer [1, 2]. Gene therapy is based upon the introduction of therapeutic genes into the target cells and organs of patients. In the previous decade, gene therapy was initially adapted to the treatment of genetic diseases, such as adenosine deaminase severe combined immunodeficiency (ADA-SCID). At present, the clinical trials on primary immunodeficiency diseases (PID) are being continued by several groups [3-5]. Recent advances in virology, biochemistry, and molecular biology allow us to approach infectious diseases at the molecular level and supply us with useful genetic information for designing therapeutic reagents. Therefore, gene therapy may prove suitable for a number of infectious diseases. Currently, the strategies against viral infections include genetic vaccination stimulating the host immune responses and the inhibition of viral replication with gene products, such as antisense DNA/RNA, RNA interference (RNAi), and DNA decoys. However, the lack of appropriate and safe gene transfer vectors has severely restricted gene therapy in the mortal diseases.

Various methods have been developed for delivering therapeutic genes to the target cells or organs. Recombinant viral vectors, like retroviruses [6] and adenoviruses [7], represent one such approach. The greatest advantage of viral vectors is their high efficiency of gene transfer. However, there are severe limitations due to the properties of wild-type viruses. The viral vectors transfer genes into cells other than the target cells because of their wide range of cell infectivity. Thus, these vectors are rarely used to *in vivo* methods and applications. Retroviral vectors, which are popular vectors in clinical trials, have the ability to integrate their genomes into the host chromosomal DNA [8-10]. This results in a long-term expression of transgenes, while it is difficult to control the insertion position in the chromosome of the infected cells, and random site integration may lead to gene inactivation or activation of oncogenes [11, 12]. In 2002, two children were found to develop leukemia during the gene therapy trials in France [13, 14]. They were treated for X-linked severe combined immunodeficiency (X-SCID) using a retroviral vector [4]. In this case, the retroviral vector has integrated its payload in proximity to T-cell oncogene *LMO2*, and led to premalignant cell proliferation with unexpected frequency.

In order to succeed in gene therapy, the gene delivery system should possess the following features; (i) effectiveness of delivering genetic materials, (ii) preciseness of delivering into the appropriate target cells and organs, (iii) preciseness of delivering into the appropriate genetic site, and (iv) overall safety for patients. Although there have been many attempts to develop a novel gene transfer system, no system has so far fulfilled these important criteria. This current situation prompted us to develop a novel gene transfer vector based on hepatitis B virus (HBV) surface antigen (HBsAg) L particle. Our previous report demonstrated the properties of HBsAg L particle are suitable for a novel vector system. In this review, we discuss HBsAg L particle as a gene transfer vector and also their potentials in treating infectious diseases.

*Address correspondence to this author at the Department of Structural and Molecular Biology, Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka, 567-0047, Japan; Tel: +81-6-6879-8462; Fax: +81-6-6879-8462; E-mail: skuroda@sanken.osaka-u.ac.jp

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WHAT IS HBsAg L PARTICLE?

HBV is a human liver-specific DNA virus, whose 3.2-kbp genome harbors three overlapping envelope (*env*) genes in a single open reading frame. Depending on the three translation initiation codons, three related transmembrane proteins are produced in human hepatocytes, designated as small (S), middle (M), and large (L). The S protein is a major *env* protein (226 amino acid residues), and the M protein comprises the entire sequence of the S protein with an additional 55 amino acids at the amino-terminal (pre-S2 peptide). The L protein has the entire sequence of the M protein with an additional 108 or 119 amino acids (depending on the HBV subtype) at the amino-terminal (pre-S1 peptide) [15]. As shown in Fig. (1), HBsAg L particle is a hollow subviral particle consisting of HBsAg L proteins and a lipid bilayer derived from the host cell [16, 17]. The pre-S1 peptide mediates the specific attachment of HBV to human and chimpanzee hepatocytes [18-20].

Previously, we demonstrated the efficient production of HBV L particles in recombinant yeast cells (up to 42% of the total soluble proteins) [21]. Owing to the high productivity, L particles were promptly purified using only ultracentrifugal procedures, which was enough to analyze their physico-chemical properties [22]. By equilibrium sedimentation, the average molecular weight of L particles was estimated to be approximately 6.4 x 10^6. Atomic force microscopy observation in a moist atmosphere had revealed L particles are large spherical particles with a diameter of 50-500 nm (average diameter is 200 nm).

HBsAg L PARTICLE AS A GENE/DRUG TRANSFER VECTOR

The HBsAg L particle is displaying a peptide that is indispensable for liver-specific infection by HBV in humans and chimpanzees. Thus, it has been expected that L particles could serve as a vector possessing high infectivity and high specificity to human and chimpanzee hepatocytes. Recently, we demonstrated that L particles could introduce genes and drugs into human hepatocytes both in cultured cells and in laboratory animals [23]. We have found that electroporation is efficient for incorporating transgenes and drugs inside the particle. The L particles containing these materials could introduce into human hepatocytes and hepatocellular carcinoma cells, but not into control non-human hepatic cells. Also, the L particles were able to deliver their payloads through the blood stream only in human hepatocellular carcinomas, but neither in other human carcinomas nor in normal mouse tissues. Intravenous injection of the L particles containing the gene for human clotting factor IX (hFIX) resulted in the production of hFIX protein in the xenograft model at levels sufficient to the treatment of moderate hemophilia B. These data indicated that L particle is an ideal vector for the specific delivery of genes and drugs *in vitro* and *in vivo*. In addition, the L particle can be overproduced easily in yeast cells without any contamination of viral genomes. These properties demonstrate that L particle is suitable for clinical trials.

In previous studies on HBV, a sequence in the pre-S1 peptide was identified responsible for specific attachment to human hepatocytes [18, 24]. We attempted to alter the specificity of the L particle by substituting a bio-recognition molecule for the human hepatocytes-binding domain. For example, the engineered particles displaying human epidermal growth factor (EGF) lost the ability for binding to human hepatocytes and gained the affinity to the EGF receptor-expressing cells. Taken together, the HBsAg L particle-based delivery system might become a novel platform for gene transfer and drug delivery systems and could possess the tailor-made tissue/cell type specificity.

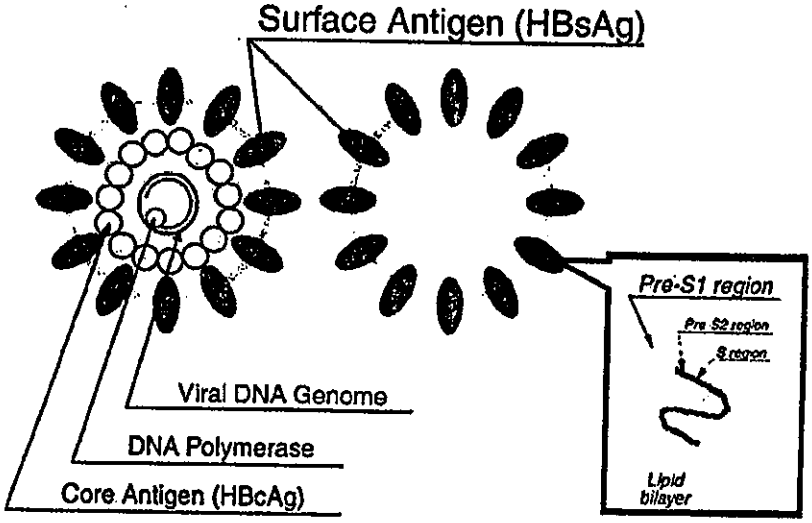

Fig. (1). Diagram of HBV and HBsAg L particle. HBV (left) contains one viral genome, one DNA polymerase, core antigens (HBcAg), surface antigens (HBsAg), and lipid bilayer. HBsAg particle (right) consisting of surface antigens and lipid bilayer is a hollow particle. The HBsAg L particle consisting of L proteins and lipid bilayer possesses the high infectivity to human liver cells without replication activity. The L protein contains pre-S1, pre-S2 and S regions (inset). The S region is a transmembrane protein indispensable for the formation of the particle. The pre-S1 region on the surface of L particle is responsible for the specific infection to human liver cells.

HBsAg L PARTICLES AS A MULTIVALENT VACCINE PLATFORM

From 1985 to 1995, the importance of the entire L protein (pre-S1 + pre-S2 + S) for the prevention of HBV infection has been recognized [18, 25-29]. We have previously reported the efficient production system for recombinant L proteins (as L particles) in yeast cells and the immunological properties of L particles in mice [21, 22]. Intraperitoneal injection of L particles efficiently and simultaneously elicits anti-S, anti-pre-S2, and anti-pre-S1 antibodies. In addition, the anti-pre-S1 rabbit antibodies were found to recognize various segments of the pre-S1 region, including the pre-S1 (21-47) segment, which is indispensable for HBV infection. These results demonstrate the ability of L particles to induce all antibodies against HBV *env* proteins. Thus, HBsAg L particles have an important role in mass vaccination against HBV, as well as for people considered at high risk of the HBV infection, especially in developing countries.

Advances in recombinant DNA technology allowed us to generate a novel vaccine against emerging infectious diseases. Human immunodeficiency virus type 1 (HIV-1) is one of the emerging viruses and has received much the attention from public [9, 31-33]. Among these studies focusing on this problem, several researchers developed a novel vaccine against HIV-1 [34, 35], which displays HIV envelope glycoproteins on the surface of HBsAg particles. While these glycoproteins *per se* induce a slow and weak immune response to HIV-1, this hybrid vaccine elicited stronger and more rapid humoral responses against both HIV-1 and HBV. Thus, HBsAg particles could boost the host immune responses as an adjuvant. Since we succeeded in producing the engineered particles displaying various molecules on the surface with the help of recombinant yeast cells, a hybrid vaccine displaying various foreign epitopes can be generated and might play an important role in mass vaccination against a number of infectious diseases.

HBsAg L PARTICLE FOR THE GENE THERAPY OF HEPATITIS B AND C

Because of the human liver-philic properties of L particles, viral hepatitis, cirrhosis and hepatocellular carcinoma are the suitable targets for the L particle-mediated gene therapy. There are two strategies for the gene therapy of hepatitis B and C. One strategy, the immunological approach is a popular therapeutic method based on the stimulation of broad humoral and cellular immune responses to viral proteins. At present, vaccination for HBV is only the way to protect efficiently against HBV infection. Gene transfers of interferon (IFN)-α and/or IFN-γ genes [36-38] are the alternative approaches to vaccination. However, systemic administration of IFN is known to induce serious side effects in whole body, which limit broad usage of this IFN therapy.

Another strategy, the antiviral approach is designed to block the viral life cycle at several critical points. The molecular biological analysis of hepatitis viruses has led us to the identification of the appropriate targets for antiviral therapy [39]. Antisense oligonucleotides [40-45], antisense RNA [46-48] and ribozymes [49, 50] have demonstrated the effective blockade of viral gene expression *in vitro*. Recently, several groups have demonstrated that RNAi is an attractive therapeutic tool for inhibiting viral replication [51-54]. RNAi is based upon the process of sequence-specific gene silencing using short interfering RNAs (siRNAs). The siRNAs, designed as sequences homologous to the viral genome, reduced the transcription of viral genes *in vivo* without affecting to the host cell.

Although the specific therapeutic genes appear to be effective in antiviral therapy, currently available vectors cannot deliver them to their targets in a pinpoint manner. Therefore, HBsAg L particle is considered to be applicable for both IFN treatment and antiviral therapy. According to our data, L particles can deliver these therapeutic materials efficiently into human hepatocytes by an intravenous injection.

Chronic infections of hepatitis B and C virus induce hepatocellular carcinoma (HCC) in patients with high frequency. Numerous gene therapy strategies have been investigated for their potential in treating HCC. They include the suicide system, the genetic immunotherapy, and the gene replacement. The suicide system is considered as one of the most powerful approaches to cancer treatment. The tumor cells, to which suicide genes coding for enzymes that convert non-toxic compounds (prodrugs) into toxic products were introduced, can be readily eliminated upon the subsequent administration of prodrug. Many suicide gene therapies have been successfully used in animal models of cancer. The most potent and widely used gene is the herpes simplex virus thymidine kinase (HSV-*tk*) [55, 56]. Since all cells transfected with HSV-*tk* gene are killed by the administration of HSV-*tk*-specific inhibitor, gancyclovir (GCV), it is necessary to achieve the cancer cell-specific expression of HSV-*tk* gene. For this purpose, the tissue-specific and the cell-specific promoters/enhancers were considered suitable for the *in vivo* targeting. Most widely used promoter/enhancer is derived from the α-fetoprotein (AFP) gene, of which the protein is produced only in the fetal liver but reproduced in large parts of HCC patients [57, 58]. Thus, AFP promoter has been expected promising for the gene therapy of HCC [59-61]. Combination with L particles might further reduce the side effect of gene therapy in tissues other than liver, and change from surgical operation to intravenous injection for the administration of therapeutic genes. It is also important to achieve the high cell specificity by dual mechanisms, inside promoter and outside cell-recognition molecule. Since 30-40% of HCC patients are non- or low-AFP producing, it is necessary to establish novel *in vivo* targeting system for all types of HCC. For examples, the L particles displaying HCC-specific antibodies and the L particles displaying dual cell-recognition molecules (EGF and wild type pre-S1 peptide), which we recently developed, are highly promising.

CONCLUSION

Based on the HBsAg L particle, we have succeeded in generating the platform technology for *in vivo* pinpoint delivery system. This system is applicable for not only genes but also drugs and proteins. Since the HBsAg S/M particles had already been developed as a recombinant HB vaccine in

the last decade, the HBsAg L particle is considered to be essentially safe in human. This system firstly facilitates the *in vivo* pinpoint delivery of genes and drugs to the desired tissues and cells, the repetitive administration without surgical operation, and the use of large size of therapeutic gene. The treatments of liver diseases (*e.g.*, viral hepatitis, cirrhosis, and HCC) with HBsAg L particle-mediated drug delivery are in the preclinical stage, which will move into the phase I clinical trial within several years. We believe this technology will be adopted by many forthcoming high-tech medicines.

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〔総 合 論 文〕

中空バイオナノ粒子を用いたピンポイントドラッグデリバリーシステム

山田 忠範[*1]・妹尾 昌治[*2,*5]・近藤 昭彦[*3,*5]
上田 政和[*4,*5]・谷澤 克行[*1]・黒田 俊一[*1]

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要 旨 B型肝炎ウイルス(hepatitis B virus; HBV)はヒト肝細胞に対し強い感染力を有する．その感染機構を担うHBV表面抗原(HBV surface antigen; HBsAg)は，組換え酵母を用いて大量生産が可能であり，酵母由来の膜成分を取り込んで，平均直径220 nmのHBsAg粒子を形成することが知られている．最近，HBsAg粒子内部に遺伝子，タンパク質および薬剤を封入して，静脈注射のみでヒト肝臓特異的送達が可能なキャリヤーとして非常に有効であることを示した．また，HBsAg粒子の表面に存在するヒト肝細胞特異的な認識部位を，他の細胞を認識する分子へ置換することにより，粒子表面の提示分子に応じた標的細胞を生体内で標的化する「中空バイオナノ粒子」の創製に成功した．これらの結果は，生体内ピンポイントデリバリーシステムの新しいキャリヤーとして，特に遺伝子治療分野で中空バイオナノ粒子が非常に有望であることを示している．

1 緒 言

遺伝子治療とは，病気の原因へ遺伝子レベルで働きかけることで症状の改善や病気の治癒を目指す治療法であり，遺伝病，癌やエイズなどの難治性疾患に対する革新的治療法として注目を集めている．1990年に臨床研究が開始されて以来，アメリカを中心に4000人以上の患者が治療を受けている．遺伝子治療では，治療に適するように設計された遺伝子を治療対象の細胞内へ効率よく導入し，その遺伝子発現を治療レベルまで高めて維持させることが必要となる．そのため，今後，遺伝子治療が成功を収めるためには標的細胞へ治療用遺伝子を的確に効率よく送達する遺伝子導入技術が不可欠であり，遺伝子の運び屋である“ベクター”と呼ばれるツールが最も重要な鍵を握っている．

通常，細胞は自身を取り巻く生体膜によって外界から遮断されており，ほとんどの分子を通過させない．したがって，遺伝子のように分子量が100万を超えるような巨大高分子は単独では細胞内へ侵入することは難しい．また，遺伝子は生体内では非常に不安定であり，速やかに分解される．このような遺伝子を細胞内へ送達するために，遺伝子工学の進展に併せて，さまざまなベクターが開発されており，すでにいくつかは国内外で臨床試験が進められている．しかしながら，利用が広まるに従い解決すべき問題が数多く判明してきた．特に，ここ数年間にアメリカ・フランスでの臨床試験中における事故が報告されるなど，ベクターに起因する副作用が発生しており，ベクターの安全性に対して大きな懸念が広がっている[1),2)]．1999年アメリカで行われたアデノウイルスベクターを使った遺伝子治療では，大量にベクターを投与された19歳の男性が投与後にショック死する事故が起きた．これは遺伝子治療における初めての死亡例となった．また，1999年にフランスで開始された重症複合型免疫不全症(severe combined immunodeficiency-X1，以下SCID-X1)に対する遺伝子治療では，レトロウイルスベクターを用いて遺伝子導入を行ったものであり，長期の症状改善がみられていたが，2002年にベクターを投与された二人の患者に白血病が発症した．これは，ベクター由来の遺伝子が患者の染色体ゲノム中の癌抑制遺伝子近傍に挿入されたことが引き金になったと考えられている．

したがって，さらに高い安全性と効率のよい遺伝子送達を実現するベクターの登場が望まれており，医学・薬学・工学の各方面よりベクター開発が進められている．

*1 大阪大学産業科学研究所(〒567-0047 茨木市美穂ケ丘8-1)
*2 岡山大学大学院自然科学研究科(〒700-8530 岡山市津島中3-1-1)
*3 神戸大学工学部応用化学科(〒657-8501 神戸市灘区六甲台町1-1)
*4 慶應義塾大学医学部外科学教室(〒160-8582 東京都新宿区信濃町35)
*5 (株)ビークル(〒701-1221 岡山市芳賀5303)

Table 1. Comparison of various vector systems

Features	Retrovirus	Adenovirus	AAV	Naked/ Lipid-DNA
Transfection efficiency	Good	Good	Poor	Poor
Pathogenicity	Yes	Yes	No	None
Immunological problems	Few	Extensive	Not known	None
Maximum insert DNA size	7-7.5 kb	~30 kb	3.5-4 kb	Unlimited
Cell/tissue specificity	No	No	No	No

2 遺伝子導入ベクターの現状

遺伝子治療で利用される生体内遺伝子導入法には大別して，感染性ウイルスを基としたウイルスベクター法とウイルスを用いない非ウイルス型導入法がある．遺伝子治療の臨床試験には主に Table 1 に示すような遺伝子導入ベクターが利用されている．

2.1 ウイルスベクター法

元来，ウイルスは自分自身の遺伝子を宿主となる細胞へ積極的に導入し，これを細胞内で増幅させることで自らを増殖させる機構をもっている．この機構を外来遺伝子の導入に利用したものがウイルスベクターである．

最もよく利用されているベクターはレトロウイルスベクターで，外来遺伝子を導入細胞の染色体ゲノムへ効率よく組込むことができ，長期間にわたる遺伝子発現が期待できる．しかし，非分裂細胞への遺伝子導入がほとんど起こらないため，生体内(以下 *in vivo*)投与による遺伝子導入は困難である．そのため，標的細胞を一旦身体より取出し，遺伝子導入して一定数まで増殖させた後，体内へ戻す体外(以下 *ex vivo*)投与法に主に利用されている．この問題を解決するために，ヒト免疫不全ウイルス(human immunodeficiency virus type 1，以下 HIV-1)を基としたレンチウイルスベクターの開発が進められている[3]．レンチウイルスベクターはレトロウイルスベクターの特長をもちながら，非分裂細胞への外来遺伝子の導入が可能である．しかし，レトロウイルスベクターおよびレンチウイルスベクターによる外来遺伝子の染色体ゲノムへの組込み部位はランダムであることから，患者の遺伝子情報を撹乱させることで正常遺伝子の機能を低下させたり，癌遺伝子を活性化させたりする危険性が常に存在する．

レトロウイルスベクターと並んでよく利用されるアデノウイルスベクターは，分裂細胞・非分裂細胞の区別なく広範囲のさまざまな細胞に効率よく外来遺伝子を導入することが可能である．しかし，*in vivo* 投与を行うと体内のあらゆる組織へ遺伝子を導入してしまい，不適切な部位での遺伝子発現は予想不能な副作用を生じさせる危険性がある．また，レトロウイルスベクターとは異なり導入された外来遺伝子の発現は一過性である．そのため，長期間の遺伝子発現を実現するには繰返し投与することが必要であるが，ベクター自身に強い細胞毒性や抗原性があり，繰返し投与を行うことは難しい．

近年，これらに代わるウイルスベクターとしてアデノ随伴ウイルス(adeno-associated virus，以下 AAV)ベクターの研究が進められている．AAV ベクターは非分裂細胞にも外来遺伝子を導入でき，ヒト第 19 番染色体の特定の位置への組込みが可能である．また，ヒトへの病原性がないので安全性は高いと考えられている．しかし，標的細胞への遺伝子導入効率が非常に低いこと，ベクターの大量生産の技術が確立していないこと，ベクターの導入できる外来遺伝子の大きさが非常に小さく限定されることなどの欠点がある．

わが国独自のベクターとして，センダイウイルス(Sendai virus，以下 SeV)ベクターの研究が進められている[4]．1997 年に開発された SeV ベクターは，SeV の膜融合能力により広範囲の細胞・組織へ高い効率で外来遺伝子を導入することが可能である．しかしながら，標的細胞への遺伝子導入後に SeV ベクターが増殖複製して，二次伝播が生じることがあり，臨床応用には早期の解決が必要である．また，*in vivo* では赤血球にトラップされるので現時点での臨床応用は困難と考えられる．

2.2 非ウイルス型導入法

非ウイルス型導入法では，治療用遺伝子のみをコードするプラスミドを利用するため，ウイルスベクター法のようにウイルス由来遺伝子が混入することがなく，患者遺伝子に対する影響は遥かに少ないと考えられる．その中でも，最も代表的な導入法はリポソームである．リポソームは古くから薬物送達システム(drug delivery system，以下 DDS)のためのキャリヤーとして利用されてきており，遺伝子のような巨大高分子の送達も可能である．すでに人体への安全性が高く，内部に封入できる遺伝子の大きさに制限がないこと，キャリヤーの作製が容易であることなど，ウイルスベクター法と異なる特長をもつことが知られている．しかし，積極的に細胞内へ感染侵入する機構がないために遺伝子導入効率が極めて低く，また特定の標的細胞への遺伝子導入は困難である．近年，リポソームを構成する脂質を工夫することで，細胞への遺伝子導入効率を向上させようとする試みがなされているが，ウイルスベクターに匹敵する効率は得られていない．

最近，プラスミド DNA を標的組織内へ局所注入する手法が行われている[5]．プラスミド DNA の局所注入は非常に簡便な投与法であり，投与可能な遺伝子の大きさに制限がない．一方，投与した遺伝子は注入した部位近傍にしか導入されずに組織全体へ拡散していかないこと

から，多くの個所へ注入しなければ治療効果が現れにくいという欠点がある．

2.3 ハイブリッドベクター

近年，従来の欠点を克服するために，ウイルスベクターと非ウイルスベクターの特長を併せもつハイブリッドベクターが登場した．代表的なものとしては，SeVとリポソームを組合せた膜融合型リポソーム(fusogenic liposome，以下FL)ベクターがある[6]．具体的には，紫外線で不活化させたSeVと治療用遺伝子をあらかじめ包含させたリポソームを融合して作製する．FLベクターはSeVの膜融合能力を保持したまま，広い範囲の細胞種へ遺伝子以外の物質も導入できることが示されている．また，FLベクターはSeVベクターと異なり，二次伝播が生じない利点がある．しかしながら，その高い膜融合能力のために，SeVベクターと同様に生体内では主に赤血球にトラップされるので，経口あるいは血流を通じた標的組織への遺伝子や薬剤の特異的導入は困難である．

以上のように，現在汎用されている生体内遺伝子導入法には，それぞれに長所と短所があり，まだ決定的なベクターは現れていない．遺伝子治療を真の成功に導くためには遺伝子を効率よく，生体内で標的細胞特異的に導入できる(生体内ピンポイントデリバリー可能な)新たなベクターが必要であり，その登場が待たれている．

3 バイオナノ粒子

私達のグループでも従来ベクターの欠点を克服するための新規遺伝子治療用ベクターの開発を進めてきた．この新規ベクターはB型肝炎ウイルス表面抗原(hepatitis B virus surface antigen，以下HBsAg)粒子を基としており，酵母を用いて生産されるナノサイズの中空粒子であることから“中空バイオナノ粒子”と呼んでいる．この中空バイオナノ粒子は，今までとは大きく異なるベクターであり，想定される短所をほぼ完全に克服しており，遺伝子治療のみならず種々のアプリケーションに応用できると期待される．

3.1 中空バイオナノ粒子の性質

中空バイオナノ粒子の骨格となるHBsAg粒子は，B型肝炎ウイルス(hepatitis B virus，以下HBV)の外殻に相当するもので，外被タンパク質(HBsAg分子)と宿主由来の脂質二重膜から成る中空状粒子である[7),8)]．HBsAg粒子は，HBVのようにウイルス本体の遺伝子や遺伝子を複製するのに必要となる酵素などを含む核となるタンパク質群を一切もたない(Figure 1)．ウイルスには外殻をもつタイプは他にも知られているが，HBsAg粒子のように外被タンパク質が単独で脂質二重膜を含む形で中空状粒子を形成するものは他にない．HBsAg分子はHBVゲノム上にコードされ，翻訳開始位置の違いによって3種類のタンパク質に分かれ，それぞれS(small)，M(middle)，L(large)と呼ばれる．Sタンパク質は226アミノ酸から成り，MタンパクはSタンパク質のN末端側に55アミノ酸から成るpre-S2領域が付加された形になっている．LタンパクはMタンパク質のN末端側に108または119アミノ酸から成るpre-S1領域が付加している．このpre-S1領域は肝細胞と直接結合する部位(約70アミノ酸)を含んでおり，ヒト肝細胞へ特異的に感染する際に中心的な役割を担っている．また，pre-S1領域の作用により，HBVのトロピズムはチンパンジーとヒトの肝臓に厳格に限定されている[9)~12)]．

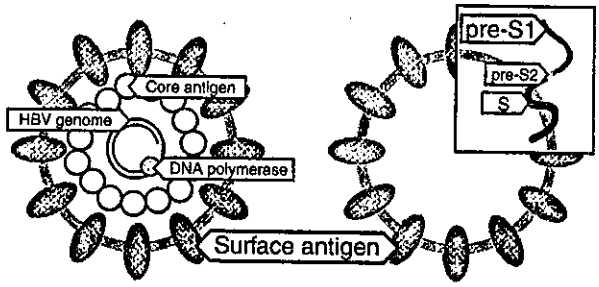

Figure 1. HBV and HBsAg particle. HBV (left) consists of DNA genome, polymerase, core antigens, surface antigens (HBsAg), and lipid bilayer. HBsAg particle (right) consisting of surface antigens and lipid bilayer is a hollow nanoparticle. The HBsAg particle retains the high infectivity to human liver cells without replication activity. The HBsAg L protein contains pre-S1, pre-S2 and S regions (inset). The S region is a transmembrane protein indispensable for the particle formation. The pre-S1 region on the surface is responsible for the specific infection to human liver cells.

HBsAg粒子はHBVの抗原として免疫原性をもち，HBVに対する抗体を誘導することから，HBVに対するワクチン用抗原として有効であることが早期から知られている．B型肝炎感染者は世界的に多いので，1980年代後半から遺伝子組換え技術による各種細胞を用いた大量生産法の開発が精力的に行われてきた．しかし，通常細胞内ではpre-S1領域がLタンパク質の生合成を阻害する作用を示すため，その多くがSタンパク質あるいはMタンパク質から成るS粒子，M粒子であった．1992年，筆者らはpre-S1領域のN末端側にシグナル・ペプチドを付加することにより，L粒子を効率よく生産することに世界で初めて成功した[13)]．このとき発現するLタンパク質は酵母の全可溶性タンパク質の42%にまで達した．現時点において，これは組換え酵母を用いたタンパク質生産の中で最も生産量の高い例である．また，このL粒子はS粒子あるいはM粒子に比べ，HBVに対する抗体の誘導能が優れていることもわかった．L粒子の物理的性質を調べたところ，HBVと同様に

熱に対して非常に安定であった．また，原子間力顕微鏡(Atomic force microscopy，以下AFM)による観察から，その平均直径は220 nmであることが判明した(Figure 2)[14]．

3.2 遺伝子治療用ベクターとしての中空バイオナノ粒子

中空バイオナノ粒子による遺伝子導入を検証するために，モデル遺伝子として，オワンクラゲ由来緑色蛍光タンパク質(green fluorescence protein，以下GFP)の発現遺伝子を用いた．L粒子内部に遺伝子を封入するために種々の手法を検討した結果，電気穿孔法を用いることで簡便に遺伝子を封入できることがわかった．GFP発現遺伝子を封入したL粒子を培養ヒト肝細胞株および数種のヒト肝癌由来細胞株の培養上澄みに血清存在下で添加すると，いずれの細胞においてもGFPに由来する蛍光を観察することができた(Figure 3A)．また，研究用遺伝子導入試薬(リポソームの一種)を用いた場合より遺伝子の使用量が1%未満で十分であった．一方，対照として数種の非肝臓系の細胞株にも同様にGFP発現遺伝子の導入を試みたが，GFPに由来する蛍光は観察されなかった．

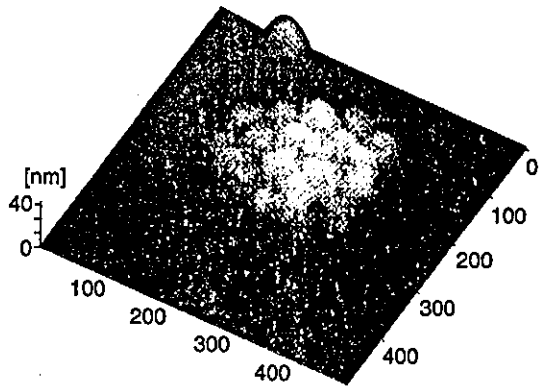

Figure 2. Shape of HBsAg L particles by AFM observation. Five microliters of a solution of 1 μg/ml of HBsAg L particles extensively dialyzed against distilled water were spotted onto mica and observed by AFM. The scan area is 500×500 nm.

中空バイオナノ粒子による生体内遺伝子導入を検討するために，ヒト肝癌由来細胞株とヒト大腸癌由来細胞株を固形癌として同じマウスの背部へ移植した担癌マウスを作製した．同様の手順で粒子内部にGFP発現遺伝子を封入したL粒子を用いて，マウス尾静脈より血流へ投与した．GFP発現遺伝子含有L粒子は，移植したヒト肝癌由来固形癌のみに到達し，その結果，組織内にてGFPの発現を観察することができた．また，対照組織ならびにマウス各臓器においてはGFPに由来する蛍光は観察されなかった．以上のことより，培養細胞・生体内かかわらず中空バイオナノ粒子(L粒子)は内包する遺伝子をヒト肝細胞・組織特異的に送達することが可能であることが示された[15]．

続いて，遺伝子治療で用いられる治療用遺伝子を用いた遺伝子治療モデルの検証実験を行った．ここでは対象疾患として血友病Bを，治療用遺伝子には血友病Bの遺伝子治療で利用されている血液凝固第9因子(blood clotting factor IX，以下FIX)発現遺伝子を用いた．FIX発現遺伝子の封入には，GFP発現遺伝子の場合と同様

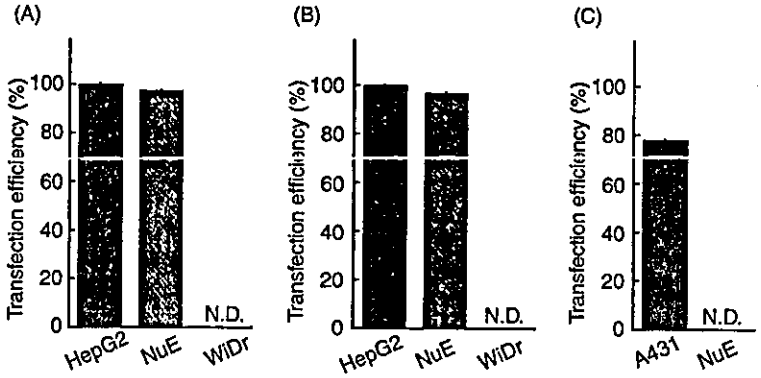

Figure 3. (A) *Ex vivo* gene transfer with L particle. The L particles containing GFP expression plasmid were transfected to two human hepatocellular carcinoma cell lines (HepG2, NuE) and human colon adenocarcinoma cell line (WiDr). Transfection efficiency was calculated with the ratio of GFP-expressing cells to all cells. (B) *Ex vivo* drug delivery with L particles. The L particles containing fluorescent dye, calcein were transfected to HepG2, NuE (both human hepatocellular carcinoma) and WiDr (human colon adenocarcinoma) cells. (C) The EGF-displayed particles containing calcein were transfected to NuE and A431 (squamous cell carcinoma) cells. Transfection efficiency was calculated with the ratio of calcein-containing cells to all cells.

に，電気穿孔法にて行った．FIX 発現遺伝子含有 L 粒子を担癌マウスの尾静脈より投与した後に経時的に採血を行い，ELISA 法にて血中 FIX 量を求めた．その結果，投与後 4 日目にマウス血中に FIX が発現し，3 週間にわたり血友病 B の重症患者に対する治療効果が期待できる発現レベルを維持した[15]．このように 1 回の静脈注射で 1 ヶ月近い治療効果が現れる血友病治療薬剤は他には存在しない．

3.3 DDS キャリヤーとしての中空バイオナノ粒子

中空バイオナノ粒子は，薬剤などの生理活性物質も内部に包含し，標的細胞へ送達させることが期待できる．私達は，モデル化合物として蛍光物質カルセインを用いて薬剤送達の検証を試みた．GFP 発現遺伝子の場合と同様に，電気穿孔法を用いることで簡便にカルセインを L 粒子内部に封入できることが判明した．カルセインを封入した L 粒子をヒト肝癌由来細胞株の培養上澄みに添加すると，カルセインに由来する蛍光を観察することができたが，対照として数種の非肝細胞系の細胞株では観察されなかった(Figure 3B)．

また，ヒト肝癌由来固形癌とヒト大腸癌由来固形癌を移植した担癌マウスの尾静脈よりカルセイン含有 L 粒子を血流へ投与した結果，移植したヒト肝癌由来固形癌の組織内にてカルセインに由来する蛍光を観察することができた．また，対照組織ならびにマウス各臓器においては蛍光を観察することができなかった．以上のことより，低分子化合物の場合でも培養細胞・生体内かかわらず中空バイオナノ粒子はヒト肝細胞・組織特異的に送達することが可能であることが示された[15]．

3.4 中空バイオナノ粒子の再標的化

中空バイオナノ粒子の骨格となっている L 粒子表面には肝細胞結合部位(約 70 アミノ酸)が提示されている．この部位が中空バイオナノ粒子に肝細胞への特異性を付与していることから，任意の生体認識分子に置換することで肝細胞以外の細胞へ標的化することが期待される．

モデル生体認識分子として上皮成長因子(epidermal growth factor，以下 EGF)を選択し，上記肝細胞結合部位とカセット式に置換した EGF 提示型 L 粒子を創製した．EGF 提示型 L 粒子の生産は，L 粒子と同様に組換え酵母を用いた生産系にて行うことが可能である．生産した EGF 提示型 L 粒子を用いてカルセインの薬剤送達を行ったところ，EGF と結合する EGF 受容体を細胞表面に発現している細胞にのみカルセインを導入することができた(Figure 3C)[15]．

以上のことより，中空バイオナノ粒子には遺伝子治療用ベクターならびに DDS に適した以下のような特長をもっている(Figure 4)．

(1) 中空バイオナノ粒子は，その表面に肝細胞結合部位を提示しており，HBV と同様にヒト肝細胞への高い親和性と高い導入効率(感染能力)を有する．

(2) 内部が中空状であるため，遺伝子・薬剤などの生理活性物質を封入することが可能．

(3) 副作用の原因となるウイルス由来の遺伝子を一切含まないため，病原性はない．

(4) 組換え酵母にて大量生産することが可能．

(5) 粒子表面に提示されている肝細胞結合部位を，他の生体認識分子へ置換することで，分子に応じた標的細胞へピンポイントで送達可能(Figure 5)．

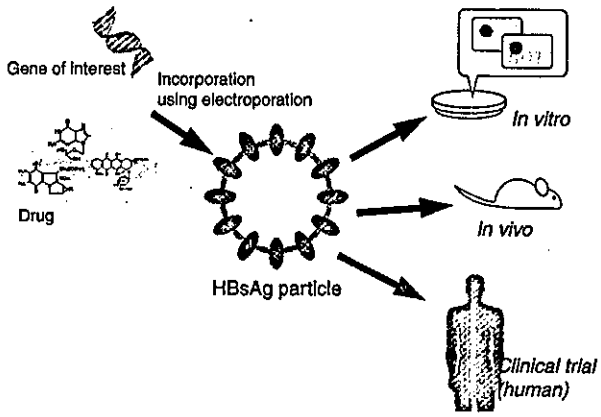

Figure 4. Concept of Hollow bio-nanoparticle technology. HBsAg particles can deliver drugs (chemicals and proteins) and genes to human liver-derived tissues and cells in cultured cells, xenograft models, and humans.

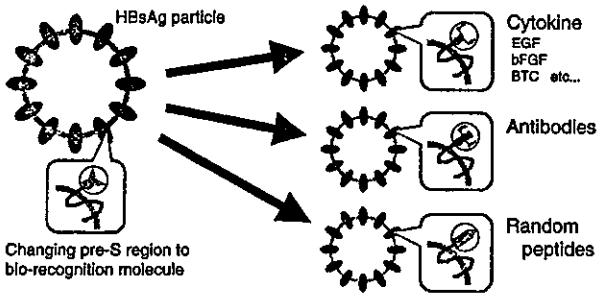

Figure 5. Strategy for changing the cell specificity of HBsAg particles from hepatophilicity to others. The pre-S region displayed on the nanoparticles can be changed by the substitution with cytokines, antibodies, and random peptides.

4 中空バイオナノ粒子の展望

中空バイオナノ粒子は，内部に封入した物質を提示する生体認識分子に応じた細胞に，生体内でピンポイントに送達することができる．それゆえ，遺伝子治療用ベクターだけではなく，DDS のキャリヤーとしても有望である．本粒子を用いたアプリケーションはさまざまなものが考えられるが，ここではそれらの一例を示したい．

4.1 肝疾患に対する治療

バイオナノ粒子は元来，肝臓指向性が高いため，肝臓を標的細胞とする治療に適している．

現在，肝臓癌に特異的に遺伝子を発現させる場合，遺伝子の発現をコントロールするためのプロモーターと呼ばれる配列を利用する．肝細胞が癌化すると多くの場合アルファフェトプロテイン(α-feto protein，以下 AFP)が産生することが知られており，AFP 発現時に作用する AFP プロモーターを用いると癌化した肝細胞にのみ特異的な遺伝子の発現が実現される[16]．しかし，肝細胞癌の中には AFP の産生を伴わないものもあり，そのような種類の癌に適用できなかった．しかし，中空バイオナノ粒子を用いることで AFP の産生の有無にかかわらず，肝細胞癌にて治療用遺伝子の発現を行うことが可能となる．加えて，癌細胞は正常細胞とは異なるタンパク質をその細胞表面に提示することが知られているため，このタンパク質に対する認識分子を提示することで，より確実な標的化を行うことが可能となる．

近年，ウイルス性肝炎に対して，インターフェロン(interferon，以下 IFN)を用いた治療が行われるようになった．しかしながら，IFN を全身投与するとさまざまな副作用が生じることが多く，その効果が十分に発揮されることが少ない．そこで，バイオナノ粒子を用いて肝臓にのみ送達させれば，これらの副作用を大きく低減させることが期待できる．

4.2 抗癌剤による癌治療

癌治療の多くはそれぞれの癌に対して有効な抗癌剤を全身投与することが行われている．抗癌剤は細胞を殺傷する能力に優れているが，癌細胞と正常細胞の区別ができないため，副作用が避けられない．したがって，副作用を低減させるために，患部に限って送達することが肝要である．現在，癌組織の細胞間間隙が大きいことに注目して，大きさに基づく DDS キャリヤーなどが開発されているが，これらは受動的送達であり，患部への積極的な薬剤送達の実現には至っていない．バイオナノ粒子を用いた薬物送達では，提示した生体認識分子に応じた標的細胞に内包させた薬剤を送達することが可能であり，DDS 技術が目的とする「必要な量」の薬剤を「必要な部位」に送達することを達成できるものと期待される．

5 結　　語

遺伝子治療や癌治療などに使用される遺伝子や薬剤は，一般に生体内では不安定で作用部位に対する特異性もない．治療効率の向上のためには，静脈注射にも対応できるピンポイント DDS 技術が不可欠である．しかしながら，現状では目的にかなった DDS 技術はなく，新しい技術の登場が望まれている．中空バイオナノ粒子は現状の DDS 技術が解決すべき問題点を大きく改善しうる可能性を秘めている技術であり，生物医学研究，先進医療を前進させることが可能であると考える．

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[Comprehensive Papers]

Pinpoint Drug Delivery System Using Hollow Bio-Nanoparticles

Tadanori YAMADA,[*1] Masaharu SENO,[*2,*5] Akihiko KONDO,[*3,*5] Masakazu UEDA,[*4,*5] Katsuyuki TANIZAWA,[*1] and Shun'ichi KURODA[*1]

[*1]*Institute of Scientific and Industrial Research, Osaka University (8-1 Mihogaoka, Ibaraki 567-0047, Japan)*

[*2]*Graduate School of Natural Science and Technology, Okayama University (3-1-1 Tsushimanaka, Okayama 700-8530, Japan)*

[*3]*Graduate School of Science and Technology, Kobe University (1-1 Rokkodai, Nada, Kobe 657-8501, Japan)*

[*4]*Keio University, School of Medicine (35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan)*

[*5]*Beacle Incorporation (5303 Haga, Okayama 701-1221, Japan)*

Hepatitis B virus envelope L proteins produced in yeast cells form hollow nanoparticles (L particles, average diameter 220 nm) displaying human liver-specific receptor. Recently, the L particles were found to incorporate genes, proteins, and drugs, and act as an efficient pinpoint delivery system to human liver-derived tissues in xenograft models. By substituting the epidermal growth factor (EGF) for human liver-specific receptor, the mutated L particles showed the affinity to the EGF receptor, not to human liver. Other similar HBV envelope proteins, *e.g.*, M and S particles, have already been commercialized for hepatitis B vaccine, strongly suggesting the safety of L particles in human. These results indicate that the hollow bio-nanoparticles are a promising candidate for the next-generation platform of DDS, especially that related to gene therapy.

KEY WORDS Hepatitis B Virus / Drug Delivery System / Gene Therapy / Yeast / *in vivo* / Nanoparticle / Hemophilia / Retargeting /

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中空バイオナノ粒子が拓く新しい医療技術

黒田俊一[*1]／山田忠範[*2]／妹尾昌治[*3]
近藤昭彦[*4]／上田政和[*5]／谷澤克行[*6]

1．はじめに

ヒトゲノム計画も終了し，Gタンパク質共役7回膜貫通型レセプター(G-protein-coupled Receptor: GPCR)に代表されるような受容体群が明らかにされ，人間の体内における全ての受容体に対する網羅的な解析と，その解析結果に基づく創薬(ゲノム創薬)が進行している．また，近年のライフサイエンスの発展に伴い，循環器病，癌，感染症，神経変性疾患など世界の死亡要因のTOPを占める各種疾患の発症機構が分子レベルで解明されはじめており，新しい創薬ターゲットが続々と増えてきている．日米欧の製薬会社を中心に，これらの新たに表れた創薬ターゲットに対する新薬開発が急速に進んでいるが，依然として薬物は低分子化合物で全身投与が主流であり，副作用の観点から新薬開発における化合物のサバイバルレートは極めて低いのが現状である．一方で，上記のような技術革新のために各製薬会社における新薬開発に必要な資金は急騰しており，製薬会社の経営資源の有効活用を目指して生き残るために世界的に大手製薬会社の統合が加速されている．以上のように，創薬の芽は非常に増えてきているが，開発される医薬品は従来どおりのコンセプトで，なかなか最終製品に到達せず，しかも，研究開発費が高騰しており各製薬会社の経営環境は厳しくなっているというのが現状である．そこで，一つの打開策として，生体内の患部特異的に必要な薬量を投与する標的化技術(Drug Delivery System：DDS)を工夫することで，著効を示すが副作用の点から開発困難であった薬剤を製品化しようとする機運が高まっている．また，このDDS技術を用いれば，これまで副作用が理由で開発が中止されてきた物質を復活させることも可能になるので，開発費の低減にも役立つことができる．

また，全く異なる話であるが，遺伝子治療は治療用遺伝子を患者に投与して，遺伝子の働きにより疾患の治療を期待するものである．このような治療法は従来の医学では考えられないことで，遺伝子治療でしか治療できない疾患も多いので社会の期待は高まっている．近年，遺伝子治療の技術レベルは非常に向上しているが，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従来の遺伝子治療に使用されてきたウイルスベクターは，ウイルス由来ゲノムやウイルス由来タンパク質を治療遺伝子と同時に患者に導入するので予期不能な副作用の危険性がある．実際に，1999年アメリカで行われたアデノウイルスベクターを使った遺伝子治療では，大量にベクターを投与された19歳の男性が投与後にショック死する事故が起きた．これは遺伝子治療における初めての死亡例

*1 Shun'ichi Kuroda 大阪大学産業科学研究所生体触媒科学研究分野 助教授 博士(農学)・株式会社ビークル
*2 Tadanori Yamada 大阪大学産業科学研究所生体触媒科学研究分野 日本学術振興会特別研究員 博士(工学)
*3 Masaharu Seno 岡山大学大学院自然科学研究科 助教授 工学博士・株式会社ビークル
*4 Akihiko Kondo 神戸大学工学部応用化学科 教授 工学博士・株式会社ビークル
*5 Masakazu Ueda 慶應義塾大学医学部外科学教室 講師 医学博士・株式会社ビークル
*6 Katsuyuki Tanizawa 大阪大学産業科学研究所生体触媒科学研究分野 教授 農学博士・株式会社ビークル
Novel Medical Technology Utilizing Hollow Bio-nanoparticles