nature biotechnology

Nanoparticles for the delivery of genes and drugs to human hepatocytes

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Hepatitis B virus envelope L particles form hollow nanoparticles displaying a peptide that is indispensable for liver-specific infection by hepatitis B virus in humans. Here we demonstrate the use of L particles for the efficient and specific transfer of a gene or drug into human hepatocytes both in culture and in a mouse xenograft model. In this model, intravenous injection of L particles carrying the gene for green fluorescent protein (GFP) or a fluorescent dye resulted in observable fluorescence only in human hepatocellular carcinomas but not in other human carcinomas or in mouse tissues. When the gene encoding human clotting factor IX was transferred into the xenograft model using L particles, factor IX was produced at levels relevant to the treatment of hemophilia B. The yeast-derived L particle is free of viral genomes, highly specific to human liver cells and able to accommodate drugs as well as genes. These advantages should facilitate targeted delivery of genes and drugs to the human liver.

Gene therapy is recognized as one of the most promising approaches to curing cancers and genetic diseases[1,2]. In the last decade, viruses including adenoviruses, adeno-associated viruses (AAV), retroviruses and lentiviruses have been adapted as gene transfer vectors by incorporating the gene of interest into the viral genome. Gene transfer techniques based on viral vectors can be applied *ex vivo* and *in vivo* with higher transduction efficiencies than most nonviral transfection methods. However, a major drawback of current viral-vector technology is that the gene is transferred not only to the desired target cells but also to other cells that are susceptible to infection. In particular, the latest-generation lentiviral and adenoviral vectors result in inadvertent transduction of antigen-presenting cells. This diminishes the overall efficacy of the viral vectors and increases the risk of inducing neutralizing antibodies against the transgene product[3,4]. The lack of specificity of viral vectors also increases the risk of inadvertent germline gene transfer after somatic gene therapy, which raises additional safety and ethical concerns[5]. The recent demonstration that AAV vectors can be detected in the semen of patients with hemophilia B who have received AAV–factor IX via hepatic artery catheterization further illustrates the lack of specificity of current technologies[5]. *In situ* gene delivery by administration of vector locally into the target tissue may to some extent reduce gene transfer into distal nontarget tissues but cannot prevent it, because local injection does not preclude vector dissemination via the circulation. Additionally, some viral vectors may trigger a potent inflammatory immune response or may be associated with an increased risk of insertional leukemiagenesis[6–8]. Finally, the mass production of recombinant viruses for clinical use is time consuming and labor intensive, and poses liability considerations for most manufacturers. These concerns prompted us to develop a gene transfer method that does not involve viral genomes and is easily amenable to mass production, while showing high transfection efficiency and high cell and tissue specificity.

Hepatitis B virus (HBV) is a human liver-specific virus whose 3.2-kilobase-pair (kbp) genome harbors three overlapping envelope (*env*) genes in a single open reading frame, encoding small (S), medium (M) and large (L) proteins[9]. Around 1990, recombinant HBV env S, M and L proteins were produced in yeast cells as hollow particles and the S and M particles were used as immunogens in hepatitis B vaccines that were proven safe for humans[10–13]. The L particles, readily purified by ultracentrifugation alone, have an average diameter of 80 nm and consist of about 110 molecules of L protein embedded in a yeast endoplasmic reticulum (ER) membrane–derived phospholipid vesicle, with no HBV genome inside[14]. The pre-S1 peptide, the N-terminal amino acid residues 108–119 of the L protein, displayed on the surface of L particles functions as the specific ligand for receptor binding on human hepatocytes and is crucial for HBV infectivity[15,16].

Here we describe the use of L particles as a safe vehicle for delivering both genes and drugs with high *ex vivo* and *in vivo* transfection efficiencies and high targeting specificity to human hepatocyte–derived cells.

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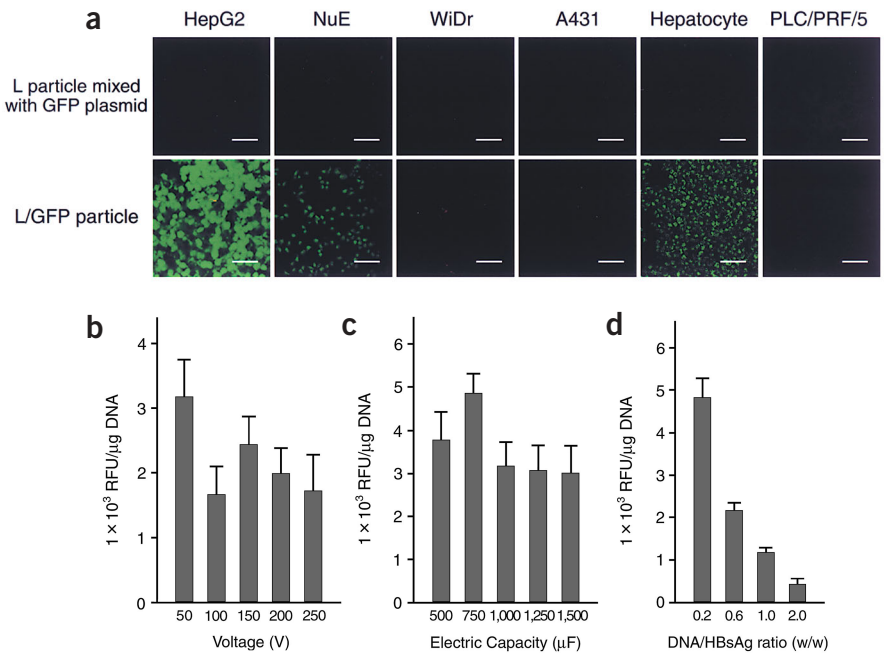

Figure 1 *Ex vivo* gene transfer with L/GFP particles. (**a**) The L/GFP particles containing 10 ng GFP expression plasmid were transfected into about 5×10^4 cells of HepG2, NuE, WiDr, A431, normal human adult-derived hepatocyte and PLC/PRF/5. Fluorescence was observed on day 3 after transfection under a LSM 5 Pascal laser scanning microscope (Carl Zeiss). As a negative control, a mixture of L particles (50 ng) and GFP expression plasmid (10 ng) was used without electroporation and added to HepG2, NuE, WiDr, A431, normal human adult-derived hepatocytes and PLC/RPF/5. Experiments were repeated at least three times. Transfection efficiency was calculated as a percentage of the GFP-expressing cells in 500 randomly selected cells. Scale bar, 100 μm. (**b**,**c**) The L particles were subjected to electroporation and a 0.2-μl aliquot was transferred to the 2-ml culture of HepG2 cells ($n = 10$). On day 3 after transfection, relative fluorescent unit (RFU) of the cells was measured with a microplate reader (Fusion; Packard). (**d**) The electroporation was carried out with various ratios of plasmid to L particle.

RESULTS

Optimization of electroporation

The L particles are efficiently synthesized in yeast cells[13] and readily purified by simple ultracentrifugal procedures[14]. Physicochemical properties of L particles have been described[14]. As the L particle is made of a yeast ER membrane–derived liposome, we incorporate genes into the particles by electroporation. The molecular mass of the L protein (about 52 kDa, as determined by SDS-PAGE) did not change upon electroporation. The average diameter of L particles (about 210 nm as determined by a dynamic light-scattering method) was also unchanged. In addition, both the size and shape of L particles were identical before and after electroporation as assessed by atomic force microscopy (data not shown). An aliquot of the mixture containing 40 ng of L particles and 10 ng of plasmid was subsequently transferred to a 2-ml culture of human hepatocellular carcinoma HepG2 cells (about 5×10^4 cells) in the presence of serum. On day 3 after transfection, nearly 100% of HepG2 cells expressed GFP (**Fig. 1a**). Based on the extent of fluorescence, optimal conditions for voltage and electric capacitance in electroporation were determined to be 50 V and 750 μF, respectively (**Fig. 1b**,**c**). However, neither voltage (50–250 V) nor electric capacitance (500–1,500 μF) substantially affected the transfection efficiency, probably because of the wide variation in the size of L particles (~50–500 nm in diameter)[14]. The weight-based ratio of DNA to L particles was then optimized in a similar fashion. 100 μg of L particles gave the best result for incorporating 20 μg of GFP expression plasmid (**Fig. 1d**). Unless otherwise mentioned, L particles carrying GFP expression plasmids (designated hereafter as 'L/GFP particles') were prepared under the optimal conditions.

Ex vivo gene transfer with L/GFP particles

L/GFP particles containing 10 ng of DNA were used to transfect various human cancer cells (about 5×10^4 cells). A mixture of L particles and GFP expression plasmid with no electroporation was used as a negative control. On day 3 after transfection, GFP fluorescence was observed specifically in human hepatocellular carcinoma HepG2 and NuE cells (**Fig. 1a**), indicating transfection efficiencies of 100 ± 0.01%, mean ± s.d. ($n = 500$) and 97.3 ± 0.03% ($n = 500$), respectively. The L/GFP particles yielded similar transfection efficiencies in other human hepatocellular carcinoma cells (data not shown), whereas human colon adenocarcinoma WiDr cells and human epidermoid carcinoma A431 cells did not express GFP after transfection with the L/GFP particles (**Fig. 1a**). Most commercial transfection reagents based on Lipofectamine (*e.g.*, FuGENE6) showed about 50% transfection efficiency in all cells examined using 1 μg of GFP expression plasmid (data not shown). Hepatocytes from normal human adults were also efficiently transfected with L/GFP particles (59.0 ± 0.02%, $n = 500$; **Fig. 1a**). However, human hepatocellular carcinoma PLC/PRF/5 cells, releasing HBV surface antigen particles containing L protein[15], were not transfected at all with L/GFP particles (**Fig. 1a**); the secreted L protein presumably competed with L/GFP particles. No expression of GFP was observed in the negative controls not subjected to electroporation (**Fig. 1a**). Treatment of the L/GFP particles with DNase I did not affect the transfection efficiency in HepG2 cells (99.5 ± 0.03%, $n = 500$), indicating that most of the GFP expression plasmids resided inside the L particles. These results demonstrate that L particles yield very high transfection efficiencies in human hepatocellular carcinoma cells and normal human hepatocytes, while retaining a high specificity of gene transfer to human liver–derived cells.

In vivo gene delivery with L/GFP particles

The specific infectivity of HBV to human and chimpanzee[11] limits *in vivo* studies of L particles using experimental animals. We therefore adopted a mouse xenograft model involving hepatic NuE and non-hepatic WiDr cell–derived tumors, both grown on the back of the same mouse to similar sizes. The L/GFP particles containing 20 μg of GFP expression plasmid were injected intravenously into the tail vein ($n = 3$). On day 7 after injection, the mice were killed for histological analyses. GFP fluorescence was observed in most cells of NuE cell–derived tumors (93.1 ± 0.35%, $n = 300$; **Fig. 2**), whereas no fluorescence was observed in WiDr cell–derived tumors (**Fig. 2**) or in mouse brain, heart, lung, liver, spleen, kidney, adrenal gland, intestine and skeletal muscle (data not shown). The control mice that received the mixture of L particles and GFP expression plasmid (without electroporation) showed no fluorescence (**Fig. 2**).

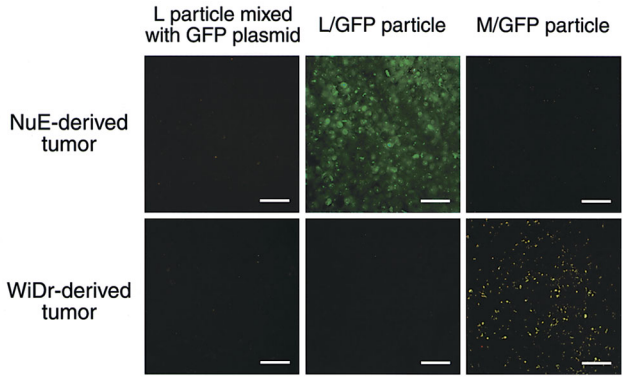

Figure 2 *In vivo* gene delivery with L/GFP particles in the mouse xenograft model. The mice bearing NuE and WiDr cell–derived tumors were injected in the tail vein with L/GFP particles (20 μg). On day 7 after injection, fluorescence was observed in sections from tumors derived from NuE cells and from WiDr cells. Mice were injected with a mixture of L particles (100 μg) and GFP expression plasmid (20 μg) without electroporation, injected with L/GFP particles (20 μg) or injected with M/GFP particles (20 μg). Experiments were repeated with three animals. Transfection efficiency was calculated as the percentage of GFP-expressing cells among 300 randomly selected cells. Scale bar, 50 μm.

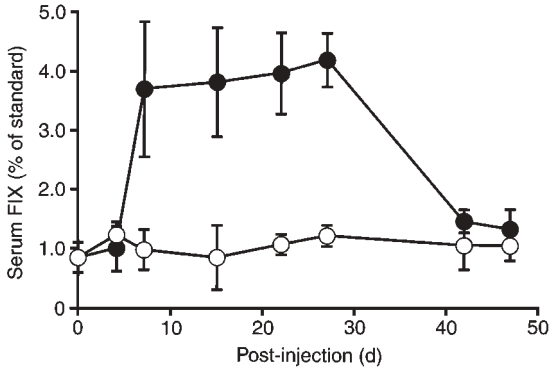

Figure 3 *In vivo* expression of *hFIX*. The mice carrying NuE cell–derived tumors received an intravenous tail vein injection of L/*hFIX* particles (20 μg) ($n = 3$). Plasma was collected by retro-orbital bleeding at different time points after injection, and the plasma hFIX level was measured using the commercial Asserachrom IX:Ag kit (Diagnostica Stago) according to the manufacturer's instructions. The hFIX levels were expressed as a percentage of the hFIX level of control sera (●). As negative controls, three mice were injected with only 20 μg of *hFIX* expression plasmid (○). The hFIX levels in normal human sera are usually 15–20%.

To assess the function of the pre-S1 region, which is displayed on the L-particle surface and contains a human hepatocyte-specific binding site that confers specific hepatocyte infectivity[10,16–18], we prepared nanoparticles that lack the pre-S1 region, called M particles, as an additional control. M particles (100 μg) were electroporated with GFP expression plasmid (20 μg) under the same conditions as described above, and the M/GFP particles were injected intravenously (20 μg/mouse) in the mouse xenograft model. In contrast to L/GFP particles, GFP-derived fluorescence was observed in the WiDr cell–derived tumor rather than in the NuE cell–derived tumor on day 7 after injection (30.9 ± 0.01%, $n = 500$; **Fig. 2**). Combined with the result obtained with PLC/PRF/5 cells (**Fig. 1a**), these data indicate that the pre-S1 region determines the specificity of L particles to human liver–derived cells.

In vivo expression of human clotting factor IX (hFIX)

The human *F9* gene (also known as the *hFIX* gene)[19,20], a therapeutic for hemophilia B and a model for gene therapy, was incorporated into L particles. L/*hFIX* particles containing 20 μg of *hFIX*-expressing plasmid[21] were injected intravenously into mice bearing a tumor derived from NuE or WiDr cells ($n = 3$). Plasma was collected for 7 weeks after the injection, and hFIX levels in the plasma were determined by ELISA. As a control, *hFIX*-expressing plasmid (20 μg) alone was also injected. Only in the plasma of the mouse carrying the NuE cell–derived tumor did hFIX synthesis, initially observed on day 7 after injection, continue for at least 1 month and disappear on day 42 after injection (**Fig. 3**). The plasma hFIX levels obtained would be sufficient to convert severe hemophilia to moderate hemophilia in humans[20]. The eventual disappearance of hFIX was likely a result of the necrosis of the NuE cell–derived tumor. *hFIX* expression was not observed either in the mouse carrying a WiDr tumor or in the negative control, indicating that the L/*hFIX* particles transferred the *hFIX* expression plasmid specifically to the hepatic tumor.

To assess the toxicity of L/*hFIX* particles, we measured the concentrations of liver cytoplasmic enzymes. The serum aspartate aminotransferase (also known as serum glutamic-oxaloacetic transaminase) of mice that received an injection of L/*hFIX* particles was 63 ± 14.2, 44 ± 11.3, 39 ± 15.5 (IU/l) on days 0, 4, and 7 after injection, respectively. Serum alanine aminotransferase (also known as glutamic pyruvate transaminase) was 40 ± 5.5, 39 ± 16.0 and 41 ± 20.5 (IU/l) on days 0, 4 and 7 after injection, respectively. When five Jcl:ICR mice (male, 4 weeks old, body weight about 25 g) received an intravenous injection of 500 μg L/*hFIX* particles (1 ml), they survived for more than 2 weeks, indicating that the median lethal dose (LD_{50}) is > 20 mg/kg. These data suggest that L/*hFIX* particles are relatively nontoxic.

Ex vivo and *in vivo* drug delivery

To test the utility of L particles for delivering proteins, we produced L/calcein particles by mixing and electroporating L particles (50 μg) and 1 mM calcein (fluorescent reporter molecule). The reaction mixture (100 μl) was transferred into the 2-ml culture medium of HepG2, NuE, and WiDr cells (about 5×10^4 cells). Substantial fluorescence was detectable in HepG2 and NuE cells at 24 h after transfection by confocal laser scanning microscopy (100 ± 0.03% and 96.2 ± 0.03%, respectively; **Fig. 4a**), which was quantified with a microplate reader (**Fig. 4b**). A relatively small amount of calcein seemed to be incorporated spontaneously by all cells, possibly because of the hydrophobic nature of the dye. As observed with M/GFP particles, the M/calcein particle showed lower cell specificity (96.7 ± 0.04%, 47.7 ± 0.11% and 22.3 ± 0.06% with HepG2, NuE and WiDr cells, respectively; $n = 500$), suggesting that the pre-S2 region contained in the N terminus of M protein plays a role in cell attachment facilitated by binding of serum albumin to the polyalbumin receptor present in pre-S2 (ref. 22). When the L/calcein particles (100 μg/mouse) were injected into the mouse xenograft model bearing NuE and WiDr cell–derived tumors, fluorescence was observed only in the NuE cell–derived tumor on day 1 after injection (4.9 ± 0.04%, $n = 300$; **Fig. 5**). These data suggest that L particles are effective for the delivery of drugs.

Alteration of the cell specificity of L particles

The N-terminal half of the pre-S1 region contains a specific host cell receptor–binding domain for human hepatocytes[16,17]. We attempted

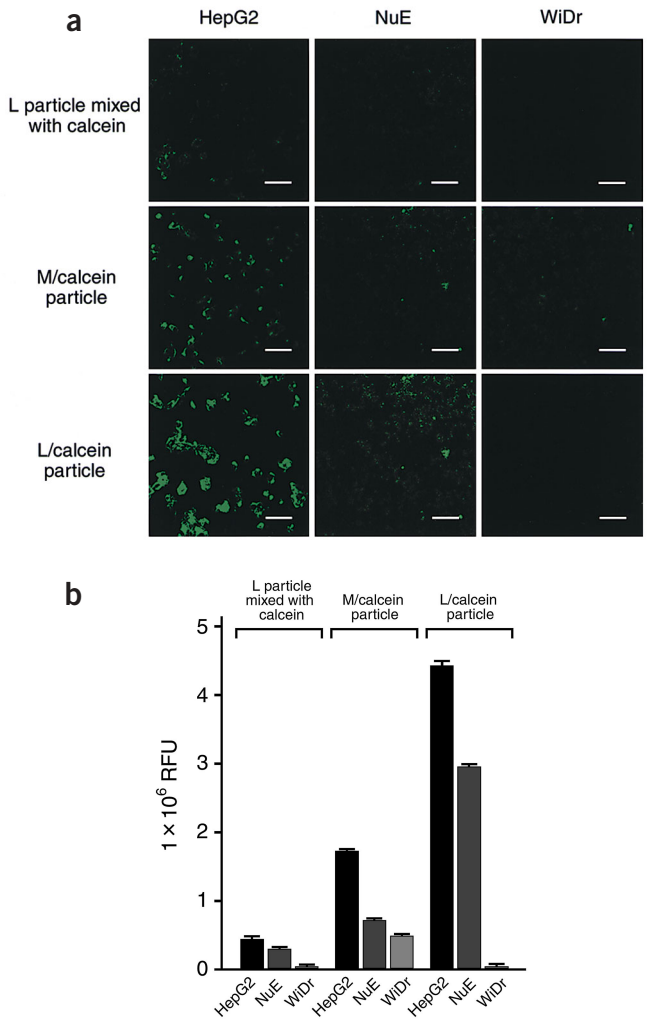

Figure 4 *Ex vivo* drug delivery with L/calcein particles. (**a**) L/calcein particles (10 μg) were added to about 5×10^4 each of HepG2, NuE and WiDr cells. Fluorescence was observed on day 1 after transfection. M/calcein particles (10 μg) were also added to the same cells. As a negative control, the mixture of L particles (10 μg) and 1 mM calcein was directly added to the same cells, without electroporation. Experiments were repeated at least three times. Transfection efficiency was calculated as a percentage of the calcein-containing cells in 500 randomly selected cells. Scale bar, 100 μm. (**b**) RFU of the cells was measured ten times with a microplate reader.

to alter the cell specificity of L particles by genetically substituting a mature form of human epidermal growth factor (EGF) for the pre-S1 region of L particles. Like the original L particles, the engineered L particles were efficiently synthesized in yeast, readily purified and found to display the EGF moiety on the surface of 'EGF particles' (data not shown). As we did with L/calcein particles, we mixed and electroporated EGF particles (50 μg) and 1 mM calcein to produce 'EGF/calcein particles.' The reaction mixture (100 μl) was transferred into the 2-ml culture medium of A431 and NuE cells (about 5×10^4 cells). A high level of fluorescence was observed in A431 cells ($78.4 \pm 0.07\%$, $n = 500$) at 24 h after transfection (**Fig. 6a**), whereas no fluorescence was observed in NuE cells (see also **Fig. 6b** for quantification). Because A431 cells express a large number of EGF receptors on the cell surface, it is likely that the EGF moiety functions to target these cells. This result demonstrates that the pre-S1 region plays a critical role in the cell recognition of L particles and that foreign biorecognition molecules can be displayed on the L particles to endow them with a new cell specificity.

DISCUSSION

We have established an *ex vivo* and *in vivo* system for gene and drug delivery using L nanoparticles. The L particles are easily produced in yeast, are devoid of potentially hazardous virions and are highly specific to human hepatic cells. These features would be advantageous in overcoming some of the limitations and risks of current viral technologies, avoiding, for example, inadvertent gene transfer into undesired cell types[3,4] or into germline cells after somatic gene therapy[5].

Another advantage of this system is that there is no practical limitation on the size of the transgene that can be incorporated into L particles[14]. Our preliminary data show successful incorporation of a ~40-kbp plasmid, far exceeding the packaging capacity of commonly used viral vectors[4,23]. Although the maximum DNA size that can be incorporated into L particles has not been established, L particles could potentially be used for delivery of episomal or integrating vectors.

Both L/GFP and L/calcein particles specifically delivered the GFP gene and calcein to the target human hepatocellular carcinoma tumors *in vivo* (**Figs. 2** and **5**). Incorporation into L particles of therapeutic genes expressed under the control of liver-specific promoters (*e.g.*, those of the genes encoding albumin and α-fetoprotein)[24] or drugs may be effective for treating not only primary but also metastatic liver cancer in humans.

The *ex vivo* transfection efficiency of L/GFP particles to HepG2 cells is about 100-fold higher than that of the common transfection reagent FuGENE6. On the other hand, an estimated multiplicity of infection of L/GFP particles for HepG2 cells was still relatively high (~10^4–10^5). To achieve high gene transfer efficiencies comparable to those of native HBV, alternative methods to incorporate genes and drugs into particles that are better than electroporation would need to be developed, substantially decreasing the required *in vivo* dose of L particles.

Yeast-derived HBV *env* S and M particles have been used clinically as immunogens in hepatitis B vaccines and have an established safety record[12]. L particles, which are structurally very similar to S and M particles, are probably also safe for humans. However, for long-term and repeated administration of L particles, it will be necessary to reduce their immunogenicity by deleting the immunogenic portion within the pre-S1 and pre-S2 regions. L particles may also be modified to be nonresponding to the human immune system by introducing amino acid substitutions found in HBV escape mutants that have been found in recipients of the hepatitis B vaccine[25,26]. Because of widespread hepatitis B vaccination programs, most people in the world have antibodies to HBV[27]. These programs have been conducted mostly with recombinant S particles as an immunogen for elicitation of an anti-S antibody, which recognizes the 'a' determinant located at amino acid residues 117–128 of S protein. The presence of unmodified S proteins in L particles may obstruct their use in individuals who have received the hepatitis B vaccine. However, HBV escape mutants carrying mutations in the 'a' determinant of the S protein propagate in vaccine recipients[25,26]. Thus, mutant L particles carrying amino acid substitutions found in HBV escape mutants are expected to facilitate the hepatocyte-specific delivery of genes and drugs in hepatitis B vaccinees.

Another advantage of L particles is that their cell and tissue specificity can be easily altered by replacing the hepatocyte-specific pre-S1 region with other targeting moieties or biorecognition molecules (*e.g.*, ligands, receptors and antibodies) without modifying the assem-

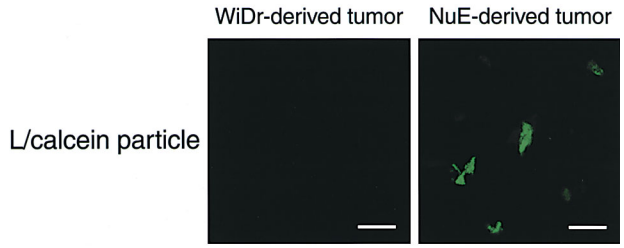

Figure 5 *In vivo* drug delivery with L/calcein particles in the mouse xenograft model. The mice carrying NuE and WiDr cell–derived tumors received an intravenous injection of L/calcein particles (100 μg) in the tail vein. On day 1 after injection, fluorescence was analyzed of WiDr cell–derived and NuE cell–derived tumor sections. Experiments were repeated with at least three animals. Transfection efficiency was calculated as a percentage of the calcein-containing cells in 300 randomly selected cells. Scale bar, 50 μm.

bly of L particles. Such engineered L particles may well be produced in yeast cells, as has been shown for the generation of a multivalent vaccine carrying foreign epitopes. For example, human immunodeficiency virus type 1 *env* protein displayed on the surface of HBV M particles was successfully expressed in yeast[28]. Here we used EGF particles to alter the specificity of L particles to EGF receptor–expressing cells (**Fig. 6**). Recently, short peptide segments produced by combinatorial biochemical methods were shown to possess high affinity for certain tissues *in vivo*[29]. These peptides could also be displayed on L particles to alter their tissue specificity. The use of liposomes coupled with biorecognition molecules for a tissue- and cell-specific DDS has already been reported (*e.g.*, antibody-coupled liposomes (immunoliposomes) and cytokine-coupled liposomes)[30]. However, there are some limitations on the availability and stability of such modified liposomes. Because the phospholipid content of yeast-derived particles (about 10%, wt/wt)[17] and the lipid fluidity within the particles are both low[31], the L particle membrane appears to be organized as a discontinuous, rigid bilayer of lipids interacting with L protein aggregates. Consequently, L particles are resistant to treatment with heat (80 °C, 5 min) and with the surfactant sodium dodecyl sulfate[14]. In view of the production criteria and particle stability, L particles may be more promising than conventional liposomes for drug delivery.

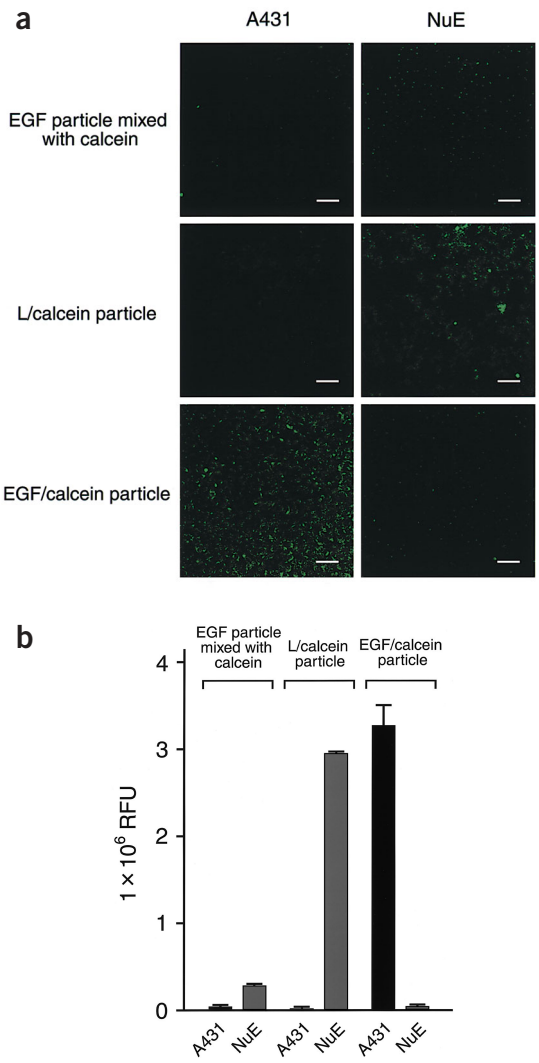

Figure 6 *Ex vivo* drug delivery with EGF/calcein particles. (**a**) EGF/calcein particles (10 μg) were added to about 5×10^4 cells of A431 and NuE cells. Fluorescence was observed on day 1 after transfection. L/calcein particles (10 μg) were also added to the same cells. As a negative control, the mixture of EGF particles (10 μg) and 1 mM calcein was directly added to the same cells, without electroporation. Experiments were repeated at least three times. Transfection efficiency was calculated as a percentage of the calcein-containing cells in 500 randomly selected cells. Scale bar, 100 μm. (**b**) RFU of the cells was measured ten times with a microplate reader.

METHODS

Experimental ethics policy. All animal experiments were approved by the committee for experimental animal science of Osaka University. Animals were treated according to the guidelines of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Cell cultures. HepG2, A431, WiDr and PLC/PRF/5 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) FBS. Human hepatocellular carcinoma NuE cells[32] were obtained from T. Tadakuma (National Defense Medical College) and maintained in RPMI-1640 medium with 10% (vol/vol) FBS. Normal human hepatocytes (originating from a 54-year-old Caucasian female) were purchased from Cambrex Bio Science and maintained in HCM medium (Cambrex Bio Science).

Preparation and electroporation of L and M particles. L particles were overexpressed in *Saccharomyces cerevisiae* AH22R$^-$ carrying L protein expression plasmid pGLDLIIP39-RcT[13] and purified by precipitation with polyethylene glycol 6000, CsCl isopycnic ultracentrifugation and sucrose density gradient ultracentrifugation[14]. About 4 mg of the highly purified L particles were finally obtained from the yeast cells (wet weight about 20 g), the purity of which was judged by SDS-PAGE followed by silver staining. Physicochemical and immunological properties of L particles were reported previously[14]. The size distribution of L particles was measured by dynamic light scattering using a DLS-7000 submicron particle analyzer (Otsuka Electronics). Mammalian GFP expression plasmid (pTB701-GFP)[33] (20 μg), the plasmid expressing hFIX under a hepatocyte-specific albumin promoter (pRRLsinPPTAlbFIXpre)[21] (20 μg) (kindly provided by L. Naldini, University of Torino Medical School), or 1 mM calcein (3,3′-bis[*N*,*N*-bis(carboxymethyl)-aminomethyl]fluorescein; Dojindo) was mixed with purified L particles (100 μg of protein) in 500 μl of PBS, and electroporated into particles with a Gene Pulser II electroporation system (Bio-Rad) in a 4-mm gap cuvette, typically at 220 V and 950 μF for about 20 ms. M particles were also prepared from the yeast recombinant cells as described previously[10,18].

Mouse xenograft model. BALB/c nude mice (*nu*/*nu*, 5 weeks old, male) were purchased from CLEA. About 1×10^7 carcinoma cells (NuE and WiDr) were subcutaneously injected into the backs of the mice. After 5–6 weeks, mice bearing tumors that were large enough (diameter about 1 cm) were injected with

100 μl of L particles containing an expression plasmid or calcein from the tail vein ($n = 3$).

Histological analyses. The mice were anesthetized with diethyl ether, killed, and tumors, brains, hearts, lungs, livers, spleens, kidneys, adrenal glands, intestines and skeletal muscles were isolated. These tissues were fixed in 4% (wt/vol) paraformaldehyde and embedded in the synthetic resin with Technovit 8100 (Kluzer). The blocks were sectioned into a width of 5 μm and then observed under a laser scanning confocal microscope.

Preparation of EGF particles. A nucleotide sequence in the L protein expression plasmid pGLDLIIP39-RcT[13], corresponding to amino acid residues 3–77 in the HBV pre-S1 region (subtype ayw) that were shown to interact directly with human hepatocytes[17], was replaced by the synthetic gene encoding human mature EGF (53 amino acids, GenBank accession number P01133) to construct a plasmid pGLDLIIP39-RcT-EGF. The engineered L particles efficiently produced in *S. cerevisiae* AH22R$^-$ cells carrying the plasmid were purified by the same ultracentrifugal procedures as the original L particles[14]. The final yield of the highly purified EGF particles was about 500 μg from 20 g (wet weight) of the yeast cells.

ACKNOWLEDGMENTS

This study was supported by the Grants-in-Aid from the Japan Science and Technology Corporation (Research Fund for Patenting), the Ministry of Education, Culture, Sports, Science and Technology, Japan (nos. 13558110, 13218080, 15025240; the 21st century Center of Excellence program), TERUMO Life Science Foundation, Kowa Life Science Foundation, Naito Memorial Foundation (to S.K.), the Fund for Scientific Research (FWO, Belgium) and a Vlaams Interuniversitair Instituut voor Biotechnologie grant from the Flemish Government (to M.K.L.C. and T.V.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 1 February; accepted 19 May 2003
Published online 29 June 2003; doi:10.1038/nbt843

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