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Specific gene transfer mediated by galactosylated poly-L-lysine into hepatoma cells

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Abstract

Plasmid DNA/galactosylated poly-L-lysine(GalPLL) complex was used to transfer luciferase reporter gene in vitro into human hepatoma cells by a receptor-mediated endocytosis process. DNA was combined with galPLL via charge interaction (DNA:GalPLL:fusogenic peptide, 1:0.4:5, w/w/w) and the resulting complex was characterized by dynamic light scattering, gel retardation assay and zeta potential analyzer to determine the particle size, electrostatic charge interaction, and apparent surface charge. The complex was tested for the efficiency of gene transfer in cultured human hepatoblastoma cell line Hep G2 and fibroblast cells NIH/3T3 in vitro. The mean diameter of the complex (DNA:GalPLL = 1:0.4, w/w) was 256 ± 34.8 nm, and at this ratio, it was positively charged (zeta potential of this complex was 10.1 mV). Hep G2 cells, which express a galactose specific membrane lectin, were efficiently and selectively transfected with the RSV Luc/GalPLL complex in a sugar-dependent manner. NIH/3T3 cells, which do not express the galactose-specific membrane lectin, showed only a marginal level of gene expression. The transfection efficiency of GalPLL-conjugated DNA complex into Hep G2 cells was greatly enhanced in the presence of fusogenic peptide that can disrupt endosomes, where the GalPLL-DNA complex is entrapped with the fusogenic peptide. With the fusogenic peptide KALA, the luciferase activity in Hep G2 cells was ten-fold higher than that of cells transfected in the absence of the fusogenic peptide. Our gene transfer formulation may find potential application for the gene therapy of liver diseases.

Keywords: Receptor-mediated gene delivery; Galactosylated poly-L-lysine; Molecular conjugate; Hepatoma cells

1. Introduction

Polyion complexes of a nucleotide with polycations are considered as a promising system for a gene transfer vector (Wu and Wu, 1987; Katayose and Kataoka, 1997). The net ionic charge (i.e. the ratio of cationic vector over anionic phosphate) governs the fate of the resulting particles (Schwartz et al., 1995) and the transfection is most efficient only when particles are cationic and thus can be taken up by adherent cells after binding to anionic cell surface proteoglycans (La-

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bat-Moleur et al., 1996). DNA has a polyanionic character and can be bound to polycations, e.g. poly-L-lysine (PLL), through electrostatic interaction. It is well known that PLL strongly binds to DNA to induce compaction of the DNA molecule (Hynes et al., 1970). However, a soluble and electrically neutral (stoichiometric) complex consisting of PLL and DNA is hardly obtained because charge neutralization usually induces the formation of insoluble precipitates. Efforts in downsizing the particles which were undertaken by complex formation in the presence of increasing concentration of NaCl (Perales et al., 1994) resulted in DNA/ligand-PLL complex of small, defined size.

The asialoglycoprotein receptor (galactose receptor) is known to be present only on hepatocytes (Ashwell and Harford, 1982) at a high density of 500 000 receptors per cell (Schwartz et al., 1980), and retained on several human hepatoma cell lines (Knowles et al., 1980; Fallon and Schwartz, 1988). Once a ligand binds to the galactose receptor, the ligand-receptor complex is rapidly internalized and the receptor recycles back to the surface (Ciechanover et al., 1983), allowing high binding capacity and efficient uptake of galactosylated ligands by liver cells.

Many studies have exploited asialoglycoprotein receptor-mediated gene transfer to cells of hepatic origin by natural or synthetic glycosylated peptide complexes in vitro or in vivo (Merwin et al., 1994; Perales et al., 1994). The structure of the DNA/ligand complex, the sugar substitution level of glycosylated peptide, plasmid size and solubility of complexes were of key importance for the successful introduction of genes into the tissues of animals by receptor-mediated endocytosis (Erbacher et al., 1995; Wadhwa et al., 1995). And also gene transfer was strongly promoted when amphipathic peptides derived from the N-terminal sequence of influenza virus hemagglutinin HA-2 (Plank et al., 1992; Midoux et al., 1993) or lysosomotropic agents such as chloroquine (Wadhwa et al., 1995) were present in these DNA complexes.

To achieve the selective delivery of plasmid DNA to the liver parenchymal cells, we prepared galactosylated poly-L-lysine (GalPLL) as a macromolecular DNA carrier. The GalPLL is bifunctional in that it possesses the cationic charge necessary for electrostatic binding with plasmid DNA and galactose residues as the targetable ligand to the hepatoma cells. The use of GalPLL instead of natural glycoprotein like asialoorosomucoid offers several advantages: it is a synthetic compound that can be rapidly prepared, easily purified and highly soluble at physiological ionic strength and pH.

In our previous studies on targeted gene delivery to hepatocytes with galactosylated albumin-PLL-DNA complex, cell binding by the complex was due to the galactosylated component (Han et al., 1999) and this complex required the fusogenic peptide for the DNA to be efficiently delivered into the nucleus. In this paper, we used GalPLL as a DNA delivery vector and KALA as a fusogenic peptide in order to achieve an effective liver targeting and transfection of plasmid DNA via galactose receptors. We observed that the GalPLL-DNA-KALA complex could mediate a high level expression of the reporter gene in the hepatoma cells.

2. Materials and methods

2.1. Materials

Lactose, sodium cyanoborohydride, PLL (average MW 19 200), asialofetuin (AF), and D-luciferin were the products of Sigma (St. Louis, MO, USA). Phosphate buffered saline (PBS), DMEM-h, FBS, and trypsin EDTA were obtained from GIBCO-BRL (Grand Island, NY).

2.2. Cells and cell culture

The human hepatoma cell line Hep G2 and the fibroblast cell line NIH/3T3 were maintained in plastic dishes containing DMEM-h plus 10% heat inactivated fetal bovine serum plus 2 mM L-glutamine plus antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C, 5% CO_2.

2.3. Reductive coupling of lactose to PLL.

Galactosylated PLL was synthesized by slight modification of coupling lactose to the lysine residues of BSA (Kim et al., 1992). In brief, a mixture of 1 μM PLL, 2.7 mM lactose and 15.9 mM sodium cyanoborohydride in 0.1 M sodium borate buffer (pH 8.5) was shielded from light by aluminum foil and incubated at 37°C for 96 h. The reaction mixture was dialyzed against double distilled water, concentrated by ultrafiltration (Ultrafiltration kit, Diaflo, Amicon Co., Beverly, MA, USA) and lyophilized. After resuspending in double-distilled water, the lactose content in the galactosylated PLL was measured by the phenol/sulfuric acid method (Dubois et al., 1956) and PLL content was assayed using UV spectrophotometer at 210 nm. The average number of galactose residues bound per PLL molecule was found to be 20.

2.4. Formation and physical characterization of plasmid DNA/galPLL complex

To determine the optimal galPLL/DNA ratio, we prepared the galPLL-condensed DNA at increasing weight ratios (0.05–0.4) by adding galPLL dropwise to DNA solution. The reaction mixture was incubated for 30 min at room temperature and aliquots were analyzed by means of gel electrophoresis in 0.8% agarose gel in Tris-acetate-EDTA buffer. The DNA was visualized with ethidium bromide. In a typical transfection experiment, the complex was prepared as follows; 36 μg of plasmid RSV luciferase contained in 216 μl of 400 mM NaCl was vortexed at medium speed in a Thermolyne vortex mixer (Thermolyne Corporation, Dubuque, IA, USA) apparatus. 14.4 μg of galPLL dissolved in 216 μl of 400 mM NaCl was added dropwise to the vortexing solution of DNA over a period of 1–1.5 h in 15 μl aliquots every 5 min. This generates a molar ratio of 1 DNA PO_4^- group to 1 lysine NH_4^+ group in the final complex. The slow addition of the polycation resulted in the formation of a turbid solution that was dissolved by the slow, stepwise addition of 3 μl aliquots of 5M NaCl. The disappearance of the turbidity was monitored by eye and the size distribution of this DNA/galPLL complex examined using laser particle size analyzer. Zeta potential was obtained by direct measurement of the rate of migration of DNA/galPLL complex (10 μg/ml as of DNA) using Zeta plus® microelectrophoresis apparatus (Brookhaven Instruments Corporation, NY, USA) and this apparatus was standardized using particles of known zeta potential.

2.5. Plasmid preparation

The plasmid RSV Luciferase was used to study the expression of luciferase. Plasmid was expanded in *Escherichia coli* by growing the bacteria to confluency, extracted by means of alkaline hydrolysis (Sambrook et al., 1989) and purified by the standard cesium chloride gradient centrifugation. Purity was confirmed by 0.7% agarose gel electrophoresis, demonstrating the absence of bacterial chromosomal DNA.

2.6. Peptide synthesis and purification

The fusogenic peptide KALA (WEAKLAKALAKALAKHLAKALAKALKACEA) (Wyman et al., 1997), GALA (WEAALAEALAEALAEHLAEALA EALAELAAGGSC) (Parente et al., 1990), INF7K1 (GLFEAIEGFIENGWEGMIDGWYGVA$(K)_{15}$), and INF6 (GLFGAI AGFIE NGWEGMIDGWYG) (Plank et al., 1994) were synthesized using a peptide synthesizer. The peptide was cleaved from the resin with trifluoroacetic acid (TFA) in the presence of phenol, ethanedithiol (EDT), and thioanisole and separated from the resin by filtration followed by precipitation with ether. The resulting white solid was dissolved in 20 mg/ml water and purified by reverse-phase HPLC on the C_{18} column using the following conditions: solvent A, 0.05% TFA in water; solvent B, 0.05% TFA in acetonitrile: water (80:20); gradient 0–20% B in 4 min and 20–60% B in 20 min and gradient 60–100% in 22 min and 100 to 0% in 30 min at a flow rate 1.5 ml/min; the eluant monitored at 220 nm.

2.7. Transfection of Hep G2 cells with DNA/galPLL-fusogenic peptide complex

DNA transfection was performed when the cells reached approximately 50% confluence. Cells were seeded in 24-well plates at 5×10^4 cells/well and incubated for 24h prior to transfection. DNA-galPLL-fusogenic peptide complex was prepared by rapidly mixing the DNA/galPLL complex (20 μg/ml as of DNA) and peptide solution (500 μg/ml) on a vortex mixer. This complex solution was diluted to a DNA concentration of 10 μg/ml in double-distilled water and then added to 1 μg (in 100 μl) per well in 300 μl of culture medium in triplicates. To determine the optimal DNA/peptide ratio, we prepared the complex at increasing weight ratio (DNA: peptide = 1:1 to 6) and checked the transfection efficiency. After the complex was added to the cells and after 16 h, the culture medium was replaced with 1 ml of fresh DMEM-h medium and the cells were incubated for another 24 h at 37°C. Finally, the cells were washed twice with PBS (Ca-, Mg-free), harvested after the addition of 0.5-ml trypsin EDTA and lysed by means of three freeze-thaw cycles.

2.8. Carrier dependent gene transfer

Hep G2 cells and NIH/3T3 cells (5×10^4 cells/well) were plated on day 0 into 24-well tissue culture plates. On day 1, after removing the medium, the solution (0.4 ml) containing the plasmid/glycoconjugate complex made with either 100 μM in chloroquine or in 5 μg of KALA, was added into the well. After 16 h incubation, the medium was removed and the cells were further incubated for 24 h at 37°C. Lysates from cells transfected with DNA prepared from the calcium phosphate precipitation method (Reston et al., 1991) were used as the positive control and those with DNA alone were used as negative controls.

2.9. Effect of galactose receptor on the transfection activity of DNA/galPLL/PLL-KALA complex

Hep G2 cells and NIH/3T3 cells were seeded in six-well plates at 3×10^5 cells/well and incubated for 24 h prior to transfection. DNA/galPLL/PLL-KALA complex was prepared by rapidly mixing the DNA/galPLL/PLL complex (1 mg/ml as of DNA) and KALA solution (0.5 mg/ml) on a vortex mixer. This complex solution was diluted to a DNA concentration of 30 μg/ml in double-distilled water and then added with 3 μg (in 100 μl) per well in 700 μl of culture medium in triplicates. To evaluate the effect of galactose receptor, we prepared the DNA/galPLL/PLL complex at increasing amounts of galPLL (0–0.4) and checked the transfection efficiency. After the complex was added to the cells and after 16 h, the culture medium was replaced with 3 ml of fresh DMEM-h medium and the cells were incubated for another 24 h at 37°C. Finally, the cells were washed twice with PBS (Ca-, Mg-free), harvested after the addition of 0.5-ml trypsin EDTA and lysed by means of three freeze-thaw cycles.

2.10. Luciferase assay

Luciferase gene expression was measured by luminescence according to the slightly modified method (De Wet et al., 1987). The culture medium was discarded and cells were washed twice with PBS and harvested with 0.4 ml of Trypsin solution (0.05% Trypsin, 0.53 mM EDTA 4Na in HBSS). After centrifugation at 4°C, 4000 rpm for 4 min, the supernatants were aspirated and the homogenation buffer (50 μl; 250 mM Tris–HCl (pH 8.0)/1 mM dithiothreitol) was added into the pellet. The suspension was shaked with a vortex mixer and freeze-thawed three times and was spun down (4°C, 15 min, 13 000 rpm). 350 μl of ATP solution (250 mM Glycylglycine (pH 7.8) 9 ml, 0.02 M ATP 100 μl, 1 M $MgSO_4$ 100 μl, deionized water 800 μl) was added to 20 μl of supernatant and the luminescence was recorded for 30 s by using a luminometer (Lumat LB9501, Berthold, Wildbach, Germany) upon automatic addition of 100 μl of a luciferin solution (25 mM Glycylglycine (pH 7.8) 4 ml, 167 μM D-Luciferin in water 1 ml).

3. Results and discussion

3.1. Uptake of radiolabeled DNA-neoglycoprotein complex by Hep G2 cells and NIH/3T3 cells

In order to demonstrate that the gene targeting by galPLL is sugar specific, we looked for the presence of various membrane lectins on the cell lines used (Hep G2 and NIH/3T3 cells) in these experiments. Indeed, while liver parenchymal cells express a galactose specific lectin on their surface (Ashwell and Harford, 1982), many hepatoma cells do not. Furthermore, upon passaging, cell lines may stop expressing a given protein. Schwartz et al. (1981) showed that one hepatoma cell line, called Hep G2 expresses a galactose-specific lectin. The presence of membrane lectins on Hep G2 and NIH/3T3 cells was checked by uptake experiment with [^{14}C-Thymidine]-labeled bovine serum albumin substituted with galactose (so called neoglycoproteins; Han et al., 1999). Serum albumin bearing galactosyl residues were recognized and internalized by Hep G2 cells but not by NIH/3T3 cells. The internalization of the galactosylated serum albumin was ascertained by showing that the cellular uptake decreased in the presence of asialofetuin which competes with the galactosylated serum albumin. On these bases, sugar-selective targeting is expected to be efficient with galactosylated polymer (e.g. galPLL) in the case of Hep G2 cells.

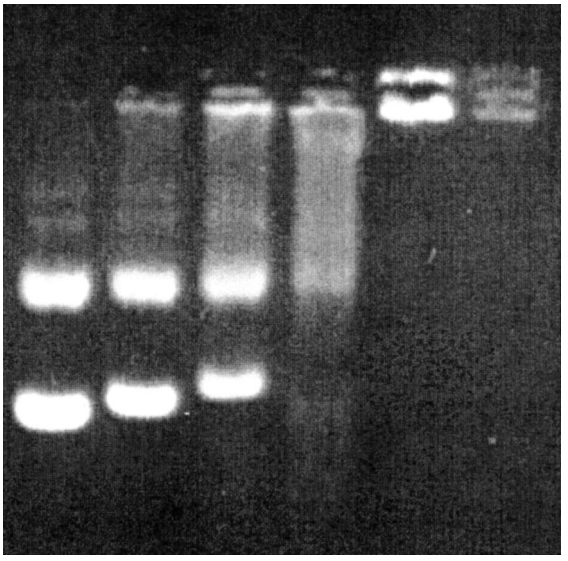

Fig. 1. Gel retardation assay of DNA complexed with galPLL

Lane #	1	2	3	4	5	6
DNA (μg)	1	1	1	1	1	1
GalPLL (μg)	0.05	0.1	0.2	0.3	0.4	0.5
GalPLL/DNA	0.05	0.1	0.2	0.3	0.4	0.5

One microgram of plasmid DNA was complexed with the indicated amount of PLL. DNA was run on a 0.8% agarose gel in Tris-acetate-EDTA buffer.

3.2. Formation and physical characterization of GPD complex

Twenty percent of the 99 lysine residues of the PLL used, was substituted with lactose residues as the recognition signals by using lactose and sodium cyanoborohydride; the remaining cationic charges of lysine residues serve as counterions to form stable complex with plasmid DNA. Condensed DNA was prepared by rapidly mixing DNA and galPLL in deionized water. Increasing the proportion of galPLL to DNA in the complex affected plasmid DNA migration in an agarose gel as can be seen in Fig. 1. As the proportion of galPLL increased, there was a decrease in the staining intensity of DNA that entered the gel (lanes 1–6) and a complete retardation was achieved at the galPLL to DNA ratios above 0.4:1. Taking into account that the galactosylated PLL possess 79 remaining cationic charges and that pRSVLuc is a plasmid of 4.1 kb, the electroneutrality of a complex between galPLL and pRSVLuc is obtained when a pRSVLuc plasmid molecule is covered by 5 galPLL molecules (galPLL: DNA = 0.63:1, w/w). Formation of an 'isoelectric' complex at this ratio is probably due to the structural organization of the condensed DNA/galPLL complex in which some phosphate groups may not be accessible to galPLL. The zeta potential of galPLL-DNA (DNA: galPLL = 1:0.4, w/w) complex was 10.1 mV and the mean particle size of this complex was 256 ± 34.8 nm. It appears that this galPLL-DNA complex is electrically neg-

ative and could interact with positively charged fusogenic peptide to form a stable net electropositive complex so that it could be easily taken up by hepatocytes (Kim et al., 1994).

3.3. Effect of the fusogenic peptide on the transfection efficiency of Hep G2 cells by RSVLuc/galPLL complex

Asialoglycoproteins internalized by their receptor have been shown to be ultimately delivered to lysosome via membrane-limited vesicles with subsequent glycoprotein degradation (Wall et al., 1980). Because recognition of our carrier system is directed by a galactosylated poly-L-lysine, our complex is expected to follow a similar fate. Chang and Kullberg (1982) showed that a conjugate consisting of an asialoglycoprotein bound to diphtheria toxin A-chain could be internalized by the galactose receptor and exert lethal effects to target cells, indicating that escape from the normal degradative pathway is possible. More recently, Midoux et al. (1993) showed that fusogenic peptide such as influenza virus hemagglutinin HA2 N-terminal polypeptide is ten times more efficient than chloroquine in increasing the luciferase activity upon transfection with the plasmid DNA/lactosylated polylysine complex. The fusogenic peptide is supposed to disrupt the endosomal membrane allowing the release of endocytosed plasmid molecules into the cytosol. Therefore, the association of plasmid DNA/galactosylated poly-L-lysine complex together with fusogenic peptides could be a very efficient way of transfecting specific cell types in vivo. KALA, a cationic amphipathic peptide known to bind to DNA and to destabilize membranes, can mediate DNA transfection (Wyman et al., 1997). This peptide was synthesized and used as a cofactor for transfection of Hep G2 cells by DNA/galPLL complex. GALA, INF6 and INF7K1 were also synthesized and compared with KALA for the fusogenic activity. Of the four kinds of fusogenic peptides tested, KALA showed an outstanding performance in facilitating the galPLL-mediated luciferase gene expression (Fig. 2). In contrast, the luciferase activity was very low and not dependent on the presence of other peptides. No luciferase activity was observed in cells incubated with DNA alone or galPLL/DNA complex without KALA (Fig. 3). Chloroquine is a weak base used in endocytosis studies in order to neutralize the acidic pH of endocytic vesicles, to inhibit hydrolase contained in lysosome, to inhibit the fusion of endocytic vesicles and lysosomes and thereby to decrease the degradation of endocytosed macromolecule (Midoux et al., 1993). The fusogenic peptide KALA was 30 times more efficient than chloroquine in increasing the luciferase activity upon transfection with the RSVLuc/galPLL complex. Because fusogenic peptides are more active than chloroquine, the association of targeted plasmid DNA/galPLL complex together with targeted fusogenic peptides could open the way to very efficient and specific cell transfection in vivo. The effect of KALA on the expression of the enzyme upon transfection with RSVLuc/galPLL complex was optimal at the DNA/KALA = 1/5 (Fig. 4). In control experiments, it was shown that four kinds of peptide at any amount up to 30 μg (10 nM) did not impair the cell growth of Hep G2 cells over a period of 3 days (data not shown).

3.4. Optimal conditions for transfection of Hep G2 cells by RSVLuc/galPLL/KALA complexes

By using dilute solution of RSVLuc/galPLL/KALA complex (galPLL to DNA molar ratio (2.5:1)), the gene transfer efficiency was dependent upon the over all concentration of the complex (Fig. 4). The luciferase activities were similar when Hep G2 cells were transfected with complexes formed with galPLL and either 3 μg DNA or 4.5 μg DNA, suggesting that above the amount of 3 μg of plasmid, the system is apparently saturated.

3.5. Specificity of RSVLuc gene transfer in HepG2 cells by using galPLL as a carrier

An expression plasmid RSVLuc encoding the firefly luciferase gene was used as a reporter gene to monitor the efficiency of gene transfer and expression when using galPLL as a carrier. The complexes were prepared by mixing 3 μg RSVLuc plasmid and 1.2 μg galPLL in 100 μl of 400 mM

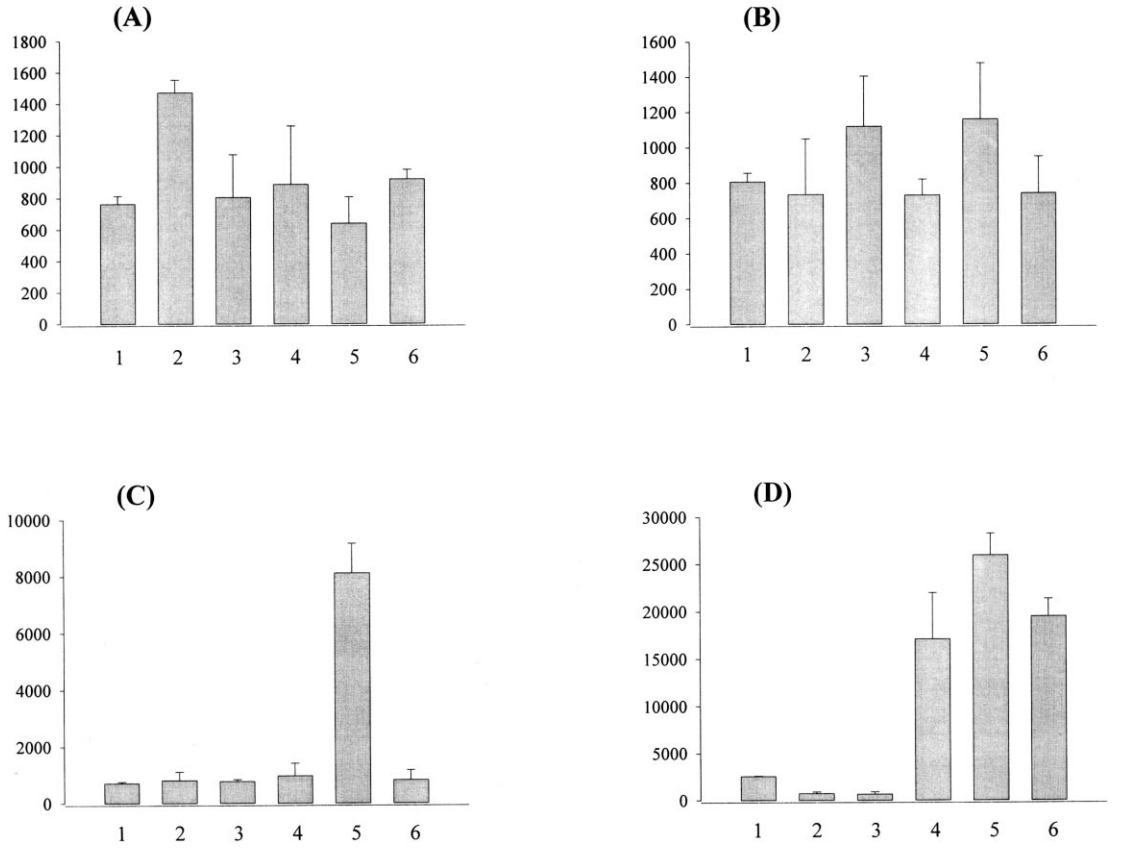

Fig. 2. Effect of various fusogenic peptides on the transfection efficiency of DNA/galPLL complex. Hep G2 cells (5×10^4/well) were incubated at 37°C in the presence of 1 μg of pRSVLuc complexed with 0.4 μg of galPLL and various amounts of peptides. After incubation for 16 h, the medium was replaced with plain culture medium and the cells were further incubated at 37°C for 24 h. The values of relative light units (RLU) shown represent the activity for 10^6 cells. Each point represents the mean value ± S.D. ($n = 2$). (A) GALA, (B) INF7K1, (C) INF6, (D) KALA. Note: *Y*- and *X*-axes represent the relative luciferase activity and the amount of peptide (μg), respectively.

NaCl solution (galPLL to DNA molar ratio close to 2.5:1). Cells were incubated at 37°C with the plasmid/galPLL complex with/without KALA, a fusogenic peptide. After 16 h incubation, cells were washed and further incubated at 37°C in KALA or chloroquine free and plasmid-free culture medium. The efficiency of the gene transfer into Hep G2 cells was evaluated 24 h later by measuring the luciferase activity in the cell lysate (Fig. 3). In the presence of chloroquine, the luciferase gene expression was much higher (RLU = 1.7×10^4) than that obtained in the absence of chloroquine (RLU = 3.6×10^2). A maximum luciferase activity was obtained with cells incubated for 16 h in the presence of RSVLuc/galPLL complexes, when KALA was added either at the same time or 1 h later. RSVLuc/galPLL/KALA complex present during the first 16 h incubation did not induce any cytotoxic effect nor cell detachment and the number of cells present in a well was identical with that of mock treated cells. With RSVLuc carried by galPLL/KALA, the luciferase expression based on emitted light, was 23-fold larger than in the case of RSVLuc complexed with non-galactosylated PLL/KALA, showing that the galactose residues present on galPLL specifically enhance the gene transfer into Hep G2 cells. When the same plasmid carrier preparations were

used with NIH/3T3 cells (that do not express the galactose specific lectin), the luciferase activity measured (RLU = 2.3×10^4) was one fourth as low as by using RSVLuc/non-galactosylated PLL/KALA complex with Hep G2 cells, confirming that the high activity obtained with Hep G2 cells was related to the presence of galactose on the carrier. The luciferase activity measured upon transfection of RSVLuc by the classical calcium phosphate precipitation method was relatively low (RLU = 8.5×10^4).

To estimate the galactose receptor-mediated transfection of RSVLuc/galPLL, the transfection of Hep G2 cells by this complex in the presence of asialofetuin (AF) as a competitor for galactose receptor (Fig. 5) was investigated. Hep G2 cells were incubated with or without AF in the presence of RSVLuc complexed with various amount ratios of PLL and galPLL. The transfection efficiency decreased to about 10% by the addition of

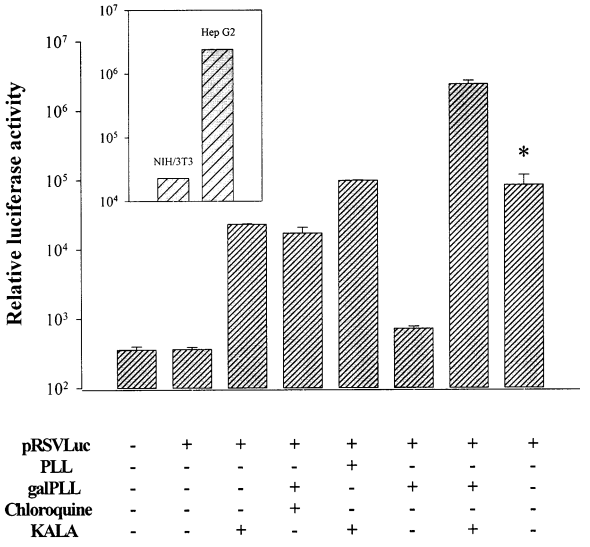

Fig. 3. Carrier dependent gene transfer. Hep G2 cells (5×10^4/well) were incubated at 37°C in the presence of 3 μg of pRSVLuc plasmid or complexes formed with 1.2 μg of either PLL or galPLL. After incubation for 16 h, the medium was removed and the cells were further incubated in complete culture medium in the absence of formulation at 37°C for 24 h. Gene expression was determined by assaying the luciferase activity of three aliquots of cell lysates. The values of relative light units (RLU) shown represent the activity of 10^6 cells. Note: *DNA was introduced by calcium phosphate co-precipitation method (Reston et al., 1991).

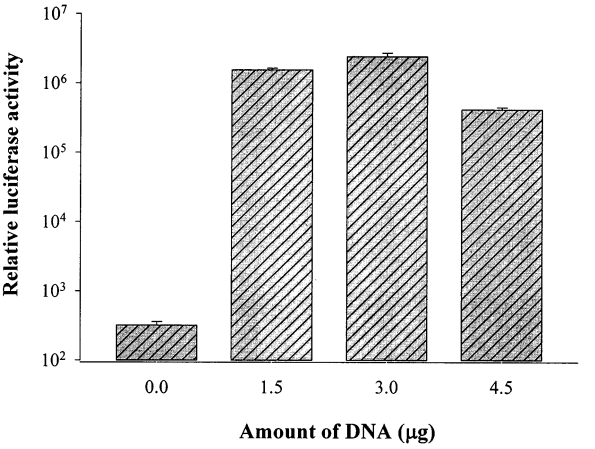

Fig. 4. Amount-dependent gene transfer by using various concentration of pRSVLuc/galPLL complex. Hep G2 cells (5×10^4/well) were incubated at 37°C for 16 h with various dilution of a pRSVLuc/galPLL (1:0.4, w/w) complex in the presence of KALA. Then, the medium was removed and the cells were further incubated in complete culture medium in the absence of formulation at 37°C for 24 h. Gene expression was determined by assaying the luciferase activity of three aliquots of cell lysates. The values of relative light units (RLU) shown represent the activity of 10^6 cells.

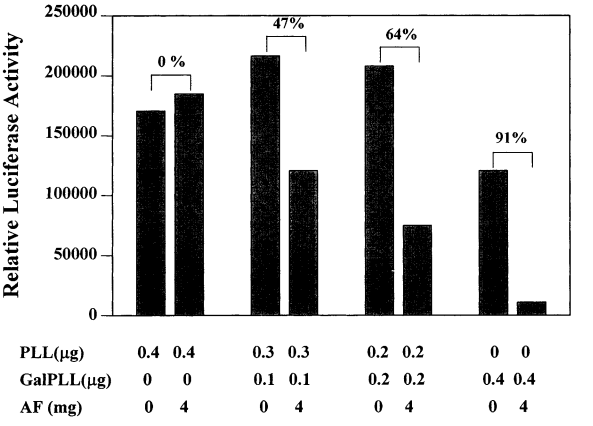

Fig. 5. Transfection efficiency of DNA-galPLL-PLL complex in the presence of asialofetuin (AF). Hep G2 cells (3×10^5/dish) were incubated at 37°C with or without AF (4 mg) in the presence of 3 μg of pRSVLuc complexed with various amounts of PLL and galPLL with KALA (15 μg). The relative light units (RLU) shown represent the activity for 3×10^5 cells. Each point represents the mean value ± S.D. ($n = 2$).

4 mg of free AF and this decreased fraction would be attributed to the inhibition of specific recognition of DNA/galPLL complex. When the experiment was repeated in NIH/3T3 cells, which do not express galactose receptor on the cell surface, AF-associated inhibition was not detected (data not shown).

In conclusion, our results showed that the gene delivery formulation consisting of DNA-galPLL-KALA complex may find potential applications for the gene transfer to the hepatic cells and tissue after a parenteral administration.

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References

Ashwell, G., Harford, J., 1982. Carbohydrate-specific receptors of the liver. Ann. Rev. Biochem. 51, 531–554.

Chang, T.M., Kullberg, D.W., 1982. Studies of the mechanism of cell intoxication by diphtheria toxin fragment A-asialoorosomucoid hybrid toxins. Evidence for utilization of an alternative receptor-mediated transport pathway. J. Biol. Chem. 257, 12563–12572.

Ciechanover, A., Schwartz, A.L., Lodish, H.F., 1983. Sorting and recycling of cell surface receptors and endocytosed ligands: the asialoglycoprotein and transferrin receptors. J. Cell. Biochem. 23, 107–130.

De Wet, J.R., Wood, K.V., De Luca, M., Helinski, D.R., Subramani, S., 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. 7, 725–737.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Calorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Erbacher, P., Roche, A.C., Monsigny, M., Midoux, P., 1995. Glycosylated polylysine/DNA complexes: gene transfer efficiency in relation with the size and the sugar substitution level of glycosylated polylysines and with the plasmid size. Bioconjug. Chem. 6, 401–410.

Fallon, R.J., Schwartz, A.L., 1988. Asialoglycoprotein receptor phosphorylation and receptor-mediated endocytosis in hepatoma cells. Effect of phorbol esters. J. Biol. Chem. 263, 13159–13166.

Han, J.H., Lim, M.J., Yeom, Y.I., 1999. Receptor-mediated gene transfer to cells of hepatic origin by galactosylated albumin-polylysine complexes. Biol. Pharm. Bull. 22, 836–840.

Hynes, M., Garrett, R.A., Gratzer, W.B., 1970. Structure of nucleic acid-poly base complexes. Biochemistry 9, 4410–4416.

Katayose, S., Kataoka, K., 1997. Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-Poly(L-lysine) block copolymer. Bioconjugate Chem. 8, 702–707.

Kim, C.-K., Lee, W.D., Park, H.K., 1992. Liver targetability and pharmacokinetics of ^{3}H-methotrexate-lactosaminated bovine serum albumin conjugates. Yakhak Hoeji 36, 591–597.

Kim, C.-K., Lee, M-K., Han, J-H., Lee, B-J., 1994. Pharmacokinetics and tissue distribution of methotrexate after intravenous injection of differently charged liposome-entrapped methotrexate to rats. Int. J. Pharm. 108, 437–446.

Knowles, B.B., Howe, C.C., Aden, D.P., 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209, 497–499.

Labat-Moleur, F., Steffan, A.-M., Brisson, C., Perron, H., Feugeas, O., Furstenberger, P., Oberling, F., Brambilla, E., Behr, J.-P., 1996. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. Gene Therapy 3, 1010–1017.

Merwin, J.R., Noell, G.S., Thomas, W.L., Chiou, H.C., DeRome, M.E., McKee, T.D., Spitalny, G.L., Findeis, M.A., 1994. Targeted delivery of DNA using YEE(GalNAcAH)3, a synthetic glycopeptide ligand for the asialoglycoprotein receptor. Bioconjug. Chem. 5, 612–621.

Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M., Roche, A.C., 1993. Specific gene transfer mediated by lactosyalted poly-L-lysine into hepatoma cells. Nucleic Acids Res. 21, 871–878.

Parente, R.A., Nir, S., Szoka, F.C. Jr, 1990. Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. Biochemistry 29, 8720–8728.

Perales, J.C., Ferkol, T., Beegen, H., Ratnoff, O.D., Hanson, R.W., 1994. Gene transfer in vivo: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. Proc. Natl. Acad. Sci. USA 91, 4086–4090.

Plank, C., Zatloukal, K., Cotton, M., Mechtler, K., Wagner, E., 1992. Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. Bioconjug. Chem. 3, 533–539.

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., Wagner, E., 1994. The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. J. Biol. Chem. 269, 12918–12924.

Reston, J.C., Gould-Fogerite, S., Mannino, R.J., 1991. Potentiation of DNA mediated gene transfer in NIH3T3 cells by activators of protein kinase C. Biochim. Biophys. Acta 1088, 270–276.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning 1. Cold Spring Harbor Laboratory Press, New York, pp. 1.33–1.52.

Schwartz, A.L., Rup, D., Lodish, H.F., 1980. Difficulties in the quantification of asialoglycoprotein receptors on the rat hepatocyte. J. Biol. Chem. 255, 9033–9036.

Schwartz, A.L., Fridovich, S.E., Knowles, B.B., Lodish, H.F., 1981. Charaterization of the asialoglycoprotein receptor in a continuous hepatoma line. J. Biol. Chem. 256, 8878–8881.

Schwartz, B., Benoist, C., Abdallah, B., Scherman, D., Behr, J.P., Demeneix, B.A., 1995. Lipospermine-based gene transfer into the newborn mouse brain is optimized by a low lipospermine/DNA charge ratio. Human Gene Therapy 6, 1515–1524.

Wadhwa, M.S., Knoell, D.L., Young, A.P., Rice, K.G., 1995. Targeted gene delivery with a molecular glycopeptide carrier. Bioconjugate Chem. 6, 283–291.

Wall, D.A., Wilson, G., Hubbard, A.L., 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. Cell 21, 79–93.

Wu, G.Y., Wu, C.H., 1987. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. J. Biol. Chem. 262, 4429–4432.

Wyman, T.B., Nicol, F., Zelphati, O., Scaria, P.V., Plank, C., Szoka, F.C. Jr, 1997. Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. Biochemistry 36, 3008–3017.