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Lactoferrin as a gene delivery vehicle to hepatocytes

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Abbreviations: pL, polylysine; Lf, lactoferrin; Tf, transferrin; bLf, bovine Lf; hLf, human Lf; CAT, chloramphenicol acetyltransferase; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

Abstract

Using lactoferrin as the specific ligand, we developed a simplified method for preparation of molecular conjugate for gene delivery. Replacement of column chromatography and dialysis by one step centrifugal filtration (Centricon, cut off size : 30,000), resulted in the rapid purification of bovine lactoferrin/polylysine (bLf/pL) and human lactoferrin/polylysine (hLf/pL) conjugates and easy separation of unconjugated polylysine. The Lf/pL conjugates prepared by this method efficiently transferred the reporter genes, CAT and LacZ gene, to HeLa and hepatic cells. The bLf/pL and hLf/pL conjugates could transfer the reporter genes to various hepatocytes including primary mouse hepatocyte, Hepa 1-6, SK-Hep1 and Chang liver, but not to NIH 3T3 mouse fibroblast cells, indicating that the Lf/pL conjugates conferred hepatocyte-specific gene transfer. The bLf/pL and hLf/pL conjugates prepared in the present study exhibited higher transfection efficiencies for mouse and human hepatocytes than the commercially available transferrin/polylysine (Tf/pL) conjugate.

Keywords: lactoferrin, poly-L-lysine, gene delivery

Introduction

Receptor-mediated gene transfer has been attempted to deliver gene to cells. In comparison with viral delivery system, the receptor-mediated gene delivery system has certain advantages. First, because of the cell type-specific distribution of various receptor, the delivery can be specific (Cristiano and Curiel, 1996). Second, since vector entry follows a normal physiological pathway, the delivery is not harmful to cell (Michael and Curiel, 1994). Third, this system avoid limitation of DNA size and sequence selection.

Receptor-mediated gene delivery vehicles possess two distinct functional domains. DNA binding domain is composed of polycation, such as polylysine (pL), which electrostatically interacts with DNA and forms toroid complex (Wagner *et al*., 1991). Ligand domain binds to a specific receptor molecule at the cell surface. Asialoglycoprotein and transferrin (Tf) are widely used to transfer foreign genes *in vivo* and *in vitro* to hepatocytes (Wu and Wu, 1987, 1988, 1992; Wagner *et al*., 1990; Zenke *et al*., 1990; Wu *et al*., 1991; Wilson *et al*., 1992; Cristiano *et al*., 1993; Liang *et al*., 1993; Stankovics *et al*., 1994). Recently various ligands, such as galactose, lactose, epidermal growth factor (EGF), folate, and malarial circumsporozoite protein, wsere used for gene delivery (Midoux *et al*., 1993; Chen *et al*., 1994; Gottschalk *et al*., 1994; Ding *et al*., 1995).

In this study, lactoferrin (Lf) was used as a specific ligand for gene delivery to hepatocyte. Lf belongs to the transferrin family of non-heme iron binding glycoprotein. Blood Lf originates from polymorphonuclear leucocyte which release Lf during exocytosis of specific granules. Since the liver rapidly clears Lf from circulation, the concentration of lactoferrin in the blood normally remain low, less than 20 nM (Imber and Pizzo, 1983). Lf has at least two functions in blood. First, lactoferrin inhibits division of macrophage and granulocyte progenitor cells in bone marrow (Zucali *et al*., 1989). Second, Lf may regulate iron retrieval and processing (MaAbee, 1995). Hepatic cells ingest iron-lactoferrin complex and store the liberated iron with ferritin.

In previous studies (Wagner *et al*., 1990, Ding *et al*., 1995), ligand-polycation conjugates were purified by cation exchange column chromatography or gel permeation chromatography. We developed a simple method for the preparation of Lf/pL conjugate using centrifugal filtration system. In comparison with the previous study, Lf/pL conjugate can be easily prepared and efficiently transfer foreign gene to hepatocytes.

Materials and methods

Cell lines and medium

HeLa (ATCC CCL2), Hepa 1-6 (ATCC CRL 1830) and OVCAR-3 (ATCC HTB 161) were cultured in Dulbecco's

Modified Eagle's Medium (DMEM) supplemented with heat inactivated fetal bovine serum. Chang liver (ATCC CCL 13), SK-Hep1 (ATCC HTB 52) and NIH 3T3 (ATCC CRL 1688) were cultured in DMEM supplemented with 10% heat inactivated calf serum. Each medium used in this study was supplemented with 120 μg/ml penicillin G (Sigma, St. Louis, MO, 1690 U/mg) and 200 μg/ml streptomycin sulfate (Sigma 750 U/mg).

Isolation of primary hepatocyte from mouse liver

Mouse hepatocytes were isolated by perfusion from the liver of mouse C57BL/6 weighing 10-20 g as previously described (Berry and Friend, 1969). The mouse was anesthetized with an intraperitoneal administration of 0.3 ml of Avertin (2,2,2-tribromoethanol, 20 mg/ml, Aldrich, Milwaukee, WI). The portal vein was cannulated with a 24-gauge catheter and inferior venae cava cut. The liver was perfused with 50 ml of perfusion medium and an additional 50 ml of perfusion medium containing 0.5 mg/ml of collagenase (Type IV; Sigma). All the above solutions were prewarmed to 37˚C and infused at a rate of 400 ml/h. Isolated hepatocytes were plated onto a collagen (Type I; Boerhinger Mannheim) coated culture dish at a density of 5×10^5 cells/6-cm dish, containing 2 ml of DMEM supplemented with 5% fetal bovine serum, 0.1 μM dexametasone and 20 nM insulin. After more than 80 % of the cells become attached to the culture dish (after about 1 h), the medium was changed. The medium was subsequently changed every 24 h.

Preparation of Lf/pL conjugate

Bovine lactoferrin (Sigma) and human lactoferrin (Calbiochem) was conjugated to pL (poly-L-lysine, average molecular mass 20,000, Sigma) by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Calbiochem) according to the protocol described by Halloran and Parker (1966). Reaction mixture contained 2.6 mg of Lf and 680 μg of pL. The pH was adjusted to 7.0 and 2.5 mg of EDC were added. The reaction mixture was incubated for 24 h at room temperature in the dark. The mixture was applied to Mono-S cation exchange column chromatography pre-equilibrated in 150 mM NaCl, 20 mM HEPES, pH 7.4 (HBS) or to the centrifugal filtration system (Centricon, cut off size: 30,000). After washing the column with HBS, bovine Lf/pL (bLf/pL) and human Lf/pL (hLf/pL) conjugate was eluted with a linear gradient of NaCl (150 mM-3 M) in HBS.

Preparation of Lf/pL/DNA and Tf/pL/DNA complex

A mammalian vector system expressing the bacterial chloramphenicol acetyltransferase (CAT) and β-galactosidase were used in this study. The plasmid pCMV-CAT and pCMV-LacZ contains CAT and LacZ gene under the control of cytomegalovirus immediate early promoter. The plasmid DNA was prepared by a Qiagen column. To prepare Lf/pL/DNA and Tf/pL/DNA complex, 80 μg of Lf/pL conjugate and 40 μg of Tf/pL conjugate in 350 μl of HBS was added to 10 μg of plasmid DNA in 150 μl of HBS and allowed to stand for 30 min at room temperature. Lf/pL//DNA and Tf/pL/DNA complex were directly added to 5×10^5 cells in 800 μl of fresh DMEM supplemented with 2% heat inactivated fetal bovine serum and 100 μM chloroquine. After 6 h incubation at 37˚C, the cells were transferred to the fresh medium.

CAT assay and LacZ staining

CAT assays were performed as previously described (Gorman *et al*., 1982). Two days after transfection, cells were harvested, washed with phosphate buffered salines (PBS), and resuspended in 0.25 M Tris-HCl (pH 7.5). Total proteins were prepared by 4 to 5 cycles of freeze/thaw followed by incubation at 65˚C for 10 min. Equivalent amounts of protein were assayed for CAT activity. The conversion of ^{14}C-chloramphenicol to its acetylated forms was determined by quantitating the intensity of each spot with phosphoimager (FUTIX BAS1000). X-gal staining was carried out as previously described (Sanes *et al*., 1986). The transfected cells were fixed with 0.5% glutaraldehyde in PBS and stained with 5-bromo-4-chloro-3-indoyl D-galactopyranoside (X-gal, Gibco BRL).

Results

Preparation of Lf/pL conjugate

hLf and bLf was conjugated to pL (average molecular mass 20,000) at a 1:1 molar ratio by using EDC. Separation of the unconjugated lactoferrin from the mixture was carried out by the use of Mono S cation exchange column chromatography. The hLf/pL or bLf/pL conjugates was eluted at 1.6 M NaCl. In contrast to the previous separation of Tf/pL conjugate (Wagner *et al*., 1990), the unconjugated Lf was not detected in flow-through fractions (Figure 1). Therefore, the centrifugal filtration using Centricon was employed to isolate the hLf/pL and bLf/pL conjugates. The unconjugated pL was easily removed from Lf/pL conjugate.

Optimization for the formation of conjugate-DNA complex for transfection

In order to determine the optimal ratio between the conjugate and DNA, a gel retardation experiment was performed (Figure 2). Migration of plasmid DNA was retarded by Lf/pL conjugate in 1% agarose gel as the concentrations of the conjugate was increased. DNA was completely retarded by Lf/pL conjugate at 2.5 mass ratio of the conjugate over DNA.

We also determined the mass ratio of conjugate over DNA for the optimal transfection efficiency. HeLa cells were transfected with pCMV-CAT plasmid conjugated to

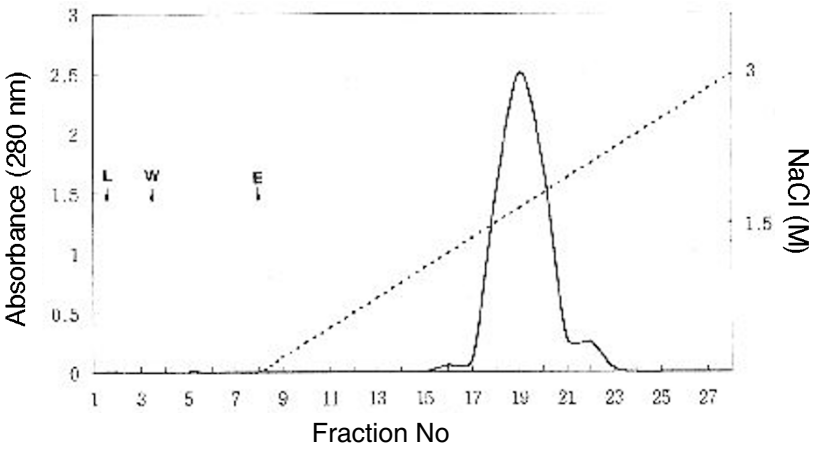

Figure 1. Purification of Lf/pL conjugate by Mono S cation exchange column chromatography. Purification was carried out as described in the Materials and Methods. Solid line, protein concentration: dotted line, NaCl concentration. L , loading: W , washing: E, elution.

hLf/pL. For the highest transfection efficiency the ratio of hLf/pL conjugate over DNA was 8 (Figure 3). This ratio was used consequently to transfect HeLa cells with pCMV-LacZ plasmid conjugate. After X-gal staining, about 10% of hLf/pCMV-LacZ transfected cells and 3% of Tf/pL/pCMV-LacZ transfected cells were stained as blue, whereas pL/pCMV-LacZ transfected cells were unstained (Figure 4). These results suggest that the transfection was specifically mediated by the ligand, i.e., Lf or Tf.

Gene delivery by Lf/pL conjugate to primary hepatocyte and various cell lines

To test whether bLf/pL conjugate is able to transfer a foreign gene to primary hepatocytes isolated from C57BL/6 mouse, cells were incubated with the bLf/pL/pCMV-CAT complex. Transfer of the gene to cells was determined by CAT assay as described in the "Materials and Methods". The results presented in Figure 5 show that the bLf/pL conjugate can transfer CAT gene to the primary hepatocytes more efficiently than Tf/pL conjugate. In order to determine hepatocyte specificity and the efficiency of Lf/pL and Tf/pL transfection, the CAT gene was transferred to various hepatic and non hepatic cell lines, such as SK-Hep1 (human hepatoma cell lines), Chang liver (immortalized human hepatocyte), OVCAR-3 (human ovarian cancer cell lines), Hepa 1-6 (mouse hepatoma cell lines), and NIH 3T3 (mouse fibroblast cell lines) (Figure 6). Compared to the nonhepatocytes (NIH 3T3 and OVCAR-3), the transfer of pCMV-CAT to the hepatocytes was dependent upon the ligand Lf or Tf. The CAT activities detected from hepatocytes were generally higher than that from NIH 3T3 cell, although the preference of ligands was different. The ligand-independency along with the similar level of CAT activities found in OVCAR-3 human ovarian cancer cells transfected with pL/pCMV-CAT, hLf/pL/pCMV-CAT and Tf/pL/pCMV-CAT suggest that the transfer of pCMV-CAT was mediated by pL rather than the ligands.

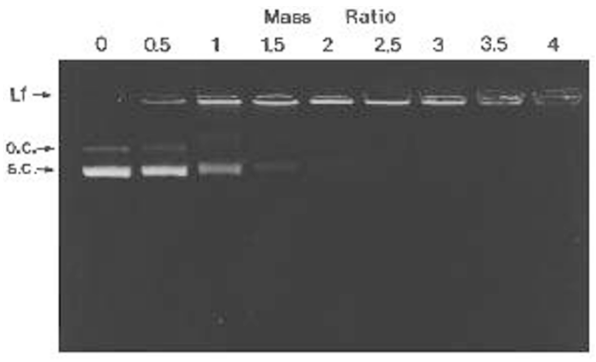

Figure 2. Retardation of Lf/pL/DNA complex. DNA/conjugate complex was formed with increasing mass ratios of Lf/pL conjugate to pCMV-CAT DNA. DNA was visualized with ethidium bromide. s.c., supercoiled DNA; o. c., open circle DNA; Lf, Lf/pL/DNA complex.

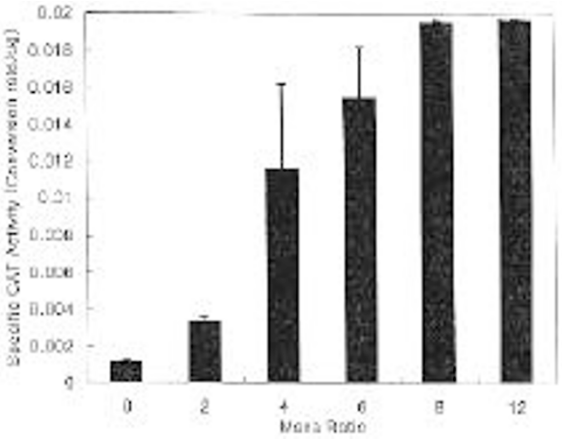

Figure 3. Determination of the optimal ratio of conjugate over DNA *in vitro*. HeLa cells transfected with the complex of various mass ratio of hLf/pL conjugate to DNA were assayed for CAT activity 48 h after transfection. Two independent mesurements were made in each mass ratio.

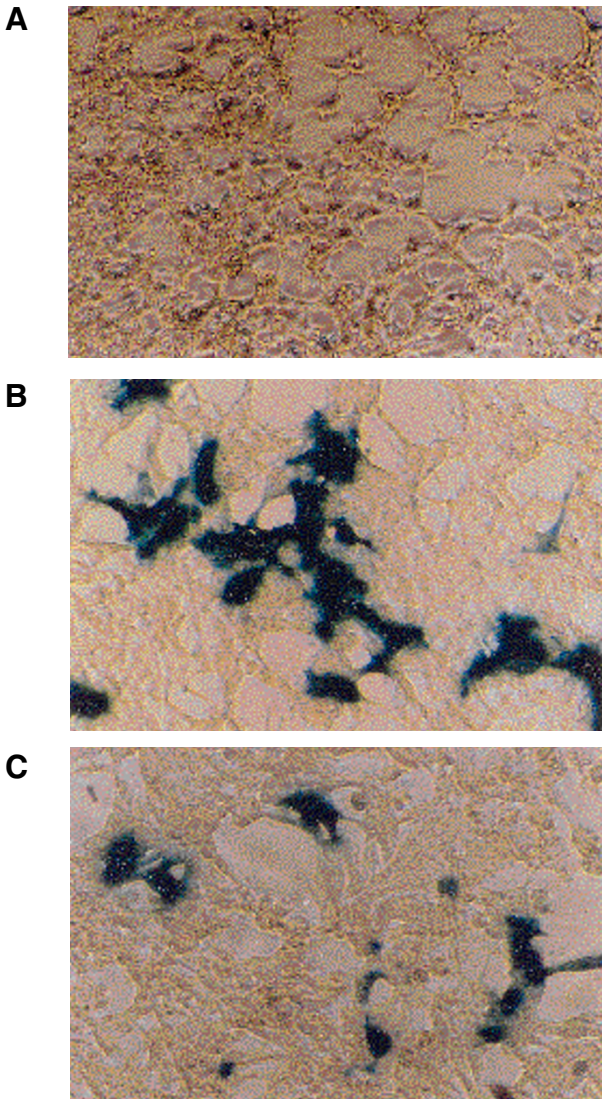

Figure 4. Visualization of transfected HeLa cells by X-gal staining. **(A)** pL/pCMV-LacZ; **(B)** Lf/pL/pCMV-LacZ complex; **(C)** Tf/pL/pCMV-LacZ complex.

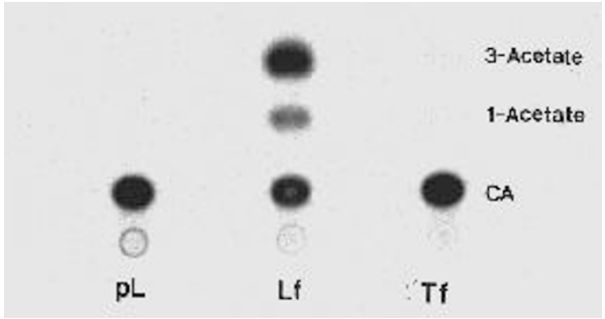

Figure 5. The expression of CAT gene in primary hepatocytes isolated from C57Bl/6 mouse. pL, polylysine/pCMV-CAT complex; Lf, Lf/pL/pCMV-CAT complex; Tf, Tf/pL/pCMV-CAT complex; CA, chloramphenicol.

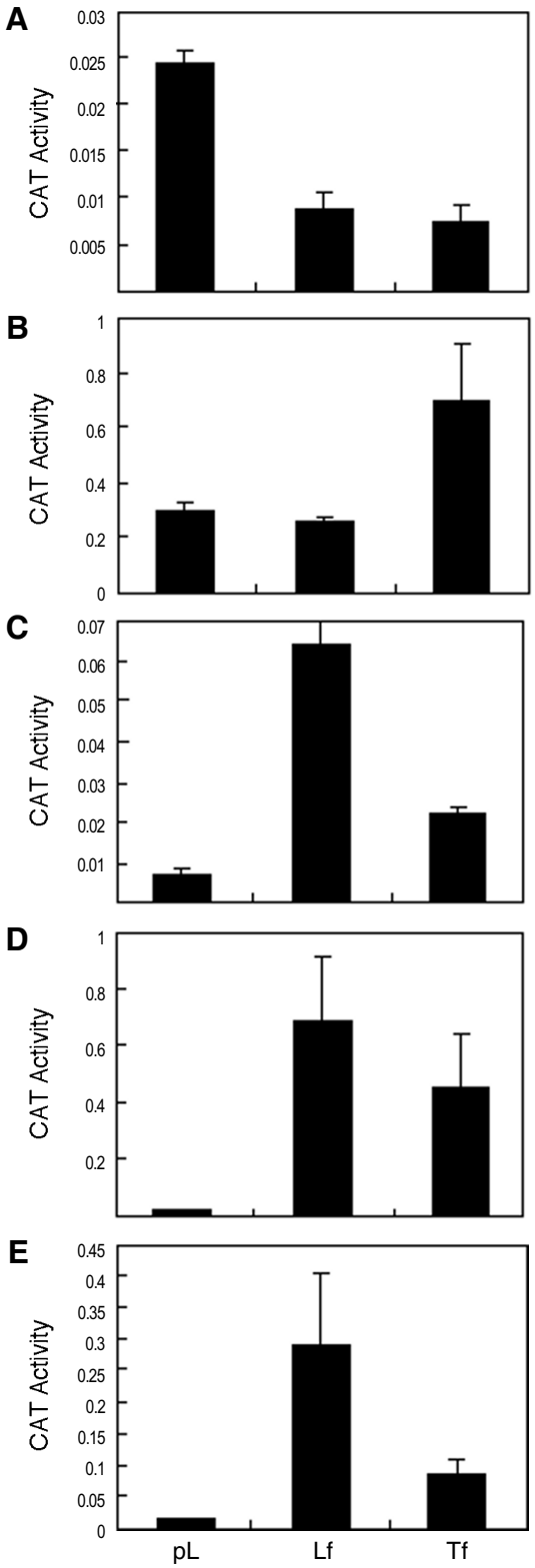

Figure 6. Expression of CAT in various cell lines. **(A)** mouse fibroblast NIH 3T3; **(B)** human ovarian cancer, OVCAR-3; **(C)** mouse hepatoma, Hepa 1-6; **(D)** human hepatoma SK-Hep1; **(E)** human immortalized Chang liver cell. pL, pL/pCMV-CAT complex; Lf, Lf/pL/pCMV-CAT complex; Tf, Tf/pL/pCMV-CAT complex. Three independent mesurements were made in each cell lines.

Discussion

This study was aimed to develop new vector systems useful for transferring a foreign genes to hepatocyte. It has been reported that significant amount (15-20 mg) of Lf which is cleared from circulation daily by human liver reappears in the nucleus (He and Furmanski, 1995). Therefore, Lf was chosen as a liver specific ligand to target foreign genes to hepatocytes.

Lf was conjugated to pL by EDC. When the material was purified by Mono S cation exchange column chromatographic procedure developed by Wagner *et al*. (1990), the unconjugated Lf was not detected in the flow-through and washing fractions, rather it was eluted as a single peak at a concentration of 1.6 M NaCl. Therefore, we employed a simplified centrifugal filtration method using Centricon (cut off size : 30,000) to purify the Lf/pL conjugate. Compared to the method described previously for the preparation of other conjugates, such as Tf/pL, asialogycoprotein/pL and circumsporozoit/pL (Wu *et al*., 1987; Wagner *et al*., 1990; Ding *et al*., 1995), our simple centrifugal filtration method using Centricon has certain advantages over the column chromatography and dialysis step. The preparation of Lf/pL is easier and more rapid. Since unconjugated pL was proved to be separated conveniently from Lf/pL conjugate, Centricon method can be applied for the preparation of other protein-pL conjugate.

The purified bLf/pL conjugate could transfer foreign genes to primary hepatocytes and the transfection efficiency was higher than that of Tf/pL conjugate. In addition, bLf/pL and hLf/pL were able to transfer DNA to other cell lines including mouse Hepa 1-6 hepatoma cells and human SK-Hep1 hepatoma cell whereas the transfer to mouse NIH3T3 fibroblast cells was inefficient. The results suggest that the use of the ligand-mediated gene transfer by pL conjugates is limited to certain types of cancer cells and hepatocyte cell lines.

Acknowledgment

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