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LECTIN CONJUGATE-DIRECTED GENE TRANSFER TO AIRWAY EPITHELIAL CELLS

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Many cell surface receptors contain covalently linked carbohydrates recognizable by lectins. This study examined the feasibility of using lectins as gene delivery vectors. Polylysine (PL) and histone (His) conjugates of concanavalin A (Con A), Maackia amurensis agglutinin (MAA), Sambucus nigra agglutinin (SNA), and wheat germ agglutinin (WGA), were employed to deliver pCMVlacZ to human airway epithelial cells (CFT1). As compared to the control (=1.0), the β-galactosidase activities expressed in CFT1 cells transfected with lectin-PL/His-DNA containing 5 μg/ml conjugate and 10 μg/ml DNA were: Con A-His, 7.7; SNA-His, 3.5; Con A-PL, 2.4; WGA-PL/His, 1.3; others, 1.0. The efficiency of gene transfer directed by Con A-His was dose dependent between 2.5 and 10 μg/ml of the conjugate. The maximal β-galactosidase activity expressed relative to the control was 42 obtained using 10 μg/ml conjugate. These results demonstrate that lectin-PL/His conjugates can be used as vectors to transfer genes to airway epithelial cells.

Cell surface receptors regulate many cellular functions by delivering extracellular growth factors, hormones, ion-binding proteins, and nutrients to the cells (1). Recently, this receptor-mediated transport mechanism has been exploited to deliver cDNAs to airway epithelial cells (2-4) and other cell types (5-7). The ligands employed for this gene delivery strategy include retrovirus (8), adeno-associated virus (9), adenovirus (10), molecular conjugates (2, 5-7, 11), and molecular conjugates linked with virus (4). Many cell surface receptors contain covalently-linked carbohydrates (8,12-15) recognizable by lectins. We hypothesize that when lectins bind to these receptor-associated carbohydrates (16), they would be internalized. This receptor carbohydrate-mediated endocytosis could allow lectins to serve as receptor-targeting vectors to deliver cDNAs into cells.

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In this study, we demonstrated that lectins-PL/His conjugates could deliver a reporter gene to human airway epithelial cells and the delivered gene could express in these cells. We also showed that among the lectin-His (and PL) conjugates examined, Con A-His was the most efficient vector for delivering this LacZ gene to airway epithelial cells.

MATERIALS AND METHODS

The materials used in this study were purchased from the following suppliers: Sigma, St. Louis, MO - human apo-transferrin (hTf) (iron poor), polylysine-hydrobromide (MW, 46,800), and histone (type V-S, lysine-rich subgroup); Pierce, Rockford, IL - N-succinimidyl 3-(s-pyridyldithio) propionate (SPDP); Fisher Biotech, Pittsburgh, PA - dithiothreitol; Clontech Laboratories, Palo Alto, CA - pCMVlacZ; Pharmacia LKB Biotechnology, Piscataway, NJ - Mono S HR 5/5 HPLC column; GIBCO/BRL Life Technologies Inc., Gaithersburgh, MD - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal); Collaborative Biomedical Products, Bedford, MD - insulin, transferrin, epidermal growth factor, triiodothyronine, cholera toxin, hydrocortisone, and endothelial cell growth supplement; Gelman Sciences, Ann Arbor, MI - 0.2 μ Z-spin units; and E-Y Laboratories, San Mateo, CA - lectins (both pure and FITC-labeled). The lectins (16) employed were: Con A, WGA, SNA, MAA, Ulex europaeus agglutinin (UEA-1), Griffonia simplicifolia (GS-I), GS-II, Bauhinia purpurea agglutinin (BPA), Glycine max agglutinin (SBA), Arachis hypogaea agglutinin (PNA), Maclura pomifera agglutinin (MPA), and Dolichos biflorus agglutinin (DBA). CFT1 cells (17) were provided by Dr. James Yankaskas at the University of North Carolina at Chapel Hill.

Binding of FITC-Lectins to CFT1 Cells CFT1 cells were cultured in a 4-chamber glass plate (Nunc, Inc., Naperville, IL) bathed in Ham's F12-7X medium (17), which contained insulin (10 μg/ml), transferrin (5 μg/ml), epidermal growth factor (25 ng/ml), triiodothyronine (3×10^{-8} M), cholera toxin (10 ng/ml), hydrocortisone (1×10^{-6} M), and endothelial cell growth supplement (3.75 μg/ml). The culture chamber was incubated at 37°C under 5% CO_2. Prior to fixation in cold methanol (4°C for 5 min), the cells were rinsed twice with PBS. The fixed cells were then washed 3X with PBS, incubated in 1% BSA in PBS at room temperature for 1 h, and exposed to FITC-lectins (1:50) at room temperature for 1 h in the dark. After rinsing 3X with PBS, the treated cells were covered with 50% glycerol in PBS and examined under a fluorescence microscope (AO Corp., Buffalo, NY).

Synthesis of Lectin-PL, Lectin-His, and hTf-PL Conjugates Lectin-PL, lectin-His, and hTf-PL were synthesized according the procedure described by Jung et al (18) and Wu and Wu (5) as diagramed in Fig. 1. In brief, the amino groups of lectins, PL, His, and hTf were modified with SPDP to form PDP derivatives. The PDP derivatives of PL and His were treated with dithiothreitol to remove thiopyridyl groups, thus exposing thiopropyl groups. The thiopropyl-PL or thiopropyl-His then reacted with PDP-lectins or PDP-hTf under N_2 to form lectin-PL/His and hTf-PL conjugates via disulfide bonding. The conjugates were then separated from unreacted PDP-lectins, PDP-hTf, thiopropyl-PL or thiopropyl-His on a Mono S HPLC column (12), which had been equilibrated in 50 mM HEPES, pH 7.9. Following the application of the samples, the column was developed with same buffer for 5 min. The conjugates retained on the column were eluted with a 0-3 M NaCl gradient in 50 mM HEPES, pH 7.9 at 1 ml/min for 25 min. The conjugates isolated were dialyzed against HBS buffer (20 mM HEPES, pH 7.3 and 100 mM NaCl), sterilized by filtration through 0.2 μ Z-spin, and quantified by ninhydrin assay (19) using PL or His as the standard.

Estimation of the Ratio of Con A and Histone in Con A-His Conjugate The Con A-His conjugate was treated with 100 mM dithiothreitol for 10 min and then applied to the Mono S HPLC column and developed as described above. The thiopropyl-Con A, which was eluted in the void volume, and thiopropyl-His, which was eluted with 2.2 M NaCl, were quantified by absorbance at 280 nm using Con A and His as the standard, respectively.

Gene Transfer Protocol CFT1 cells were cultured in F12-7X medium in a 12-well plate (Flow Laboratories, Inc., Mclean, VA) at 2×10^5 cells/well. The confluent cultures were

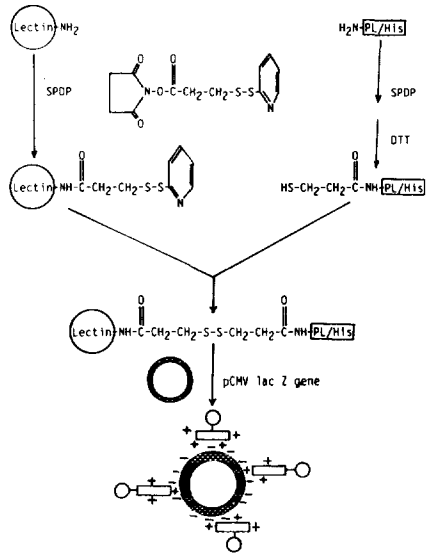


Figure 1. Synthesis of lectin-PL/His conjugates and preparation of lectin-PL/His-DNA complex. Lectins and PL/His were treated with SPDP to form PDP-derivatives. PDP-lectins were then reacted with thiopropyl PL/His to form lectin-PL/His conjugates. The lectin-PL/His-DNA complex was prepared by mixing lectin-PL/His with pCMVlacZ prior to the transfection experiments.

exposed for 1 h at 37°C to 500 μl HBS containing DNA-conjugate. These complexes, which contained the conjugates at specified concentrations and 20 μg per ml of the DNA in HBS buffer, were prepared 30 min prior to the experiment. CFT1 cells tnat were exposed to lectin (5 μg/ml or concentrations comparable to the lectin portion of the conjugates) + PL/His (5 μg/ml or concentrations comparable to the PL/His portion of the conjugates) + DNA (10 μg/ml) served as the control. At the end of the exposure period, one ml of Ham's F12-7X medium was added to each well. After cultured for 24 h, the cells were fixed in 4% paraformaldehyde-0.25% glutaraldehyde at 4°C for 5 min, rinsed 4X with PBS, and exposed to 1 ml X-gal (1 mg/ml in 100 mM sodium phosphate, pH 7.3 containing 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$ and 3 mM $K_4Fe(CN)_6$) at 37°C for 24-48 h. The cells were then examined under a phase contrast microscope (Nikon, Japan) for blue stained cells, which represent positively transfected cells. For initial assessment of transfection efficiency, the number of blue-colored cells out of 100 cells was measured in a hemacytometer after the cells had been released from the culture dish by trypsin treatment. However, this assay procedure could not detect the transfected cells expressing low level of β-galactosidase activity. For quantitative assessment of the transfection efficiency, we directly measured the β-galactosidase activity in the transfected cells. The transfected cells that had been cultured for 24h were recovered by trypsinization, pelleted by low speed centrifugation (2000 x g, 5 min.), and then lysed by 3 cycles of freezing and thawing. The cell lysates prepared from three separate culture dishes were measured for β-galactosidase activity by a fluorimetric method (20) on a Lumat LB9501 spectrofluorimeter (EG&G Berthold, NaShua, NH). Data were expressed as light units per 5 μg protein (Mean $\pm$ SEM). Protein was measured using the Bio-Rad protein assay kit employing BSA as the standard.
Statistical Analysis The significance of the difference of the means between the control and the test groups was analyzed by an unpaired Student's t-test program provided in the GraphPAD (ISI Software, San Diego, CA). The difference in the means was considered statistically significant when p value was $\leq$ 0.05.

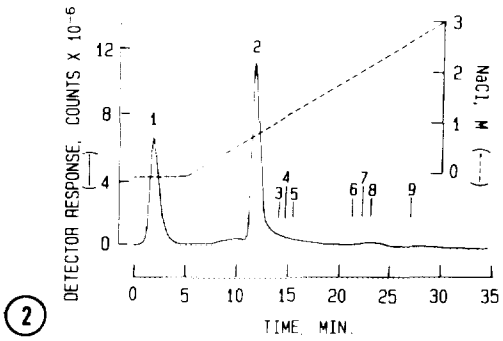
RESULTS

FITC-Lectin Binding to Airway Epithelial Cells Twelve fluorescein-conjugated lectins which include Con A, WGA, SNA, MAA, UEA-1, GS I, GS II, BPA, SBA, PNA, MPA, and DBA were tested for binding to cultured human airway epithelial cells. Only Con A, MAA, SNA, and WGA exhibited strong binding as demonstrated by intense cell-associated fluorescence (Data not shown).

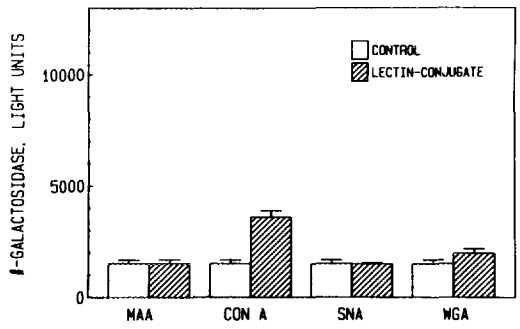
Purification and Characterization of Lectin-PL, Lectin-His, and hTf-PL Conjugates PL and His conjugates of these 4 lectins were synthesized for gene transfer experiments. hTf-PL was also synthesized to serve as a positive control. As shown in Fig. 2, PDP-lectins and PDP-hTf were recovered in the void volume fractions while lectin-His and lectin-PL in fractions between 0.8 and 2.1 M NaCl and thiopropyl-His and thiopropyl-PL in fractions at 2.2 and 2.6 M NaCl, respectively.

All the lectin-PL/His conjugates and hTf-PL did not enter 2% PAGE gel, suggesting that the molecular weights of these conjugates were greater than 1×10^6 daltons. In addition, the ratio of Con A subunit to His in Con A-His conjugate was estimated to be 1.0.

Expression of β-Galactosidase in CFT1 Cells Transfected with Lectin-PL-pCMVlacZ Staining of the lectin-PL-pCMVlacZ transfected CFT1 cells for β-galactosidase activity with X-gal showed occasional blue-colored cells (< 0.1 %) for Con A-PL and WGA-PL (5 μg/ml) but none for the conjugates of the other two lectins. However, cytotoxicity was observed for PL conjugates of Con A and WGA at ≥10 μg/ml. On the other hand, unconjugated Con A and WGA were not toxic at concentrations <120 μg/ml. The β-galactosidase activities in lectin-PL-pCMVlacZ-transfected CFT1 cells were 2.4 (p=0.003) and 1.3 times (p = 0.08) the control for Con A-PL and WGA-PL conjugates, respectively. No increase in β-galactosidase activity was found for MAA-PL and SNA-PL conjugates (Fig. 3).



(2)



(3)

Figure 2. HPLC separation of PDP-Con A (1), Con A-His (2), and thiopropyl-His (3). After the sample was applied to the column (Mono S HR 5/5), the column was washed for 5 min with 50 mM HEPES, pH 7.9 and then with a 0-3 M NaCl gradient in the same buffer for 25 min at 1 ml/min. The retention times for PDP-lectins, lectin-His/PL, hTf-PL, and thiopropyl-His/PL were: 1) PDP-lectin and PDP-hTf; 2) Con A-His; 3) SNA-PL, MAA-His, MAA-PL; 4) SNA-His; 5) Con A-PL; 6) hTf-PL; 7) WGA-PL; 8) thiopropyl-His; 9) thiopropyl-PL.

Figure 3. Lectin-PL directed gene transfer. β-Galactosidase activities (light units/5 μg protein, mean±SEM, n=3) were measured in the CFT1 cells treated with lectin (5 μg/ml) + PL (5 μg/ml) + DNA (10 μg/ml)(control) and with DNA complexes of MAA-PL (5 μg/ml), Con A-PL (5 μg/ml), SNA-PL (5 μg/ml), and WGA-PL (5 μg/ml).

Expression of β-Galactosidase in CFT1 Cells Transfected with Lectin-His-pCMV Lac Z

Gene transfer experiments were also performed using these lectins conjugated with His, a naturally occurring cationic nuclear protein, histone. CFT1 cells transfected with Con A-His-DNA expressed β-galactosidase activity 7.7 times ($p < 0.0001$) the control, while CFT1 cells transfected with SNA-His-DNA and WGA-His-DNA generated only 3.5 ($P < 0.0001$) and 1.3 ($P = 0.03$) times the β-galactosidase activity of the control, respectively (Fig. 4). MAA-His-DNA exhibited only background gene transfer efficiency. In addition, the efficiency of Con A-His directed gene transfer was dose dependent between 2.5 and 10 μg/ml of the conjugate. The Con A-His-directed gene transfer was abolished after pretreatment of the conjugate with 0.1-0.2 M mannose for 30 min. Maximal expression of β-galactosidase was observed at 10 μg/ml Con A-His (Fig. 5) while SNA-His and WGA-His at 10 μg/ml had only background gene transfer efficiency. At this concentration of Con-His, the β-galactosidase activity in the transfected cells was 42 times ($p < 0.0001$) the control with 1.3 % blue-colored cells. These values were higher than the transfection efficiency, i.e. 39-fold β-galactosidase activity with 1.2 % blue-colored cells, shown in the CFT1 cells transfected with hTf-PL-DNA in a contemporaneous experiment. However, at a higher Con A-His concentration, e.g. 20 μg/ml, the expressed β-galactosidase activity was only 4.0 times ($p = 0.0003$) the control value. At this concentration, Con A-His-DNA showed cytotoxicity.

DISCUSSION

The present study shows that out of 12 lectins examined for binding to human airway epithelial cells, only Con A, MAA, SNA, and WGA were bound, suggesting that the

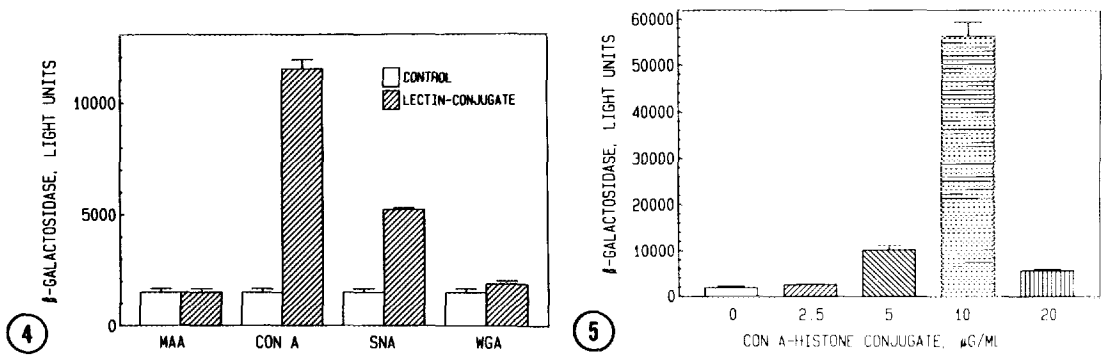


Figure 4. Lectin-His directed gene transfer. β-Galactosidase activities (light units/5 μg protein, mean±SEM, n=3) were measured in the CFT1 cells treated with lectin (5 μg/ml) + His (5 μg/ml) + DNA (10 μg/ml) (control) and with DNA complexes of MAA-His (5 μg/ml), Con A-His (5 μg/ml), and SNA-His (5 μg/ml).

Figure 5. Con A-His directed gene transfer at conjugate concentrations between 2.5 and 20 μg/ml. Ten μg/ml of DNA was used in this experiment. The β-galactosidase activities (light units/5 μg protein, mean ± SEM, n=3) at "0" conjugate were measured in the CFT1 cells exposed to 5 μg/ml Con A + 5 μg/ml His + 10 μg/ml DNA. The β-galactosidase activities measured in the CFT1 cells exposed to unconjugated Con A (2.5 - 10 μg/ml) + His (2.5 - 10 μg/ml) + DNA (10 μg/ml) were the same.

surface of these cells contained significant amounts of asparagine-linked carbohydrate, sialic acid α2,3/6Gal, sialic acid α2,6GalNAc, and/or N-acetylglucosamine at non-reducing termini (16). Our results also suggest that binding of these lectins to the cell surface glycoconjugates does not necessarily lead to internalization, a necessary step for receptor-mediated gene transfer. For example, MAA, which recognizes sialic acidα2,3Gal (16), bound to the airway epithelial cells, but no significant gene transfer was observed for MAA-PL/His conjugates. On the other hand, PL/His conjugates of Con A, SNA, and WGA could deliver a reporter gene to these cells. Therefore, sialic acidα2,3Gal structure probably is not receptor-associated while the asparagine-linked carbohydrate and sialic acidα2,6Gal(NAc) are. This observation confirms that the surface of airway epithelial cells contain many different glycoconjugates, some are associated with receptors and some are not.

Among the 4 lectin conjugates examined for gene transfer, Con A-His was the most efficient conjugate. Con A recognizes the core mannose oligosaccharides in asparagine-linked carbohydrates which are found in a variety of membrane-bound cell surface glycoproteins (13-15). These glycoproteins include receptors (13-15) and transporters (21). The Con A-His conjugate-directed gene transfer is mediated by receptor-associated asparagine-linked carbohydrates, because 0.1-0.2 M mannose blocked this event. The efficiency of this gene transfer was concentration-dependent between 2.5 and 10 μg/ml Con A-His, but cytotoxicity was observed at a higher concentration (20 μg/ml). The apparent dose-response relationship suggests that lectin-His conjugate would have a definable therapeutic safety-toxicity profile. Although binding of Con A-PL/His-DNA to cell surface non-receptor glycoproteins may perturb some important cellular functions, such as chloride conductance (21), this is probably not the primary mechanism for the observed cytotoxicity. This suggestion was supported by the observation that Con A, PL/His, and DNA at concentrations up to 100 μg/ml did not cause apparent cytotoxicity. Understanding the mechanism of cytotoxicity elicited by Con A-His-DNA should help the development of more efficient gene transfer protocols using lectin-PL/His conjugates.

Current vectors employed for somatic cell gene therapy, with the exception of retrovirus (8) and adeno-associated virus (9) vectors, produced transient expression of the genes in the target cells lasted for the duration of the life span of the cells. In order to achieve sustained therapeutic efficacy, repeated dosing is required. Therefore, cell type-specific vectors could prove to be advantageous by minimizing adverse cell-mediated immune responses as observed in adenovirus-mediated gene therapy (25-27). As discussed above, Con A may be used as a non-specific, receptor-mediated gene transfer vector. To deliver genes to specific cell types, lectins which recognize specific cell types need to be identified. Ciliated cells from human (22) and ferret (23) airway epithelium contain a unique surface carbohydrate structure (sialic acidα2,6Gal), which is recognized by influenza virus (23) and SNA (24). In addition, the cystic fibrosis transmembrane conductance regulator (CFTR) is found in the ciliated cells of human airway epithelium (29,30). If the sialic acidα2,6Gal structure is found to be associated with the apical surface receptors, it may be feasible to use a SNA-PL/His-CFTR gene complex to deliver the CFTR cDNA to the cili-

ated airway epithelium to correct the gene defect found in patients with cystic fibrosis. The cell type-specific targeting should decrease the dosage required for efficient delivery of CFTR cDNA and other therapeutic agents. This strategy should also decrease the risk associated with introducing a gene into cells in which this gene is not normally expressed, such as delivery of CFTR gene to goblet cells. Furthermore, it should be possible to further minimize or even eliminate the host immune response if human lectins (7,30) with similar carbohydrate specificities can be identified. CFT1 cells do not have ciliated cells, therefore they are not expected to contain abundant amounts of the sialic acidα2,6Gal structure, which is evident from the low gene transfer efficiency of SNA-PL/His (Fig. 3 &4). To test this gene transfer strategy, we need to perform the SNA-His directed gene transfer in tracheal primary cell and organ cultures or in the respiratory tract.

The lectin-PL/His-directed gene transfer strategy is not limited to airway epithelial cells. It is known that some tumor cells contain unique surface carbohydrates, which can be recognized by monoclonal antibodies or lectins (31). PL/His conjugates of these monoclonal antibodies or lectins are potentially useful as malignant cells-targeting vectors to deliver therapeutic DNAs or drugs to control or inhibit the growth of tumor cells.

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