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ScienceDirect
Biomaterials 28 (2007) 3448–3455
Biomaterials
www.elsevier.com/locate/biomaterials

Characterization of lactoferrin as a targeting ligand for nonviral gene delivery to airway epithelial cells

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Received 25 January 2007; accepted 2 April 2007
Available online 13 April 2007

Abstract

In this study lactoferrin (Lf) was investigated as a targeting ligand for receptor-mediated gene delivery to human bronchial epithelial cells. A high number of lactoferrin receptors (LfRs) were detected on bronchial epithelial (BEAS-2B), but not on alveolar epithelial (A549) cells by fluorescence microscopy and FACS measurements, suggesting potential targeting selectivity for bronchial epithelial cells. Molecular conjugates with ratios of Lf to branched polyethylenimine 25 kDa (PEI) ranging from 4:1 to 1:40 (mol/mol) were synthesized and analyzed for complexation of plasmid DNA (pDNA), transfection efficiency, and cytotoxicity. Whereas particle size increased with the degree of Lf coupling from 45 to 225 nm, surface charge was not significantly influenced. Transfection studies on BEAS-2B cells revealed that Lf-PEI 1:20 exhibited the highest luciferase gene expression which was 5-fold higher at an N/P ratio (molar ratio of PEI nitrogen to pDNA phosphate) of 4 than PEI and could be inhibited by an excess of free Lf. With A549 cells, no significant enhancement in transfection efficiency between Lf-PEI/pDNA and PEI/pDNA complexes could be observed. Increasing the degree of Lf coupling to PEI resulted in reduced transfection efficiency in both alveolar and bronchial epithelial cells. Cell viability assays resulted in significantly lower cellular toxicity of Lf-PEI/pDNA compared with PEI/pDNA complexes. We suggest that Lf represents a potent targeting ligand for receptor-mediated gene delivery to bronchial epithelial cells and might be a promising candidate for lung gene transfer *in vivo*.

Keywords: Lactoferrin; Polyethylenimine; Gene delivery; Receptor-mediated; Lung

1. Introduction

Gene therapy is receiving increasing attention and could represent an attractive approach for novel treatment or prevention of many common acquired or inherited human diseases, where conventional clinical procedures have poor efficacy. The lung is an important target organ for gene therapy strategies of many acute and chronic diseases such as cancer, asthma, and cystic fibrosis. One great advantage of gene delivery to the lung is its relatively noninvasive accessibility for drug application using well-developed delivery technologies, in particular via aerosol inhalation. Many studies have demonstrated the feasibility of pulmonary gene delivery and a variety of viral and nonviral gene transfer agents have been evaluated in the lung [1,2]. However, suitable vector systems, which should be safe, efficient, and inexpensive, are still an objective of intensive research efforts.

Within the group of nonviral vectors, polyethylenimine (PEI) is one of the most effective, commercially available polymeric gene transfer agent which has been successfully used for gene delivery both *in vitro* and *in vivo* [3,4]. PEI is a polycation capable of binding and compacting plasmid DNA (pDNA) into nanoparticles and protecting it from nuclease degradation. In addition, it has been shown to be more efficient in gene transfer into respiratory epithelial cells *in vivo* than other gene transfer agents, e.g., cationic lipids [5]. Therefore, it represents an ideal candidate molecule for the design of more sophisticated gene delivery systems.

Recent studies have shown that nonviral gene delivery with PEI/pDNA nanoparticles is able to efficiently

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doi:10.1016/j.biomaterials.2007.04.011

transfect airway epithelial cells in the murine lungs after aerosol delivery [6]. Nevertheless, gene expression in lung tissue *in vivo* is unsatisfactory due to phagocytosis of transfection complexes by alveolar macrophages which results in their clearance from the lungs [7,8]. To circumvent this problem, internalization kinetics of PEI/pDNA nanoparticles by lung cells needs to be improved. This could be achieved by the development of ligand-associated delivery gene vectors which take advantage of the natural process of receptor-mediated endocytosis pathway. These systems allow targeted gene delivery to selected cell types which is also a key aspect for the purposes of therapeutic applications with respect to the specificity towards the cells of interest. In addition, undesirable side effects, including immune, inflammatory, and cytotoxic responses, caused by the expression of exogenous genes in nontarget cells, could be limited. Many Ligand–receptor systems have been investigated to date for nonviral gene delivery. Ligands that have been applied to target PEI/pDNA polyplexes include antibodies [9], peptides [10], transferrin [11], sugars [12], mannuronic acid [13], folate [14], and growth factors such as EGF [15].

Since previous studies demonstrated that lactoferrin receptors (LfRs) are expressed on the apical surface on bronchial epithelial cells [16], lactoferrin (Lf) may serve as a suitable targeting ligand for receptor-mediated gene delivery to the lung. Lf (also called lactotransferrin) is an 80 kDa, single-chain glycoprotein which is folded into two lobes that show sequence homology with each other and can each reversibly bind one ferric ion with high affinity, even at low pH [17,18]. Furthermore, it is closely related to the iron-transport protein transferrin, although its affinity for iron is somewhat higher ($K_D \sim 10^{-20}$ M). Lf is a commonly expressed protein in the human body, being secreted in an iron-free form from epithelial cells into most exocrine fluids. High concentrations occur in human milk with varying concentrations of 1–7 mg/ml, whereas the concentration in blood is normally very low (<1 μg/ml) and probably originates from degradation of polynuclear leukocytes [19]. A large amount of research on Lf over the last years has revealed a high number of possible physiological functions, for example, antimicrobial, immunmodulatory, antiviral, and anti-tumor metastasis activity [18]. Additionally, it plays a major role in physiologic bacterial defense mechanisms within the respiratory tract [20].

In the present study, we explored the use of Lf as a targeting ligand to improve nonviral polymeric gene delivery to human bronchial epithelial cells. Gene transfer efficiency, cytotoxicity, and biophysical properties of conjugates consisting of Lf and PEI with varying conjugation degrees were characterized.

2. Materials and methods

2.1. Materials

Lf (from human milk), branched PEI (average molecular weight 25 kDa), fluorescein-5-thiosemicarbazide (FTSC), and all other chemicals were obtained from Sigma-Aldrich (Schnelldorf, Germany) and used without further purification. Branched PEI was diluted in double-distilled water and adjusted to pH 7 with HCl. The plasmid pCMVLuc containing the *Photinus pyralis* luciferase gene under the control of the cytomegalovirus immediate early promotor (CMV) was kindly provided by Prof. E. Wagner (Department of Pharmacy, Ludwig Maximilians University, Munich, Germany). This plasmid was propagated in *Escherichia coli* and purified by PlasmidFactory GmbH & Co. KG (Bielefeld, Germany). The purity of this plasmid was ⩽0.1 EU/μg pDNA, the amount of supercoiled pDNA ⩾90% ccc.

2.2. Synthesis of Lf-FTSC

Lf was labeled with FTSC by a reductive amination procedure according to Leveugle et al. [21]. Briefly, 15 mg of Lf (0.18 μmol) was dissolved in 690 μl 100 mM sodium phosphate, 10 mM sodium chloride, pH 5.6, and mixed with 300 μl sodium periodate solution (0.018 M). After incubation for 10 min at 4 °C, the reaction was stopped by the addition of 30 μl ethylene glycol, followed by purification and desalting on a Sephadex G-25M PD-10 Column (GE Healthcare, Uppsala, Sweden) in 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150 mM NaCl, pH 7.4. Subsequently, 5 mg of the oxidized Lf was incubated with 10 μl of 1.32 mM FTSC (5.5 mg in 100 μl DMF) for 20 h under gentle shaking at room temperature. To reduce the hydrazone bonds to more stable linkages, 10 μl of sodium cyanoborohydride (4 M) was added and the mixture was incubated for additional 2 h. Finally, unreacted fluorescent dye was removed by size-exclusion chromatography on a Sephadex G-25M PD-10 Column in 20 mM HEPES, 150 mM NaCl, pH 7.4. The ratio of fluorescein to protein was calculated to be 1:1 by measuring the absorbance at 495 and 280 nm, and the concentration was adjusted to 20 μM.

2.3. Synthesis of Lf-PEI

Coupling of Lf to PEI was performed with a comparable strategy like the conjugation to FTSC. About 25 mg (0.3 μmol) of human Lf was diluted in 1 ml of 30 mM sodium acetate buffer at pH 5.0. After addition of a 3-fold molar excess of sodium periodate, the mixture was incubated on ice for 90 min in the dark. Low-molecular-weight products were removed from oxidized Lf solution using a Sephadex G-25M PD-10 Column in sodium acetate buffer. The resulting solution yielded an amount of 21.5 mg oxidized Lf. Subsequently, oxidized Lf (0.0625 mol per synthesis) was mixed with different amounts of branched PEI 25 kDa, resulting in ratios from 4:1 to 1:40 (mol/mol). After 30 min of incubation, four portions of sodium cyanoborohydride (1 mg per 10 mg Lf) were added at 1 h intervals. Incubation was continued for additional 20 h, followed by purification of the reaction mixture on a cation-exchange column (POROS HS) using a BioLogic HR FPLC system (BioRad, Munich, Germany) with a salt gradient from 0.5 to 3.0 M sodium chloride and a constant content of 20 mM HEPES. The conjugate eluted between 2.5 and 3.0 M salt was pooled. Combined conjugate fractions of each sample were desalted and concentrated by a triple ultracentrifugation with Amicon Ultra-15 5k Centrifugal Filter Columns (Millipore, Bedford, USA). PEI concentration of each fraction was determined by ninhydrin assay and the Lf content was calculated by measuring the protein absorption at 280 nm. Finally, the samples were diluted to 1 mg/ml PEI concentration, divided into convenient small aliquots, and kept at −80 °C until further use.

2.4. Cell lines

A549 and BEAS-2B cells were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The cells were grown in minimum essential medium (MEM, Gibco-BRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Gibco-BRL) at 37 °C in a 5% CO_2 humidified air atmosphere.