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Targeting of the prostacyclin specific IP_1 receptor in lungs with molecular conjugates comprising prostaglandin I_2 analogues

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ABSTRACT

Molecular conjugates comprising targeting ligands hold great promise for site-specific gene delivery to distant tumors and individual organs including the lung. Here we show that prostaglandin I_2 analogues can be used to improve gene transfer efficiency of polyethylenimine (PEI) gene vectors on bronchial and alveolar epithelial cells *in vitro* and lungs of mice *in vivo*. Prostacyclin (IP_1) receptor expression was confirmed in pulmonary epithelial cell lines by western blot. Iloprost (ILO) and treprostinil (TRP), two prostaglandin I_2 analogues, were conjugated to fluorescein-labeled BSA (FLUO-BSA) and compared for IP_1 receptor binding/uptake in different lung cell lines. Binding of FLUO-BSA-ILO was 2–4-fold higher than for FLUO-BSA-TRP and could be specifically inhibited by free ILO and IP_1 receptor antagonist CAY10449. Internalization of FLUO-BSA-ILO was confirmed by confocal microscopy. Molecular conjugates of PEI and ILO (PEI-*g*-ILO) were synthesized with increasing coupling degree (F_{ILO} (ILO:PEI) = 2, 5, 8, 16) and analyzed for DNA binding, particle formation and transfection efficiency. At optimized conditions (N/P 4, $F_{ILO} = 5$), gene expression using PEI-*g*-ILO was significantly up to 46-fold higher than for PEI gene vectors and specifically inhibited by CAY10449. Gene expression in the lungs of mice after aerosol delivery was 14-fold higher with PEI-*g*-ILO $F_{ILO} = 5$ than for PEI. We suggest that targeting of IP_1 receptor using ILO represents a promising approach to improve pulmonary gene transfer.

1. Introduction

Gene transfer holds great promise for the treatment of acquired and inherited lung diseases and may further offer new perspectives for vaccination [1,2]. One great advantage of gene delivery to the lung is its relatively noninvasive accessibility by using well-developed delivery technologies such as aerosol or dry powder inhalation. These technologies allow uncomplicated repeated dosing which is a precondition for the treatment of chronic pulmonary diseases such as cystic fibrosis [3]. Numerous studies have demonstrated successful gene transfer to the lungs using a variety of viral and non-viral gene transfer agents after aerosol delivery to the lungs of mice [4], rabbits [5] and sheep [6]. As of yet, only one non-viral gene transfer agent, a cationic lipid formulation based on Genzyme lipid 67, has been successfully aerosolized to the lungs of patients in a phase I clinical trial [7].

Among non-viral vectors branched polyethylenimine 25 kDa (PEI) has been shown to be effective both in cell culture and *in vivo* [8]. PEI is a highly polycationic molecule which efficiently condenses plasmid DNA (pDNA) into nanoparticles [9] and protects DNA from nuclease degradation [8]. Nevertheless, its low gene transfer efficiency compared to viral vectors and its high toxicity due to high polymer doses needed for optimal gene transfer, limit the use of PEI, especially in terms of *in vivo* application. A variety of ligands including transferrin [10], folate [11], lactoferrin [12], clenbuterol [13], and growth factors such EGF [14], have been investigated to enhance PEI-mediated gene delivery in terms of cell specificity and reduction of cell toxicity.

The prostacyclin (IP_1) receptor is a seven transmembrane G-protein coupled receptor, identified in many tissues including the lungs [15–17]. Binding of IP_1 receptor agonists leads to endosomal internalization of receptor/ligand complexes via clathrin-mediated

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process [18,19] and thus, iloprost (ILO) may serve as suitable targeting ligand to enhance gene delivery in a receptor-specific manner. Various IP_1 receptor agonists like ILO and treprostinil (TRP) are used in patients for the treatment of pulmonary arterial hypertension via intravenous and aerosol application [20].

In the present study, IP_1 receptor was investigated as novel target for receptor-mediated gene transfer into lung epithelial cells. We analyzed IP_1 receptor expression in lung cells by western blot and further examined if ILO and TRP were capable of mediating cellular binding and uptake of model cargo conjugates. For this purpose, ILO and TRP were conjugated to fluorescein-labeled bovine serum albumin (FLUO-BSA) and their binding and cellular uptake were examined on various lung cell lines by flow cytometry and confocal laser scanning microscopy. In the next step, ILO was conjugated to PEI and the resulting PEI-*g*-ILO conjugates were characterized for pDNA binding and transfection of various lung cells *in vitro*. Finally optimized formulations were investigated for gene delivery to the lungs of mice after aerosol administration.

2. Materials and methods

2.1. Chemicals and plasmids

Iloprost, treprostinil and CAY10449 were purchased from Cayman Chemical (Michigan, USA), branched polyethylenimine (average molecular weight of 25 kDa), *N*-hydroxysulfosuccinimide (sulfo-NHS), bovine serum albumin (BSA), sodium phosphate, picrylsulfonic acid solution, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and heparan sulfate were obtained from Sigma Aldrich (Schnelldorf, Germany). PEI was diluted in double-distilled water (water for injection, B. Braun Melsungen AG, Melsungen, Germany), and adjusted to pH 7 with hydrochloric acid. Sodium phosphate was dissolved in double-distilled water to a concentration of 0.5 mM and adjusted to pH 7.5 with sodium hydroxide. HEPES was dissolved in distilled water to a concentration of 0.1 M and adjusted to pH 7.4 with sodium hydroxide. Heparan sulfate was dissolved in double-distilled water to a concentration of 5 mg/ml. Ethanol p.a. and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were purchased from Merck (Darmstadt, Germany). MOPS was dissolved in double-distilled water to a concentration of 0.1 M and adjusted to pH 6 with hydrochloric acid. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 5-(and 6-)carboxyfluorescein succinimidyl ester (fluorescein-NHS) were obtained from Pierce (Rockford, USA). Dithiothreitol (DTT) was purchased from Amersham Biosciences (South San Francisco, USA). D-luciferin was purchased from Synchem OHG (Flensberg/Altenburg, Germany). The plasmid pCMV-luc 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). pCpG-luc was constructed by Manfred Ogris (Department of Pharmacy, Ludwig-Maximilians-University Munich, Germany). Both plasmids were propagated in *Escherichia coli* and provided in a highly purified form (LPS content $\leq$ 0.1 E.U./μg DNA) by PlasmidFactory GmbH (Bielefeld, Germany). The amount of supercoiled pDNA was $\geq$90% ccc (covalently closed circular) for pCMV-luc and greater than 98% ccc grade for pCpG-luc.

2.2. Cell lines

A549 (human alveolar epithelial) cells were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). BEAS-2B (human bronchial epithelial), H441 (human bronchiolar epithelial) cells were purchased from the ATCC (American Type Culture Collection). 16HBE14o- (human bronchial epithelial) cells were kindly provided by Prof. D. C. Gruenert (University of Vermont, Burlington, USA). A549, BEAS-2B and 16HBE14o- cell lines were grown in Minimum Essential Media (MEM, Gibco-BRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Gibco-BRL, Karlsruhe, Germany) at 37 °C in a 5% CO_2 humidified air atmosphere. H441 cell line was grown in Roswell Park Memorial Institute media 1640 (RPMI 1640, Gibco-BRL, Karlsruhe, Germany) supplemented with 10% FCS at 37 °C in a 5% CO_2 humidified air atmosphere.

2.3. Animals

Fourteen-week-old female BALB/c mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained under specific pathogen-free conditions. Mice were acclimatized to the environment of the animal facility for at least seven days prior to the experiments. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

2.4. Western blot analysis

A549, BEAS-2B and 16HBE14o- cells were washed with PBS and lysed on ice in lysis buffer containing 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.05% sodium deoxycholate. Protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and 1 mM DTT were added fresh before use. Protein concentrations were determined using BioRad Protein Assay (BioRad, Munich, Germany). For each cell line, 40 μg of protein was diluted in SDS sample loading buffer (62.5 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 2% DTT, 0.001% bromphenol blue) boiled for 5 min, separated on a 7.5% Tris/HCl gel (BioRad, Munich, Germany) and transferred to PVDF membrane (Millipore, Schwalbach, Germany). Membranes were blocked with TBS-T (20 mM Tris/HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20) containing 5% skimmed milk powder (Sigma Aldrich, Deisenhofen, Germany) for 1 h at RT. The primary polyclonal antibody (diluted 1:500) for IP_1 receptor (Cayman Chemical, Michigan, USA) was incubated overnight in 0.5% skimmed milk. Membranes were washed with TBS-T and incubated with an anti-rabbit HRP-conjugated secondary antibody (diluted 1:15,000; BioRad, Munich, Germany) for 1.5 h at RT in 0.5% skimmed milk. After several wash steps with TBS-T, chemiluminescence detection was done using ECL detection kit (PIERCE, Rockford, USA) following manufacturer's instructions.

2.5. Synthesis of fluorescein-BSA-iloprost (FLUO-BSA-ILO) and fluorescein-BSA-treprostinil (FLUO-BSA-TRP)

20 mg (0.3 μmol) of BSA was diluted in 2.5 ml of sodium phosphate buffer pH 7.5 and mixed with a 10-fold molar excess of fluorescein-NHS. After stirring 1 h at RT, the mixture was purified on a PBS equilibrated Sephadex G-25 M PD-10 column (GE Healthcare, Uppsala, Sweden). Either 0.7 mg (1.8 μmol) of ILO or 0.8 mg (1.8 μmol) of TRP were dissolved in 130 μl ethanol p.a. and mixed with 370 μl of MOPS buffer 0.1 M pH 6. 0.5 mg (5 mM) of sulfo-NHS (in MOPS buffer) and 0.2 mg (2 mM) of EDC (in MOPS buffer) were added and stirred for 15 min at RT. Afterwards 5 μl (20 mM) of DTT (in distilled water) was added and immediately 3 mg (45.2 nmol) of FLUO-BSA in 190 μl and 210 μl of phosphate buffer 0.5 M were pipetted to the reaction mixture. After stirring for 2 h at RT the mixture was purified on a PBS equilibrated Sephadex G-25 M PD-10 column (GE Healthcare, Uppsala, Sweden). BSA amounts were quantified by BioRad Protein Assay using a BSA standard curve. Coupling efficiencies of the final and intermediate products were determined by TNBS-Assay [21] and measuring the absorbance at 495 nm. Coupling degree of BSA-ILO and BSA-TRP resulted in 10 mol ILO or TRP per mol BSA.

2.6. Synthesis of PEI-graft-iloprost polymers (PEI-g-ILO)

Different coupling degrees of PEI-*g*-ILO were synthesized by variation of EDC amounts given to the reaction mixture. 1 mg (2.8 μmol) of ILO was diluted in 100 μl ethanol p.a., mixed with 68 nmol PEI in 900 ml HEPES buffer 0.1 M pH 7.4 and 1 mg (5 mM) sulfo-NHS. Different amounts of EDC to a final concentration of 25 mM, 50 mM, 60 mM and 100 mM were added and incubated under stirring for 4 h at RT. The reaction mixture was purified on a double-distilled water equilibrated Sephadex G-25 M PD-10 column (GE Healthcare, Uppsala, Sweden). Concentration of PEI was determined with $CuSO_4$ assay according to Ungaro et al. [22]. ^1H–^1D NMR spectra of PEI-*g*-ILO were recorded in D_2O on a Bruker AV 250 MHz spectrometer. Coupling degrees of PEI-*g*-ILO were calculated via integration of the broad multiplett of PEI (CH_2–CH_2–NH–) at δ (1H) = 2.5–3.1 ppm and the singulett of the terminal methyl group of ILO (–CΞC–CH_3) at δ (1H) = 1.73 ppm. Covalent conjugation of ILO to PEI resulted in four different coupling degrees (F_{ILO} (mol ILO per mol PEI) = 2, 5, 8, 16). PEI-*g*-ILO constructs were divided into small aliquots, snap-frozen in liquid nitrogen and kept at −80 °C until further use.

2.7. Incubation experiment of FLUO-BSA-ILO and FLUO-BSA-TRP

Receptor binding/uptake of FLUO-BSA-ILO was investigated in A549, H441, 16HBE14o- and BEAS-2B cells. For FACS measurement experiments, 100,000 cells per well were seeded in 24-well plates (TPP, Trasadingen, Switzerland) 24 h prior to addition of the conjugates. FLUO-BSA-ILO, FLUO-BSA-TRP and FLUO-BSA conjugates, respectively, were diluted in MEM to a concentration of 0.5 μM and incubated on the cells for 4 h at 37 °C. After washing the cells with PBS, cells were dislodged from the wells by trypsin treatment and FACS measurements were performed using a Becton Dickinson FACS Scan (San Jose, USA). For confocal laser scanning microscopy, experiments were performed in 4-chamber BD Falcon Culture Slides (BD Biosciences San Jose, USA) with 25,000 cells per chamber. Incubation of FLUO-BSA-ILO and FLUO-BSA was performed as described before. Cells were rinsed and fixed in 4% paraformaldehyde, followed by staining of cell nuclei with 0.33 μM DAPI (4′,6-diamidino-2-phenylindole) and F-actin with Alexa Fluor® 568 phalloidin (Invitrogen GmbH, Karlsruhe, Germany) using standard protocols. The slides were covered with mounting media (Vectashield, Vector Laboratories Inc., Burlingame, USA) and images were taken with a confocal laser scanning microscope (Leica, Solms, Germany).