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In vitro cytotoxic and immunomodulatory profiling of low molecular weight polyethylenimines for pulmonary application

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ABSTRACT

Polyethylenimines (PEI) are potent non-viral nucleic acid delivery vehicles used for gene delivery and RNA interference (RNAi). For non-invasive pulmonary RNAi therapy the respiratory tissue is an attractive application route, but offers particularly unwanted side-effects like cytotoxicity as well as inflammatory and immune responses.

In the current study, we determined the most crucial issues of pulmonary applications for two low molecular weight PEIs in comparison to the well-known lung toxic crystalline silica. Cytotoxic effects and inflammatory responses were evaluated in three murine pulmonary target cell lines, the alveolar epithelial (LA4), the alveolar macrophage (MH-S) and the macrophage-monocyte-like (RAW 264.7) cell line.

For both PEIs, cytotoxicity was detected most prominently in the alveolar epithelial cells and only at high doses. Cytokine responses, in contrast were observed already at low PEI concentrations and could be divided into three groups, induced (i) by free PEI (IL-6, TNF-α, G-CSF), (ii) by PEI/siRNA complexes (CCL2, -5, CXCL1, -10), or (iii) unaffected by either treatment (IL-2, -4,-7, -9, and CCL3).

We conclude that even for the respiratory tissue both PEIs represent powerful siRNA delivery tools with reduced cytotoxicity and minor proinflammatory potency. However, in relation to response levels observed upon crystalline silica exposures, some PEI induced proapoptotic and proinflammatory responses might not be considered completely harmless, therefore further *in vivo* investigations are advisable.

1. Introduction

RNA interference (RNAi) represents a powerful method for specific gene silencing and RNAi therapeutics achieve a fundamentally new way to treat human diseases by activating selective mRNA cleavage for efficient ablation of the expression of any target gene (Aigner, 2006; Fougerolles de et al., 2007). Due to their instability and poor tissue and cell penetration, the delivery of RNAs to their sites of action is one of the most challenging aspects, particularly *in vivo*. The use of nanoparticles for encapsulating therapeutic agents represents an advanced class of drug delivery systems for both conventional drugs as well as for nucleic acids, i.e. plasmid DNA, coding for therapeutically relevant genes, antisense oligonucleotides and small interfering RNA (siRNA). For the induction of siRNA-mediated gene-targeting *in vivo* the efficient protection of siRNAs against enzymatic or non-enzymatic degradation is most important due to their short biological half-life. One promising strategy is the complexation of siRNAs with polyethylenimine (PEI). Initially, PEI was introduced as an efficient non-viral transfection reagent for the delivery of plasmid DNA, demonstrating high transfection efficiency *in vitro* and *in vivo* (Boussif et al., 1995). In more recent studies, the PEI-mediated delivery of nucleic acids was extended towards small RNA molecules, i.e. siRNAs (Urban-Klein et al., 2005). Due to its high cationic charge density and a large number of protonable nitrogen atoms, PEI is able to form stable, water-soluble non-covalent complexes with nucleic acids. These complexes are efficiently taken up by cells through endocytosis and subsequently, based on the so-called 'proton-sponge effect' (Behr, 1997), intracellularly released without the support of endosome disruptive agents for lysosomal escape. Since a high efficacy of nucleic acid delivery generally requires an excess of PEI

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDH, lactate dehydrogenase; LMW, low molecular weight; mRNA, messenger RNA; NaCl, sodium chloride; PEI, polyethylenimine; RNAi, RNA interference; siRNA, small interfering RNA.

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thus leading to a net positive surface charge, non-specific interactions of the complexes with negatively charged cellular structures may result in decreased efficiency and increased toxicity. To reduce PEI cytotoxicity, several studies have introduced modified PEIs such as block or graft copolymers containing cationic and hydrophilic non-ionic components (Park et al., 2006). Furthermore, increased biocompatibility without modifications of the polymer structure was achieved by employing low molecular weight PEIs, which displayed significantly reduced cytotoxicity (Fischer et al., 1999; Kunath et al., 2003). Additionally, it was shown that certain low molecular weight PEIs provide increased transfection efficacies for DNA as well as for siRNA. This is particularly true for the commercially available linear 22 kDa jet-PEI (Polyplus, France) as well as for the branched 4–10 kDa PEI F25-LMW, which was obtained through size fractionation of commercially available 25 kDa branched PEI by gel permeation chromatography (Werth et al., 2006).

With regard to the therapeutic application in humans, high efficacy as well as safety and biocompatibility are among the most critical issues for any gene delivery system including PEI. Therefore, in this study we focused on the two low molecular weight PEIs of different structure described above, which have already been employed *in vitro* and *in vivo*, and thus have been established as relevant delivery reagents for systemic application (Urban-Klein et al., 2005; Grzelinski et al., 2006; Werth et al., 2006; Hobel et al., 2008). Targeted delivery of polyplexes to the lung is an emerging area of gene therapy research. The main attraction of using sub-micron-sized particles, particularly below 300 nm in size for pulmonary delivery, is the observation that these particles tend to escape the detection by the macrophage clearance system and remain in the lung sufficiently long enough to release their 'payload' in a controlled manner (Oberdorster et al., 2005b). However, instilled non-biodegradable polystyrene nanospheres with small diameters and thus large surface areas have been shown to induce pulmonary inflammation (Brown et al., 2001). In comparison to micron-sized particles, a given mass of the sub-micron fraction is considered to be in particular hazardous and might be more reactive due to their increased surface area-to-mass ratio, since they not only escape clearance mechanisms resulting in increased retention time (Oberdorster et al., 2005b). In addition, upon pulmonary delivery of positively charged amine-polystyrene particles even systemic effects like enhanced thrombosis via platelet activation have been observed (Nemmar et al., 2003). Thus, *in vitro* toxicity studies are considered as an important adjunct to *in vivo* studies (Oberdorster et al., 2005a) and, due to possible toxic or inflammatory effects of the particles employed, need to precede any *in vivo* study also with regard to identifying optimal and maximum dosages.

To study cytotoxic and proinflammatory effects of nanoplexes in the lung, three cell lines were selected: the alveolar epithelial cells (LA4), the alveolar macrophages (MH-S) and the macrophage-monocyte-like cell line RAW 264.7. This selection of relevant cell lines is based on the fact that (i) for inhaled particles, the fragile, alveolar epithelium represents the first barrier, and (ii) macrophages are vital to the regulation of immune response and the development of inflammation.

In our study, cell-based assays for cytotoxic and proinflammatory endpoints were performed with two already established PEIs, namely branched PEI F25-LMW and linear jet-PEI, and their corresponding PEI/siRNA complexes, since the complexes may dissociate making the toxicity of free PEI a relevant issue (Akhtar and Benter, 2007). To benchmark putative adverse effects related to PEI exposure, we included respirable crystalline silica particles (α-quartz) as lung toxic positive control, which are considered as a well recognized health hazard known to cause pulmonary inflammation and severe lung diseases (Oberdorster et al., 2005b; Sayes et al., 2007).

Hence, the goal of this study was to evaluate the (dose-dependent) inflammatory and toxic effects of PEI and PEI/siRNA complexes, in order to assess the safety of these nanoplexes formulated as therapeutic aerosols. The correlation of these data with physicochemical particle properties may also help to design novel, optimized, non-viral vector systems for pulmonary application and to establish, which molecules are relevant to estimate.

2. Materials and methods

2.1. Polymers and particles

PEI F25-LMW was purified from 25 kDa branched PEI (Al 25-kDa, free base, water free, Sigma–Aldrich, Taufkirchen, Germany) by gel permeation chromatography as described previously (Werth et al., 2006). The obtained low molecular weight fraction of 25 kDa PEI was characterized by a size of 4–10 kDa and accordingly termed PEI F25-LMW. PEI F25-LMW featured highest transfection efficiency for different stable tumor cell lines (SKOV-3, SW-13, and ME-180) which was comparable to the commercially available jetPEI. For details see (Werth et al., 2006; Hobel et al., 2008). JetPEI was purchased from Polyplus (Ilkirch, France), and represents a linear polyethylenimine (PEI) with a molecular weight of ~22 kDa according to the informations provided by the manufacturer. Min-U-Sil 5 (crystalline silica, α-quartz) was obtained from US Silica Company, Berkeley Springs, WV, USA. According to the datasheet this frequently used reference material is characterized by a median diameter of 1.7 μm and a purity of 98% SiO_2.

2.2. Cell culture

Cell culture experiments were carried out using murine alveolar epithelial – like type II cells (LA4; ATCC No. CCL-196™) and murine alveolar macrophages (MH-S; ATCC No. CRL-2019) as well as the mouse leukaemic monocyte macrophage cell line RAW 264.7 (ATCC No. TIB-71). LA4 cells were grown in HAM's F12 medium with stable L-glutamine (Biochrom AG, Seromed, Germany) containing 15% fetal bovine serum (FBS, Gibco, Germany) and 1% non essential amino acids (Biochrom AG, Seromed, Germany) and 100U/ml penicillin and 100 mg/ml streptomycin (Biochrom AG Seromed, Germany), MH-S cells were cultured in RPMI 1640 (Biochrom AG, Seromed, Germany) with stable L-glutamine supplemented with 10% fetal bovine serum (FBS, Gibco, Germany), 50 μM 2-mercaptoethanol (Gibco, Germany), 10 mM HEPES (Gibco, Germany), 1 mM Na-pyruvate (Gibco, Germany), 50 U/ml penicillin and 50 mg/ml streptomycin (Biochrom AG Seromed, Germany), RAW 264.7 cells were grown in Dulbecco's modified Eagle's medium with stable L-glutamine (DMEM, Biochrom AG, Seromed, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco, Germany) and 100 U/ml penicillin and 100 mg/ml streptomycin (Biochrom AG Seromed, Germany). All cells were grown in a humidified incubator at 37 °C and 5% CO_2 and passaged every 2–3 days.

2.3. Exposure experiments

Cells were exposed to pure polymer (PEI F25-LMW, jetPEI), the corresponding polyplexes with siGL3, or Min-U-Sil 5 particles as reference particle. Polyplexes were prepared with the siRNA siGL3 (Sense: 5′ – CUU-ACG-CUG-AGU-ACU-UCG-ATT-3′; Antisense: 5′ – UCG-AAG-UAC-UCA-GCG-UAA-GTT-3′ MWG, Ebersberg, Germany) as described previously (Urban-Klein et al., 2005; Werth et al., 2006; Hobel et al., 2008). Briefly, appropriate amounts of siGL3 (10 μg) and PEI solution were each separately diluted in 50 μl HEPES (10 mM) buffered sodium chloride solution (150 mM), pH