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Cationic polyaspartamide-based nanocomplexes mediate siRNA entry and down-regulation of the pro-inflammatory mediator high mobility group box 1 in airway epithelial cells

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ABSTRACT

High-mobility group box 1 (HMGB1) is a nonhistone protein secreted by airway epithelial cells in hyperinflammatory diseases such as asthma. In order to down-regulate HMGB1 expression in airway epithelial cells, siRNA directed against HMGB1 was delivered through nanocomplexes based on a cationic copolymer of poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) by using H441 cells. Two copolymers were used in these experiments bearing respectively spermine side chains (PHEA-Spm) and both spermine and PEG_{2000} chains (PHEA-PEG-Spm).

PHEA-Spm and PHEA-PEG-Spm derivatives complexed dsDNA oligonucleotides with a w/w ratio of 1 and higher as shown by a gel retardation assay. PHEA-Spm and PHEA-PEG-Spm siRNA polyplexes were sized 350–650 nm and 100–400 nm respectively and ranged from negativity/neutrality (at 0.5 ratio) to positivity (at 5 ratio) as ζ potential. Polyplexes formed either at a ratio of 0.5 (partially complexing) or at the ratio of 5 (fully complexing) were tested in subsequent experiments. Epifluorescence revealed that nanocomplexes favored siRNA entry into H441 cells in comparison with naked siRNA. As determined by flow cytometry and a trypan blue assay, PHEA-Spm and PHEA-PEG-Spm allowed siRNA uptake in 42–47% and 30% of cells respectively, however only with PHEA-Spm at w/w ratio of 5 these percentages were significantly higher than those obtained with naked siRNA (20%). Naked siRNA or complexed scrambled siRNA did not exert any effect on HMGB1mRNA levels, whereas PHEA-Spm/siRNA at the w/w ratio of 5 down-regulated HMGB1 mRNA up to 58% of control levels (untransfected cells). PEGylated PHEA-Spm/siRNA nanocomplexes were able to down-regulate HMGB1 mRNA levels up to 61% of control cells. MTT assay revealed excellent biocompatibility of copolymer/siRNA polyplexes with cells.

In conclusion, we have found optimal conditions for down-regulation of HMGB1 by siRNA delivery mediated by polyaminoacidic polymers in airway epithelial cells in the absence of cytotoxicity. Functional and in-vivo studies are warranted.

Abbreviations: DPBS, dulbecco phosphate buffered saline; HMGB1, high-mobility group box 1; PHEA, β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide; PHEA-Spm, PHEA-spermine; PHEA-PEG-Spm, PHEA-polyethylene glycol-spermine; COPD, chronic obstructive pulmonary disease; MUC 8, mucin 8; DLS, dynamic light scattering; dsDNA, double stranded DNA; siRNA, short interfering RNA; DAPI, 4′6-diamino-2-phenylindole; PBS, phosphate buffer saline; EDTA, ethylenediaminetetracetic acid; FACS, fluorescence-activated cell sorting; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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1. Introduction

Nucleic acid-based drugs (NABDs) are a novel class of pharmaceuticals including small interfering RNA (siRNA) (Durcan et al., 2008; Pestourie et al., 2005), oligonucleotides that base their activity on gene silencing process. In the last years, RNA interference has been shown as a powerful tool for post-transcriptional gene silencing (Fujita et al., 2013) and has opened new avenues in gene therapy. However, its application and development in the medical field is currently limited by the lack of optimal delivery systems (Grassi et al., 2010). Indeed, entry of siRNAs into the cells is hampered by their negative charges on the phosphate groups which interact with negative phospholipids of the plasma membrane (Raemdonck et al., 2008). Moreover, once entered in the cytosol, naked siRNAs are rapidly degraded by intracellular nucleases (Mantei et al., 2008). Therefore, siRNAs require an appropriate vector to trespass the plasma membrane, evade nuclease degradation, and finally exert their activity.

HMGB1 (high mobility group protein B1), originally identified as a nuclear nonhistone protein with DNA-binding domains, can be secreted by cells and now regarded as an important endogenous danger signaling molecule. Besides as a signal of tissue injury, HMGB1 is considered a mediator of inflammation and high levels of HMGB1 are found in various inflammatory conditions such as sepsis, cystic fibrosis and rheumatoid arthritis (Karlsson et al., 2008; Rowe et al., 2008; Taniguchi et al., 2003; Wang et al., 2001). Interestingly, the concentrations of HMGB1 in asthmatic and COPD (chronic obstructive pulmonary disease) patients positively and significantly correlated with neutrophils counts and percentage of neutrophils in sputum (Hou et al., 2011). In another study, it has been demonstrated that HMGB1 neutralizing antibody attenuates neutrophilic inflammation in vivo by using a murine model of neutrophilic asthma induced by ovalbumin plus lipopolysaccharide (Zhang et al., 2014). However, the role of HMGB1 in respiratory diseases is still elusive but nevertheless these studies suggest an involvement of this cytokine in their pathogenesis.

Based on these considerations, the aim of this paper was that to explore the possibility to use two polyaminoacid copolymers based on the α,β-poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA), already successful tested for the in vitro delivery of plasmid, as delivery system for a siRNA-based approach aimed at silencing HMGB1.

In effect in the field of biocompatible synthetic polymers, PHEA has been used as drug carrier in the synthesis of macromolecular conjugates polymeric (Cavallaro et al., 2004a, b, 2006b), starting polymer for the synthesis of polymeric micelles (Caliceti et al., 2001; Cavallaro et al., 2003, 2004c; Salmaso et al., 2008), hydrogels (Casadei et al., 2008; Pitarresi et al., 2007) and polycations for gene therapy (Cavallaro et al., 2006a, 2009; Licciardi et al., 2006). However, considering that PHEA-copolymers bearing permanently positive charged groups in general showed a great condensing ability, excellent biocompatibility, but a not high DNA release capability, oligoamines (such as spermine), bearing amino groups protonable at physiological conditions, have been chosen to prepare PHEA copolymers in order to better modulate the DNA release (Cavallaro et al., 2008, 2010).

In this paper, polycation/siRNA polyplexes obtained at different weight ratios were characterized from the physico-chemical point of view in terms of ζ potential and size. The efficiency of siRNA delivery into H441 airway epithelial cells by PHEA-Spm and PHEA-PEG-Spm based polyplexes was also evaluated. We found that PHEA-based nanocomplexes down-regulated HMGB1 mRNA levels with the absence of cytotoxic effects.

2. Materials and methods

The synthesis of α,β-poly(*N*-2-hydroxyethyl)-co-[*N*-2-(spermidylcarbamate) ethylen]-D,L-aspartamide derivative (PHEA-Spm and PHEA-PEG-Spm) have been previously described (Cavallaro et al., 2008, 2010).

Active siRNA pool directed against HMGB1, scrambled negative control siRNA and Quasar 570-conjugated hGAPDH siRNA were purchased from Riboxx GmbH, Radebeul, Germany.

2.1. Dynamic light scattering measurement and zeta potential analysis

Dynamic light scattering studies (DLS) were performed at 25 °C with a Malvern Zetasizer Nano ZS instrument fitted with a 532 nm laser at a fixed scattering angle of 173°, using the Dispersion Technology Software 7.02. The PHEA-Spm and PHEA-PEG-Spm/siRNA polyplexes were prepared in Dulbecco's modified phosphate buffered saline (DPBS) nuclease free (pH 7.4), by adding to 50 μl of siRNA solution, at a concentration of 0.008 μg/μl, the same volume of PHEA-Spm or PHEA-PEG-Spm solutions at various concentration in order to obtain weight ratios in the range of 0.1–5. The intensity-average hydrodynamic diameter (nm), and polydispersity index (PI) were obtained by cumulative analysis of the correlation function.

Polyplex containing solutions were then diluted with 400 μl of DPBS nuclease free and used to determine the ζ potential of PHEA-Spm and PHEA-PEG-Spm/siRNA complexes at the above mentioned weight ratio ranges. Zeta potential measurements were performed by aqueous electrophoresis measurements, recorded at 25 °C using the same apparatus. The ζ potential values (mV) were calculated using the Smoluchowski relationship.

2.2. Gel retardation assay

One volume of solvent (water molecular biology grade, Euroclone, Milan, Italy) containing double strand DNA 21-nt oligonucleotide (ODN; AT content = 52%, GC content = 48%) at a concentration of 0.1 μg/μl was mixed with one volume of the same solvent containing various amount of polymer (PHEA-Spm or PHEA-PEG-Spm) so as to obtain weight ratios ranging from 0.1 to 10. After 30 min of incubation at 37 °C, complexes were loaded onto 0.7% agarose gel containing ethidium bromide using uncomplexed ODN as running control.

2.3. Transfections

Cells were seeded on to 24-well plates at 5–6 × 10^4 per well to obtain 70–80% confluence after 48 h. Complexes prepared with 30 nM siRNA in 100 μl of water molecular biology grade transfection solution at room temperature for 30 min, then added with 400 μl of Opti-MEM (Life Technologies, Milan, Italy) and finally added to each well. In the epifluorescence and flow cytometry experiments, cells were incubated for 4 h in Opti-MEM and then analysed. For silencing experiments, cells were incubated for 4 h and then the transfection medium was substituted with RPMI medium (Sigma–Aldrich, Milan, Italy), containing 10% FBS, 1% streptomycin and 1% penicillin (complete RPMI medium) for further 20 h.

2.4. Epifluorescence

H441 cells (1 × 10^5) were plated on glass coverslips (12 mm circles, fit in 24-well dishes) in complete RPMI 24 h before transfection. The day after, cells were transfected. After 4 h, cell nuclei were labelled with the 4′6-diamidino-2-phenylindole (DAPI) fluorescent dye for 5 min, then cells were overlayed with a drop of fluorescent mounting medium (Dako, Milan, Italy) followed by a coverslip. Samples were analyzed with a Nikon