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GENE DELIVERY

Reduced *in vitro* and *in vivo* toxicity of siRNA-lipoplexes with addition of polyglutamate

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ABSTRACT

We previously designed a new siRNA vector that efficiently silences genes *in vitro* and *in vivo*. The vector originality is based on the fact that, in addition to the siRNA molecule, it contains two components: 1) a cationic liposome that auto-associates with the siRNA to form particles called "lipoplexes" and, 2) an anionic polymer which enhances the lipoplex's efficiency. This anionic polymer can be a nucleic acid, a polypeptide or a polysaccharide. We show here how the nature of the added anionic polymer into our siRNA delivery system impacts the toxic effects induced by siRNA lipoplexes. We first observed that: (i) siRNA lipoplexes-induced toxicity was cell line dependent, tumoral cell lines being the more sensitive; and (ii) plasmid DNA-containing siRNA lipoplexes were more toxic than polyglutamate-containing ones or cationic liposomes. We next determined that the toxicity induced by plasmid-containing lipoplexes is a long-lasting effect that decreased cell survival capacity for several generations. We also found that treated cells underwent death following apoptosis pathway. Systemic injection to mice of siRNA lipoplexes, rather than of cationic liposome, triggered a production of several cytokines in mice and replacement of plasmid by polyglutamate reduced the elevation of all assayed cytokines.
In order to enhance siRNA lipoplexes efficiency, the addition of polyglutamate as anionic polymer should be preferred to plasmid DNA as far as *in vitro* as well as *in vivo* toxicity is concerned.

1. Introduction

RNA interference, a natural cellular process that regulates gene expression through a highly precise mechanism of sequence-directed gene silencing, could theoretically be used to target any disease-associated pathogenic gene of interest. The most difficult hurdle in developing RNAi-based therapies, however, is the delivery of the RNAi molecule (also called siRNA, for small interfering RNA) to the target tissue. As small synthetic RNA duplexes, naked siRNAs face rapid degradation in the extracellular environment and are not efficiently internalized into cells. As a consequence, the use of siRNA delivery vehicles is essential for practical siRNA-mediated silencing. Since the first report of *in vivo* delivery of siRNA [1], there have been numerous studies of *in vivo* gene silencing with siRNAs (reviewed in [2]), relying on the development of siRNA delivery systems, including hydrodynamic injection of synthetic siRNA, delivery using lipid-based agents and various carriers, such as atelocollagen, a protamine-antibody fusion protein and polyethyleneimine, as well as local administration. Over the past decade at least 21 siRNA therapeutics have been developed for various diseases, including cancer, infectious diseases or genetic disorders [3].

Nevertheless, during the development of these siRNA-based therapies, it has become clear that siRNAs commonly delivered to induce RNAi can also induce multiple nonspecific effects, such as a saturation of cellular proteins involved in RNAi and miRNA pathways, a sequence-specific targeting of unintended transcripts (off-target effect) and an activation of cellular sensors of foreign RNA and their downstream effects leading to interferon induction and cell death [4,5]. Indeed, nucleic acids are key structures sensed by the innate immune system, which comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner [6]. The corresponding receptors for foreign nucleic acids include members of Toll-like receptors (TLR), RIG-I-like receptors, and intracellular DNA sensors [7,8].

The choice of delivery strategy also impacts whether a siRNA will induce an innate immune response. In trafficking from the extracellular environment, through endosomal compartments and to the cytoplasm, there are multiple points at which recognition of siRNA by the innate immune system may occur. TLR-mediated recognition of

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siRNA takes place in endosomes. A siRNA may be immunologically inert when delivered as a naked siRNA but will stimulate immunity when complexed with a delivery vehicle. Such effects have been observed using cationic lipids, such as DOTAP [9].

We have previously shown that the pre-mixture of an anionic polymer with siRNA before forming a nucleic acid–cationic lipid complex, or lipoplex, by association with a cationic liposome led to more efficient complexes than the ones formed with siRNA alone [10,11]. Hence, several types of anionic polymer can be added in the formulation, namely polypeptide, polysaccharide or nucleic acid. Among these polymers, the most powerful to enhance siRNA lipoplexes efficiency is plasmid DNA (pDNA), as evidenced both in *in vitro* and *in vivo* experiments [11,12]. However, at high concentrations these pDNA-containing siRNA lipoplexes are far more toxic than siRNA lipoplexes containing other polymers, as evidenced by MTT-assay on mouse melanoma B16 cells [11]. In this study, we attempted to evaluate how the nature of the added anionic polymer into our siRNA delivery system impacts the toxic effects induced by siRNA lipoplexes. We then characterized the siRNA lipoplexes induced toxicity in transfected cell lines and in i.v. treated mice.

2. Materials and methods

2.1. Cell culture

B16-F0 (ATCC-CRL-6322), NIH-3T3 (ATCC-CRL-1658), J774A.1 (ATCC-TIB-67) and CT26.WT (ATCC-CRL-2638) cells were from LGC Promochem and were grown in DMEM with Glutamax (Gibco), streptomycin (100 μg/ml) and penicillin (100 U/ml), and 10% fetal calf serum (FCS) for B16-F0, J774.A1A.1 and CT26.WT and 10% calf serum for NIH-3T3. Cultures were maintained at 37 °C in a 5% CO_2/air incubator.

2.2. siRNA, plasmids and polymers

Unmodified and non-silencing siRNA (5′ UUC UCC GAA CGU GUC ACG UdTdT 3′) were obtained from Eurogentec. Plasmids added in lipoplexes were purified with a QIAGEN Plasmid Endofree Maxi Kit. Endotoxin contaminants were estimated by limulus amoebocyte lysate (LAL) assay, and were found to represent less than 0.1 EU/μg of plasmid, a concentration usually considered endotoxin-free. Various plasmids were used: (i) pSL301 (Invitrogen) non-coding and containing 5 CpG motifs known to be optimal sequences for activating mouse TLR9, (ii) pXL3296 [13] non-coding and containing no optimal CpG sequences for activating mouse TLR9, (iii) pVaxLuc [14] encodes the engineered cytoplasmic luciferase gene (Luc+) under CMVβ promoter, (iv) pVaxLuc w/o promoter is pVaxLuc deleted from its promoter, and (v) pSL301 + promoter is pSL301 with addition of CMVβ promoter. Synthetic oligonucleotides were from Eurogentec. Sodium poly-L-glutamate (P1818) and polyIC (P1530) were obtained from Sigma-Aldrich. Yeast tRNA was obtained from Invitrogen. Genomic DNA was prepared from mammalian or bacterial cell culture and prepared as described [15].

2.3. Preparation of cationic liposomes and siRNA lipoplexes

Cationic liposome was prepared as described [11] from an equimolar mixture of DMAPAP (2-{3-[bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecyl carbamoyl methyl-acetamide) cationic lipid and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, Avanti Polar Lipids). Lipoplexes were prepared by mixing an equal volume of the siRNA/anionic polymer (ratio 1/1; w/w) solution diluted in 150 mM NaCl (*in vitro* transfection) or 5% glucose (*in vivo* injection) to the cationic liposome suspension also in 150 mM NaCl or 5% glucose and rapidly mixed by vortexing. Lipoplexes were allowed to form for 30 min at room temperature before use. We have previously showed that the addition of anionic polymers such as polyglutamate to siRNA lipoplexes led to the formation of particles with similar characteristics to crude siRNA lipoplexes in terms of size, structure and stability [11]. We have also evaluated the siRNA loading capacity of lipoplexes prepared with polyglutamate or pDNA by gel retardation [11] or by measuring the accessibility of the fluorescent dye Picogreen to nucleic acid present in the complexes (supplementary figure), as described in [10], and showed that in these particles siRNA is 100% encapsulated. The ratio of (1/1 w/w) for siRNA/polyglutamate ratio was previously identified as the optimal ratio in terms of gene knock-down efficiency [39]. Upon incubation to cells, siRNA lipoplexes prepared with polyglutamate were taken up in cytosolic vesicles as was observed to classical siRNA lipoplexes [39]. The charge ratio was calculated as the molar ratio of positive charges (3 positive charges per molecule of DMAPAP) to the molar ratio of negative charges from the mixture of siRNA and anionic polymer molecules (3.08 nmol negative charges per μg of nucleic acid molecules and 6.62 nmol negative charges per μg of sodium polyglutamate). When it was necessary to compare lipoplexes containing the same amount of cationic lipid, lipoplexes without anionic polymer were prepared with twice as much siRNA as lipoplexes with anionic polymer, to compensate for the final amount of lipid.

2.4. Cytotoxicity assays

MTT assay: Cells were plated onto 96-well plates at 4000 cells per well in 100 μl of culture medium. Twenty-four hours after plating, 100 μl of transfection medium to be tested, prepared as described above, was added to the cells (in triplicate) and incubated as described above. After a 48 h-exposure period, cell viability was assayed using the MTT test, as described [10]. Appropriate controls with DMEM only and MTT were run to subtract background absorbance. Results were expressed relative to non-transfected cells. Trypan Blue assay: Cells were plated onto 24-well plates at 40,000 cells per well in 1 ml of culture medium. Twenty-four hours after plating, 1 ml of transfection medium to be tested, prepared as described above, was added to the cells (in duplicate) and incubated as described above. After a 48 h-exposure period, cells were detached, incubated in Trypan Blue (0.2% in PBS), and the total number of cells and the number of stained cells were counted on microscope. Results were expressed relative to non-transfected cells. Plating efficiency assay: Viable cells identified in Trypan Blue assay (see above) were seeded onto Petri dishes (diameter 9 cm) at 100 viable cells/10 ml of culture medium and incubated until colonies formed. Cells were then fixed in absolute methanol and stained for 10 min in 1% crystal violet. After washing and drying, colonies were counted and results were expressed relative to non-transfected cells. Attachment assay: Viable cells identified in Trypan Blue assay (see above) were seeded onto 96-well plates at 25,000 cells per well in 200 μl of culture medium and incubated for 20 min at 37 °C. Non-adherent and loosely attached cells were removed by gentle wash and attached cells were fixed by addition of glutaraldehyde (5%, w/v). After washing, cells were stained with 0.1% crystal violet for 60 min. After washing, dye was solubilized in 10% (v/v) acetic acid and absorbance was measured at 570 nm. Spreading assay: Cells were prepared as above (attachment assay) and fixed at the end of incubation by direct addition of 50% glutaraldehyde, without any washing step. Morphology of cells was evaluated using an inverted phase contrast microscope.

2.5. Apoptosis assay

B16F0 cells were treated as in Trypan Blue assay (see above). At 12 h, 24 h or 48 h after the onset of transfection, cells were detached and the percent of late apoptotic cells was determined using FlowCellect™ Annexin Red Kit and Guava Easycyte™ (Millipore).