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Transferrin-bearing polypropylenimine dendrimer for targeted gene delivery to the brain

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ABSTRACT

The possibility of using genes as medicines to treat brain diseases is currently limited by the lack of safe and efficacious delivery systems able to cross the blood–brain barrier, thus resulting in a failure to reach the brain after intravenous administration.
On the basis that iron can effectively reach the brain by using transferrin receptors for crossing the blood–brain barrier, we propose to investigate if a transferrin-bearing generation 3-polypropylenimine dendrimer would allow the transport of plasmid DNA to the brain after intravenous administration.
In vitro, the conjugation of transferrin to the polypropylenimine dendrimer increased the DNA uptake by bEnd.3 murine brain endothelioma cells overexpressing transferrin receptors, by about 1.4-fold and 2.3-fold compared to that observed with the non-targeted dendriplex and naked DNA. This DNA uptake appeared to be optimal following 2 h incubation with the treatment.
In vivo, the intravenous injection of transferrin-bearing dendriplex more than doubled the gene expression in the brain compared to the unmodified dendriplex, while decreasing the non-specific gene expression in the lung. Gene expression was at least 3-fold higher in the brain than in any tested peripheral organs and was at its highest 24 h following the injection of the treatments.
These results suggest that transferrin-bearing polypropylenimine dendrimer is a highly promising gene delivery system to the brain.

1. Introduction

Gene therapy has emerged as a promising strategy to treat cerebral diseases such as glioma, Alzheimer's and Parkinson's diseases, which affect a large percentage of the world's population and hardly respond to intravenously administered, small molecule treatment [1–4]. Although the genetic basis for many of these diseases is known, the possibility of using genes as medicines is currently limited by the lack of safe and efficacious delivery systems able to cross the blood–brain barrier (BBB) and to deliver DNA to the brain after intravenous administration.

The BBB acts as an entrance gateway, restricting the movement of ions and nutrients to the central nervous system while protecting the brain against harmful blood-borne substances and invading organisms [2,5]. Its permeability properties prevent the delivery of more than 98% of drugs, including nucleic acids, to the brain [2,3]. In addition, locally administered treatments fail to achieve a widespread gene expression in the target cells throughout the entire brain, which is necessary for a successful treatment of most cerebral pathologies [2,3,6].

However, the BBB does possess specific receptor-mediated transport mechanisms that can potentially be exploited as a means to target drugs and genes to the brain. The transferrin receptor (TfR) is of particular interest because it is overexpressed on the brain capillary endothelial cells [7]. The antibodies that bind to the TfR have been shown to selectively target the brain microvascular endothelium due to the high levels of TfR expressed by these cells [8–10]. This strategy has been widely investigated for the delivery of drugs and genes to the brain [11].

Several strategies have been explored to formulate TfR-targeted delivery systems able to transport nucleic acids to the brain following intravenous administration [11]. Numerous non-viral gene delivery systems are currently under development, due to their low immunogenicity, stability, unrestricted plasmid size and versatility in types of modifications [12,13]. Among these delivery systems, generation 3-diaminobutyric polypropylenimine dendrimer (DAB) appears to be particularly promising. We recently prepared a transferrin (Tf)-bearing generation 3-diaminobutyric polypropylenimine dendrimer (DAB-Tf), able to increase the cellular uptake and gene expression of DNA by cancer cells overexpressing transferrin receptors compared to non-targeted delivery systems, *in vitro* and *in vivo* [14]. Importantly, the treatment was well tolerated by the animals, with no apparent signs of toxicity.

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Building on this study, we now would like to investigate if this Tf-bearing gene delivery system could improve the delivery of DNA to the brain, *in vitro* and *in vivo* following intravenous administration.

2. Materials and methods

2.1. Cell lines and reagents

Human holo-transferrin, generation 3-diaminobutyric polypropylenimine dendrimer (DAB), dimethylsuberimidate and all other chemicals and reagents that are not specifically mentioned below were obtained from Sigma-Aldrich (Poole, UK). The expression plasmids encoding β-galactosidase (pCMVsport β-galactosidase) and tdTomato (pCMV-tdTomato) were respectively purchased from Invitrogen (Paisley, UK) and Clontech (Mountain View, CA). They were propagated in *Escherichia coli* and purified using an Endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany). Vectashield® mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) came from Vector Laboratories (Peterborough, UK). Passive lysis buffer, Label IT® Cy3- and fluorescein-Nucleic Acid Labeling kits were respectively obtained from Promega (Southampton, UK) and Cambridge Biosciences (Cambridge, UK). bEnd.3 murine brain capillary endothelial cell line was purchased from LGC Standards (Teddington, UK), while cell culture media were obtained from Invitrogen (Paisley, UK).

2.2. Synthesis and characterization of transferrin- bearing DAB dendrimer

Transferrin (Tf) was conjugated to generation 3-diaminobutyric polypropylenimine dendrimer (DAB) by using dimethylsuberimidate (DMSI) as a cross-linking agent, as previously reported [14,15]. DAB (24 mg) was added to transferrin (6 mg) and dimethylsuberimidate (12 mg) in triethanolamine HCl buffer (pH 7.4, 2 mL). The reaction took place for 2 h at 25 °C while stirring. The conjugate was purified by size exclusion chromatography using a Sephadex G75 column and freeze-dried. The conjugation of Tf to DAB was assessed by ^{1}H NMR spectroscopy using an Oxford NMR AS 400 spectrometer (Jeol, Peobody, MA).

2.3. In vitro biological characterization

2.3.1. Cell culture

Immortalized bEnd.3 cells overexpressing Tf receptors were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 0.5% (v/v) penicillin-streptomycin. Cells were cultured at 37 °C in a humid atmosphere of 5% carbon dioxide.

2.3.2. Cellular uptake

Imaging of the cellular uptake of the DNA carried by DAB-Tf was carried out using epifluorescence microscopy. Labeling of the β-galactosidase-encoding plasmid DNA with the fluorescent probe Cy3 was performed using a Label IT® Cy3 Nucleic Acid Labeling kit, as described by the manufacturer. bEnd.3 cells were seeded on coverslips in 6-well plates (10^4 cells/well) and grown at 37 °C for 72 h. They were then incubated for different durations (15, 30, 45, 60, 120, 240 min) with Cy3-labeled DNA (2.5 μg DNA/well) complexed to DAB-Tf at the dendrimer:DNA weight ratio of 10:1. The cells were then washed three times with PBS and fixed with methanol for 10 min. Upon staining of the nuclei with DAPI, the cells were examined using an E600FN Upright Epifluorescence microscope (Nikon, Tokyo, Japan). DAPI was excited with the 365 nm CoolLED pE excitation system (bandwidth: 435-485 nm), whereas Cy3 was excited with the 470 nm CoolLED pE excitation system (bandwidth: 515-555 nm).

Once the treatment duration allowing maximal DNA uptake was determined, a similar procedure was performed to compare the cellular uptake of Cy3-labeled DNA (2.5 μg/well) complexed to DAB-Tf and DAB (dendrimer:DNA weight ratios respectively of 10:1 and 5:1) [14,16] during the optimized treatment duration. Control samples were treated with naked DNA or remained untreated.

Quantification of cellular uptake was performed using flow cytometry. Labeling of plasmid DNA with the fluorescent probe fluorescein was performed using a Label IT® Fluorescein Nucleic Acid Labeling kit, as described by the manufacturer. bEnd3 cells were grown in 6-well plates (1.6×10^5 cells/ well) at 37 °C for 72 h. The cells were then treated with fluorescein-labeled DNA (5 μg DNA/well), alone or complexed to DAB-Tf and DAB (dendrimer:DNA weight ratios respectively of 10:1 and 5:1). Untreated cells served as a negative control. After 2 h incubation with the treatments, single cell suspensions were prepared, washed (2 mL PBS pH 7.4 per well) and pelleted (378 g for 8 min) 3 times, before being analyzed using a FACSCanto® flow cytometer (BD, Franklin Lakes, NJ). Ten thousand cells (gated events) were counted for each sample. Their mean fluorescence intensity was analyzed with FACSDiva® software (BD, Franklin Lakes, NJ).

2.3.3. Mechanisms of cellular uptake of DNA complexed to DAB-Tf dendriplex

The mechanisms involved in the cellular uptake of DNA complexed to DAB-Tf dendriplex were investigated by treatment with uptake inhibitors and escalating concentrations of free Tf. Cells were seeded and grown as described above. After removal of the medium, they were then pre-treated with phenylarsine oxide (10 μmol/L), filipin (5 μg/mL), colchicine (10 μmol/L), poly-L-lysine (400 μg/mL) and various concentrations of free Tf ranging from 2.5 to 20 μmol/L for 10 min at 37 °C. The cells were then treated with Cy3- or fluorescein-labeled DNA (respectively 2.5 and 5 μg/well for qualitative and quantitative analysis) complexed to DAB-Tf for 2 h, before being washed and processed for fluorescence microscopy and flow cytometer analysis as described above.

2.3.4. In vitro transfection

Transfection efficacy of the DNA carried by DAB-Tf dendrimer was assessed with a plasmid DNA encoding β-galactosidase (pCMV βgal), using a β-galactosidase transfection assay. bEnd.3 cells were seeded at a density of 2000 cells/well in 96-well plates (n = 15). After 72 h incubation, the cells were treated with the DAB-Tf dendriplex at the dendrimer:DNA weight ratio of 10:1, which has previously been shown to give the highest transfection on other cancer cell lines [14,15]. DNA concentration (10 μg/mL) was kept constant for all the formulations tested. Naked DNA served as a negative control, DAB-DNA (dendrimer:DNA weight ratio 5:1) served as a positive control. After 72 h incubation, cells were lysed with 1× passive lysis buffer (PLB) (50 μL/well) for 20 min. The cell lysates were subsequently analyzed for β-galactosidase expression. Briefly, 50 μL of the assay buffer (2 mM magnesium chloride, 100 mM mercaptoethanol, 1.33 mg/mL o-nitrophenol-β-galactopyranoside, 200 mM sodium phosphate buffer, pH 7.3) was added to each well containing the lysates. After 2 h incubation at 37 °C, the absorbance of the samples was read at 405 nm with a Multiscan Ascent® plate reader (Thermo Scientific, Waltham, MA).

2.4. In vivo study

2.4.1. Animals

Female BALB/c mice were housed in groups of five at 19 °C to 23 °C with a 12-h light-dark cycle. They were fed a conventional diet (Rat and Mouse Standard Expanded, B&K Universal, Grimston, UK) with mains water ad libitum. The *in vivo* experiments described below were approved by the local ethics committee and performed in accordance with the UK Home Office regulations.

2.4.2. Biodistribution of gene expression

The biodistribution of gene expression was visualized by bioluminescence imaging, using an IVIS Spectrum® (PerkinElmer, Waltham, MA).