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Tumor regression following intravenous administration of lactoferrin- and lactoferricin-bearing dendriplexes

Li Ying Lim, MPharm[a], Pei Yin Koh, MPharm[a], Sukrut Somani, MSc[a], Majed Al Robaian, MPharm[a], Reatul Karim, MRes[a], Yi Lyn Yean, MPharm[a], Jennifer Mitchell, MPharm[a], Rothwelle J. Tate, PhD[a], RuAngelie Edrada-Ebel, PhD[a], David R. Blatchford, CBiol, MBS[a], Margaret Mullin[b], Christine Dufès, PhD[a,*]

[a]*Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom*
[b]*College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom*

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Abstract

The possibility of using gene therapy for the treatment of cancer is limited by the lack of safe, intravenously administered delivery systems able to selectively deliver therapeutic genes to tumors. In this study, we investigated if the conjugation of the polypropylenimine dendrimer to lactoferrin and lactoferricin, whose receptors are overexpressed on cancer cells, could result in a selective gene delivery to tumors and a subsequently enhanced therapeutic efficacy. The conjugation of lactoferrin and lactoferricin to the dendrimer significantly increased the gene expression in the tumor while decreasing the non-specific gene expression in the liver. Consequently, the intravenous administration of the targeted dendriplexes encoding TNFα led to the complete suppression of 60% of A431 tumors and up to 50% of B16-F10 tumors over one month. The treatment was well tolerated by the animals. These results suggest that these novel lactoferrin- and lactoferricin-bearing dendrimers are promising gene delivery systems for cancer therapy.

Key words: Cancer therapy; Gene delivery; Dendrimer; Lactoferrin; Lactoferricin

Introduction

Despite numerous advances in the field of cancer gene therapy, the use of therapeutic genes in cancer treatment is still limited by the lack of safe, intravenously administered delivery systems able to carry therapeutic DNA selectively to the tumors, without secondary effects to healthy tissues.[1]

In order to remediate to this problem, numerous non-viral gene delivery systems are currently under development, due to advantages such as their low toxicity, stability and high flexibility regarding the size of the transgene delivered.[2,3] Among these delivery systems, generation 3 diaminobutyric polypropylenimine dendrimer (DAB) appears to be particularly promising. We have recently demonstrated that the intravenous administration of this dendrimer conjugated to transferrin (Tf), whose receptors are overexpressed on cancer cells, resulted in gene expression mainly in the tumors after intravenous administration.[4] Thus, DAB-Tf dendrimer complexed to a TNFα-encoding DNA led to a rapid and sustained tumor regression over one month, resulting in complete suppression of 90% of the tested A431 tumors and regression of the remaining 10%.[4] Importantly, the treatment was well tolerated by the animals, with no apparent signs of toxicity.

Building on this study, we now would like to develop a novel gene-based therapeutic system with improved tumor targeting and therapeutic efficacy. To do so, we propose to replace the

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*Corresponding author at: Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom.
E-mail address: C.Dufes@strath.ac.uk (C. Dufès).

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transferrin moiety by other promising tumor-targeting ligands of the same family that have been shown to have intrinsic anti-tumoral activity, such as lactoferrin and lactoferricin.

Lactoferrin (LF) and lactoferricin (LFC) are iron-binding members of the transferrin family, able to bind the transferrin receptors. In addition to their tumor delivery properties, these iron-carriers have recently been shown to have anti-cancer properties themselves, which make them highly attractive as part of a gene medicine.

LF has been shown to inhibit the proliferation of many cancer cell lines through induction of cell cycle arrest and modulation of the mitogen-activated protein kinase signaling pathway *in vitro*.[5] The inhibition of tumor cell growth by LF may also be related to the ability of this protein to induce apoptosis of cancer cells by activating the Fas signaling pathway in cancerous cells.

Like LF, LFC has been shown to exert anti-tumor effects against a number of cancer cell lines. LFC is a potent inducer of apoptosis in various cancer types.[6] LFC has also been reported to exert potent *in vivo* anti-tumor activity in mouse models of cancer. For example, direct injection of LFC into solid Meth A tumors causes tumor cell lysis and reduction in tumor size.[7] In addition, subcutaneous administration of LFC inhibits tumor metastasis by metastatic murine L5178Y-ML25 lymphoma cells and B16-F10 melanoma cells.[8] We therefore hypothesize that using LF and LFC as tumor-targeted ligands could improve the overall efficacy of the DAB delivery system.

The objectives of this study were therefore 1) to prepare and characterize lactoferrin- and lactoferricin-bearing DAB dendrimers and 2) to evaluate their targeting and therapeutic efficacy on cancer cells *in vitro* and *in vivo* after intravenous administration.

Methods

Cell lines and reagents

Lactoferrin and lactoferricin, generation 3-diaminobutyric polypropylenimine dendrimer (DAB) and the other chemicals were purchased from Sigma Aldrich (Poole, UK). The expression plasmids encoding Tumor necrosis factor (TNF) α (pORF9-mTNFα) and β-galactosidase (pCMVsport β-galactosidase) were obtained respectively from InvivoGen (San Diego, CA) and Invitrogen (Paisley, UK) and were purified using an Endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany). Passive lysis buffer was from Promega (Southampton, UK). Quanti-iT™ PicoGreen® dsDNA reagent and tissue culture media were obtained from Invitrogen (Paisley, UK). Bioware® B16-F10-luc-G5 mouse melanoma was obtained from Caliper Life Sciences (Hopkinton, MA). A431 human epidermoid carcinoma and T98G human glioblastoma were purchased from the European Collection of Cell Cultures (Salisbury, UK).

Synthesis and characterization of lactoferrin- and lactoferricin-bearing DAB dendrimers

Conjugation of lactoferrin and lactoferricin to DAB

Lactoferrin (LF) and lactoferricin (LFC) were conjugated to generation 3- diaminobutyric polypropylenimine dendrimer (DAB) in a similar manner to that we previously reported for the preparation of other conjugates.[4,9–12] DAB (24 mg) was added to lactoferrin or lactoferricin (6 mg) and dimethylsuberimidate (12 mg) in triethanolamine HCl buffer (pH 7.4, 2 mL). The coupling reaction was allowed to take place for 2 h at 25 °C whilst stirring. The conjugates were purified by size exclusion chromatography using a Sephadex G75 column and freeze-dried. The grafting of lactoferrin and lactoferricin to DAB was assessed by ^{1}H NMR spectroscopy using a Jeol Oxford NMR AS 400 spectrometer.

Characterization of dendriplex formation

The ability of DNA to form complexes with DAB-LF and DAB-LFC dendrimers was assessed by PicoGreen® assay, following the protocol provided by the supplier. PicoGreen® reagent was diluted 200-fold in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.5) on the day of the experiment. One milliliter of PicoGreen® solution was added to 1 mL of dendrimer–DNA complexes prepared at various dendrimer:DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1, 0:1). The DNA concentration in the complexes (10 μg/mL) was kept constant during the experiment. The fluorescence intensity of the complexes was analyzed at various time points with a Varian Cary Eclipse Fluorescence spectrophotometer (Palo Alto, CA) (λ_{exc}: 480 nm, λ_{em}: 520 nm). Results were represented as percentage of DNA condensation and compared with those obtained for DAB-DNA complex (dendrimer:DNA weight ratio 5:1) (n = 4).

DNA condensation ability of DAB-LF and DAB-LFC was also assessed by agarose gel retardation assay (Supplementary data). Nanoparticles of DAB-LF and DAB-LFC complexed with DNA were also visualized by transmission electron microscopy[10] (Supplementary data).

Dendriplex size and zeta potential measurement

Size and zeta potential of DAB-LF and DAB-LFC dendriplexes prepared at various dendrimer:DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1, 0:1) were measured by photon correlation spectroscopy and laser Doppler electrophoresis using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK).

In vitro biological characterization

Cell culture

A431, T98G and B16-F10-luc-G5 cell lines overexpressing Tf receptors were grown as monolayers in DMEM (for A431 and T98G cells) or RPMI-1640 medium (for B16-F10-luc-G5 cells) 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.

In vitro transfection

Transfection efficacy of the DNA carried by DAB-LF and DAB-LFC dendrimers was assessed by a β-galactosidase transfection assay, using a plasmid DNA encoding β-galactosidase. A431, B16-F10 and T98G cells were seeded in quintuplicate at a density of 2 000 cells/well in 96-well plates. After 72 h incubation, the cells were treated with the DAB-LF and DAB-LFC dendriplexes at the following dendrimer:DNA weight ratios: 20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1, 0:1. 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