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Cationic liposome–DNA complexes as gene delivery vectors: Development and behaviour towards bone-like cells

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Abstract

Modulation of the biological pathways responsible for fracture repair and osteogenisis may accelerate regeneration. Gene therapy is an alternative method for the release of osteogenisis-stimulating proteins into tissues. The development of vectors for gene release is still a problem in terms of ethics and techniques. In this work we evaluated whether cationic liposomes constitute a valuable strategy for the release of genetic material into bone tissue cells as non-viral vectors. Liposomes were prepared with 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)–2-dioleoyl-sn-glycero-3-phosphatidylethanolamine and DOTAP–cholesterol, and characterized according to their size, zeta potential, DNA protection capacity and cytotoxicity. Transfection studies were also carried out using pCMVβ-gal plasmid in two osteoblastic cell lines (MG63 and MC3T3-E1) and in the 294T line, varying the charge ratio and the applied DNA dose. Inclusion of transferrin to increase the expression was also tested. The results suggest that there is great dependency between the transfection activity and the lipid formulation, the charge ratios of the complexes, the applied DNA dose and the cell type. There were even some differences concerning both osteoblastic lines under study. The cells of the MC3T3-E1 line present greater expression levels than the cells of the MG-63 line. The conjugation of the transferrin with the complexes contributes to the increase in transfection levels, possibly due to an increase in internalization of complexes. It is thus a good strategy for inducing the expression of specific genes in osteoblast-like cells.

Keywords: Cationic liposomes; Gene therapy; Osteogenesis; Osteoblast

1. Introduction

The molecular mechanism underlying bone formation and repair is a complex and highly coordinated process that is not completely understood. Despite this, bone is one the few organs that retains the potential for regeneration into adult life. Fracture repair and segmental bone defect healing are commonly attempted procedures in orthopaedic surgery. Aged-induced bone illnesses, like osteoporosis, may produce bone defects whose dimensions limit the mechanisms of self-repair [1,2]. There are many clinical options for treating bone defects, including bone allograft, bone autograft, biomaterial implants and amputation. However, there are many problems associated with these procedures: lack of sufficient material for bone autografts; risk of pathological transmission and impaired immune responses in bone allograft and xenographs; the failure of biomaterials; and biological incompatibility [3–5]. These difficulties have resulted in the search for another method to repair skeletal defects, namely gene therapy. Although the molecular mechanism underlying bone formation is yet to be defined, it is known that bone morphogenic proteins (BMPs) are essential to osteoinduc-

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tion, which is the activation of various growth factors to attract osteoblasts to the repair site and induce them to produce bone [6]. In such cases, BMPs have been reported to be effective in enhancing bone deposition. However, several problems are associated with it, in particular: requirement of large doses; a short half-life and thus short-term bioavailability; and the lack of a practical method for sustained delivery of these exogenous proteins [5,7]. Gene therapy could provide an alternative method for the delivery of BMPs into tissues, thus stimulating osteogenesis.

Gene therapy is defined as the introduction of exogenous genetic material into cells or tissues in order to cure a disease or to avoid associated symptoms [8]. Research into osteogenic factor delivery for bone repair has shown endogenous BMP production by transfected cells at the fracture site to be more efficient than exogenous delivery of recombinant proteins, as indicated by the smaller amount of BMP required to stimulate healing [9,10]. Both non-viral and viral vectors have been used to mediate the transfer of genes encoding BMPs into target cells, with the aim of promoting bone regeneration. It is also crucial that gene delivery vectors are able: to accommodate an unlimited size of DNA; to be available in a concentrated form; to be easy to scale up; to be targeted to specific cells or tissues; to ensure high levels and long term gene expression; and to be non-toxic and non-immunogenic. A number of viral delivery systems are available for gene delivery, in particular retroviral and adenoviral systems [11–14]. The usefulness of viral vectors is limited, however, by host immune and inflammatory reactions (in the case of adenovirus), the difficulty of large-scale production, the size limit of the exogenous DNA (in the case of adeno-associated virus), random integration into the host genome (in the case of retroviruses), and the risks of inducing tumorigenic mutations and generating active viral particles through recombination [15,16]. In contrast to the viral-based vectors, liposome–DNA complexes and direct administration of pure DNA complexes can transfer expression cassettes with almost no size limitations. Additionally, these systems offer advantages such as proven stability under a variety of conditions and utilization in a number of delivery systems, and are less immunogenic than the viral vectors [17]. The major drawback of these systems is that gene transfer is very inefficient, requiring large quantities of materials, and sustained site or repetitive administration to achieve clinical success [13].

Transfection efficiency is determined by the limiting barriers involved in this process, such as the entry of lipoplexes into the cell, their escape from the endosome, dissociation of the plasmid from the lipid, translocation into the nucleus and finally transcription of the transgene [18].

The in vitro transfection ability of lipoplexes depends on many parameters, such as their physico-chemical characteristics (size and zeta potential), lipid/DNA charge ratio, type of cells, incubation conditions and mode of lipoplex preparation [19]. Recently it has been demonstrated that the association of transferrin (Tf) with cationic liposomes followed by complexation with DNA promotes a significant enhancement of transfection as compared to conventional lipoplexes [20,21]. In these studies Tf-lipoplexes encapsulation efficiency, size and zeta potential were characterized.

In this work, the potential of cationic liposome–DNA complexes associated or not with transferrin to mediate gene transfer into osteoblast-like cells (MG-63 and MC3T3-E1 cells lines) was evaluated using 293T cells as positive control. Transfection activity, cytotoxicity and dependence of DNA dose of non-viral plasmid gene transfection were evaluated using two different lipoplex formulations: 1,2-dioleoyl-3-trimethylammonium propane–2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOTAP–DOPE) and DOTAP–cholesterol (DOTAP–Chol). Lipoplexes were produced and characterized in terms of size, charge ratio (lipid/DNA) and DNA protection capacity.

2. Materials and methods

2.1. Preparation of cationic liposomes and their complexes with DNA

Small unilamellar cationic liposomes (SUV) were prepared from a 1:1 (mol ratio) mixture of DOTAP–DOPE or DOTAP–Chol, by extrusion of multilamellar liposomes (MLV). Briefly, lipids (Avanti Polar Lipids, Alabaster, AL) dissolved in $CHCl_3$ were mixed at the desired molar ratio and dried under nitrogen. Any remaining solvent was removed under vacuum for 8 h. The dried lipid films were hydrated with deionized water to a final lipid concentration of 4 μM. The tube was vortexed for 10 min and the resulting MLV were then sonicated, for 3 min before being extruded, 21 times, through two polycarbonate filters of 50 nm pore diameter using a mini-extruder (Avanitilipids). The resulting SUVs were then diluted five times with deionized water. The cationic phospholipid concentration was determined by Fiske–Subbarow Method and Infinity Cholesterol Liquid Stable Reagent (Thermo Electron Corporation). Liposomes were stored at 4 °C under nitrogen and used within 1 month after preparation.

Complexes were prepared by sequentially mixing 100 μl of a HEPES-buffered saline solution (HBS) (100 mM NaCl, 20 mM HEPES, pH 7.4) with liposomes (volume was dependent on the (±) charge ratio) and with 100 μl of HBS solution containing 1 μg of pCMVlacZ plasmid. The mixture was further incubated for 15 min at room temperature. The Tf-lipoplexes were obtained by gently mixing 100 μl of the HBS and liposomes with 100 μl of human transferrin solution (320 μg per ml of HBS) (Holo-transferrin Human, Sigma–Aldrich) 15 min prior to the addition of 100 μl DNA solution, and further incubating the resulting mixture for 15 min. Lipoplexes were prepared immediately before experiments. Complexes prepared from the commercially available formulations (Lipofectamin®, Invitrogen) were obtained in a manner similar to that described above.

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