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Application of galactose-modified liposomes as a potent antigen presenting cell targeted carrier for intranasal immunization

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ABSTRACT

The mucosal immune system produces secretory IgA (sIgA) as the first line of defense against invasion by foreign pathogens. Our aim was to develop a galactose-modified liposome as a targeted carrier which can be specifically recognized by macrophage, one of the most important antigen presenting cells. First, galactose was covalently conjugated with 1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine (DLPE) to give a targeted ligand, a galactosyl lipid. The galactosyl lipid was then incorporated into a liposomal bilayer to form a galactosylated liposome carrier. Further, the ovalbumin (OVA) was encapsulated into the galactosylated liposome carriers and mice were intranasally immunized. Confocal laser scanning microscopy and flow cytometry analysis showed that the targeted galactosylated liposome carrier had a higher uptake rate than unmodified liposomes. The targeted galactosylated liposome induced higher levels of tumor necrosis factor-α and interleukin-6 production than unmodified liposomes ($P < 0.05$). Furthermore, 6-week-old BALB/c female mice immunized with the OVA-encapsulated targeted galactosylated liposome had significantly higher OVA-specific s-IgA levels in the nasal and lung wash fluid ($P < 0.05$). In addition, the targeted galactosylated liposome simultaneously augmented the serum IgG antibody response. In summary, the OVA-encapsulated targeted galactosylated liposome induced significantly higher mucosal IgA and systemic IgG antibody titers and is a potential antigen delivery carrier for further clinical applications.

1. Introduction

Mucosal surfaces, such as those of the respiratory, gastrointestinal and genital tract, are the main entry site for most environmental antigens. Thus mucosal immunity is believed to play a critical role in preventing the attachment of and initial infection by many microbial pathogens. [1]. Not surprisingly, many researchers have focused on developing effective mucosal vaccines capable of inducing both a mucosal and systemic immune response [2]. In the past the use of live attenuated virus vaccines produced promising antibody responses to viral invasion through the mucosal immune pathway, however, there are several disadvantages to using live viruses, such as the inherent risk of reversion to virulence. As a result a number of approaches have been investigated to develop "antigen vaccines", such as killed viruses, subunit vaccines, and DNA vaccines. However, the use of antigen vaccines has proven problematical in the case of mucosal immunization due to poor immunogenicity when inoculated via the mucosal route. To enhance the effectiveness of antigen vaccines through mucosal inoculation it is particularly important to develop effective adjuvants or carriers.

Many researchers have attempted to improve antibody responses to antigens delivered mucosally by encapsulating the antigen in liposomes [3,4]. Past findings have demonstrated the effectiveness of liposomes as an antigen vector in the immune transport system [5]. Liposomes are capable of improving the delivery of antigens across the mucosal membranes in mammalian cells [6] and play important roles in animals in the enhancement of adaptive immune responses [7,8]. Our previous research proved that the nasal administration of inactivated vaccines encapsulated within a liposome can increase the production of antibodies [9]. One of the primary mechanisms used to induce immune responses

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through intranasal administration is the capture of antigens by microfold cells (M cells) located on nasal-associated lymphoid tissue (NALT), and transporting them to antigen-presenting cells (APCs), such as dendritic cells and macrophages, eventually resulting in both systemic and mucosal responses [10,11]. Therefore, a method to improve the capture and transport of antigens is crucial to inducing an effective immune response. Several mucoadhesive liposomal preparations have been evaluated regarding their effectiveness as vaccine delivery systems through the intranasal route [12]. The factors that contribute most to the immuno-stimulating effect of liposomes have been identified and bioadhesive gels used during intranasal inoculation have improved uptake by the nasal mucosa and prolonged exposure to the antigen. In a previous study we demonstrated that co-administering vaccines with bioadhesive materials on mucosal surfaces successfully promotes efficient immune responses [13].

Recently liposomes modified with receptor-specific ligands (so-called "homing devices") on their surfaces have been used to enhance the delivery of genetic payloads to specific cells. It has been reported that carbohydrate recognition increases the binding and recognition between liposomes and immune cells. Saccharification modification enhances the activity of vaccines, which subsequently increases the immunity they impart [14]. C-type lectins are molecules that recognize carbohydrates and are believed to play a significant role in cellular recognition through carbohydrate ligands. [15]. Macrophage galactose type C-type lectins (MGLs) are type II transmembrane glycoproteins containing a single carbohydrate recognition domain. They have the capacity to bind to galactose and N-acetylgalactosamine as a monosaccharide. Recent studies have shown that terminal galactose has the highest affinity for mouse MGL receptors (mMGLl) on murine macrophages [16]. Based on these findings, galactose is believed to be a good candidate ligand for targeting macrophages, one of the most important cells involved in the presentation of antigens to helper T-cells. Although studies on the target effects of galactose-modified liposomes on liver cells have been conducted in animal models [17], no vaccination trials using the intranasal administration of galactose-modified liposomes have been conducted.

In this study we first conjugated a galactose molecule with 1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine (DLPE) as a targeting ligand and then incorporated it on the surface of a liposome to form a galactosylated liposome carrier. Ovalbumin (OVA) was subsequently encapsulated in the galactosylated liposome. This study investigated the effects of galactosylated liposome carrier uptake by macrophages and analyzed the cytokine profile of stimulated macrophages. Mucosal and humoral antibody titers were also assessed using enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Synthesis of galactosyl lipid

A 50 ml, two necked, round bottomed flask was fitted with a glass tube to admit ozone, a calcium chloride drying tube, a glass stopper, and a magnetic stirring bar, and was charged with 170 mg α-allylic galactoside (0.77 mmol) and 10 ml of methanol. The flask was cooled to −78 °C (acetone–dry ice) and ozone gas was bubbled through the solution with stirring. When the solution turned blue ozone addition was stopped. Nitrogen gas was passed through the solution until the blue color was discharged and then the cold bath was removed. The drying tube and ozone inlet were replaced with a stopper and rubber septum, and 1 ml of dimethyl disulfide was added. The solution was allowed to warm to room temperature with stirring under a nitrogen atmosphere for 30 min. The heterogeneous mixture was then concentrated by rotary evaporation. The residue was subjected to column chromatography (silica gel, methanol/ethyl acetate (1/9–1/3)) to give the aldehyde intermediate (quantitative yield). To this aldehyde intermediate (100 mg, 0.45 mmol) was added methano/chloroform solution (10 ml, 1:1), DLPE (261 mg, 0.45 mmol), and sodium cyanoborohydride ($NaBH_3CN$, 37.8 mg, 0.57 mmol). After being stirred for 2.5 h the reaction mixture was concentrated by rotary evaporation. The residue was subjected to column chromatography (silica gel, methanol/dichloromethane (1/8–1/6)) to give α-galactosyl-DLPE (compound **1** in Scheme 1) as an oil (76% yield). The synthesis of β-galactosyl-DLPE (**2**) was similar to that of compound **1**.

2.2. Preparation and characterization of liposomes

Multilamellar vesicles (MLVs) were prepared according to a method described in a previous study [13]. Briefly, thin films were obtained through the rotary evaporation of chloroform solutions with a lipid molar ratio of 95% phosphatidylcholine (PC) (Degussa, Hamburg Germany), cholesterol (Sigma-Aldrich, St. Louis, MO), galactosyl lipid (α-galactosyl-DLPE or β-galactosyl-DLPE) 4:0.5:0.5 (galactosyl-DLPE-liposome), using a final lipid concentration of 20 mM. The lipid films were maintained under vacuum for an additional 60 min in order to remove residual organic solvents. The lipid films were subsequently rehydrated with 10 ml of OVA solution (1 mg ml^{-1}) and sonicated using a bath sonicator (frequency 40 kHz, Ultrasonic Steri-Cleaner, Taiwan) for 30 min at room temperature to form liposomes incorporating α-galactosyl-DLPE (α-Gal-liposome) and liposomes incorporating β-galactosyl-DLPE (β-Gal-liposome). Unmodified liposomes were also prepared according to the above procedure. Equivalent quantities of galactose were added to unmodified liposomes to form galactose-mixed liposomes. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Sigma Aldrich)-labeled liposomes were also prepared by adding 0.04 mol.% DiI (against total lipid moles), while the lipids were dissolved in chloroform for the preparation of liposomes. The particle size and zeta potential of the resulting preparations (200 ml diluted in 4 ml of double distilled water) were confirmed by dynamic light scattering (DLS) analysis using a particle analyzer (BIC 90 Plus, Brookhaven Instruments, Holtsville, NY).

2.3. Cell and culture medium

The RAW264.7 murine macrophage cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The RAW264.7 cells were cultured at 37 °C under a 5% CO_2 atmosphere in complete RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (Gibco), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Gibco).

2.4. Cellular uptake assay

The RAW264.7 cells were seeded at a density of 10^4 cells cm^{-2} on the coverslips of 6-well tissue culture plates (Nunc). The cells were incubated with various DiI-labeled liposomal formulations at 37 °C, 5% CO_2. After 6 h the cells were washed with cold phosphate-buffered saline (PBS) three times and then fixed using 0.5% paraformaldehyde (Sigma Aldrich) in PBS for 15 min. The cells were stained in the dark at room temperature for 60 min using anti-mouse CD11b–FITC antibodies (Miltenyi Biotec, Gladbach, Germany) in 3% bovine serum albumin (BSA)/PBS. The slides were subsequently examined for liposome uptake using a confocal spectral microscope imaging system (Leica TCS SP5).

Quantitative analysis of cellular uptake was determined using flow cytometry. RAW264.7 cells were seeded at a density of 10^6 cells per well in a 24-well dish and incubated with various liposome formulations at 37 °C for 6 h, under 5% CO_2, at 95% humidity.

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