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Liposomal encapsulation of dexamethasone modulates cytotoxicity, inflammatory cytokine response, and migratory properties of primary human macrophages

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Abstract

The encapsulation of drugs into liposomes aims to enhance their efficacy and reduce their toxicity. Corticosteroid-loaded liposomes are currently being evaluated in patients suffering from rheumatoid arthritis, atherosclerosis, colitis, and cancer. Here, using several different fluorophore-labeled formulations, we comprehensively studied the impact of liposome encapsulation of the prototypic corticosteroid dexamethasone on various primary human cells *in vitro*. Liposomal dexamethasone targeted several primary cell types in a dose and time-dependent manner, but specifically reduced cytotoxicity against human fibroblasts and macrophages in comparison to the solute drug. Furthermore, macrophage maturation and polarization markers were altered. Interestingly, liposomal dexamethasone induced proinflammatory cytokine secretion (specifically TNF, IL1β, IL6) in unstimulated cells, but reduced this response under inflammatory conditions. Monocyte and macrophage migration was significantly inhibited by dexamethasone-loaded liposomes. The findings indicate that the encapsulation of dexamethasone into liposomes modulates their cellular mechanism of action, and provides important indications for follow-up *in vivo* investigations.

Key words: Liposomes; Corticosteroids; Glucocorticoids; Dexamethasone; Primary human macrophages; Immune cells; Cell migration; Cell activation; Cytokine release; Inflammation

Background

Liposomes are in clinical use as nanocarriers of glucocorticoids (GC) that are intended to suppress inflammatory diseases such as arthritis, colitis, and atherosclerosis.[1,2] GC act via the GC receptor and lead to the downregulation of proinflammatory mediators.[3] Liposomes are approved for autologous stem cell transplantation induction regimes,[4] autoimmune encephalomyelitis,[3] and cancer treatment.[5] Liposomal encapsulation of vaccines has also been done to increase vaccination efficiency,[6] a process that strongly relies on antigen-presenting cells such as monocytes and macrophages. The pharmaceutical advantages of encapsulated

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glucocorticoids are based on the antiinflammatory activities of GC. The decoration of nanoparticle surfaces with polyethylene glycol (PEG) is known to increase the circulation time of nanoparticles,[2,7] and we could correlate this increased circulation with a decreased clearance of PEGylated particles by macrophages.[8,9]

There are two major subpopulations of macrophages that can be classified as either classically (proinflammatory) or alternatively (antiinflammatory) activated. Classical activation of macrophages is induced by bacterial products such as lipopolysaccharides or immune cell-derived interferon γ and the corresponding cells are referred to as M1 macrophages. In contrast, alternative activation is a response to stimulation with glucocorticoids such as dexamethasone (Dex)[10] and the designated cells are defined as M2 macrophages. The M2 subtype is known to express typical markers of alternative activation like CD163.[11] A recent study has shown that liposomal encapsulation of prednisolone increases M2 marker expression by macrophages.[3] However, the effects of encapsulation of the more frequently used GC dexamethasone specifically on immune cell activation would be of great interest for clinicians.

Furthermore, it is known that Dex can have cytotoxic effects.[12] This might be especially relevant to cell compartments in the liver, where GC as well as nanocarriers are typically cleared from the circulation.[9] For experimental purposes, fluorescent labeling of nanocarriers might assist in verifying their cell targeting capacities and facilitates their detection in fluorescence-based applications such as immunofluorescence microscopy or flow cytometry.

In order to address these unknown aspects of liposome-based GC targeting, we generated several different formulations: we equipped liposomes with a diameter of 100 or 400 nm with a fluorophore resulting in a green fluorescent signal, additionally PEGylated them and loaded them with Dex. We studied the cytotoxicity of solute and encapsulated Dex and of liposomes using four different primary cell types. The uptake of different liposomal formulations by the most relevant scavenging cells was studied using flow cytometry, and cellular morphology changes were studied in macrophage cell cultures. Changes in macrophage phenotype were monitored using flow cytometric detection of surface markers. Inflammatory cytokine expression was studied using quantitative real-time-PCR and cytokine multiplex assays. Using these sophisticated methods on primary cell populations, our study revealed distinct effects of liposomal versus free Dex on cell-specific targeting, cytotoxicity and interactions with immune cell function and migration.

Methods

Liposome preparation and characterization

Liposomes were prepared according to the film-method.[13] In brief, Dipalmitoyl phosphatidylcholine (DPPC) and PEG-(2000)-distearoyl phosphatidylethanolamine (PEG-(2000)-DSPE) were obtained from Lipoid (Ludwigshafen, Germany), cholesterol was obtained from Sigma (St. Louis, MO, USA) and (*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-snglycero-3-phosphoethanolamine, triethyl-ammonium salt) (NBD-PE) was obtained from Molecular Probes (Grand Island, New York, USA). All other chemicals were of reagent grade. A mixture of chloroform/methanol (10:1 volumetric ratio) containing DPPC, PEG-(2000)-DSPE, NBD-PE and cholesterol was prepared at a molar ratio of 1.85:0.15:0:1. As a fluorescent marker 1 mol % of NBD-PE was added to the organic phase in relation to the total amount of lipid, including cholesterol. The organic phase was evaporated with a rotavapor (BUCHI Labortechnik AG, Flawil, Switzerland), followed by nitrogen flushing for removal of residual organic solvent. The lipid film was hydrated at 50 °C in an aqueous solution of dexamethasone phosphate in a concentration of 100 mg/mL at a phospholipid concentration of 100 mM. Empty liposomes without dexamethasone phosphate were dispersed in phosphate buffered saline (PBS). The liposomes were sequentially extruded through two stacked polycarbonate filters with pore sizes of 600, 200, and 100 nm (Nuclepore, Pleaston, USA) under nitrogen pressure, using a Lipex high pressure extruder (Lipex, Nortern Lipids, Vancouver, Canada). Unentrapped dexamethasone phosphate was removed by dialysis at 4 °C against PBS using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, USA) with a molecular cut-off of 10 kD.

The mean particle size was determined by dynamic light scattering with an ALVCGS-3 system (Malvern Instruments, Worcestershire, United Kingdom). Zeta potential was determined using a Zetasizer Nano Z (Malvern Instruments Ltd., Worcs, UK). The phospholipid content was determined with a phosphate assay[14,15] on the organic phase after extraction of the liposomal preparation with chloroform. The aqueous phase after extraction was used to determine the liposomal dexamethasone phosphate concentration of the liposomes by high performance liquid chromatography using a mobile phase of acetonitrile/water of pH 2 in a ratio of 25/75 and monitoring the eluents with a UV-detector at 254 nm. The batch of 100 nm PEGylated liposomes that was used in this study had a zeta potential of 5.1 +/− 1.4 mV, and its loading capacity was 0.13 mg dexamethasone phosphate per μmol of phospholipid. Earlier studies have shown that these liposomes are physically and chemically stable for up to 1 year at temperatures up to 40 °C, with no loss of the encapsulated corticosteroid, either upon dilution, or upon interaction with body fluids.[2,16] The formulations were extensively dialyzed, according to previously established procedures,[17] to remove all free and surface-associated corticosteroid. A limulus amebocyte lysate (LAL) assay QCL-1000 was obtained from Lonza (Walkersville, MD, USA) to test for possible endotoxin contaminations of the liposomes. The kit was used according to the instructions of the manufacturer.

Cell isolation and culture

Human primary blood leukocytes were isolated using dextran sedimentation and purification of peripheral blood mononuclear cells (PBMC) was done using Ficoll-based density gradient centrifugation as reported earlier.[8] To isolate monocytes for macrophage culture, PBMC were incubated at 37 °C on bacterial grade Petri dishes at a density of three million cells per mL in RPMI1640 (Sigma-Aldrich, St. Louis, MA, USA) containing 5% human autologous serum for 35 min in a humidified incubator with 5% CO_2. During this period monocytes become adherent