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Glucocorticoid-loaded liposomes induce a pro-resolution phenotype in human primary macrophages to support chronic wound healing

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ABSTRACT

Glucocorticoids are well established anti-inflammatory agents, however, their use to treat chronic inflammatory diseases is limited due to a number of serious side effects. For example, long-term local treatment of chronic wounds with glucocorticoids is prohibited by dysregulation of keratinocyte and fibroblast function, leading to skin thinning. Here, we developed and tested liposome formulations for local delivery of dexamethasone to primary human macrophages, to drive an anti-inflammatory/pro-resolution phenotype appropriate for tissue repair. The liposomes were loaded with the pro-drug dexamethasone-phosphate and surface-modified with either polyethylene glycol or phosphatidylserine. The latter was used to mimic phosphatidylserine-harboring apoptotic cells, which are substrates for efferocytosis, an essential pro-resolution function. Both formulations induced a dexamethasone-like gene expression signature in macrophages, decreased IL6 and TNFα release, increased secretion of thrombospondin 1 and increased efferocytosis activity. Phosphatidylserine-modified liposomes exhibited a faster uptake, a higher potency and a more robust phenotype induction than polyethylene glycol-modified liposomes. Fibroblast and keratinocyte cell cultures as well as a 3D skin equivalent model showed that liposomes applied locally to wounds are preferentially phagocytosed by macrophages. These findings indicate that liposomes, in particular upon shell modification with phosphatidylserine, promote dexamethasone delivery to macrophages and induce a phenotype suitable to support chronic wound healing.

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1. Introduction

Chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, chronic obstructive lung disease (COPD), autoimmune diseases and chronic skin wounds still represent a major unmet medical need. To a large extent, the inflammatory state is regulated by the innate immune system, with macrophages playing a central role [1]. Chronic inflammatory conditions are characterized by a constant influx of monocytes, a sustained high number of active pro-inflammatory macrophages, combined with a relative lack of anti-inflammatory/pro-resolution macrophages that actively support the resolution of inflammation and promote tissue repair [2–4].

In the skin, chronic wounds are generally characterized as open

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wounds in a chronic inflammatory state [5–9], with an important bacterial colonization, leukocyte entrapment and prolonged pro-inflammatory mediator secretion, including TNFα, IL6 and IL1β [10–12]. This pro-inflammatory environment induces macrophage phenotypes with deficient phagocytic activity resulting in the build-up of necrotic debris. Wound fluid also contains high levels of proteases such as matrix metalloproteinases (MMPs) and low levels of tissue inhibitor of metalloproteinases (TIMPs), due to the sustained presence of neutrophils [13,14], which favors extracellular matrix degradation [7]. In such environments keratinocytes and fibroblasts tend to become senescent, and their migration and proliferation capacities are impaired [15,16]. This leads overall to a persistent inflammatory state that prevents resolution and tissue repair.

Glucocorticoids (GCs) are highly potent, clinically routinely used anti-inflammatory agents acting on macrophages via complex mechanisms of direct and indirect transrepression or transactivation of gene expression mediated by the GC receptor (GR) [17]. The GC-induced phenotype not only exhibits a decrease in inflammatory activities, but also the induction of processes involved in the resolution of inflammation and wound healing. The anti-inflammatory action of GCs is mediated by interfering with specific signaling pathways, including a reduced production of pro-inflammatory cytokines such as IL6 or TNFα. On the other hand, a number of genes positively regulated by the GR contribute to the pro-resolution and regenerative activity of macrophages [18].

Efferocytosis, the removal of apoptotic neutrophils before they undergo secondary necrosis, is a critical macrophage activity limiting tissue damage and supporting recovery [19,20]. GCs stimulate efferocytosis by upregulating the membrane receptor MerTK which is involved in the recognition of the phospholipid phosphatidylserine (PS) exposed on apoptotic cells [21–23]. Of note, the process of phagocytosing apoptotic cells itself contributes to the anti-inflammatory function of macrophages by inducing TGFβ1 and IL10 release [24–27] and by suppressing pro-inflammatory cytokine production [19,23,24,26,28]. Hence, GC-induced macrophage polarization and efferocytosis generate a positive feedback loop for resolution.

However, as GRs are expressed in most cell types and since GCs have a large volume of distribution, treatments with GCs lead to side effects that limit their use in systemic applications, in particular for long term treatments at high doses. In the context of skin wounds, skin atrophy and impaired healing are typical side effects of both systemic and topical GC therapies [29–32]. Skin atrophy is characterized by thinning of the epidermal layer (decreased keratinocyte numbers), loss of elasticity, increased permeability (disrupted skin barrier function), dermal atrophy due to low number of fibroblasts and decreased levels of extracellular matrix (e.g. collagen, hyaluronan proteins) [32–34]. Strategies to overcome these limitations include the use of nanomedicine formulations, such as liposomes, to improve cell-specific delivery and sustain on-site drug availability.

In this study, we set out to compare two formulations, based on liposomes containing in their lipid bilayer either 10% PS or polyethylene glycol (PEG) exposed at the surface. GCs were encapsulated in both formulations in the form of the pro-drug dexamethasone phosphate (DexP), which is known to be processed in phagocyte lysosomes to deliver active dexamethasone (Dex) into the cell cytoplasm. PS-containing liposomes may be able to mimic PS-harboring apoptotic cells and their resolution enhancing properties. We first evaluated these formulations *in vitro* with regards to efficacy of delivery, and their ability to induce an anti-inflammatory state and pro-resolution functions. In a second step we evaluated the targeting of liposome-mediated dexamethasone delivery to macrophages, as compared to liposome uptake by keratinocytes and fibroblasts, in 2D cultures and in a 3D skin equivalent model. The preferential liposome uptake by macrophages suggests that local delivery of GCs specifically to monocytes/macrophages via liposomes represents a new therapeutic avenue for the treatment of chronic wounds.

2. Materials and methods

2.1. Liposome preparation and characterization

Dipalmitoylphosphatidyl choline (DPPC), PEG-(2000)-distearoylphosphatidyl ethanolamine (PEG-(2000)-DSPE) and Dioleylphophatidyl serine sodium salt (DOPS) were obtained from Lipoid (Steinhausen, Switzerland) and Cholesterol HP was obtained from Dishman (Veenendaal, Netherlands). All chemicals were of reagent grade. Liposome formulations were prepared with the film method. For PEG liposomes, DPPC, cholesterol and PEG-(2000)-DSPE were mixed at a molar ratio of 1.85:0.15:1 and dissolved in ethanol. For PS liposomes, DPPC, cholesterol and DOPS were mixed at a molar ratio of 1.7:0.3:1 (equivalent to 10 mol% DOPS relative to the total lipid amount) and dissolved in ethanol. The final lipid concentration was 100 mM for both formulations. The organic phase was evaporated with a rotavapor (BUCHI Labortechnik, Flawil, Switzerland) until a lipid film was obtained. Residual organic solvent was removed by placing the films overnight on a tabletop lyophilisator (Christ Alpha1-2 LD, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). For dexamethasone 21-phosphate disodium salt (DexP) (Sigma-Aldrich, Buchs, Switzerland) loaded liposomes, the lipid films were hydrated at 50 °C with a 50 mg/mL DexP aqueous dextrose solution (Hospira, Lake Forest IL, USA). Drug-free formulations where hydrated with 5% dextrose. The liposomes were extruded at 50 °C (Lipex, Northern Lipids, Vancouver, Canada) under nitrogen pressure through Whatman polycarbonate membranes with a pore size of 1 μm and then 100 nm (GE Healthcare, Glattburg, Switzerland). Non-encapsulated DexP was removed by dialysis at 4 °C against 5% dextrose using a Float-a-Lyzer G2 (Sigma-Aldrich) with a cut-off of 100 kDa. Liposomes were diluted 1:100 in sterile-filtered phosphate-buffered saline (PBS) pH 7.4 (Sigma-Aldrich) before use *in vitro*. DexP liposomes were prepared freshly every second week. Fluorescent drug-free DiD and DiI liposomes were prepared as the other formulations, with an additional 1 mol% of DiD or DiI (Biotium, Fremont CA, USA) relative to the total lipid concentration added to the lipid mix in ethanol.

The mean particle size, polydispersity index (PDI) and zeta potential were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) (Supplement Fig. 1). Target size for liposomes was between 100 and 150 nm. Expected zeta potential values are close to neutral for PEGylated liposomes and negative for PS-containing liposomes.

The DexP amount retained inside the liposomes was determined by High Performance Liquid Chromatography (HPLC) (1100 Series, Agilent Technologies, Basel, Switzerland) using a mobile phase of acetonitrile and water (35:65) at pH 2 (pH adjustment with 0.1% v/v phosphoric acid). Eluents were measured with a UV-detector at 242 nm, passing through a ZORBAX SB-C18 column 1.8 μm; 4.6 × 100 mm (Agilent Technologies).

For assessing the cytotoxicity of liposomes, monocytes were seeded in 96-well plates at 2×10^5 cells per well and treated as described for 24 h. The assay was performed immediately, following the manufacturer's protocol (Cayman Chemicals, Hamburg, Germany). The absorbance of the solution (dissolved formazan crystals) was measured at 570 nm on a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale CA, USA) and reported as a percentage of viability/metabolism relative to the

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