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Fluorescent cell-traceable dexamethasone-loaded liposomes for the treatment of inflammatory liver diseases

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ABSTRACT

Liposomes are routinely used carrier materials for delivering drug molecules to pathological sites. Besides in tumors and inflammatory sites, liposomes also strongly accumulate in liver and spleen. The potential of using liposomes to treat acute and chronic liver disorders, however, has not yet been evaluated. We here explored the therapeutic potential of dexamethasone (Dex)-loaded liposomes for inflammatory liver diseases, using experimental models of acute and chronic liver injury in mice. Fluorescently labeled liposomes predominantly accumulated in hepatic phagocytes, but also in T cells. Importantly, Dex-loaded liposomes reduced T cells in blood and liver, more effectively than free Dex, and endorsed the anti-inflammatory polarization of hepatic macrophages. In experimental chronic liver damage, Dex-loaded liposomes significantly reduced liver injury and liver fibrosis. In immune-mediated acute hepatitis Dex-loaded liposomes, but not free Dex, significantly reduced disease severity. T cells, not macrophages, were significantly depleted by Dex liposomes in liver disease models *in vivo*, as further supported by mechanistic cell death *in vitro* studies. Our data indicate that Dex liposomes may be an interesting treatment option for liver diseases, in particular for immune-mediated hepatitis. The depletion of T cells might represent the major mechanism of action of Dex liposomes, rather than their macrophage-polarizing activities.

1. Introduction

Liposomes are nano-sized biodegradable drug delivery systems that can be used for a targeted, cell-specific administration of drugs with reduced systemic side effects. The increased efficacy and reduced toxicity of drugs upon liposomal encapsulation has been convincingly established for anti-cancer chemotherapeutics, for instance, for the cytostatic drug doxorubicin [1]. Liposomal drug delivery is also considered to be potentially beneficial for the treatment of inflammatory diseases [2], because the systemic administration of anti-inflammatory drugs regularly results in systemic immunosuppression rendering patients susceptible for infections such as sepsis [3]. We have previously shown that liposomal encapsulation of the anti-inflammatory drug dexamethasone allows for a reduction of unspecific effects of the encapsulated compounds on human primary cells [4].

Corticosteroids are routinely used to treat inflammatory liver diseases, such as autoimmune hepatitis and alcoholic hepatitis [5,6]. At present, no liposomal corticosteroid formulations have been approved for the treatment of liver diseases yet. As large quantities of nanoparticles translocate to the liver upon systemic application [7], nanoparticle-based drug delivery could represent a promising strategy for combatting liver diseases [8]. The accumulation of nanoparticles in the liver can be partially related to their internalization by hepatic macrophages, such as Kupffer cells,

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which are located at the fenestrated endothelium and which are in direct contact with blood [9]. Subsequent to their internalization by macrophages [10], nanoparticles are known to influence the state of hepatic macrophage polarization [11].

Macrophages exhibit a high plasticity with respect to their functions in regulating inflammation [12]. They can be polarized into either a proinflammatory M1 or an anti-inflammatory M2 macrophage subtype. Key molecular markers of M1 macrophages are tumor necrosis factor α (TNFα) and interleukin 1β (IL1β), whereas characteristic M2 markers are Arginase 1 (Arg1), resistin like alpha (Retnla, FIZZ-1) and IL4 [13]. M1 macrophages in diseased livers mainly originate from monocytes that translocate into the liver upon injury, and they express F4/80, CD11b and the Ly6C antigen on their surface, whereas Kupffer cells are resident liver macrophages, expressing high levels of F4/80 but low levels of Ly6C and CD11b [14]. Alternatively activated M2 macrophages exhibit characteristic surface markers such as the IL4 receptor α (CD124), which inhibits inflammation [15], the mannose receptor (CD206) and the macrophage C-type lectin domain family 10, member A (CLEC10A, CD301), all of which can potentially be regulated by targeted nanotherapeutics [11].

While many reports have underlined the importance of macrophages for interactions with nanoparticles *in vitro*, it has not been studied in great detail to which extent these observations translate into disease models *in vivo*. We have recently demonstrated that liposomes loaded with the corticosteroid dexamethasone block migratory and inflammatory functions of primary human macrophages *in vitro* [4]. We now investigated the effects of liposomal encapsulated dexamethasone on macrophages and other immune cell populations *in vivo*. Using fluorescently labeled and/or dexamethasone (Dex)-loaded liposomes for intravenous administration in mice, we characterized the biodistribution, toxicity, impact on immune cell numbers, and functions in the circulation and in the liver. We further evaluated the potential of Dex-loaded liposomes as therapeutics for treating hepatitis and liver fibrosis, employing experimental models of acute Concanavalin A (ConA)-based hepatitis and chronic toxic carbon tetrachloride (CCl_4)-based liver injury, respectively. As these data unexpectedly indicated that Dex liposomes primarily exert their anti-inflammatory actions via depleting T cells *in vivo*, we furthermore isolated primary murine lymphocytes as well as macrophages and explored cell-specific mechanisms of Dex liposome induced cell death.

2. Materials and methods

2.1. Liposome preparation and characterization

Liposomes were prepared based on the film-method [16]. Briefly, Dipalmitoyl phosphatidylcholine (DPPC) and PEG-(2000)-distearoyl phosphatidylethanolamine (PEG-(2000)-DSPE), obtained from Lipoid (Ludwigshafen, Germany), cholesterol, 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 mix of chloroform and methanol (volumetric ratio of 10:1) containing DPPC, PEG-(2000)-DSPE, NBD-PE, and cholesterol was prepared at a molar ratio of 1.85:0.15:0:1. One 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. Hydration of the lipid film was done at 50 °C in an aqueous solution of dexamethasone phosphate in a concentration of 100 mg/mL and a phospholipid concentration of 100 mM. 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), retrieving liposomes sizing 100 nm. Unencapsulated dexamethasone phosphate was cleared by dialysis at 4 °C against PBS using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, USA) with a molecular cut-off of 10 kD [11].

The mean particle size was determined by dynamic light scattering with an ALVCGS-3 system (Malvern Instruments, Worcestershire, United Kingdom). Thezeta potential was determined using a Zetasizer Nano Z (Malvern Instruments Ltd., Worcs, UK) and that of the PEGylated liposomes was 5.1 ± 1.4 mV. The phospholipid content was determined with a phosphate assay [17,18] 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 at a ratio of 25/75 and monitoring the eluents with a UV-detector at 254 nm. A limulus amebocyte lysate (LAL) assay QCL-1000 obtained from Lonza (Walkersville, MD, USA) was used to test for possible endotoxin contaminations of the liposomes. The kit was used according to the instructions of the manufacturer.

2.2. Mice

C57BL6/J wild-type mice were housed in a specific pathogen-free environment. All experiments were done with male animals at 8–12 weeks of age under ethical conditions approved by the appropriate authorities according to German legal requirements. In an earlier *in vitro* study, we have used Dex at a concentration of 10 μg/mL with human primary immune cells. This concentration resembled a Dex concentration of 1 mg/kg body weight, based on the assumption that one mouse has a total body fluid volume (where liposomes can accumulate) of 2.5 mL, a comparative study that we have done earlier with gold nanorods [11,19]. For the cell culture experiments, the liposomes without Dex were diluted with sterile 0.9% sodium chloride solution to a phospholipid (PL) concentration of 77 nmol/mL, corresponding to a concentration of 77 μM PL in the cell culture medium. Thus, one mouse weighing 25 g received 192.5 μM of liposomes, what corresponds to a concentration of 7.7 mM PL/kg body weight (25 g × 40).

2.3. Fluorescence reflectance imaging

Organs (liver, kidneys, spleen, intestine, lung, heart, muscle, brain, and bone) of mice that were sacrificed 72 h after the injection of 80 nM/kg liposomes were scanned *ex vivo* (at 750 nm) for liposomal accumulation using a 2D Fluorescence Reflectance Imaging (FRI) (FMT2500 LX, PerkinElmer, Waltham, Massachusetts, USA). The total fluorescence of each organ was quantified in counts/energy (normalized to 100 mm^2/tissue).

2.4. Liver injury models

Liposomes and control solutions were injected intravenously at a volume of 100 μL. ConA-based hepatitis was induced after 40 h by injecting ConA (Sigmaaldrich, St. Louis, USA) at 15 mg/kg intravenously, and sacrificing mice eight hours later. Chronic liver injury was induced using repetitive intraperitoneal CCl_4 (Merck, Darmstadt, Germany) challenge twice weekly for six weeks [11]. Control animals received the same volume of vehicle (corn oil). Mice were sacrificed 48 h after the last injection of CCl_4.

2.5. Liver enzymes, histology, and immunohistochemistry

ALT was assessed at 37 °C in serum using the Modular Preanalytics System (Roche, Penzberg, Germany). Hematoxylin and Eosin (H&E) and Sirius Red stainings were done following established protocols. Sirius Red stained sections were analyzed by morphometrical assessment of the area fraction. Staining of CD45 and F4/80, and α smooth muscle actin (αSMA) was done according to optimized protocols [20].

2.6. Cell isolation, flow cytometry, and fluorescence microscopy

Blood was taken from the right ventricle. Red blood cell lysis was done using Pharm Lyse (BD, Franklin Lakes, USA) and was stopped using Hank's buffer salt solution (HBSS) supplemented with 5 μM ethylenediaminetetraacetic acid and 0.5% bovine serum albumin. Hepatic leukocytes were isolated from liver as described earlier [11]. Single cell suspensions were filtered using a 100 μM mesh, and stained for flow cytometry as described earlier in detail [11]. Additionally, count beads (calibrite beads, BD, Franklin Lakes, USA) were added to organ cell suspensions to determine cell numbers in different organs. Flow cytometric data are given as percentages of leukocytes or as absolute numbers calculated from organ weight or blood volume. To prepare sections for fluorescence microscopy, cryosections sizing 20 μM were prepared and analyzed using a Zeiss Axio Observer Z1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.7. Cell viability and cell death assessment

Live-dead staining was done in 48 well plates according to the instructions of the manufacturer (Life Technologies, Carlsbad, CA, USA). To determine the mode of cell death, we used an Annexin V (conjugated with allophycocyanin) apoptosis detection kit (BD, Franklin Lakes, USA). Propidium iodide was used to stain necrotic cells in parallel (BD, Franklin Lakes, USA).