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Contribution of Kupffer cells to liposome accumulation in the liver

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ABSTRACT

The liver is a major barrier for site-specific delivery of systemically injected nanoparticles, as up to 90% of the dose is usually captured by this organ. Kupffer cells are thought to be the main cellular component responsible for nanoparticle accumulation in the liver. These resident macrophages form part of the mononuclear phagocyte system, which recognizes and engulfs foreign bodies in the circulatory system. In this study, we have compared two strategies for reducing nanoparticle accumulation in the liver, in order to investigate the specific contribution of Kupffer cells. Specifically, we have performed a comparison of the capability of pegylation and Kupffer cell depletion to reduce liposome accumulation in the liver. Pegylation reduces nanoparticle interactions with all types of cells and can serve as a control for elucidating the role of specific cell populations in liver accumulation. The results indicate that liposome pegylation is a more effective strategy for avoiding liver uptake compared to depletion of Kupffer cells, suggesting that nanoparticle interactions with other cells in the liver may also play a contributing role. This study highlights the need for a more complete understanding of factors that mediate nanoparticle accumulation in the liver and for the exploration of microenvironmental modulation strategies for reducing nanoparticle-cell interactions in this organ.

1. Introduction

Patient survival and quality of life are highly dependent on biodistribution of administered drugs. For many diseases, including metastatic cancer, it is challenging or impossible to locally administer therapeutic agents, necessitating the use of the circulatory system for drug delivery. Strategies that increase the accumulation of systemically administered drugs in diseased tissues improve therapeutic efficacy and minimize side effects. For instance, nanocarriers can enhance localized delivery of therapeutic agents by incorporating transport enhancing components, such as targeting ligands and sustained/triggered-release systems, and by taking advantage of transport phenomena that appear on the nanoscale, *e.g.* reduced renal clearance and the enhanced permeability and retention effect [1,2]. Studies have demonstrated that nanodelivery can substantially increase the tumor accumulation of small molecules [3,4]. However, in most cases, less than 1% of the intravenously injected nanoparticle dose reaches the intended location [5] and up to 90% accumulates in the liver [6]. Despite the fact that the liver is a major barrier for drug delivery, mechanisms for nanoparticle accumulation in this organ are poorly understood. In particular, it is unclear to what extent each component of the liver contributes to nanoparticle deposition, and it is thought that both cells and physical features play a role [7]. Specifically, the physical organization of the vascular network in the liver is likely to be a major contributing factor [8,9]. In regards to cellular components, Kupffer cells, which are the resident macrophages of the liver, are considered to be largely responsible for cellular uptake of nanoparticles in this organ [6]. These phagocytes engulf damaged cells and foreign material, such as bacteria, viruses, and nanoparticles [10], and make up 80–90% of the total macrophage population in the body [11]. In the circulatory system, nanoparticles interact with plasma proteins, which form a protein corona around the particle surface [12]. Opsonins such as immunoglobulins and complement proteins trigger phagocytosis by binding to membrane receptors on Kupffer cells [12]. The most widely used strategy for reducing nanoparticle interactions with macrophages is to coat the particle surface with polyethylene glycol (PEG) [13]. PEG attracts water molecules that form a hydration layer, which reduces protein binding and cell interactions. Notably, the stealth effect is not spe-

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cific to macrophages, since this hydration layer reduces interactions with all types of cells [14,15].

In this study, we compared two different strategies for decreasing nanoparticle accumulation in the liver (Fig. 1). The first strategy entailed pegylation of nanoparticles, while the second strategy involved Kupffer cell depletion prior to nanoparticle administration. The nanoparticles used in this study were liposomes as they represent one of the largest categories of clinically approved nanodrugs. There are currently over a dozen liposomal drug formulations on the market used for treatment of various conditions, including fungal infections and cancer [16,17]. The objective of this study was to investigate the specific contribution of Kupffer cells to cell-mediated accumulation of liposomes in the liver. This goal was achieved by using pegylation as a control for examining the role of Kupffer cells in nanoparticle uptake, since PEG reduces interactions with all cell types.

2. Materials and methods

2.1. Materials

Materials were acquired from the following sources: dioleoyl-phosphatidylcholine (DOPC)/cholesterol (CHOL) liposomes (F60103F-TR) and DOPC/CHOL/mPEG-distearoyl-phosphoethanolamine (DSPE) liposomes (F60203F-TR) labeled with Texas Red-dihexadecanoyl-phosphoethanolamine from FormuMax Scientific; clodronate liposomes from Encapsula NanoSciences; Prigrow II Medium and immortalized rat Kupffer cells from Applied Biological Materials; Raw 264.7 mouse macrophage cells from American Type Culture Collection (ATCC); fetal bovine serum (FBS) from Atlas Biologicals; 96-well flat clear bottom black polystyrene TC-treated microplates and Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine & sodium pyruvate from Corning; penicillin-streptomycin solution from Sigma-Aldrich; ethylenediaminetetraacetic acid (EDTA) and phosphate buffered saline (PBS; HyClone) from Thermo Fisher Scientific; F4/80: Alexa Fluor 647 antibody from Bio-Rad (MCA497A488); CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay from Promega; Microtainer Tubes with K2E from BD; Amicon Ultra-15 Centrifugal Filter Device 100 K from Millipore Sigma; Tissue-Tek optimum cutting temperature (O.C.T) compound form VWR; Vectashield Antifade Mounting Medium with DAPI (4′,6-diamidino-2-phenylindole) and normal horse serum from Vector Laboratories.

2.2. Nanoparticle characterization and stability

Dynamic light scattering and laser Doppler microelectrophoresis were used to measure the size and zeta potential of liposomes (0.5% v/v in distilled water), respectively, using a Zetasizer Nano ZS (ZEN 3600, Malvern Instruments) as previously reported [18–20]. The fluorescence intensity (Ex/Em of 590 nm/620 nm) of serial dilutions of the liposome stock solution was measured in black clear-bottom 96-well microplates with a Synergy H4 Hybrid Microplate Reader (BioTek). Dynamic light scattering was used to measure liposomal stability at various time points under physiological conditions. Liposomes were incubated in Prigrow II Medium with 10% FBS (2.7% v/v) on a shaker at 37 °C and the size was measured in distilled water (2% v/v) as described above. The detachment of fluorophore from liposomes was also measured at 37 °C on a shaker (0.2% v/v in Prigrow II Medium with 10% FBS). At various time points, centrifugation (4000 × *g*; 30 min) of the media solution was performed in Amicon Ultra-15 Centrifugal Filter Device 100 K. The fluorescence intensity of the ultrafiltrate was measured as described above.

2.3. Liposome uptake and cell viability in vitro

Kupffer cells and Raw 264.7 cells were cultured in PriGrow II Media and DMEM, respectively. The media was supplemented with 1% penicillin (10,000 units/mL)-streptomycin (10 mg/mL) solution and 10% FBS and cells were incubated at 37 °C and 5% CO_2. Experiments were performed with cells grown in culture for less than ten passages. Cells were grown to 80% confluency in 96-well plates and incubated with fluorescent non-pegylated and pegylated liposomes (200 μM of lipids) for 3 h. Cells were washed three times in PBS and liposome uptake in live cells was visualized with an Eclipse Ti Inverted Fluorescence Microscope (Nikon). Quantitative measurements of fluorescence intensity (Ex/Em of 590 nm/620 nm) were performed on a H4 Hybrid Microplate Reader (BioTek). The background fluorescence from untreated cells was subtracted from the obtained values. Cell viability measurements were then performed with a CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay according to the manufacturer's instructions.

2.4. Liposome biodistribution

Animal studies were conducted in accordance with the guidelines of the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals following a protocol approved by the Institutional Animal Care and Use Committee at the Houston Methodist Research Institute. Athymic nude mice purchased from Charles River (female; 6–8 weeks; ~22 g) were intravenously injected with PBS (control mice) or clodronate liposomes (clodrolip; 50 mg/kg clodronate) [21]. Mice were intravenously administered with fluorescent pegylated or non-pegylated liposomes (100 μL/mouse; 50 mM lipids). The liver, spleen, and plasma were collected 24 h post-injection. Blood collection was performed through cardiac puncture with needles pre-rinsed in EDTA (0.5 M; pH 8) and plasma was obtained through centrifugation in Microtainer Tubes with K2E (10 min; 3000 × *g*). A T25 Digital Ultra Turrax Homogenizer (Ika) was used to homogenize preweighed organs (1 g tissue/3 mL PBS). The fluorescence intensity (Ex/Em of 590 nm/620 nm) of serial dilutions of the samples was measured in black clear-bottom 96-well plates with a Synergy H4 Hybrid Microplate Reader (BioTek). Homogenized organs and plasma samples from untreated mice served as a background signal that was subtracted from the liposome samples.

2.5. Immunofluorescence staining

Mice were sacrificed 24 h post-injection of PBS (control mice) or clodrolip (50 mg/kg clodronate). Livers were placed in Tissue-Tek optimum cutting temperature (O.C.T) compound on dry ice. Immunofluorescence staining was performed on frozen acetone fixed liver sections (6 μm). The slides were blocked in 2.5% normal horse serum, incubated with a F4/80-Alexa Fluor 647 antibody (1:10 dilution) overnight at 4 °C, and mounted with Vectashield Antifade Mounting Medium with DAPI. The slides were visualized using a Nikon A1 Confocal Imaging System.

3. Results and discussion

3.1. Liposome characteristics and stability

Prior to assessing nanoparticle uptake *in vitro* and biodistribution *in vivo*, the size, polydispersity index (PDI), zeta potential, and fluorescence intensity of the liposomes was measured. Dynamic light scattering revealed that the non-pegylated and pegylated liposomes had a size of 111.7 ± 4.2 nm and 99.4 ± 1 nm, respectively (Fig. 2a), which is similar to the size of liposome formulations on