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In vivo hepatocyte MR imaging using lactose functionalized magnetoliposomes

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ABSTRACT

The aim of this study was to assess a novel lactose functionalized magnetoliposomes (MLs) as an MR contrast agent to target hepatocytes as well as to evaluate the targeting ability of MLs for *in vivo* applications. In the present work, 17 nm sized iron oxide cores functionalized with anionic MLs bearing lactose moieties were used for targeting the asialoglycoprotein receptor (ASGP-r), which is highly expressed in hepatocytes. Non-functionalized anionic MLs were tested as negative controls. The size distribution of lactose and anionic MLs was determined by transmission electron microscopy (TEM) and dynamic light scattering (DLS). After intravenous administration of both MLs, contrast enhancement in the liver was observed by magnetic resonance imaging (MRI). Label retention was monitored non-invasively by MRI and validated with Prussian blue staining and TEM for up to eight days post MLs administration. Although the MRI signal intensity did not show significant differences between functionalized and non-functionalized particles, iron-specific Prussian blue staining and TEM analysis confirmed the uptake of lactose MLs mainly in hepatocytes. In contrast, non-functionalized anionic MLs were mainly taken up by Kupffer and sinusoidal cells. Target specificity was further confirmed by high-resolution MR imaging of phantoms containing isolated hepatocytes, Kupffer cell (KCs) and hepatic stellate cells (HSCs) fractions. Hypointense signal was observed for hepatocytes isolated from animals which received lactose MLs but not from animals which received anionic MLs. These data demonstrate that galactose-functionalized MLs can be used as a hepatocyte targeting MR contrast agent to potentially aid in the diagnosis of hepatic diseases if the non-specific uptake by KCs is taken into account.

1. Introduction

Liver biopsies and serum biomarkers are the gold standard for predicting disease stage and prognosis in a wide range of liver diseases. However, interobserver variability, rare but possible complications of the invasive biopsy collection and the lack of quantitative information at various time points are some of the unsolvable limitations of these techniques [1,2]. In order to monitor the dynamic changes associated with liver diseases (such as hepatocarcinoma (HCC), fibrosis and cirrhosis) but also to monitor the success or failure of novel therapeutic approaches (for example stem cell therapy), the development of non-invasive imaging techniques would be beneficial for diagnosis and staging of liver diseases.

Many (pre-) clinical studies have already indicated MRI as the preferred methodology for liver imaging [2–5]. Apart from anatomical information, contrast agents have been utilized for several decades in clinical liver MRI in particular for the diagnosis of liver tumors [6–10]. Clinically approved contrast agents for hepatic MRI include non-specific gadolinium chelates, which are distributed in the extracellular interstitial space [11], reticulo-endothelial system (RES) specific contrast agents like functionalized superparamagnetic iron oxide particles (SPIO) that are selectively taken

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up by Kupffer cells (KCs) and endothelial cells in the liver [12,13] and as a third category, hepatocyte specific contrast agents, which target functional hepatocytes [14]. However, none of these can predict the functional status of the liver longitudinally [6,14]. It has been reported that the uptake of hepatocyte targeted contrast agents is reduced in hepatitis [15] and hereby also reflects hepatic function [16,17]. Therefore, determining hepatic function using hepatocyte targeted contrast agents would be of great benefit to diagnose liver diseases at an early stage.

After intravenous injection of SPIOs most are rapidly cleared from the blood by KCs, which are part of the RES. These particles then accumulate in several organs such as the liver, spleen and lymph nodes through the actions of the mononuclear phagocytic system. Hepatocyte targeting can be achieved by the introduction of cell recognizing ligands on the SPIOs surface. Frequently used receptors for hepatocyte targeting includes the asialoglycoprotein receptors (ASGP-r), which are abundantly present on the surface of hepatocytes [18]. ASGP-r recognizes galactose or N-acetylgalactosamine residues of desialylated glycoprotein and the uptake of galactosylated imaging probes correlates well with hepatic function [15–17,19]. To achieve this, addition of lactobionic acid [20], PVLA (polyvinylbenzyl-O-β-D-galactopyranosyl-D-gluconamide) [21], lactosylated amine [22], polycaprolactone-g-dextran [23], chitosoan – linoleic acid [24,25] and lactose functionalized anionic MLs [26] have been introduced to target ASGP-r from either freshly isolated hepatocytes or hepatocarcinoma cell lines. These studies have also demonstrated the feasibility of galactosyl coupled imaging probes for hepatocyte targeting. However, none of them address the long-term fate and distribution of the functionalized imaging probes in different hepatic cell types *in vivo* after systemic administration.

Liposomes incorporating iron oxide cores are generally referred to as MLs and have been used frequently as MR contrast agents [27]. The inner layer of the phospholipid bilayer is strongly chemisorbed onto the iron oxide core, while the outer layer is loosely adsorbed which allows to a limited degree exchange of these lipids with lipids from vesicles co-incubated with the MLs [28]. In this study, anionic MLs bearing 5% DMPG have been used as negative control. These anionic MLs were further functionalized by incorporation of 1% DOPE-lac; where the lactose is conjugated to the lipid by its glucose residue, leaving a terminal galactose residue accessible for ASGP receptor mediated uptake by hepatocytes. Previously, it was shown that galactosylated anionic MLs could specifically target ASGP-r present on HepG2 cells [26]. However, their specificity to target primary hepatocytes *in vitro* or *in vivo* has not been evaluated.

Here, we report on the target specificity and the label retention of lactose-functionalized MLs and non-functionalized anionic MLs *in vivo* in healthy mice using MRI, transmission electron microscopy (TEM) and histological staining. The future aim of this approach is the usage of lactose MLs as an agent for hepatocyte imaging and magnetic separation, and ultimately for contrast enhancement in the diagnosis of liver disease using MRI.

2. Materials and methods

2.1. MLs materials and characterization

Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-lactosyl (DOPE-lac) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The MLs were synthesized as described earlier [26]. For the hydrodynamic diameter measurements, particles were diluted with TES buffer (5 mM, pH 7.0) and were measured with Zetasizer Nano (Malvern, UK) at 25 °C. The average value of hydrodynamic diameter was determined by three independent measurements using three different runs of different particle batches. Electrophoretic mobility was determined with a Zetasizer Nano instrument (Malvern,UK) at 25 °C. Respective iron and phosphate contents were measured as described previously [28].

2.2. Hepatic cell isolation

Six animals were used for parenchymal (hepatocytes) and non-parenchymal (Kupffer cells and stellate cells) hepatic cell isolations performed on day two and four post lactose or anionic MLs intravenous administration. After intraperitoneal anesthesia, the liver was perfused through the portal vein for 5 min with pre-perfusion buffer (SC1, containing 8000 mg/L NaCl, 400 mg/L KCl, 88.17 mg/L NaH_2PO_4, 120.45 mg/L Na_2HPO_4, 2380 mg/L HEPES, 350 mg/L $NaHCO_3$, 190 mg/L EGTA, 900 mg/L glucose, pH 7.3) at 37 °C until it was completely discolored and then for 5 min with perfusion buffer (SC2, containing 8000 mg/L NaCl, 400 mg/L KCl 88.17 mg/L NaH_2PO_4 H_2O, 120.45 mg/L Na_2HPO_4, 2380 mg/L HEPES, 350 mg/L $NaHCO_3$, $CaCl_2$.$2H_2O$, pH 7.3) along with 0.25 mg/mL collagenaseP (Roche Applied Science, Mannheim, Germany). Centrifugation for 2 min at 50 *g* was performed to separate hepatocytes from the non-parenchymal cell fraction. HSCs and Kupffer cells were collected from the non-parenchymal fraction by fluorescent activated cell sorting (FACS Aria; Becton–Dickinson, Erembodegem, Belgium) using the endogenous UV in HSCs and an APC coupled F4/80 antibody (Life Technologies Corporation, Carlsbad, California, USA). Mouse hepatocytes were further purified by gradient centrifugation on a 25% Percoll gradient (GE Healthcare Life Science, Diegem, Belgium).

2.3. MR imaging

All experimental protocols were approved by the Institutional Animal Care Commission and Ethical Committee of the Katholieke Universiteit Leuven and performed in accordance with international standards on animal welfare.

2.3.1. In vivo imaging

30 male C57bl6 (weight 25–30 g) mice received lactose MLs and anionic MLs through intravenous injection of 200 µl (at 200 µg Fe/ml) MLs diluted in PBS. Mice were scanned under 1–2% isoflurane (carrier gas O_2) with a 9.4T Bruker Biospec small animal MR scanner (Bruker Biospin, Ettlingen, Germany; horizontal bore 20 cm) equipped with actively shielded gradients (600 mT m^{-1}). A quadrature radio-frequency resonator (transmit/receive; inner diameter 7 cm, Bruker Biospin) was used for image acquisition. Animals were scanned on the day of the injection and on day two, four, six and eight post particle injections. The *in vivo* MR imaging protocol used for liver imaging consisted of 2D T_2*-weighted fast low-angle shot (FLASH) and a multi-slice-multi-echo (MSME) sequence. The FLASH sequence (TE = 2.3 ms, TR set to 203 ms for six slices with thickness of 1 mm each and an in-plane resolution of 117 μm^2) was used to determine the decrease in the signal intensity (SI) post injection. T_2 values (maps) were determined from the MSME experiments and were used for a semi-quantitative analysis. Parameters for the MSME sequences were TR at least 3000 ms, echo spacing of 7 ms, with 234 μm^2 in plane resolution with six slices of thickness 1 mm each. In order to evaluate particle distribution post intravenous administration in other organs, mice were subjected to whole body scan with a Rapid Acquisition with Relaxation Enhancement (RARE) sequence (TE = 15.88 ms, TR = 6000 ms, spatial resolution of 200 μm^2, slice thickness = 0.5 mm with 50 slices) was performed. Mice were monitored using a monitoring and gating model (type 1030) from SA Instruments Inc. (Stony Brook, NY, USA) for controlling physiological parameters. Temperature (with rectal probe) and respiration were monitored and maintained during the acquisition at 37 ± 1 °C and 60–90 min^{-1}, respectively. All *in vivo* MRI measurements were respiration triggered. The study design is schematically illustrated in Fig. 1.

2.3.2. Ex vivo imaging

Isolated hepatic cell fractions were scanned by MRI in order to assess the targeting specificity and MRI detectability limits using different amounts of cells (from 5000 to 25,000 cells/µl). Samples were prepared by suspending isolated cells in 100 µl PBS followed by mixing with 1.5% agarose (Sigma) in a 1:1 ratio. Agar and cell suspensions were quickly transferred to 500 µl microcentrifuge tube one-third prefilled with solidified agar. All microcentrifuge tubes containing different hepatic cells at different cell density were assembled in a custom made plastic container completely filled with agarose. Upon solidifying, the agarose gel phantoms were scanned using a 9.4T MRI scanner. Similar coil configuration was used for phantom imaging as for animal imaging. 3D high resolution, T_2*-weighted MR images were acquired using a gradient echo sequence (FLASH, TR = 200 ms, TE = 15 ms with field of view 6.0 × 6.0 × 2.25 cm resulting in an isotropic resolution of 234 μm^3). T_2 maps were acquired using the MSME sequence also used for *in vivo* experiments (FOV and matrix adjusted for the size of the agar phantom).

2.3.3. Image processing

All images were processed with Paravision 5.1 (Bruker Biospin, Ettlingen, Germany). *In vivo* images were analyzed on a blind basis, irrespective of the particles injected. Reduction in *in vivo* SI was determined by selecting ROI on the liver image. White circles in Fig. 2 indicate the ROIs chosen on the liver section to calculate the mean SI. The SI was normalized using two same size ROIs drawn in the muscle region (SI of muscle = 100%). The relative mean SI was determined as SI Liver/SI muscle*100. T_2 values were determined from MSME scans. Pixel wise T_2 maps were calculated for a slice of interest using $A + c^*\exp(-t/T_2)$ with A, c and T_2 as variables. The whole liver was manually delineated as ROI on the T_2 maps. Thus T_2 values