A NOVEL MACROPHAGE IMAGING STRATEGY USING APOPTOTIC LIPOSOMES INCORPORATING PHOSPHATIDYLSERINE AND AZ-CHOLESTEROL

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Introduction: Macrophage inflammation is an important attribute of plaque instability in atherosclerosis. Molecular imaging approaches to detect macrophage content may provide early identification of this attribute and play a role in the clinical care of atherosclerosis. Imaging approaches for macrophages have involved the use of paramagnetic/supermagnetic particles (Gd- or Fe-oxide-based) conjugated to antibodies or ligands that recognize epitopes specific for macrophages, delivered as immunomiceles, liposomes, or lipoproteins. These approaches are hampered by complicated conjugation chemistry, expense, toxicity of the constituents and the need for higher field imaging owing to limited SNR. Herein, we present a simple and efficient Gd-based contrast agent strategy that takes advantage of macrophage engulfment of apoptosis that is simple and does not pose toxicity concerns. Exteriorized phosphatidylserine (PS) residues of cell membranes and oxidized lipids are apoptotic cues to engage macrophage engulfment. In order to express these cues on a liposomal surface to provide a target for macrophage engulfment, we synthesized a novel cholesterol derivative (cholesteryl-9-carboxynonanoate) that was included in a liposomal formulation along with PS and Gd (Az-Chl). The efficacy of this approach was assessed through in-vitro uptake experiments in cultured human macrophages and in-vivo studies in the Watanable Heritable Hyperlipidemic (WHHL) rabbit model along with pharmacokinetics studies and compared with control liposomes (without cholesteryl-9-carboxynonanoate).

Methods: Az-Chl was synthetized from cholesterol and azelaic acid. The paramagnetic liposomes consisted of 15 mol% Az-Chl, 20% cholesterol, 5% PS, 20% Gd-DTPA-SA, 39.75% phosphatidylcholine conjugated to 0.25% rhodamine phosphatidylethanolamine to allow for confirmatory flow-cytometry and confocal microscopy studies. Vesicles were made by lipid hydration followed by sonication. Control liposomes were prepared without Az-Chl. WHHL Rabbits (n=5) were fed a normal chow for 12 months. Anesthetized animals were injected IV with Az-Chl (0.02mmol/kg of Gd) or control liposomes and their uptake in atherosclerotic plaque was assessed by MRI. The abdominal aorta was imaged on a 1.5 T clinical MRI scanner (Avanto, Siemens) using T1-weighted gradient echo approach spanning the iliac bifurcation to the superior pole of the topmost kidney (≅30 slices, TR/TE=260/5.91, NEX=3, flow and fat suppression). Images were acquired at pre-contrast, 1, 12 and 24 hrs post-contrast administration time points. The same time points were used for blood collection from each animal to study pharmacokinetics of Az-Chl and control liposomes. Next, animals were sacrificed and abdominal aortas were removed. Sectioned aortic tissues were stained with RAM11 macrophage specific antibody and nuclear dye followed by confocal microscopy. Gd Concentrations in blood samples were determined by ICP-MS while Az-Chl levels were quantitated using MRM method on ESI-mass spectrometer in combination with liquid chromatography. In vitro studies to assess uptake of Az-Chl and the receptor mechanisms were performed on cultured macrophages isolated from human monocytes differentiated by macrophage-colony stimulating factor (M-CSF) and subjected to various antibodies to block apoptotic clearance pathways.

Results: A simple one-step synthetic procedure allowed production of large quantities of Az-Chl (74% yield after purification on SiO_2). Mean diameter of Az-Chl and control liposomes were 92 and 104 nm respectively. Longitudinal relaxivities of studied formulations were 5.9±0.1 and 5.7±0.1 $mM^{-1}s^{-1}$ for Az-Chl and control liposomes respectively. Typical MR images at different time points along with confocal microscopy data of matched plaque region are shown on Fig. 1A and B. Delineation of vessel wall was clearly seen event at 24 hrs post contrast injection with Az-Chl but not with control liposomes (Fig. 1B). These findings were confirmed by rhodamine fluorescence from Az-Chl liposomes and co-localization with macrophages (green RAM-11 staining) by confocal microscopy, wherein Az-Chl liposomes laden macrophages appeared yellow on merged images. Normalized enhancement (NENH) values showed signal enhancement with both Az-Chl and control vesicles (compared to pre-injection), with the differences between these formulations becoming statistically significant at later time points (12 and 24 hrs), proving prolonged retention with Az-Chl (Fig. 1C). Pharmacokinetic studies demonstrated rapid clearance from circulation with control liposomes compared to Az-Chl, which was detected in plasma > 24 hrs after injection. Pharmacokinetic trends were confirmed for Az-Chl liposomes by quantitation of Az-Chl concentrations in plasma using MRM ESI-MS suggesting integrity of Az-Chl particles in-vivo (Fig. 1D). In-vitro uptake studies were performed on human macrophages and demonstrated preferential rapid uptake of Az-Chl particles compared to control resulting in ~5-fold increase in uptake after 2 hrs of incubation (Fig. 2A and B). Macrophage uptake of Az-Chl was reduced by antibodies targeting αvβ3 ($p<0.000001$) and αvβ5 V ($p<0.002$) but not by antibodies targeting CD36 or scavenger receptor A. Macrophages ingesting Az-Chl had lower TNF-α expression compared with control liposomes (Fig. 2C).

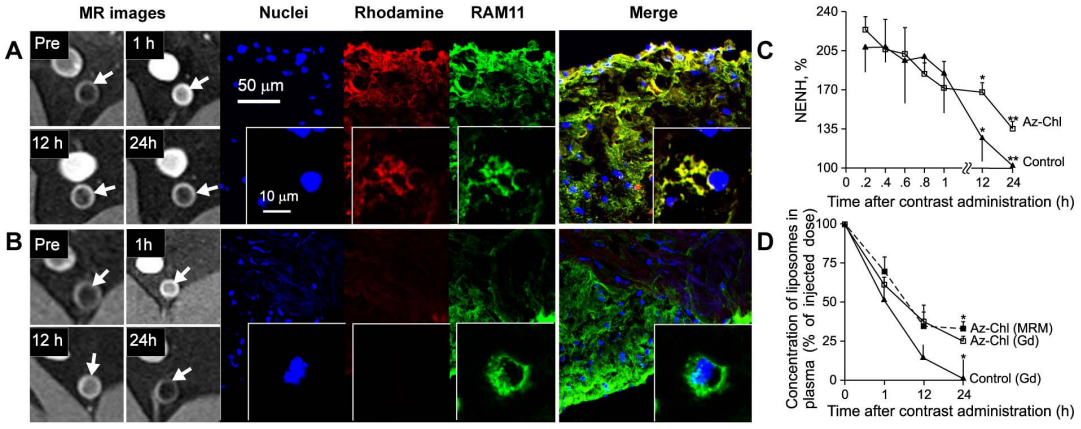

Fig. 1. In-vivo testing of Az-Chl. MR images pre and post Az-Chl (**A**) and control liposomes (**B**) administration, and confocal microscopy images of matched regions. **A and B:** arrows indicate abdominal aorta enhancement at different time points. **C:** NENH values of wall enhancement for Az-Chl and control. **D:** The amount of Gd in plasma was quantified with ICP-MS (Az-Chl-Gd and Control-Gd) and with MRM ESI-MS (Az-Chl-MRM). Liposomes concentration in plasma is shown as a percentage of the dose injected.

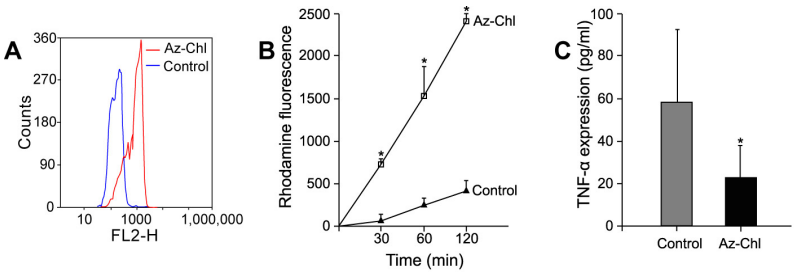

Fig 2. In-vitro study of uptake of Az-Chl liposomes by human macrophages as compared to control. A: Flow-cytometry histogram shows higher fluorescent intensity of Az-Chl when compared to control after 30 min of incubation. **B:** Time course of Az-Chl uptake. **C:** TNF-α expression in macrophages exposed to Az-Chl or control.

Conclusions: Gd containing Az-Chl liposomes to mimic apoptotic particles allow for in-vivo imaging of macrophages in atherosclerosis. Az-Chl liposomes demonstrate favorable pharmacokinetics over a 24 h period and are rapidly taken up by human macrophages by an αvβ3 and αvβ5 dependent mechanism. Our findings have important implications for identification of unstable plaque.