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Immuno-magnetoliposomes targeting activated platelets as a potentially human-compatible MRI contrast agent for targeting atherothrombosis

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ABSTRACT

To detect unstable atherosclerotic plaques early and noninvasively would be of great clinical interest. Activated platelets are an interesting molecular target for detecting early lesions or unstable plaques. We therefore developed an MRI contrast agent consisting of magnetoliposomes (ML) linked to an antibody (anti-LIBS) specifically targeting the ligand-induced binding site of the activated GPIIb/IIIa receptor of platelets. ML were prepared by dual centrifugation (DC). ML pegylation up to a total PEG content of 7.5 mol% positively influenced the stability and amount of entrapped SPIOs, and also reduced SPIO-membrane interactions, while higher PEG contents destabilized PEG-ML. Stable anti-LIBS-ML with high amounts of entrapped SPIOs (~86%, ~0.22 mol Fe/mol liposomal lipid) and high MRI sensitivity (relaxivity $r_2 = 422\ s^{-1}\ mM^{-1}$ and $r_2^* = 452\ s^{-1}\ mM^{-1}$) were obtained by coupling anti-LIBS to ML in a two-step post-insertion technique. We confirmed specific binding to the GPIIb/IIIa receptor's activated conformation on activated human platelets and cell lines expressing activated GPIIb/IIIa receptor *ex vivo*. The immuno-ML obtained in this study constitute an important step towards developing a potentially human-compatible MRI contrast agent for the timely detection of plaque rupture by targeting activated platelets.

1. Introduction

Characterized by plaque formation in arterial vessels, atherosclerosis is one of the most widespread diseases in the western world. While stable plaques may be present without causing any harm for years, unstable plaques can be life-threatening. Unstable plaques develop during atherosclerosis progression due to intraplaque inflammatory processes [1]. When a plaque ruptures, a subsequent rapid thrombosis often leads to acute myocardial infarction or stroke [2]. To reduce the incidence of infarction or stroke, a non-invasive, rapid and rather simple method for detecting unstable plaques would be highly desirable.

So far, much research has focused on the active imaging of different stages during atherosclerosis progression. Potential targets are activated endothelial cells, ox-LDL in the plaques, macrophages as inflammatory markers, apoptotic cells, activated platelets, or acute thrombosis [3,4]. Activated platelets are of special interest since they can be found in plaques at early asymptomatic stages of plaque inflammation and during arterial thrombus formation after plaque rupture [5,6]. We have previously described the imaging of activated platelets by targeting their ligand-induced binding sites (LIBS), which are only exposed upon

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activation of the platelet glycoprotein IIb/IIIa-receptor (GPIIb/IIIIa). Techniques in these approaches included ultrasound, SPECT-CT, or MRI [7–10], but all these methods have advantages and disadvantages. However, MRI as a tool for molecular imaging has key clinical advantages over other imaging approaches: it involves no radiation, enables the sensitive detection of paramagnetic contrast agents, and provides anatomical information thanks to good spatial resolution [3]. Superparamagnetic iron oxide particles (SPIOs) have special magnetic properties which cause hypointense contrast in MRI [11]. Furthermore, SPIOs offer good biocompatibility, low toxicity, and have already been used for the passive imaging of liver disease [12]. Thus SPIOs are an ideal superparamagnetic MRI contrast agent.

We previously described the application of microparticles of iron oxide (MPIOs) coated with anti-LIBS-scFv (anti-LIBS-MPIO) for active MRI-imaging of activated murine platelets *in vivo* and of activated human platelets *ex vivo* [9,10,13]. Despite being useful as sensitive MRI contrast agent for basic research of murine platelet behavior during atherosclerosis, MPIOs are unsuitable for human application due to their size (~1 μm) and being coated with a tetradentate cobalt chelator.

Immuno-SPIOs, which are much smaller and more suitable for future human application, have already been used to actively image activated platelets in *ex vivo* and *in vivo* models. Unfortunately, resolution of the immuno-SPIOs was too low for active vascular imaging *in vivo* by MRI [14]. MRI sensitivity and therefore the r_2 and r_2^* relaxivity of SPIOs can be increased by agglomeration [12]. One strategy for controlled agglomeration is to entrap the SPIOs into liposomes, generating magnetoliposomes (ML), which are useful contrast agents [18]. Entrapping SPIOs in liposomes increases their relaxivity r_2 up to five-fold [15]. Furthermore, ML are useful as a platform for several applications [16,17]. Adding an active compound, ML may be used as theranostica as well [18]. Having been in use for years in cancer therapy, there is ample evidence that liposomes in general are efficient drug carriers. The active targeting of liposomes or ML is also easily achieved by modifying their surface with special ligands [19–21].

ML preparation via the passive entrapment of SPIOs in liposomes is achievable with the simple film-hydration method followed by extrusion [18,22,23], reverse phase evaporation method [24,25], or by sonication [23]. A new technique for the fast and simple preparation of liposomes with high entrapment efficiency is the dual centrifugation (DC) method. In contrast to classic centrifugation, during DC, the samples additionally rotate around their own vertical axes, which results in a unique combination of two movements of the sample material with and against the centrifugal force. The resulting inner friction causes high shear forces and thus, highly effective homogenization. To prepare liposomes, molecular disperse lipid mixtures (40 mg) are hydrated with a small amount of aqueous phase (60 μl) and homogenized in a dual centrifuge to achieve viscous vesicular phospholipid gel (VPG). VPG is a highly concentrated, creamy liposome dispersion in which the amount of water outside and inside the liposomes is nearly identical, which results in high entrapment efficiency up to 50% for compounds dispersed in the water phase. After preparation, VPG can be easily diluted to a liposome dispersion. Furthermore, the DC can be used under aseptic conditions; it permits the entrapment of sensitive compounds [26,27]. Since high concentrations of SPIO in ML are a prerequisite for high MRI sensitivity [15], we developed ML preparation by DC.

To actively target activated platelets with ML, a previously described single chain antibody (anti-LIBS-scFv) [9,10,13] appears suitable since it binds specifically to the LIBS of activated murine and human GPIIb/IIIa on platelets. Essentially, anti-LIBS-scFv does not block the fibrinogen binding site, thereby allowing clot formation [28,29].

In this study, we developed a simple, highly reproducible and speedy method for preparing stable immuno-ML (anti-LIBS-ML) with high amounts of entrapped SPIO and high MRI-sensitivity measured as relaxivity r_2 and r_2^*. Furthermore, we confirmed the specific binding of these anti-LIBS ML to the activated GPIIb/IIIa receptor *in vitro*.

2. Material & methods

2.1. Material

2.1.1. Cells expressing activated and non-activated GPIIb/IIIa

Previously described Chinese hamster ovary (CHO) cell lines stably expressing the activated and non-activated GPIIb/IIIa receptor were used as a reliable test platform for immunofluorescence experiments with immuno-ML [28,30]. The cells were cultivated at 37 °C under 5% CO_2 atmosphere.

2.2. Human platelet rich plasma

Citrate-blood samples were collected from volunteers with no relevant medical history and not taking any medications affecting platelet activity for at least ten days. The blood was centrifuged at 4 °C and 100 × g for 5 min. The collected supernatant was centrifuged again at 4 °C and 100 × g for 5 min to obtain human platelet rich plasma (PRP). The fresh PRP was immediately used for the flow cytometry experiments.

2.3. Anti-LIBS and control scFv

We generated codon-optimized DNA encoding targeted (anti-LIBS) or non-targeted (control) scFv directed against GPIIb/IIIa expressed on activated platelets. Plasmid integrity was confirmed by sequencing. Drosophila S2 cells were cultured in Express Five SFM medium containing L-glutamine (f.c. 18 mM) and 1% penicillin/streptomycin (all from Invitrogen). Cells were maintained at 28 °C in ventilated polycarbonate Erlenmeyer flasks (Corning) under constant rotation. Cells were diluted to 1×10^6 cells/ml and mixed with 80 ng/ml DNA preincubated with 250 ng/ml dimethyldioctadecylammonium bromide for 30 min. Cells were then incubated for 2 days before inducing protein production with $CuSO_4$. After 6 days, the cell supernatant was centrifuged at 15,000 × g for 15 min. Cell supernatant was applied to a chelating Sepharose fast flow column (20 ml bed volume, 5 ml/min flow rate, GE Healthcare). The column was washed to baseline with PBS, 0.5 M NaCl and 10 mM imidazole in 50 mM Tris, pH 8.0 to remove non-specifically bound proteins. Elution was carried out with 50 mM imidazole in 50 mM Tris, pH 8.0. Fractions of 2 ml were collected. Fractions containing significant amounts of product were pooled and dialyzed against PBS. For further purification, the scFv was concentrated to 10 mg/ml (10 kDa cut-off spin column, Millipore) and 1 ml loaded onto a HiPrep 16/60 Sephacryl S-200 HR (GE Healthcare) with a flow rate of 1 ml/ml PBS (without Ca/Mg). The main peak was collected, again concentrated and stored at −80 °C until further use. SDS-PAGE analysis revealed a homogenous band with about 92% purity. Protein concentration was determined with a BCA Protein Assay Kit (Pierce) with bovine serum albumin as a standard.

3. Methods

3.1. Preparation of ML by DC

The ML were prepared according to the previously-described DC method [26]. Therefore, the lipids POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine Corden Pharma Switzerland LLC), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine Lipoid GmbH, Ludwigshafen, Germany), m-PEG_{2000}DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) Avanti Polar Lipids, Inc, Alabaster, AL, USA) and cholesterol (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) were dissolved in chloroform (Sigma Aldrich Chemie GmbH, Steinheim, Germany). When indicated, 0.5 mol% of the fluorescence dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate Life Technologies, Darmstadt, Germany) was added. A total amount of 40 mg of lipid was mixed in a 2 ml twist top vial (Sorenson Bio Science, Inc, Salt lake City, UT, USA) and the mixture was dried under constant N_2 stream at 60 °C. Afterwards, the lipid film was dried for several hours at high vacuum. The lipid films were stored at −20 °C under argon atmosphere until they were used.

To prepare ML by DC, the lipid films were rehydrated with 60 μl SPIO solution (SHP-10; Ocean Nano Tech, Springdale, AR, USA), and 300 mg of Silibeads® (ø 0.6–0.8 mm, Sigmund Lindner GmbH, Warmensteinach Germany) were added. The lipid-SPIOs-mixture was homogenized in a dual centrifuge (ZentriMix 380R, Andreas Hettich GmbH & Co KG, Tuttlingen) for 30 min at 2350 rpm or in a dual centrifuge (DC 150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany) for 30 min at 3540 rpm. The resulting viscous vesicular phospholipid gel VPG was than diluted with 200 μl of 10 mM Hepes (Carl Rhoth GmbH & Co. KG, Karlsruhe, Germany) buffer pH 7.4 and was again homogenized for 5 min at 3540 rpm to achieve liposome dispersion.