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A molecular intravascular ultrasound contrast agent allows detection of activated platelets on the surface of symptomatic human plaques

Alexander Maier [a], Patricia Plaza-Heck [a], Fabian Meixner [a], Felix Guenther [a, b], Beat A. Kaufmann [c], Mara Kramer [a], Timo Heidt [a], Andreas Zirlik [a], Ingo Hilgendorf [a], Jochen Reinöhl [a], Peter Stachon [a], Peter Bronsert [d], Ralf Birkemeyer [e], Irene Neudorfer [a], Karlheinz Peter [f], Christoph Bode [a], Constantin von zur Mühlen [a, *]

[a] Department of Cardiology and Angiology I, Heart Center Freiburg University, Faculty of Medicine, University of Freiburg, Germany
[b] MediClin Staufenburg Klinik, Durbach, Germany
[c] Department of Biomedicine, University of Basel, Switzerland
[d] Institute of Pathology & Comprehensive Cancer Center, University Medical Center Freiburg, Faculty of Medicine, University Freiburg, Germany
[e] Herz-Klinik Ulm, Ulm, Germany
[f] Baker IDI Heart & Diabetes Institute, Melbourne, Australia

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ABSTRACT

Background and aims: Activated platelets are amongst the most attractive imaging targets in atherosclerosis due to their important role in early processes of atherogenesis and thrombus formation. We developed a molecular intravascular ultrasound (IVUS) approach to detect activated platelets *ex vivo* on the surface of human plaques, using an IVUS system applied in clinical routine.
Methods: Human carotid endarterectomy specimens were obtained directly from the operating room and exposed to artificial arterial flow conditions for incubation with the contrast agent. This consists of microbubbles (MB), which are linked to an antibody against the ligand induced binding site (LIBS) of the activated platelet glycoprotein IIb/IIa, and a sialyl Lewis polymer (SL), which mediates binding to selectins (LIBS-SL-MB). IVUS was performed pre and post incubation with LIBS-SL-MB and after rinsing with PBS. In comparison, IVUS was performed pre and post incubation with MBs linked to an unspecific control antibody and a dysfunctional polymer (control-MB). All imaging results were correlated to histology findings.
Results: IVUS imaging showed a high signal enhancement after administration of LIBS-SL-MB. After rinsing with PBS, the signal enhancement remained stable. Immunofluorescence and immunohistochemistry confirmed significant binding of microbubbles to thrombi on the plaque surface. Moreover, thrombus size and number of bound MBs correlated well.
Conclusions: LIBS-SL-MB allows *ex vivo* IVUS imaging of even small numbers of activated platelets on the surface of human carotid endarterectomy specimens. This diagnostic approach could deliver valuable additional information for risk stratification of atherosclerotic plaques, especially since we apply a clinically well-established IVUS imaging system.

1. Introduction

Ruptured atherosclerotic plaques or endothelial erosions are seen as the precipitating events for thrombus formation, resulting in acute coronary syndromes or stroke [1,2]. Activated platelets are the key to thrombus formation, but also play an important role in the development of vascular inflammation and therefore atherosclerosis [3], since they adhere to the endothelial surface of vulnerable or recently ruptured plaques even in small numbers, contributing to the inflammation in early stages of atherosclerosis [4]. Due to this important pathophysiologic role, imaging of aggregated or adhering platelets would be an attractive opportunity for further characterization of atherosclerotic lesions and/or

* Corresponding author. University Heart Center Freiburg, Hugstetter Strasse 55, 79106 Freiburg, Germany.
E-mail address: constantin.vonzurmuehlen@universitaets-herzzentrum.de (C. von zur Mühlen).

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plaque erosions, especially in patients with culprit non-obstructive plaques or acute coronary syndromes. According to current guidelines, intravascular ultrasound (IVUS) can be used to detect stent-related mechanical problems, to optimize stent implantation, or to assess mechanical stent failure [5]. So far, approaches of IVUS combined with molecular imaging ultrasound contrast agents have not been widely investigated [6], although dedicated contrast agents for vascular surface characterization would add an additional diagnostic dimension. Since platelets are small cells not discernible with the relatively low spatial resolution of IVUS, construction of specific contrast agents is challenging. Gas filled microbubbles (MBs) are well established as an ultrasound contrast agent in clinical routine [7], and have a diameter of 4–5 μm. Coupling of such MBs with platelet specific markers is promising for molecular characterization and detection of rupture prone or already ruptured plaques with IVUS. Recent animal studies have used microbubble-based molecular ultrasound contrast agents for detection of inflammation in atherosclerosis [8,9] and myocarditis [10]. However, studies describing this application in human vascular pathologies are missing, especially applying commercially available IVUS catheters already used in clinical routine.

In this project, we report the application of a platelet specific ultrasound contrast agent in symptomatic human carotid thrombendarterectomy specimens under arterial blood flow conditions. We use a unique dual-targeted contrast agent, which specifically detects activated platelets by targeting P-selectin via the sialyl Lewis polymer (SL), and the ligand induced binding sites (LIBS) of the activated GP IIb/IIIa receptor with an IgG antibody. These two molecular markers are linked to decafluorobutane gas filled autofluorescent microbubbles (MBs), resulting in the dual-targeted contrast agent (LIBS-SL-MB). The LIBS epitope was used successfully in several projects by our group for targeting activated platelets in magnetic resonance imaging (MRI) [11–14], positron emission tomography (PET) [15], molecular ultrasound [16,17] and fluorescence computed tomography (FLECT) [18] imaging, and is responsible for the specific binding of the contrast agent construct. On the other hand, P-selectin is involved in “platelet rolling“ and loose contact between circulating platelets and vascular endothelium, supporting the efficient binding of the large MBs to the areas of interest.

2. Materials and methods

2.1. Contrast agent

2.1.1. Platelet specific contrast agent (LIBS-SL-MB)

2.1.1.1. LIBS-IgG purification:. The monoclonal LIBS-IgG antibody was produced in hybridoma cells. Cells from the clone anti-LIBS-145-mAb were cultivated under sterile conditions in 100 ml of culture medium at 37 °C and 5% CO_2. Every three days for one month, 50 ml of the culture medium were removed and exchanged with fresh medium. The exchanged medium was stored at −20 °C. After defreezing at 4 °C, the collected suspension was centrifuged for 15 min at 1000 rpm. LIBS-IgG was purified from the supernatant with the affinity chromatography Nab™ Protein A/G Spin Kit (#89980, Thermo Scientific, Rockford, USA). After using the elution buffer, protein concentration measurements were performed with a NanoDrop 2000c Spectrophotometer (NanoDrop products, Wilmington, USA). The purity of the antibody elutions with the highest protein concentrations was tested using Laemmli's Sodiumdodecylsulfat-Polyacrylamidgel Electrophoresis. 50 μl protein samples in PBS without Ca^{2+}/Mg^{2+}, with a concentration of 0.2 μg/μl, was reduced with a 5× Laemmli buffer, denaturized for 5 min at 95 °C and placed in the electrophoresis chamber (Bio-Rad Laboratories GmbH, München, Germany) for 30–45 min at 200 V power. Protein bands were made visible using Coomassie-Blue solution (Thermo Scientific, Rockford, USA) and then rinsed with 6% acetic acid (Merck, Darmstadt, Germany). The eluates with high protein concentration were pooled together and remeasured using NanoDrop. These were then placed into Amicon filter devices (Amicon Ultra-15 Centrifugal Filters, Millipore, Billerica, USA) with a molecular weight cut off of 100 kDa for their respective concentration and dialysis with PBS without Ca^{2+}/Mg^{2+}. Finally, the protein concentration was measured one last time. For evaluating binding purposes, the antibody was incubated for 60 min at room temperature with the appropriate volume of a biotin solution (EZ-Link™- Sulfo-NHS-LC-Biotinylation kit, Thermo Scientific, Rockford, USA). The excess biotin was removed using Zeba Spin Desalting Column (Thermo Scientific, Rockford, USA). The HABA assay (Thermo Scientific, Rockford, USA) was used for quantification of the level of incorporated biotin.

2.1.1.2. Microbubbles. Biotinylated, lipid shelled microbubbles (MB) containing decafluorobutane gas were stabilized with red fluorescent distearoyl phosphatidylcholin (dioctadecyltetramethylindocarbocyanine, excitation maximum 549 nm, emission maximum 563 nm). Preparation of the MBs is described in detail elsewhere [19].

The binding of the biotinylated antibodies with the biotinylated microbubbles was achieved through streptavidin bridging (3 μg streptavidin/10^7 MB, Sigma-Aldrich, St. Louis, USA). For binding with sLE^a (01–044, Gaithersburg, MD, USA) or dysfunctional control-PAA (01-000, Gaithersburg, MD, USA) and LIBS-IgG or a non-specific control-IgG (248.896.0145, Innovative Research, Inc., Novi, Michigan, USA), the MBs were incubated with 0.8 μg sLE^a/control-PAA per 10^7 MBs for 10 min, and thereafter with 30 μg LIBS-IgG or control-IgG per 10^7 MBs for 10 min.

A schematic graph of the contrast agent is given in Supplemental Fig. 2.

2.2. Formation of platelet aggregates

Platelet rich plasma (PRP) was activated with ADP, actin and calcium chloride to build thrombi, which were incubated with either LIBS-SL-MB or control-MB for 15 min and washed with PBS for 10 min. Thrombi were then fixed with 4% formalin solution and embedded using OCT (Sakura Finetek, Netherlands). 10 μm cryosections were stained for CD61 as described elsewhere [20] and for fluorescence staining with the ABC-AP reagent (Vectastain ABC-AP Kit, Vector Laboratories, Burlingame, USA). The binding of the contrast agent was evaluated microscopically.

2.3. IVUS

We used a standard VOLCANO intravascular ultrasound catheter (VOLCANO Revolution catheter, 45 MHz, Volcano, San Diego, USA) as it is used in routine interventional cardiology. The catheter was pulled manually with a constant pull back rate of 1 mm/s through the agarose gel or the plaque, respectively. IVUS videos were analyzed visually at the site of the largest thrombus of a plaque with nine frames per thrombus. In each frame hyperechoic „spheres“ and „double layer sandwiches“ were counted after incubation with LIBS-SL-MB or control-MB, and after rinsing with PBS. Binding affinity was quantified as the quotient of means of the nine frames pre and post rinsing with PBS.

2.4. Agarose gel with activated platelets

2.5% agarose-gels (Biozym Sieve GP Agarose, #850080, Biozyme, Oldendorf, Germany) with a column of activated platelets from PRP