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High-frequency ultrasound-guided disruption of glycoprotein VI-targeted microbubbles targets atheroprogressison in mice

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ABSTRACT

Targeted contrast-enhanced ultrasound (CEU) using microbubble agents is a promising non-invasive imaging technique to evaluate atherosclerotic lesions. In this study, we decipher the diagnostic and therapeutic potential of targeted-CEU with soluble glycoprotein (GP)-VI *in vivo*. Microbubbles were conjugated with the recombinant fusion protein GPVI-Fc (MB_{GPVI}) that binds with high affinity to atherosclerotic lesions. MB_{GPVI} or control microbubbles (MB_C) were intravenously administered into $ApoE^{-/-}$ or wild type mice and binding of the microbubbles to the vessel wall was visualized by high-resolution CEU. CEU molecular imaging signals of MB_{GPVI} were substantially enhanced in the aortic arch and in the truncus brachiocephalicus in $ApoE^{-/-}$ as compared to wild type mice. High-frequency ultrasound (HFU)-guided disruption of MB_{GPVI} enhanced accumulation of GPVI in the atherosclerotic lesions, which may interfere with atheroprogression. Thus, we establish targeted-CEU with soluble GPVI as a novel non-invasive molecular imaging method for atherosclerosis. Further, HFU-guided disruption of GPVI-targeted microbubbles is an innovate therapeutic approach that potentially prevents progression of atherosclerotic disease.

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1. Introduction

Atherosclerosis is a chronic disease with silent and gradual progression until it becomes clinically relevant [1–3]. Atherosclerotic lesions are characterized by enhanced lipid accumulation, inflammation, protease activity, and extracellular matrix reorganization [3]. Vulnerable atherosclerotic plaques are prone to rupture and thrombus formation primarily triggered by collagen exposure towards the direction of blood stream or circulation [1–3]. Platelets accumulate at sites of vulnerable atherosclerotic lesions, critically regulate apoptosis and survival of infiltrating cells, and determine thrombus formation as well as atheroprogression [4,5]. Platelet adhesion to atherosclerotic lesions is primarily mediated through the platelet-specific collagen receptor glycoprotein VI (GPVI) [6]. Binding of collagen to GPVI receptor on platelets favors platelet adhesion, activation and secretion which is governing mechanisms that contribute to atherothrombosis [7]. The soluble dimeric GPVI receptor has high affinity for immobilized collagen within atherosclerotic plaques [8]. Soluble GPVI receptor competes for collagen binding sites with surface associated platelet GPVI receptor and thereby inhibits platelet adhesion onto collagen *in vitro* and *in vivo* [8,9]. Thus, systemic administration of soluble GPVI inhibits thrombus formation and progression of atherothrombosis in mice [10–12].

Targeted contrast-enhanced ultrasound (CEU) using microbubble (MB) agents has been shown to be a promising non-invasive imaging technique to evaluate the degree of atherosclerosis in mice [13–17]. In these studies, specific monoclonal antibodies such as anti-vascular cell adhesion molecule 1 (VCAM-1) -antibodies were conjugated to the surface of MB in order to direct the latter towards the site of lesions enriched with the specific epitope [13]. Attachment of targeted MB to the respective lesions can finally be quantified by non-invasive CEU; a method with high potential for early risk stratification [13]. However, targeted MB agents can also be destructed using focused high-frequency ultrasound (HFU) [18].

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This therapeutic approach enables localized drug delivery at target site [19,20].

In the present study, we provide novel experimental evidence that ultrasound-guided molecular imaging with GPVI-targeted MB (MB_{GPVI}) can detect atherosclerotic lesions in mice. Moreover, we show that HFU-mediated disruption of MB_{GPVI} substantially suppresses accumulation of platelets at sites of atherosclerotic lesions that might have an impact on atheroprogression.

2. Materials and methods

2.1. Protein biotinylation

Synthesis of soluble GPVI (GPVI-Fc) was performed as previously described [8]. Biotinylation was performed with the EZ-Link™ Sulfo-NHS-Biotinylation Kit (Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturer's instructions and the concentration of protein was measured with Bradford reagent. As control, bovine serum albumin (BSA) was biotinylated with the same kit.

2.2. ELISA

To confirm successful biotinylation an Immuno HB 96-well plate (Thermo Fisher, Schwerte, Germany) was coated overnight at 4°C with 1 μg collagen (type I bovine, BD Biosciences, San Jose, CA, USA) in 100 μl coating buffer (1.59 g/l Na_2CO_3, 2.93 g/l $NaHCO_3$ and 0.2 g/l NaN_3 pH 9.6). To detect biotin, streptavidin horseradish peroxidase (DakoCytomation, Glostrup, Denmark) and 3,3′,5,5′-tetramethylbenzidine (Serva, Heidelberg, Germany) were used and absorbance was measured at 450 nm against reference wavelength 570 nm using an ELISA 550 plate reader (Bio-Rad, Munich, Germany).

To evaluate the functionality of the biotinylated GPVI-Fc a 96-well plate was coated as described and blocked overnight at 4°C with 1% BSA (30% solution; PAA Laboratories GmbH, Pasching, Austria). The plate was incubated for 1 h at room temperature with biotinylated GPVI-Fc or biotinylated BSA in concentrations as indicated. GPVI-Fc binding to collagen coated surface was analyzed with anti-human IgG-HRP (Jackson Immuno Research Europe, Newmarket, UK) 1:10 000 in PBS.

2.3. Generation of GPVI-targeted microbubbles

Microbubbles Vevo MicroMarker™ Target-ready contrast agent (VisualSonics Inc., Toronto, Canada) were used according to manufacturer's instructions. Microbubbles were reconstituted with 400 μl of saline, gently agitated and allowed to sit at room temperature for 5 min. Reconstituted microbubbles were incubated either with biotinylated GPVI-Fc or biotinylated BSA at a concentration of 0.05 mg/ml at room temperature for 15 min.

2.4. Transmission electron microscopy (TEM)

For immunogold labeling subsequent transmission electron microscopy, microbubbles were reconstituted as described above, but in the absence of a ligand. For native staining, 3 μl of 1:10 diluted MB were put on a 400 mesh. For immunoelectron microscopy, reconstituted MB were incubated with biotinylated goat anti-mouse IgG antibody (Vector laboratories, Burlingame, CA, USA) and counterstained with gold labeled donkey anti-goat IgG-6 nm (Jackson ImmunoResearch laboratories, West Grove, PA, USA). Samples were examined using a Zeiss LIBRA 120 transmission electron microscope (Zeiss, Oberkochen, Germany) operating at 120 kV.

2.5. Flow cytometry

Biotinylated GPVI-Fc and BSA were labeled with fluorescein-isothiocyanate (FITC) (Sigma–Aldrich, Steinheim, Germany) and fractions were separated in PD10-columns (Sephadex G-25M, Pharmacia, Uppsala, Sweden). Microbubbles were reconstituted as described above and incubated either with biotinylated and FITC-labeled GPVI-Fc or BSA. Pure microbubbles were used as control and samples were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences, Heidelberg, Germany).

2.6. Flow chamber assay

Coverslips were coated with 100 μg/ml collagen (Horm®, Takeda, Linz, Austria) overnight at 37°C and blocked for 1 h with 1% bovine serum albumin (Albumin Fraction V, Applichem GmbH, Darmstadt, Germany) at room temperature. The flow chamber was inverted and a syringe pump (KDS-100- CE, KD Scientific, Holliston, MA, USA) was used to perfuse the microbubbles through the flow chamber. Microbubbles were reconstituted and incubated as described. Microbubbles (1×10^6/ml) were perfused at a low shear rate (50 s^{-1}) for 5 min. The shear rate was gradually increased first up to 1000 s^{-1} for an additional 5 min and then up to 1750 s^{-1}. The flow chamber was flushed with 1 ml of saline at a shear rate of 1000 s^{-1} for 5 min and rest of the saline (0.4 ml) at a shear rate of 1750 s^{-1}. Images were captured after flushing with NIS-Elements BR 2.1 (Nikon, Düsseldorf, Germany) and binding of MB_{GPVI} and MB_C was counted.

2.7. Animals

Male C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany) and were used for the experiments at an age of 12 months. $ApoE^{-/-}$ mice (B6.129P2-ApoE ^{tm1Unc}/J) at an age of 12 months (12 m) and 3 months (3 m) were used from our own breeding facility. Breeders were obtained from Charles River (Sulzfeld, Germany). All animal experimentations were performed in accordance with the guidelines for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), the guidelines for the use of living animals in scientific studies, and the German law for the protection of animals and approved by the local authorities (Regierungspräsidium Tübingen).

2.8. Binding of MB_{GPVI} to the plaque area in aortic arch and truncus brachiocephalicus visualized by ultrasound

MB_{GPVI} and MB_C were generated as described above. 12 m ($n = 16$), 3 m -old $ApoE^{-/-}$ and C57/BL6 ($n = 8$) mice were analyzed. Ultrasound was performed with a Vevo 2100 high-resolution imaging system (VisualSonics Inc., Toronto, Canada). Animals were anesthetized with Isofluran (Isofluran CP; CP Pharma, Burgdorf, Germany) at 5% in an anesthesia box. Animals were placed on the VisualSonics imaging station and a tail vein cannulation was performed using Vevo MicroMarker™ TVA (Tail Vein Access; VisualSonics Inc. Toronto, Canada). The aortic arch was scanned from a modified right parasternal view with a 38 MHz MicroScan transducer (MS 400). The transducer was changed to a 24 MHz MicroScan transducer (MS 250) to perform contrast mode images. A bolus of 50 μl of MB_{GPVI} (12 m old $ApoE^{-/-}$, 3 m old $ApoE^{-/-}$, C57/BL6 ($n = 8$, each)) or MB_C (12 m old $ApoE^{-/-}$ ($n = 8$)) was initially injected. For 2 min, enhancement of microbubbles was observed, allowing GPVI binding to collagen. Next, microbubbles were destroyed twice at intervals of approximately 50 frames (around 4 s). After this procedure, the contrast intensity was recorded. Images were analyzed using a linear contrast agent imaging software (VisualSonics Inc., Toronto, Canada). Contrast intensities of aortic arch and truncus brachiocephalicus were analyzed at defined time points. Subsequently, the animals were sacrificed by bleeding to death under Isofluran anesthetic at 3 Vol. % (Isofluran CP; CP Pharma, Burgdorf, Germany) and the aortic arch was removed for histological processing.

2.9. Influence of MB_{GPVI} on plaque size in the carotid artery

12 m old $ApoE^{-/-}$ mice ($n = 12$) were prepared as described above. Vevo MicroMarker™ Target-ready contrast agent (VisualSonics Inc., Toronto, Canada) were incubated with biotinylated GPVI-Fc or BSA at a concentration of 0.76 mg/ml (dose of 2 mg/kg) and the experimental procedure took place as described above. A bolus of 50 μl of MB_{GPVI} was administered and 5 cycles of scanning and destroying took place. This treatment was repeated one week later. After another week the mice were sacrificed by bleeding to death under Isofluran anesthetic at 3 Vol. % (Isofluran CP; CP Pharma, Burgdorf, Germany) and the carotid arteries were removed and analyzed.

2.10. En-face lipid staining

Vessels (aortic arch and carotid arteries) were fixed in 4% paraformaldehyde and stained in oil red G (Sigma–Aldrich, Steinheim, Germany) for 20 min. Plaque areas were measured with an image analysis program (Carl Zeiss AxioVision Rel. 4.5, Oberkochen, Germany). Plaque area was calculated as percentage of total specimen area.

2.11. Scanning ion conductance microscopy (SICM)

Aortic arches were cut into pieces, fixed in 4% paraformaldehyde and glued (with the endothelium facing upwards) onto petri dishes using tissue adhesives. The samples were washed and imaged at room temperature in PBS using a custom-built SICM setup in backstep/hopping mode. Borosilicate glass nanopipettes with opening radii of 100–150 nm were used.

2.12. Immunohistology

Aortic arches and carotid arteries were embedded in paraffin, cut into 5 μm sections and stained with Hematoxylin–Eosin and Masson–Goldner reagent according to the standard protocol. Some aortic arches were used for cryo sectioning for GPIbα staining.

Paraffin-sections and cryo-sections were stained with an avidin-biotin-immunperoxidase system (LSAB + System HRP, DakoCytomation, Glostrup, Denmark) using anti-hIgG antibody (Jackson Immuno Research, West Grove, PA, USA), goat anti-biotin antibody (Thermo Fisher Scientific, Schwerte, Germany) or rat anti-mouse GPIbα (CD42b) antibody (emfret, Eibelstadt, Germany). Isotype control antibodies were used according to standard protocol. Corresponding secondary antibodies (Dako, Glostrup, Denmark) were used for visualization.

2.13. Immunofluorescence

Paraffin-embedded 5 μm sections from aortic arches and carotid arteries of WT and $ApoE^{-/-}$ mice were immunostained to detect collagen (extracellular matrix

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