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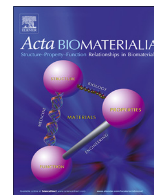

Leukocyte mimetic polysaccharide microparticles tracked in vivo on activated endothelium and in abdominal aortic aneurysm

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ABSTRACT

We have developed injectable microparticles functionalized with fucoidan, in which sulfated groups mimic the anchor sites of P-selectin glycoprotein ligand-1 (PSGL-1), one of the principal receptors supporting leukocyte adhesion. These targeted microparticles were combined with a fluorescent dye and a T_2^* magnetic resonance imaging (MRI) contrast agent, and then tracked in vivo with small animal imaging methods. Microparticles of 2.5 μm were obtained by a water-in-oil emulsification combined with a cross-linking process of polysaccharide dextran, fluorescein isothiocyanate dextran, pullulan and fucoidan mixed with ultrasmall superparamagnetic particles of iron oxide. Fluorescent intravital microscopy observation revealed dynamic adsorption and a leukocyte-like behaviour of fucoidan-functionalized microparticles on a calcium ionophore induced an activated endothelial layer of a mouse mesentery vessel. We observed 20 times more adherent microparticles on the activated endothelium area after the injection of functionalized microparticles compared to non-functionalized microparticles (197 ± 11 vs. 10 ± 2). This imaging tool was then applied to rats presenting an elastase perfusion model of abdominal aortic aneurysm (AAA) and 7.4 T in vivo MRI was performed. Visual analysis of T_2^*-weighted MR images showed a significant contrast enhancement on the inner wall of the aneurysm from 30 min to 2 h after the injection. Histological analysis of AAA cryosections revealed microparticles localized inside the aneurysm wall, in the same areas in which immunostaining shows P-selectin expression. The developed leukocyte mimetic imaging tool could therefore be relevant for molecular imaging of vascular diseases and for monitoring biologically active areas prone to rupture in AAA.

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1. Introduction

Abdominal aortic aneurysm (AAA) has a prevalence of 4–7% in men over the age of 65 and a mortality rate associated with its rupture of >80% [1,2]. New non-invasive techniques to improve diagnosis of this pathology to predict the risk of its rupture are needed [3]. Magnetic resonance imaging (MRI) provides high spatial and temporal resolution observations and has become the imaging method of choice for diagnosing AAA [4,5]. However, it only gives anatomical information and the size of an AAA has been reported as insufficient information to predict its rupture [6]. To increase the capabilities of MRI, research is needed to develop injectable contrast agents that enable molecular characterization and identification of the condition [7].

The progression of aneurysm is known to be associated with proteolytic degradation of the vascular wall. This mainly involves the infiltration of thrombus-trapped blood-borne and leukocyte-derived proteases into the arterial wall, which results in a local and massive proteolytic activity [8]. It has been reported that these highly biologically active sites lead to focal wall weakening and finally to rupture [9,10]. Monitoring leukocyte accumulation is therefore interesting for non-invasive assessment of aneurysm rupture risk.

The recruitment of inflammatory cells into the arterial wall is mediated by adhesion molecules, expressed by the endothelium on activation, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and P- and E-selectins [11]. Among these key molecules which constitute

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relevant targets for molecular imaging of vascular diseases, we selected P-selectin since it is also expressed by activated platelets, the major cell constituent of the AAA thrombus. P-selectin is thus linked with both the renewal and growth of biologically active arterial thrombi and the inflammatory process which is associated with aneurysm expansion [12,13].

Several molecular magnetic resonance imaging (MRI) tools have been developed to highlight acute inflammatory sites. Nanomicelles encapsulating superparamagnetic particles of iron oxide (SPIOs) targeted toward ICAM-1 permitted in vivo imaging of acute inflammation [14]. Macrophage lesion areas on an atherosclerosis mouse model have been revealed with dual-ligand anti-VCAM-1 and anti-P-selectin functionalized microparticles of iron oxide (MPIOs) [15]. For the conception of these tools, the use of a microparticle platform has been reported to exhibit several advantages. It appears that the binding efficiency of functionalized spheres targeted toward injured endothelium from blood flow generally increases with increasing particle size from 100 nm up to 10 μm [16]. Moreover, MPIOs bring a local magnetic field homogeneity which results in a detectable contrast significantly higher than that provided by ultrasmall particles of iron oxide (USPIOs) [17]; this property is known as the contrast "blooming effect".

In the present study, we propose a leukocyte mimetic probe targeted toward P-selectin. For this purpose, we created injectable polysaccharide microparticles from a water-in-oil emulsion process combined with a previously described crosslinked hydrogel preparation [18,19]. We functionalized the microparticles with fucoidan, a seaweed-derived polysaccharide whose sulfated chains mimic the anchor sites of P-selectin glycoprotein ligand 1 (PSGL-1) and have a strong affinity for P-selectin [20,21]. PSGL-1 is present on the surface of leukocytes and mediates, though the binding to P-selectin, their rolling followed by their transmigration into the aneurismal wall [12,22]. We also incorporated fluorescein isothiocyanate (FITC) dextran for fluorescent intravital microscopy and dextran-coated USPIO as T_2^*-weighted MRI contrast agents in order to assess in vivo the behaviour of the developed leukocyte mimetic probe.

We herein describe the fabrication, in vitro characterization and in vivo assessment, on two different vascular disease models with two imaging modalities, of fucoidan-functionalized polysaccharide microparticles. We demonstrate the ability of these microparticles to bind to activated cells on a calcium ionophore induced endothelium activation mouse model and to localize into the degraded arterial wall in an elastase-induced AAA rat model. These experiments revealed the in vivo behaviours of the fucoidan-functionalized microparticles to be similar to leukocyte rolling on activated endothelium and to leukocyte migration though the aneurysmal wall.

2. Material and methods

2.1. Microparticle preparation

Polysaccharide microparticles (MPs) were obtained via a new method [23] using a water-in-oil emulsification process combined with a previously described crosslinking protocol [24]. Pullulan (9 g, MW 200,000 g mol^{-1}, Hayashibara, Okayama, Japan), dextran (3 g, MW 500,000 g mol^{-1}, Sigma Aldrich, Fallavier, France) and FITC-dextran (100 mg, MW 500,000 g mol^{-1}, Sigma Aldrich) were solubilized either in 40 ml of purified water or in 40 ml of dextran-coated ultrasmall particles of iron oxide suspensions ([Fe] = 0.9 M, USPIO Sinerem®, Guerbet, Villepinte, France) to obtain MPs and MPIOs, respectively. To prepare functionalized microparticles (MP-fucoidan or MPIO-fucoidan), 1.2 g of fucoidan (MW 23,000 g mol^{-1}, Sigma Aldrich) was blended into the mixture. Under alkaline condition (2.3 M NaOH), 300 mg of the blend was mixed with 30 μl of STMP (30% (w/v) in water, Sigma Aldrich). The aqueous phase was slowly injected into 30 ml of colza oil containing 1.5% (w/v) of a surfactant mixture of Span 80 (Sigma Aldrich) and Tween 80 (Fluka, Fallavier, France) in a ratio of 75/25, and dispersed with a homogenizer (Polytron PT 3100, dispersing aggregate PT-DA 07/2EC-B101, Kinematica, Luzernerstrasse, Switzerland). Next, this emulsion was transferred to an oven (50 °C) wherein the crosslinking step took place for 20 min. The oil phase was then removed by phase separation and the resulting MPs were washed in PBS. The suspensions were filtered in aqueous medium (0.9% NaCl) through 5 μm nylon filters (SEFAR NITEX, 03-5/1 115 cm, Thal, Switzerland) with a vibrating sieve machine (AS 200, Retsch, Eragny sur Oise, France) then centrifuged (BR4i, JOUAN SA, Saint Herblain, France) for 10 min at 3,000g. The resulting pellets were suspended at 1.5% (w/v) in saline buffer and stored at 4 °C until use.

2.2. Microparticle characterization

The surface morphology of MP, MP-fucoidan, MPIO and MPIO-fucoidan particles was imaged using scanning electron microscopy (SEM; Philips XL 30 ESEM-FEG, Amsterdam, the Netherlands) on dried samples coated with a thin gold layer. The mean diameter and size distribution were determined by image analysis of fluorescent microscopy images (Nikon Eclipse E400, Nikon, Tokyo, Japan). Microparticle snapshots were first converted to binary images and diameter distributions were obtained with the "Analyse Particles" function of ImageJ software (ImageJ, NIH, USA). The mean diameter and the percentage of microparticles <10 μm were calculated for each snapshot. Three snapshots from three different samples of each microparticle type were analyzed. The presence of S and Fe on the surface of microparticles was demonstrated by energy-dispersive X-ray spectroscopy (EDX; Philips XL 30 ESEM-FEG). Global S content was quantified by UV fluorescence spectroscopy (THERMO TN-TS 3000, Thermo Fisher Scientific, Pittsburgh, PA, USA) on freeze-dried samples of fucoidan and microparticles ($n = 4$). The fucoidan content in the microparticles was calculated from their S content and the S content of fucoidan. The overall Fe content was measured by flame spectroscopy on MPIO and MPIO-fucoidan suspensions ($n = 5$).

2.3. In vitro binding assay

The affinity of fucoidan-functionalized microparticles for P-selectin expressed on the surface of activated human platelets was assessed by flow cytometry. For this purpose, we measured by flow cytometry the interaction between three groups of PE-Cy5 labelled platelets (non-activated platelets (PRP), activated platelets (PRP + TRAP) and P-selectin blocked activated platelets (PRP + TRAP + CD62P)) and four groups of FITC microparticles (MP, MPIO, MP-fucoidan, MPIO-fucoidan).

Blood from healthy adult volunteers was collected in sodium citrate 3.8% (w/v). Platelet-rich plasma (PRP) was obtained by centrifugation at 120g for 15 min and platelet concentration was adjusted to 2×10^8 ml^{-1} with autologous platelet-poor plasma (PPP). Activated PRP was obtained by stimulation of PRP with 20 μM of TRAP (thrombin receptor-activating-peptide). Activated then P-selectin-blocked PRP was obtained by incubation with a non-labelled anti-human CD62P at high concentration (1 mg ml^{-1}, Ancell). Before assessing the interaction with microparticles, tubes of PRP, PRP + TRAP and PRP + TRAP + CD62P were prepared and incubated with an anti-human CD62P-FITC (0.11 mg ml^{-1}, Ancell, Bayport, MN, USA) to assess P-selectin expression at the platelet surface. An additional tube of PRP was also incubated with a mouse IgG1/FITC (Ancell) at a similar concentration to verify that the FITC

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