Targeting P-Selectin by Gallium-68–Labeled Fucoidan Positron Emission Tomography for Noninvasive Characterization of Vulnerable Plaques

Correlation With In Vivo 17.6T MRI

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Objective—Nuclear imaging of active plaques still remains challenging. Advanced atherosclerotic plaques have a strong expression of P-selectin by the endothelium overlying active atherosclerotic plaques, but not on the endothelium overlying inactive fibrous plaques. We propose a new approach for noninvasive in vivo characterization of P-selectin on active plaques based on 68Ga-Fucoidan, which is a polysaccharidic ligand of P-selectin with a nanomolar affinity.

Approach and Results—68Ga-Fucoidan was tested for its potential to discriminate vulnerable plaques on apolipoprotein E–deficient mice receiving a high cholesterol diet by positron emission tomography and in correlation with 17.6T MRI. Furthermore, 68Ga-Fucoidan was evaluated on endothelial cells in vitro and ex vivo on active plaques using autoradiography. The cellular uptake rate was increased ≈2-fold by lipopolysaccharide induction. Interestingly, on autoradiography, more intensive tracer accumulation at active plaques with thin fibrous caps and high-density foam cells was observed in comparison with a weaker focal uptake in inactive fibrous plaque segments (R=1.7±0.3; P<0.05) and fatty streaks (R=2.4±0.4; P<0.01). Strong uptake of radiotracer colocalized with increased P-selectin expression and high-density macrophage. Focal vascular uptake (mean of target to background ratio=5.1±0.8) of 68Ga-Fucoidan was detected in all apolipoprotein E–deficient mice. Anatomic structures of plaque were confirmed by 17.6T MRI. The autoradiography showed a good agreement of 68Ga-Fucoidan uptake with positron emission tomography.

Conclusions—Our data suggest that 68Ga-Fucoidan represents a versatile imaging biomarker for P-selectin with the potential to specifically detect P-selectin expression using positron emission tomography and to discriminate vulnerable plaques in vivo. (Arterioscler Thromb Vasc Biol. 2014;34:1661-1667.)

Key Words: atherosclerosis ■ Ga-68-Fucoidan ■ P-selectin ■ PET-imaging ■ plaque

Several criteria are required to evaluate atherosclerotic plaques, such as inflammatory activity, calcified nodules, yellow appearance, luminal stenosis, intraplaque hemorrhage, and structure.1–4 At advanced stages of atherosclerosis, plaques most commonly present with luminal stenosis, lipid aggregates, large necrotic cores, intensive macrophages infiltration, and a thin, collagen-poor fibrous cap. Luminal stenosis is the most common indicator of plaque burden in clinical trials. However, plaque rupture is more closely correlated with the extent of inflammation in the plaque.5–7

Nuclear imaging techniques such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT) provide novel methods to understand the pathophysiology and propagation of disease. Numerous metabolic or pathophysiological biomarkers associated with plaque vulnerability were used as imaging targets. Among them, 99mTc-labeled matrix metalloproteinase (MMP) inhibitors were successfully used to assess MMP activation in atherosclerosis noninvasively8; macrophage apoptosis may be detected by using 99mTc-c′,c′-labeled annexin-V9; 18F-galacto-RGD was used to visualize angiogenesis within the plaque by targeting αvβ3 integrin.10 Furthermore, the somatostatin receptor-avid radiotracer 68Ga-[1,4,7,10-tetraazacyclododecane-N,N′,N″,N‴-tetraacetic acid]-d-Phe1,Tyr3-octreotate (DOTATATE) detected macrophages in atherosclerotic plaque both in human and animal studies.11,12 In clinical and preclinical studies, 18F-fluorodeoxyglucose (18F-FDG) is the most commonly used tracer to assess inflamed plaques by evaluating the glucose metabolism of activated macrophage.13

P-selectin is an adhesion molecule, which is highly expressed on the surface of active endothelium and platelets. It plays a
pivotal role in recruiting leukocytes to the sites of injury.14-16 This interaction is mediated by P-selectin glycoprotein ligand 1, expressed by monocytes, neutrophils, and platelets.17,18 Most active atherosclerotic lesions remain undetected until plaque rupture and thrombosis occur. A previous study found that inflamed atherosclerotic plaques have a strong expression of P-selectin on the overlying endothelium, but much less in normal arterial endothelium or in endothelium overlying inactive fibrous plaques.19 Consequently, P-selectin is thought to be an effective biomarker for assessing the bioactivity of active plaques.

Fucoidan is a synthetic sialyl-lewis X mimic, which is the natural ligand of P-selectin and is found on leukocytes.20,21 It is mainly derived from brown seaweed with an efficient binding of P-selectin as well.21 In previous studies, a high specific affinity of fucoidan for P-selectin was confirmed.22 We developed a novel PET tracer by an efficient introduction of the positron emitter 68Ga into the Fucoidan moiety. The 68Ga-Fucoidan obtained this way was used to validate the P-selectin expression in vivo on an established apolipoprotein E−/− mouse model of atherosclerosis by anatomic reference. MRI can assess plaque stability by evaluating the size of the lipid core, fibrotic caps, and calcifications;19 1,7T MRI was also performed as anatomic reference.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Synthesis of 68Ga-Fucoidan, In Vitro Stability, and Pharmacokinetics Analysis**

For optimization of the radiolabeling conditions, different amounts of Fucoidan (10–250 μg), reaction times (5–45 minutes), and reaction temperatures (22–95°C) were tested. It was found that Fucoidan could be labeled with 68Ga at 90°C <10 minutes using 100 μg of Fucoidan at pH 3.4. 68Ga-Fucoidan was obtained in an overall decay-corrected reaction yield of 90±5% and 99% radiochemical purity. The total synthesis time including column purification on a reversed-phase cartridge and quality control were completed after 40 minutes. To determine the stability of 68Ga-Fucoidan in injection solution (PBS), additional thin layer chromatography and high-performance liquid chromatography controls were performed, at 30 minutes and 1, 2, 3, and 4 hours after pharmacological formulation of 68Ga-Fucoidan. Radioactivity profiles of both thin layer chromatography and high-performance liquid chromatography identified only 1 radioactive product. The radiochemical purity of the product was still >99%, and thus 68Ga-Fucoidan showed an excellent in vitro stability in an injection solution (PBS) for further biological investigations (chromatograms are shown in Figure I in the online-only Data Supplement).

To assess the biodistribution of 68Ga-Fucoidan in mice, all data were corrected for decay and residual activity at the injection site; the tracer uptake was expressed as the percentage of injected dose per gram of tissue after correction for decay (Figure 1). Biodistribution data are expressed as %ID/g: kidney=5.9±1.4, heart=5.7±1.3, spleen=4.1±0.7, liver=4.7±1.2, blood=16.8±3.2, bone=1.4±0.2, lung=3.1±0.9, brain=1.2±0.2, fat=0.9±0.1, muscle=0.8±0.1, and skin=0.8±0.1.

**Lipopolysaccharide-Enhanced 68Ga-Fucoidan Cellular Uptakes**

Incorporation of 68Ga-Fucoidan into lipopolysaccharide-treated and nontreated bEnd.3 endothelial cells was determined using a high-energy gamma counter. Compared with noninduced endothelial cells, lipopolysaccharide-induced cells showed a 2-fold increased uptake of 68Ga-Fucoidan (Figure 2A). The radioactivity incorporation in endothelial cells after a 30-minute incubation at 37°C varied ±35% of the total loaded activity per 10⁶ cells (522–546 counts per minute per 1000 cells). We examined the expression of P-selectin proteins in response to the inflammation inducer lipopolysaccharide. An upregulation of P-selectin on lipopolysaccharide-induced bEnd.3 cells was observed in comparison to cells of untreated controls; this was confirmed by Western blot (Figure 2B).

**Cell Viability Assessment**

To determine whether Fucoidan is able to induce apoptosis, early-passage endothelial cells were incubated with different concentrations of Fucoidan in triplicate; the cells were dual-stained with annexin-V Pacific Blue and 7-AAD and subsequently analyzed by flow cytometry. Apoptosis was evaluated by determining the percentage of cells binding annexin-V and 7-AAD. The dead cell ratio amounted to 1.15% without incubation with Fucoidan as compared with 1.73% after incubation of Fucoidan (40 μg/mL) for 48 hours. No significantly diminished cell viability was observed (Figure 2C). To determine toxicity of Fucoidan in vivo, we injected intramuscularly a high dose of Fucoidan (5 mg/kg...
Ex Vivo Detection of P-Selectin Expression on Atherosclerotic Plaques

68Ga-Fucoidan specifically accumulates in unstable plaques with high-density macrophage infiltration, but much less in fibrous plaques with low-density macrophage presence assessed by autoradiography. Representative autoradiography images are shown in Figure 3. Plaque morphology and activity were assessed by H&E and DAB histological staining. Immunohistochemical staining of adjacent aorta sections indicated an enhanced P-selectin expression in high-lipid atherosclerotic plaques with high-density macrophage interaction, but weaker expression in fibrotic plaques with lower density of macrophage (Figure 3).

In the assessment of ex vivo accumulation of 68Ga-Fucoidan of apoE−/− mice, autoradiographic images were evaluated. We studied 267 lesions (152 active lipid-rich plaque segments with high-density macrophages, 66 inactive fibrous plaque segments with lower-density macrophages, and 49 nonplaque areas with fatty streaks or healthy endothelium), which were selected according to histology. The tracer uptake in active soft plaque areas was higher than in inactive fibrous plaque area (R=1.7±0.3; P<0.05) in each single apoE−/− mouse and also significantly higher than in the non-plaque endothelial segments (R=2.4±0.4; P<0.01). Furthermore, double immunofluorescence staining revealed P-selectin expression on the endothelium overlying soft inflamed plaques as indicated by von Willebrand factor staining and colocalization with macrophages within the active plaque (Figure 4).

In Vivo Detection of P-Selectin Expression

Dynamic PET studies (n=10) determined the optimum imaging time. Because of high level of radiotracer in the blood and difficulty in localizing the focal uptake at early time points, PET images were assessed by collection of 6 frames in duration from 50 minutes to >100 minutes. Subsequent work showed that 60 to 70 minutes could be used without significant degradation of the arterial signal. Data from each frame are independently analyzed (Figure 5). For statistics, micro-PET images were obtained at 60 minutes from apoE−/− mice (n=20); regions of interest were assigned to the aortic arch, descending aorta, and abdominal aorta. Representative micro-PET images showed tracer accumulation in the atherosclerotic lesions at coronal view matching the Sudan IV staining (Figure 5). Clear localized focal uptake of 68Ga-Fucoidan at time points of 60 minutes was detected in all apoE−/− mice with standardized uptake value of plaque=5.1±0.5 (mean), standardized uptake value of muscle=1.0±0.2 (mean), and mean of target to background ratio=5.1±0.8.

The specific accumulation of radiotracer at atherosclerotic plaques was also correlated with histological lipid staining, noninvasively with 17.6T MRI. The representative micro-PET, high-resolution MRI and autoradiography of aorta arch section showed good agreement between uptake of 68Ga-Fucoidan and plaque vulnerable morphology (Figure 6). No specific vascular uptake was detected at control mice (data not shown). Significant uptake in the myocardium was observed in all apoE−/− mice. Another representative image on a brachiocephalic artery section is given in Figure III in the online-only Data Supplement.

In Vivo and Ex Vivo Blocking Studies

Receptor blockage using sialyl-lewis X, the nature ligand of P-selectin, resulted in a significant decrease of tracer accumulation in aorta sections from apoE−/− mice, which indicated that the accumulation of the radiotracer was especially mediated by cellular P-selectin expression. The signal intensities characterized by target to background ratio were significantly decreasing (P<0.01) from 5.2±0.7 with no inhibition to 2.6±0.4 with inhibition. A strong decrease at plaque was observed after incubation of sialyl-lewis X on atherosclerotic aorta sections. This blocking effect was obvious on the
corresponding in vivo (Figure 6) and ex vivo autoradiographic images (Figure II in the online-only Data Supplement).

**Discussion**

Despite many efforts and great advances in technologies, the detection of vulnerable plaque at risk of rupture still remains challenging. Noninvasive detection of upregulation of P-selectin on active endothelium could become an effective tool to evaluate the risk of atherosclerosis and thrombosis because of increased P-selectin expression on vulnerable atherosclerotic plaques.

In this study, we described, for the first time to our knowledge, a P-selectin affinity PET tracer, $^{68}$Ga-Fucoidan, as a promising imaging probe to visualize murine atherosclerosis.
Fucoidans are structurally complex, largely because of their high and polydisperse molecular weight, heterogeneous sugar composition, complex sulfation patterns, and linkages. Therefore, their structural characterization has hitherto challenged analysts and pharmacologists. In the past, the structure and composition of fucoidans have been extensively investigated, and many aspects of their biological activity have been elucidated.\(^{25,26}\) Fucoidan used for radiolabeling in our study was derived from Fucus vesiculosus and purchased commercially from Sigma-Aldrich with a purity of >95%. Fucoidan from F vesiculosus has been one of the fucoidans that has been fully characterized in the last decade.\(^{27–29}\) Its chemical composition is relatively simpler because it is mainly composed of α-D-fucose and sulfate residues. On the basis of the results of methylation, fractionation, and on recent gas chromatography–mass spectrometry data,\(^{27–29}\) it was suggested that the core region of Fucoidan was primarily a polymer of α-(1→3)–linked L-fucose with sulfate groups substituted at the C-4 position on some of the fucose units; fucose was also attached to this polymer to form branched points, one for every 2 to 3 fucose residues within the chain. Although the main goal of our study was to provide a stable \(^{68}\)Ga-Fucoidan for imaging P-selectin expression on plaques rather than to characterize its structure, it is assumed that in the stable \(^{68}\)Ga-Fucoidan complex the \(^{68}\)Ga3+ cation, which was derived from \(^{68}\)GaCl3 (3x positively charged), might be coordinated and stabilized by 3 surrounding sulfate (SO\(_4^–\)) groups to form 1 stable \(^{68}\)Ga-Fucoidan complex in analogy with an other well-known Fucoidan metal complex, \(^{99}\)Tc-Fucoidan.\(^{27}\) A further characterization of a chemical structure requires, beside gas chromatography/electrospray ionization–mass spectrometry, a 3-dimensional crystallographic analysis. This is not applicable for radiopharmaceuticals labeled by short half-life PET radionuclides such as 68Ga. A nonradioactive analogue of \(^{68}\)Ga-Fucoidan (eg, Ga-Fucoidan) is required for further chemical characterizations.

\(^{68}\)Ga-Fucoidan was tested in vitro, in vivo, and ex vivo. \(^{68}\)Ga-Fucoidan showed high binding efficiency in the cell study. In micro-PET study, high specificity and sensitivity were confirmed by strongly pronounced uptake at aortic atherosclerotic region with high target to background ratio at time point of 60 minutes postinjection.

Recently, Rouzet et al\(^{30}\) reported on a new SPECT tracer, \(^{99}\)mTc-Fucoidan, which was developed to detect P-selectin’s upregulation in 2 different animal models of myocardial ischemia reperfusion injury and platelet-rich thrombus. In this study, a good targeting efficiency of \(^{99}\)mTc-Fucoidan was confirmed. Focal uptake of \(^{99}\)mTc-Fucoidan was found in all mice with endothelial activation with an uptake ratio of 4.1. Introduction of \(^{99}\)mTc-Fucoidan presents practical advantages in terms of availability of the generator-based isotope \(^{99}\)mTc and because of the wide availability of SPECT scanners in every nuclear medicine department. However, the current development of PET/MRI scanners and the superiority of an absolute image quantification of PET as compared with SPECT\(^{31}\) suggests that a Fucoidan-based PET tracer might improve the clinical implications. Furthermore, PET has a superior spatial resolution as compared with SPECT. Overall, \(^{68}\)Ga-Fucoidan-PET imaging holds sensitive, specific, and economic capability, improving its potential to be used as a clinical agent in the future.

In comparison with other potential radiotracers for assessing plaque vulnerability, \(^{68}\)Ga-Fucoidan offers more advantages: \(^{18}\)F-FDG is the most commonly used radiotracer in both clinical and preclinical studies of atherosclerotic plaques. However, the high myocardial and brain uptake of \(^{18}\)F-FDG remains a severe
In a previous study using the somatostatin receptor-avid tracer 68Ga-DOTATATE to image macrophages, the aorta uptake of 68Ga-DOTATATE likely shows greater specificity to the vascular inflammation related to 18F-FDG.12-22 Because P-selectin expression is closely associated with increased macrophage infiltration into plaques, further correlation between 68Ga-DOTATATE-PET, 18F-FDG-PET, and 68Ga-Fucoidan-PET would be of great interest. In addition, matrix-digesting enzymes (MMPs 2, 3, 9, etc) in the fibrous cap also play a vital role in the rupture of plaque; MMPs secreted by macrophages are specific indicators of the macrophage activity rather than only macrophage density. Noninvasive quantification of MMP activity in atherosclerotic plaques by PET or SPECT has been already published in preclinical models.6,33 Massive P-selectin migrates from an inner cell to the endothelial surface in response to the inflammatory mediators.25 Therefore, P-selectin and MMPs PET have specific superiority to detect the inflammatory activity of plaques. In a previous work, PET imaging of other adhesion molecules for atherosclerosis detection was investigated. Resembling P-selectin, vascular cell adhesion molecule 1 expresses in the early progress of atherosclerosis and is favorably localized on endothelial cells overlying on inflamed plaque as well. Likewise, Nahrendorf et al34 developed a PET tracer 18F-4V for characterization of vascular cell adhesion molecule 1 on atherosclerotic apoE-/- mice and wild-type mice with myocardial infarction and heart transplantation. In addition, they used PET/MRI to investigate signaling pathways of monocytes and their progenitors after therapy of myocardial infarction in atherosclerotic mice with myocardial infarction.35

The pharmacological effect of Fucoidan in processing anti-inflammatory and antithrombotic impact was approved.36 In our study, the specificity of 68Ga-Fucoidan was confirmed by in vivo micro-PET imaging with low background signal and in vitro receptor blocking study. The reason we used the standardized uptake value in muscle instead of blood pool to calculate target to background ratio is the inherent limitation of long blood half-life. However, clear uptake at vascular wall was observed at many aortal segments. So far, no reliable method exists to detect early inflammatory atherosclerotic lesions.37 Within early atherosclerosis-prone regions of the vasculature, active endothelial cells express P-selectin as well. It is thought to be an effective marker to assess the processes in early atherosclerotic endothelial dysfunction.18,30-41 Additionally, the expression of P-selectin is increased in active platelets during vascular thrombus formation, which is subsequent for growth and stabilization of the thrombus by forming large stable platelet–leukocyte complexes.23,41 Consequently, because P-selectin plays an important role in cardiovascular inflammation and hemostasis, a validated 68Ga-Fucoidan-PET imaging holds promising potential in various clinical cardiovascular settings, such as thrombosis, myocardial ischemia, and evaluation for ischemic injury therapies. However, imaging coronary artery atherosclerosis presents special challenges. These arteries are smaller than the PET resolution. In our study, unspecific myocardial accumulation of 68Ga-Fucoidan was not confirmed, from cardiac endothelium, coronary artery, or blood and requires more symmetrical assessments in coming studies.

Limitations
Because of high inherent sensitivity, PET imaging holds superiority in the detection of diseased lesions as small as atherosclerotic plaques, but lacks structural information. In combination with CT or MRI, it can have a great potential to assess both the activity and the anatomic details of diseases. Therefore, hybrid scanners such as PET/MRI or PET/CT should be used for further preclinical and clinical studies. Additionally, to adequately reflect the real situation, younger apoE-/- mice, which are at an early stage of atherosclerosis, are also significant for the study. Furthermore, in our study, 68Ga-Fucoidan-PET shows promising capability to recognize active plaques in the apoE-/- mice model. Nevertheless, these plaques are not going to rupture, which can cause thrombosis. Long blood half-life of radiotracer is also an inherent limitation in plaque imaging.

Conclusions
The P-selectin affinity PET tracer, 68Ga-Fucoidan, according to this preclinical validation study, may hold the potential to discriminate between active and inactive atherosclerotic plaques. However, further studies are necessary to elucidate the role of this new radiotracer.

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Disclosures
None.

References
radiotracer was assessed in vitro, ex vivo, and in vivo. High specificity and sensitivity of 68Ga-Fucoidan to the active plaque were confirmed by myocardial ischemia, and evaluation for ischemic injury therapies as well.

Fucoidan positron emission tomography imaging holds promising potential in various clinical cardiovascular settings, such as thrombosis, triggers its binding to activated human platelets. 

of enzymes active toward this class of polysaccharide. 

functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide.

endothelial receptor for lymphocytes, the mucosal vascular addressin, is expressed on platelets and can mediate platelet-endothelial interactions in vivo.

The Peyer's patch high adhesive activities of nine different fucoidans from brown seaweeds.

Sulfated fucans, fresh perspectives: structures, adhesive activities of nine different fucoidans from brown seaweeds as studied by electrospray ionization mass spectrometry (ESIMS) and small angle X-ray scattering (SAXS) techniques.

Structure for fucoidan may explain some of its biological activities.

Table: 1667

Significance

Nuclear imaging of active atherosclerotic plaques remains challenging. In this study, we described, for the first time to our knowledge, a P-selectin affinity positron emission tomography tracer, 68Ga-Fucoidan, as a promising imaging probe to visualize murine atherosclerosis. The radiotracer was assessed in vitro, ex vivo, and in vivo. High specificity and sensitivity of 68Ga-Fucoidan to the active plaque were confirmed by strongly pronounced uptake at aortic atherosclerotic region. High specificity and sensitivity and low cost and toxicity are required for translation to clinical application. 68Ga-Fucoidan positron emission tomography seems to possess the potential to achieve noninvasive evaluation of P-selectin-related diseases. Because P-selectin plays an important role in cardiovascular inflammation and hemostasis, a validated 68Ga-Fucoidan positron emission tomography imaging holds promising potential in various clinical cardiovascular settings, such as thrombosis, myocardial ischemia, and evaluation for ischemic injury therapies as well.
Targeting P-Selectin by Gallium-68–Labeled Fucoidan Positron Emission Tomography for Noninvasive Characterization of Vulnerable Plaques: Correlation With In Vivo 17.6T MRI
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Supplemental figure I: Radioactivity profiles of HPLC (A) and TLC (B) at 60 minutes after preparation of $^{68}$Ga-Fucoidan. HPLC was assessed using 0.01 M aqueous NaOH/acetonitrile (9:1) at flow rate of 1.0 ml/min as mobile phase.
on a column (Dionex CarboPak-PA10, 250x4.5 mm, 10 µm) as stationary phase. Thin-layer chromatography (TLC) was performed, applying the analyte to an ITLC-SG strip (Varian, Lake Forest, USA) using 0.1M sodium citrate as mobile phase. Radioactivity profiles of both TLC and HPLC identified only one radioactive product with a radiochemical purity > 99%.

**Supplemental figure II.** Pre-incubation of Sialyl-LewisX target to P-selectin inhibited uptake of $^{68}$Ga-Fucoidan in atherosclerotic plaques from ApoE$^{-/-}$ mice. A, B, Autoradiography images show the lower uptake in the aorta pre-incubated with P-selectin targeted antibody. Histology demonstrated the co-localization of atherosclerotic plaque with the area of increased $^{68}$Ga-Fucoidan uptakes by (C) H&E staining and (D) DAB staining of P-selectin.
Supplemental figure III. Representative noninvasive images, brachiocephalic trunk from an ApoE−/− mouse on Western diet for 38 weeks. All red arrows indicate regions of interest. A, In vivo ultra-high-field MRI of the aortic arch using an ECG-triggered Multi-Slice-Multi-Spin-Echo sequence. B, Micro-PET imaging at transverse view suggesting specific uptake of ⁶⁸Ga-Fucoidan by the plaque. C, Corresponding autoradiography image of the aortic arch. D, H&E staining of corresponding plaque section. Thin cap (bright outer layer), large lipid core (dark inner part) of plaque were characterized on MR images and histology, whereas in accordance with high uptake of ⁶⁸Ga-Fucoidan confirmed by micro-PET and autoradiography.
Materials and Methods

Preparation of $^{68}$Ga-Fucoidan

Fucoidan was purchased commercially from Sigma-Aldrich (Deisenhofen, Germany) and was used without further purification for radiolabeling. The synthesis of $^{68}$Ga-Fucoidan was carried out on a computer-assisted synthesis-module (Scintomics, Fürstenfeldbruck, Germany). $^{68}$Ga for radiolabeling was eluted with 0.1M HCl in form of $^{68}$GaCl$_3$ from a $^{68}$Ge/$^{68}$Ga-generator (Obninsk, Russia). The labeling procedure was optimized concerning the amount of Fucoidan, reaction time and reaction temperature. Optimization led to the following standard labeling protocol: $^{68}$GaCl$_3$ (typically 500-750 MBq) was eluted with 0.7 mL of 0.1 M HCl directly in a reaction vial (Thermo Scientific, Germany) containing 300 µL of 0.1 M acetate buffer (pH = 4.8) and 50 µg (50 µl) of Fucoidan from a solution consisting of 1.0 mg Fucoidan/1mL saline). The solution was allowed to react for 10 min at 90°C, following by a purification of the crude product on a Waters-Sep-Pak-C18 cartridge (Eschborn, Germany), diluted with PBS (pH 7.0; Braun) and filtrated through a 0.22µm sterile filter (Millipore, Cork, Ireland) into a sterile vial (IBA, Berlin, Germany) for biological evaluations. Quality control and in vitro stability were assessed at different time points (30 min, 1, 2, 3 and 4h) after preparation by means of HPLC (Shimadzu, Duisburg, Germany) and TLC-scanner (mini-GITA®, Raytest, Straubenhardt, Germany).

Cellular study

To stimulate cellular inflammation, bEnd.3 cells were treated with 1 µg/ml of LPS (E. coli. 026:B6, Sigma) for 48 hours. Upregulation of P-selectin protein was analyzed by Western Blot. The immunoblot was detected using the enhanced chemiluminescence system (GE Healthcare). The cellular uptake of $^{68}$Ga-Fucoidan was tested on bEnd.3 endothelial cells with and without LPS stimulation. Radioactive substances which were diluted to 1.5*10$^6$ counts per minute (cpm) / 1mL culture medium were added to the 1*10$^5$ attached cells and incubation in a humidified 95% air/ 5% CO$_2$ incubator for different time intervals (5min, 15min, 30min, 45min) at 37°C. After incubation, interaction between cells and tracers was interrupted by placing samples on ice. Then, the cells were washed thrice with cold PBS (0.1mol/L, PH 7.4), and trypsinized in 0.5mL for measurement. Cellular uptake was measured using a high-energy gamma counter (Wallac, Rodgau, Germany). To determine apoptosis induction, endothelial cells were treated with Fucoidan at concentration of 40µg/mL for 48 hours, and were subsequently double-staining with annexin V– pacific blue (BD Bioscience) in combination with 7aad (BD Bioscience). Harvested dual stained cells and subsequently analyzed by flow cytometry. The percentage of cellular apoptosis and necrosis.
was determined by examining 200,000 cells and counting the cells that were characterized by nuclear condensation and fragmentation.

**Animal studies**

ApoE<sup>−/−</sup> mice were used in our experiments. In order to induce development of severe atherosclerotic plaques, apoE<sup>−/−</sup> mice were fed with a high cholesterol diet (1.25% cholesterol, 7.5% cocoa butter, 7.5% casein) for 34 weeks starting at the age of 4 weeks. As controls, C57BL/6 mice were fed with normal chow. All experimental protocols were in compliance with the German animal protection law and were approved by the local district government of Unterfranken (AZ: 55.2-2531.01-19/07).

**Biodistribution and Pharmacokinetics**

The biodistribution of 68Ga-Fucoidan was determined in C57/B6 mice (46 weeks old, n=6). Mice were sacrificed 60 minutes after intravenous injection of the radiotracer (4.9±0.6 MBq), and perfused with 50 µL of saline, blood, heart, lung, kidney, brain, bone, leg skeletal muscle, fat, skin, spleen and liver were harvested and weighted. Radioactivity was subsequently measured by high-energy gamma counter (Wallac, Rodgau, Germany).

**Magnetic Resonance Imaging**

ApoE<sup>−/−</sup> mice (n=4) with severe atherosclerotic plaque were assessed by high resolution MRI on a 17.6 T vertical bore MR system (Bruker Biospin GmbH, Germany), using a homebuilt radiofrequency coil (birdcage design, ID: 27 mm). The images were acquired with an ECG-triggered and respiratory gated T1-weighted Multi-Slice-Multi-Spin-Echo-Sequence with following parameters: repetition time TR=1 second; echo time, TE=9 milliseconds; bandwidth, 83 kHz; in-plane resolution 78 µm × 78 µm; slice thickness 0.6 mm; total acquisition time= 20 minutes<sup>2</sup>. Images were analyzed with OsiriX DICOM viewer software.

**PET imaging**

68Ga-Fucoidan was administered intravenously into apoE<sup>−/−</sup> mice (7.4±1.0 MBq) (n=20) under anesthesia with 1.5% isoflurane post MRI scan. Imaging was performed 60 minutes later using a micro-PET system (Inveon, Siemens healthcare, Germany) with a 10 minutes acquisition time. For imaging reconstruction, an OSEM2D algorithm was used. To quantify 68Ga-Fucoidan uptake, all micro-PET scans were reviewed for anatomic localization and amount of focal uptake. As controls, six C57BL/6 mice were administered intravenously with 68Ga-Fucoidan (6.5±0.8 MBq). Representative segments of large arteries were analyzed, aortic arch, descending thoracic aorta, and
abdominal aorta. Regions of interest (ROIs) of 0.8 mm in diameter were drawn around the wall of the selected aortic segments. The mean standardized uptake value (SUV) was extracted for the final calculation of target-to-background ratios (TBRs). Background was determined by the mean SUV of six different ROIs (diameter of 0.8 mm) in the foreleg muscle. Transverse, coronal and sagittal views were analyzed. The relative tracer uptake is characterized as the TBR and SUV respectively. Quantitative dynamic PET studies were performed in order to understand tracer kinetics; the PET imaging data was acquired from tracer injection to 100 minutes after injection. In order to estimate in vivo specificity of $^{68}$Ga-Fucoidan, four apoE$^{-/-}$ mice were pre-injected of 1 mg sLe$^X$ (Merck Millipore) mixed in 100 µL PBS respectively.

**Ex vivo autoradiographic study**

All apoE$^{-/-}$ mice (n=6) were sacrificed after the PET scan. Accumulation of $^{68}$Ga-Fucoidan in the aorta was assessed using 20 µm sections of sample tissues with a digital autoradiography system (CR 35 BIO, Duerr Medical, Bietigheim Bissingen, Germany). Regions of interest (ROIs) were drawn to assess activity ratios for the active plaque areas relative to the inactive plaque areas and the uptake ratios to the non-plaque were determined as well. Active atherosclerosis was defined by intensive infiltration of blood-derived monocytes into arterial intima. Active vulnerable plaques were characterized by thin fibrous caps and high-density macrophage infiltration; fibrous plaques were classified as inactive plaque; Fatty-streak were classified as non-plaque lesions.

**Histology and Immunohistochemistry**

After autoradiography, aortal sections from apoE$^{-/-}$ mice were harvested for immunohistochemistry testing. Double immunofluorescence staining visualized P-selectin together with von Willebrand factor (vWF) for endothelial cells. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for visualization of nuclei. Diaminobenzidine (DAB) staining was also performed to determine the localization of P-selectin within the atherosclerotic aorta together with macrophages. For correlation with in vivo PET imaging, murine aortas were excised, cut open longitudinally and stained with Sudan IV (Sigma-Aldrich). All images were obtained using a fluorescent microscope (Zeiss Axiovision).

**Statistics**

Statistical Package for Social Sciences (SPSS version 11.0; SPSS Inc.) was used for statistical analyses. Continuous variables with a normal distribution were recorded as mean ± standard deviation (SD). A two-tailed T-test was
used to evaluate statistical significance between groups. P-values of less than 0.05 were considered statistically significant.
Expanded methods

Assessment of $^{68}$Ga-Fucoidan

Quality control and stability of $^{68}$Ga-Fucoidan were assessed at different time points (30 min, 1, 2, 3 and 4h) after preparation by means of TLC and HPLC. Radioactivity profiles of both TLC and HPLC identified only one radioactive product, which was additionally stable in injection solution (PBS) for at least 4 h (equivalent of 3.5 x half-lifes of $^{68}$Ga) after preparation. Details on the optimized synthesis of $^{68}$Ga-Fucoidan are given in the Methods and Results sections of the manuscript. The preparation of $^{68}$Ga-Fucoidan was reproducible in providing one stable product, as shown by HPLC and TLC analysis. In addition, examples of radioTLC and radioHPLC profiles of the new radiotracer are presented in supplemental figure I.

Cell culture and western blot

The murine endothelial cell line b.End.3 was purchased from American type Culture Collection. The adherently growing cells were cultivated under standard conditions at 37°C in a humidified 95% air/ 5% CO₂ incubator, maintained in the complete growth medium which mainly consisted of Dulbecco's Modified Eagle's Medium (DMEM)(Gibco), supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). The bEnd.3 cells were used at passages 20 to 25. To activate inflammation, bEnd.3 cells were treated with 1 µg/ml of LPS (E. coli 026:B6, Sigma) for 48 hours. Stimulated and non-stimulated b.End 3 cells were lysed in 100 µL ice-cold protein extraction reagent type 4 lysis buffer (Sigma-Aldrich), the lysates were centrifuged at 12,000g at 4°C for 5 minutes to remove cellular debris. The supernatant was collected. 40 µL of supernatant containing the total cell proteins was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. After blocking with TBST containing 5% nonfat dry milk for 1h, the membrane was further incubated with 5ml TBST/5% nonfat dry milk solution containing 5 µL rat anti mouse P-selectin primary antibody (antibody online) for 24h at 4°C. After washing three times, the membranes were incubated with 5mL TBST solution containing 5 µL HRP-labeled ECL anti-mouse IgG secondary antibody (GE Healthcare) for 1h. The immunoblot was developed using the ECL detection system (GE Healthcare).

Viability of the cells

Apoptosis induction experiments were performed in 24-well culture plates (Costar). Cells were treated with Fucoidan at concentration of 1µg/ml, 5µg/ml,
10µg/ml, 20µg/ml and 40µg/ml for 48 hours, subsequently harvested, and ready for apoptosis analysis. To determine the morphologic nuclear changes, Phosphatidylserine exposure was determined using annexin V– pacific blue (BD Bioscience) in combination with 7aadd (BD Bioscience). Cells were harvested, washed, incubated with annexin V– pacific blue and 7aadd for 15 minutes in the dark. As control, unstained cells, cells stained with annexin V– pacific blue (no 7aadd) and cells stained with 7aadd (no AnnexinV- pacific blue) were prepared to assess as well.

**Histology**

Aortas were excised and frozen in Tissue-Tek O.C.T Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) on dry ice, sliced in sequential longitudinal sections (n=300) and transverse sections (n=200) of 8 µm and 20 µm at -22°C (Leica Cryotome CM1850, Leica Microsystems) and mounted onto microscope slides. The 20 µm sections underwent autoradiography and were apposed to an imaging plate over night for autoradiography. Autoradiographic images of longitudinal sections mainly included descending aorta, abdominal aorta, they were obtained in order to compare the uptake of activated and in inactive plaques. Transverse sections mainly include right and left subclavian artery, brachiocephalic artery, ascending aorta arch of aorta and part of descending aorta, Autoradiographic images of these arteries were used to correlate tracer’s uptake with anatomic MR imaging. The 8 µm sections were used for staining with H&E to demonstrate morphology and for immunohistology. P-Selectin primary antibody (ABIN670131, Antibodies online, Aachen, Germany), was used for visualization of early atherosclerosis, followed by a biotinylated secondary antibody (BA-1000, Vector Laboratories, Burlingame, UK) and streptavidin (ABC KIT, PK-6100, Vector Laboratories, Burlingame, UK) and stained with Diaminobenzidine (DAB substrate KIT, abcam ab94665, Cambridge, U.K.). The sections were counterstained with Hematoxylin, dehydrated in Ethanol and Xylene and mounted with Entellan (Merck 1.07960, Darmstadt, Germany). For immunofluorescence double staining the sections were incubated with primary antibody against P-selectin (ABIN670131, rabbit anti mouse, Antibodies online, Aachen, Germany), and vWF (ab11713, sheep anti mouse, Abcam, Cambridge, U.K.) or CD68 (MCA1957, rat anti mouse, AbDSerotec, Düsseldorf, Germany) as secondary primary antibody. After a two step staining procedure with biotinylated secondary antibody (BA-1000 against P-selectin, BA-6000 against vWF, and BA-9400 against macrophages, all Vector Laboratories, Burlingame, UK) and streptavidin in two different fluorescent colours, Alexa Fluor 546 (S11225, invitrogen, Karlsruhe, Germany) and (Streptavidin-FITC, SA-5001 Vector Laboratories), 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. Images were taken with a Zeiss Axioskop 2 plus fluorescence microscope.
For Sudan IV staining, aortae were fixed in 4% formaldehyde. The aortae were opened longitudinally, from the aortic arch to the iliac arteries. The primary incision followed the inner curvature of the arch. To obtain a flat imaging, an incision was made along the outer curvature of the arch. The aortae were then pinned on a black wax surface using stainless steel pins 0.25 mm in diameter. After being rinsed with water, the aorta was exposed to isopropanol (60%) for 2 min, followed by 35 minutes of staining with a Sudan IV solution, which contained 5 grams of Sudan IV, dissolve into 500mL acetone & 500mL 70% EtOH. The excess stain was removed with 80% EtOH and running water subsequently. Whole aortas were imaged using a microscope with a digital camera (Leica).

In vitro inhibition study

Sialyl-Lewis\(^x\)-like glycan, which is nature ligand of P-selectin \(^4\) and strongly expressed in circulating leukocytes. In order to evaluate specificity of \(^{68}\)Ga-Fucoidan uptake, tissue sections (n=40) from ApoE\(^{-/-}\) mice were incubated with overdose Sialyl-LewisX for 24 hours before incubation with \(^{68}\)Ga-Fucoidan in vitro at 4°C. Subsequent autoradiography was performed to confirmed the decreased radiotracer’s uptake at plaque. Representative autoradiography images and corresponding histology images were shown in supplemental figure II.

References