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Upregulated Sphingosine 1-Phosphate Receptor 1 Expression in Human and Murine Atherosclerotic Plaques

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Abstract

Purpose—Dysregulation of sphingosine 1-phosphate receptor 1 (S1PR1) signaling contributes to inflammation-related pathophysiological changes in cardiovascular diseases including atherosclerosis (AS). S1PR1-targeting compounds significantly reduce lesion size in murine models of AS. Therefore, characterization of S1PR1 expression *in vitro* and *in vivo* in atherosclerotic plaque could enable mechanistic studies and inform S1PR1 targeted therapies.

Procedures—H&E staining and immunostaining studies were performed on variably diseased human femoral endarterectomy plaque specimens, as well as mouse aortic sections from ApoE^{−/−} mice maintained on a high-fat diet (AS mice). *In vitro* autoradiography study in human femoral plaques was used to confirm the tracer specificity. Micro positron emission tomography (PET) and *ex vivo* autoradiography studies were conducted in AS mice and their controls using a S1PR1-specific radioligand [¹¹C]TZ3321 for *in vivo* and *ex vivo* quantification of S1PR1 expression in mouse aortic plaques.

Results—Increased S1PR1 expression was observed in areas of human femoral endarterectomy plaque specimens with foam cell accumulation compared with control tissue; *in vitro* autoradiography study indicated that SEW2781, a S1PR1 compound was able to reduce the uptake of [¹¹C]TZ3321 by 56 %. S1PR1 levels were also upregulated in AS mouse aortic plaques. MicroPET data showed the aorta-to-blood tracer uptake ratio in AS mice was approximately 20 % higher than that in controls. Autoradiographic study also revealed elevated tracer accumulation in AS mouse aorta.

Conclusions—Upregulated S1PR1 expression in human and mouse atherosclerotic plaques was successfully identified by immunostaining and radioligand-based methods. This data demonstrates that [¹¹C]TZ3321 PET provides great promise in imaging S1PR1 expression in atherosclerotic plaques.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Keywords

Sphingosine 1-phosphate receptor 1; Atherosclerosis; Immunostaining; MicroPET; Autoradiography; [^{11}C]TZ3321

Introduction

Although there are a number of imaging modalities that provide high-resolution morphologic information on atherosclerotic plaques and vessel wall integrity [1], *e.g.*, ultrasound, X-ray computed tomography (CT), magnetic resonance imaging (MRI), and optical coherence tomography (OCT), these modalities do not provide information on the biology related to plaque vulnerability [2]. Plaque imaging using molecular imaging modalities, such as positron emission tomography (PET), provides attractive opportunities to visualize plaque biology [3, 4]. 2-Deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG), a widely used PET tracer, has been used clinically to determine areas of enhanced cellular metabolism in atherosclerotic plaque. However, the shortcoming of [^{18}F]FDG is its lack of specificity to atheroprotection and plaque vulnerability [5].

A growing body of evidence supports an important role for sphingosine 1-phosphate and its receptors (S1P/S1PRs) in the development and regulation of the circulatory system and pathogenesis of vascular disease. S1P is a biologically active sphingolipid that affects cardiac function, vascular development, immune cell function, and inflammation [6]. Five subtypes of S1P responsive receptors (S1PR1–5) have been identified, whereas S1PR1 is expressed ubiquitously in the cardiovascular system [7]. S1PR1 couples with the G-protein G_i that inhibits adenylate cyclase (AC), and activates downstream mitogen-activated protein kinase (MAPK) and phospholipase C/phosphatidylinositol 3-kinase/protein kinase B (PLC/PI3K/Akt) signaling pathways. As a result, these signaling mechanisms can lead to endothelial nitric oxide synthase (eNOS) activation, smooth muscle cell (SMC) relaxation, vasodilatation, permeability, migration, proliferation, and tubulogenesis of endothelial cells. A conditional S1PR1 knockout causes ventricular non-compaction and ventricular septal defects and results in perinatal lethality [8]. Transgenic mice that lack plasma S1P show vascular leakage, which is attenuated by a S1PR1 agonist AUY954 [9]. S1PR1 reporter mice demonstrate enhanced activation of S1PR1 under lipopolysaccharides (LPS)-mediated systemic inflammatory condition [10]. The role of S1P/S1PR1 in vascular inflammation has also been studied in stroke-prone spontaneously hypertensive rats (SHRSPs). S1P stimulates inflammatory signaling pathways *via* transactivation of receptor tyrosine kinase (RTK) through S1PR1, leading to increased expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1) and promotes monocyte adhesion [11]. Moreover, high S1PR1 expression has been found in endothelial cells, macrophages, and proliferated vascular smooth muscle cells (VSMCs), which are major components of atherosclerotic plaques [7]. This is in agreement with a study that demonstrated that a specific S1PR1 agonist treatment reduced lesion size in low-density lipoprotein receptor (LDLR)-deficient mice [12]. Therefore, prior evidence suggests that S1PR1 is a promising target for molecular imaging of atherosclerotic lesions and may serve as a potential therapeutic target that decreases atheroprotection.

Besides recent human transcriptome analysis that demonstrated increased expression of S1PR1 in neointimal atherectomy specimens [13], there is currently little known about the expression of S1PR1 in human atherosclerotic plaques. In this study, we aimed to determine the level of S1PR1 expression in variably diseased human femoral endarterectomy specimens, as well as mouse aortic plaques with both immunostaining and PET/CT using a novel highly specific S1PR1 radioligand, [¹¹C]TZ3321 (3-((2-fluoro-4-(5-(2'-methyl-2-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)-1,2,4-oxadiazol-3-yl)benzyl)-([¹¹C]methyl)amino)propanoic acid (Fig. 1c).

Methods

S1PR1 Immunostaining Studies of Human Femoral Plaque Samples

Preparation of Human Femoral Plaque Tissue—To characterize S1PR1 expression in human atherosclerotic plaque, femoral artery atherectomy samples were obtained from four patients (male, 50–60 years old) with high-grade and > 70 % stenosis of the femoral artery, who underwent an open surgical femoral endarterectomy procedure at Washington University School of Medicine-affiliated Barnes-Jewish Hospital. Specimens were transported immediately following excision to the laboratory for subsequent analysis under an Institutional Review Board-approved vascular surgery tissue banking protocol. All plaque segments harvested for analysis were separated into minimally and maximally diseased segments, half were snap-frozen and stored in – 80 °C for autoradiography. The other half were fixed in 10 % formaldehyde/0.1 M PBS (pH 7.4) for 3 h and cytoprotected in 30 % sucrose. Following standard optimum cutting temperature compound (OCT) embedding, 10 μm tissue sections were cut and collected for immunofluorescence (IF) as well as staining with hematoxylin and eosin (H&E).

H&E and Immunofluorescence Staining—H&E staining was performed for morphological characterization of the tissue. S1PR1 expression in human femoral plaques was examined by IF staining as follows. Endogenous peroxidase activity was quenched before blocking and incubation with a mouse anti-human S1PR1 (clone 8B7.1) antibody (1:200, 4 °C, overnight, Millipore, Billerica, MA). The secondary fluorescently labeled antibody, Dylight 594 horse anti-mouse antibody (1:100, Vector Laboratories, Burlingame, CA), was added in dark for 60 min, and cover slipped using mounting media containing DAPI. Negative control sections were stained without the primary antibody. A Nikon E600 microscope coupled with a charge-coupled device camera was used to obtain all photomicrographs.

In vitro Autoradiography—To confirm the specificity of the radiotracer [¹¹C]TZ3321 binding toward S1PR1, *in vitro* autoradiography study was conducted. Twenty micrometer slices from snap-frozen femoral plaque tissues were incubated with 2 μCi/ml [¹¹C]TZ3321 at room temperature for 30 min, following a 15-min pre-incubation in the binding buffer (50 mM HEPES-Na (pH 7.5), 5 mM MgCl₂, and 1 mM CaCl₂, 0.5 % fatty acid-free BSA). A S1PR1-specific compound SEW2871 (10 μM) was used as a block agent to check the binding specificity of [¹¹C]TZ3321 toward S1PR1. Following this incubation, sections were washed two times in assay buffer at 4 °C and rinsed once in distilled water at 4 °C to remove

buffer salts. Then, the slides were dried and exposed to the storage phosphor screen (BAS-IP-MS-2025) in an imaging cassette for 4 h in -20°C at dark. The distribution of radioactivity was visualized by a Fuji Bio-Imaging Analyzer FLA-7000 (Fuji Photo Film, Tokyo, Japan).

Characterization of S1PR1 Expression in Murine Models of Atherosclerosis

Atherosclerosis-Prone Mouse Models—To further characterize S1PR1 expression in the plaque and explore the potential application of the S1PR1-specific radioligand [^{11}C]TZ3321 in detecting the change of S1PR1 levels *in vivo*, apolipoprotein E knockout (ApoE $^{-/-}$) mice on a high-fat diet (AS mouse model) and normal wild type mice were used for small-animal microPET, autoradiography, and immunostaining studies. Male ApoE $^{-/-}$ mice were fed a high-fat Western diet (Harlan Teklad) (42 % fat) for 6.5 months starting at the age of 6 weeks; age-matched wild-type C57BL/6J mice (WT mice) receiving a normal chow diet served as controls. Atherosclerotic plaques in the aorta were found at 8 months old AS mice [14]. Therefore, one cohort of AS mice and controls was used for microPET studies, followed by autoradiography studies, at 8 months old ($n = 3$). Another cohort of AS mice and controls ($n = 3$, 8 months old) was deeply anesthetized and euthanized by bilateral thoracotomy followed by terminal exsanguination for histological studies.

In addition to the regular AS mouse model, a wire-injury-induced accelerated mouse model of AS [15] was also used for immunohistochemical study to further confirm the S1PR1 expression in the plaque. The femoral artery wireinjury procedure was performed as described in our previous report [16]. Male ApoE $^{-/-}$ mice received wire injury at 6 weeks old and used at 12 weeks old, with the Western diet for 8 weeks; age-matched wild-type mice with normal diet without wire injury served as controls ($n = 3$).

All animal experiments were conducted under compliance with protocols approved by Washington University Institutional Animal Care and Use Committee and performed in accordance with the Guideline for the Care and Use of Laboratory Animals. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Production of [^{11}C]TZ3321—Radiosynthesis of [^{11}C]TZ3321 was carried out according to our previously published procedure [16]. The radiochemical yield was 40–50 % (decay corrected to end of bombardment) with a radiochemical purity of $> 99\%$, chemical purity of $> 95\%$, and specific activity $> 2000\text{ Ci/mmol}$ (decay corrected to end of synthesis).

MicroPET Scans and Data Process—Small-animal microPET imaging was performed on an Inveon PET/CT system (Siemens Inc., Knoxville, TN). PET scans were conducted under halogenated gas anesthesia (2 % isoflurane in oxygen) delivered by a nose cone. AS mice and wild-type controls ($n = 3$) were individually weighed, placed in an induction chamber, and once anesthetized; 26 gauge catheters were placed in the lateral tail vein. Then, mice were secured to a custom-designed acrylic bed with nosecones and placed in transaxial position with their whole bodies inside the Inveon PET/CT system. Following a transmission scan and a brief CT for attenuation, animals received a bolus injection (0.8–1.0 mCi) of the radiotracer [^{11}C]TZ3321 *via* the tail vein catheter. A list-mode protocol was used with 60 min dynamic data acquisition. Subsequently, 100–200 μl (100 $\mu\text{l}/20\text{ g body}$

weight) of a long-lasting intravascular contrast media agent, eXIA 160XL (Binitio Biomedical, Inc., Ottawa, Canada) was administered through the same catheter. Five minutes later, a high-resolution CT scan was acquired for anatomical co-registration. Anesthetized mice were euthanized after the imaging procedure for *ex vivo* autoradiography as described below.

Regions of interests (ROIs) were drawn by co-registration of PET data with high-resolution CT image and whole-body CT image (Fig. 2), and the data was analyzed using the Inveon Research Workstation built in software IRW 4.2 program (Siemens Inc., Knoxville, TN) to calculate the standard uptake value (SUV) by the following equation: $SUV = nCi/cm^3 \times \text{weight (g) dose injection (nCi)}$. Time-activity curves were generated accordingly and shown in the electronic supplementary material (Fig. S1). The ratio of tracer uptake in mouse aorta to that in blood was then obtained. For statistical analysis, one-tail unpaired Student's *t* test was applied, and significant difference is defined as *P* value < 0.05 for microPET data.

Ex vivo Autoradiography—AS mice and controls (*n* = 3) remained anesthetized after microPET/CT scan (~75 min post-injection of the radiotracer) and were euthanized by bilateral thoracotomy followed by terminal exsanguination. The aorta was dissected with care taken not to include any surrounding tissue, immediately placed onto a BAS storage phosphor screen in a phosphorimaging cassette and exposed in the dark for 4 h at – 80 °C before capturing the image from the phosphorimaging screen. Photostimulated luminescence (PSL) was quantified with Multi Gauge v3.0 software (Fuji Photo Film Co., Tokyo, Japan). Data were background-corrected and expressed as photo-stimulated luminescence signals per square millimeter (PSL/mm²).

H&E and Immunohistochemistry Staining—In addition to the cohort of regular AS mice and controls (*n* = 3) used for H&E and immunohistochemistry (IHC) staining, a separate histological study was also performed in the wire-injury-induced AS model and controls (*n* = 3) following the same staining procedures. The aortic root, aorta, and/or femoral arteries from both regular AS mice and the wire-injury-induced accelerated AS mice were dissected and fixed in 10 % formalin for 48 h before embedding in paraffin. Adjacent serial 5 µm sections were taken for H&E and IHC staining. For IHC, slides were first deparaffinized and hydrated. Endogenous peroxidases were quenched using 0.3 % hydrogen peroxide in PBS. Sections were incubated in blocking buffer for 60 min. The primary antibody, rabbit anti-mouse S1PR1 antibody (1:50, Santa Cruz biotechnology, Santa Cruz, CA, USA) was added and incubated with the slides at 4 °C overnight. Positively labeled cells were visualized using an anti-rabbit HRP-DAB staining kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Results

S1PR1 Expression in Human Femoral Plaques

For human femoral plaque specimens, maximally and minimally diseased segments of the plaque were identified based on H&E staining of adjacent sections. IF was used to evaluate differential distribution of S1PR1 expression in the atherosclerotic plaque of the variably diseased arterial segments. We observed accumulation of S1PR1 (red, indicated by white

arrow) in maximally diseased femoral plaque segments where foam cell accumulation is histologically evident (Fig. 1a). Foam cells were identified by H&E staining and appear as round mononuclear cells with oval nuclei and abundant amounts of vacuolated cytoplasm [17]. Quantification of positive cell counts revealed 73.38 ± 14.35 S1PR1 positive cells per $5000 \mu\text{m}^2$ in the plaque and 2.71 ± 1.17 in the control (Fig. 1b). *In vitro* [^{11}C]TZ3321 autoradiography data also revealed differential distribution of tracer uptake in the plaques. The blockade of tracer uptake ($\sim 56\%$) by $10 \mu\text{M}$ of the selective S1PR1 agonist SEW2781 further suggested the specificity of [^{11}C]TZ3321 binding toward S1PR1.

MicroPET/CT Imaging

With the use of contrast reagent and high-resolution CT scan, the mouse aorta was identified and ROIs in the aorta were drawn accordingly (Fig. 2). To exclude the change of tracer uptake in mouse blood, the ROIs of blood were drawn inside the aorta lumen. The aorta-to-blood uptake ratio was used to indicate the radioactivity from vessel wall and the plaque. Dynamic analysis of aorta-to-blood uptake ratio from 0 to 60 min showed [^{11}C]TZ3321 had higher retention in AS mouse plaque-containing vessel walls than that in healthy controls (Fig. 3a). The averaged aorta-to-blood uptake ratio from 10 to 60 min revealed $\sim 20\%$ increase in the aorta of AS mice, compared with age-matched control mice ($n = 3$, $P < 0.05$, Fig. 3b).

Ex vivo Autoradiography

To further confirm the increased uptake of [^{11}C]TZ3321 in the aorta of AS mice, *ex vivo* autoradiography studies were performed following microPET studies. Plaques were clearly visible in the aorta of AS mice after microsurgery dissection (Fig. 4a, b). The quantitative data showed that the uptake of [^{11}C]TZ3321 was $\sim$ six-fold higher in the AS mouse aorta relative to controls (7.15 ± 0.60 vs 1.30 ± 0.27 PSL/ mm^2 , Fig. 4c–e).

S1PR1 Expression in Rodent Aortic Plaques

H&E staining was performed using aortic root and aorta from high-fat fed AS mice (8 months old), as well as AS mice (3 months old) that underwent a wire injury on unilateral femoral artery. In both groups, we observed pathological changes of atherosclerosis (Figs. 5 and 6). IHC results also indicated that compared to control wild-type mice, S1PR1 positive cells were more abundant in the aortic plaques of both high-fat fed AS mice as well as AS mice that underwent a femoral wire injury (Figs. 5 and 6). In the wire-injury AS model, the injured site at the femoral artery also showed increased S1PR1 positive staining 6 weeks post-injury, although the morphology of the wire-injured femoral artery only showed intimal-medial thickening, without foam cell accumulation and cholesterol deposition (Fig. 6).

Discussion

In the current study, we observed upregulation of S1PR1 expression in areas of maximally diseased femoral artery plaque segments with foam cell accumulation, as well as increased S1PR1 expression in the plaques of murine models that are prone to atheroprotection. Moreover, using a S1PR1-specific radioligand, [^{11}C]TZ3321, an increase of S1PR1

expression in the aorta of AS mice with high-fat diet was detected by microPET and further confirmed by *ex vivo* autoradiography. This is the first report of the expression pattern of S1PR1 in human atherosclerotic plaques and reveals the potential of implementation of S1PR1-specific PET radioligands for imaging atherosclerosis *in vivo*.

Despite the fundamental role of S1P/S1PR1 signaling in regulating various vascular functions, relatively, few studies have directly addressed the effect of S1P/S1PR1 on atherosclerosis. The non-specific S1PR modulator FTY720 reduced the size of atherosclerotic lesion in LDLR^{-/-} mice and ApoE^{-/-} mice, which were fed a high-cholesterol diet [18, 19]. Interestingly, no protective effects of FTY720 on atherosclerosis were observed in chow-fed ApoE^{-/-} mice, suggesting that this S1PR modulator is contingent on severity of atherosclerotic disease [20]. Relative to this, cholesterol-fed LDLR^{-/-} mice treated with the specific S1PR1 agonist, KRP-203, demonstrated less atherosclerosis, lymphopenia, and macrophage activation [12]. These studies indicated that S1PR played a critical role in atherosclerosis and modulation of S1PR expression and activity impacts atheroprogession.

Our current study expands the understanding of the distribution of S1PR1 in atherosclerotic plaque. Specifically, we observed that S1PR1 was highly expressed in the foam cell-accumulated regions of maximally diseased human femoral artery endarterectomy plaques, while minimal diseased segments of the plaque showed lower levels of S1PR1 expression. This suggests that S1PR1 expression positively correlates with the severity of atherosclerotic plaque.

Similarly, in mouse models of atherosclerosis, the wire-injury-induced accelerated AS mice developed plaques at earlier age than the regular ApoE^{-/-} mice (3 vs 8 months old). Immunohistochemical staining in both mouse models confirmed the finding that S1PR1 expression was elevated in atherosclerotic plaques compared to normal aortas. In addition, the wire-injured site in mouse femoral arteries also showed increased S1PR1 expression, which was consistent with our previous finding in early phase of wire injury in mouse femoral arteries [16]. Although the differences in the morphology of mouse and human atherosclerotic plaques and the rarity of plaque rupture in the mouse model limit its application in studying superimposed thrombosis [21], the similarity between S1PR1 expression pattern in the plaque of the mouse model and patients permits the use of AS mice for conducting S1PR1-related mechanistic, therapeutic, and imaging studies.

Interestingly, S1P/S1PR1 pathway functions closely with high- and low-density lipoproteins, as well as apolipoproteins (ApoE and ApoM). The plasma S1P level has a significant positive correlation with LDL cholesterol concentration [22]. However, ApoE deficiency results in a suppressed synthesis of LDL [23], but an upregulated concentration of S1P level in the plasma was detected [24]. A recent study revealed that LDL receptor overexpression reduced the plasma S1P levels in wild-type mice, but not in ApoE^{-/-} mice, suggesting that ApoE might be a ligand for the LDL receptor during the clearance of S1P [24]. Another study focused on the ApoM-S1P-S1PR1 signaling axis, which is proved to restrain the lymphocyte compartment and, subsequently, adaptive immune responses [25]. Specifically, in aortic primary endothelial cells, the anti-inflammatory effects of the ApoM-S1P complex

are mediated by S1PR1 [26]. It is worth noting that patients with coronary artery disease have lower plasma S1P and HDL-S1P levels than controls [27]. In contrast, the S1P/HDL-cholesterol ratio was upregulated in patients with coronary artery disease when plasma S1P levels were adjusted by HDL-cholesterol levels [27]. Therefore, the increase of S1PR1 in atherosclerotic plaque in ApoE^{-/-} mice might be partly associated with discoordination of S1P/S1PR1 and apolipoproteins in response to ApoE deficiency. This should be taken into consideration when conducting translational studies of atherosclerosis from transgenic rodent models to human.

Currently, several potent S1PR1-specific ligands have been synthesized and radiolabeled [16, 28–31]. However, none of them has been applied in animal models of atherosclerosis or other related diseases. Our group previously reported the radiosynthesis of [¹¹C]TZ3321, which has a nanomolar binding potency and good selectivity for S1PR1 [16]. [¹¹C]TZ3321 has been validated in three rodent models of inflammation: a mouse wire-injury model of neointimal hyperplasia [16], a rat EAE model of multiple sclerosis [32], and a rat carotid injury model of acute vascular inflammation [33]. In the current study, we confirmed the specificity of [¹¹C]TZ3321 by *in vitro* autoradiography in human femoral plaque tissue and further validated the radioligand in the ApoE^{-/-} mouse model of atherosclerosis for detecting the plaque. Tracer uptake in mouse aorta was successfully quantified by co-registration of contrast-enhanced high-resolution CT and microPET images. Additionally, *ex vivo* autoradiography studies were performed after precise microdissection of mouse aorta and confirmed that tracer retention was higher in the AS model mice than the age-matched wild-type controls. Unfortunately, due to the high tracer uptake in the heart and lung, the attempt to visualize mouse aorta by microPET was very challenging. Further investigation of [¹¹C]TZ3321 PET imaging in atherosclerosis will be conducted using large animals such as rabbits and swines or targeting peripheral arterial disease in which the inflamed regions are distantly from thoracic and abdominal organs such as heart, lung, liver, and kidney to avoid over shadowing. Also, a clinical PET scanner is sufficient for imaging assessment of plaques in patients with peripheral arterial disease, such as femoral plaques [34]. Our immunostaining data in human femoral plaques also suggested high possibility in quantification of S1PR1 expression in human femoral plaques; additional investigations of PET imaging human atherosclerotic plaque are currently underway. *In vitro* autoradiography study in human plaques using its tritiated S1PR1 radioligand [³H]TZ3321 will provide more accuracy than [¹¹C]TZ3321 in measure of S1PR1 expression within human atherosclerotic plaque, which will provide additional support for PET assessment of atherosclerotic plaques by measuring S1PR1 expression in the inflamed binding sites.

Conclusion

Upregulated S1PR1 expression in human femoral atherosclerotic plaques and aortic plaques in the murine model of atherosclerosis was observed by immunostaining methods. Moreover, PET and *ex vivo* autoradiography with [¹¹C]TZ3321 also detected the increase of S1PR1 level in AS mouse aorta. The data suggest that [¹¹C]TZ3321 PET has possibility in assessment of atherosclerotic plaques by measuring the change of S1P1 expression, particularly for peripheral arterial disease, in which the regions of interests are distantly from thoracic and abdominal regions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

S1PR1 expression in human femoral plaque. **a** Representative H&E and IF staining images of human femoral plaque and control tissue from the same patient. H&E staining and immunofluorescence studies were performed using adjacent sections. Higher S1PR1 expression (red spots, indicated by white arrow, in the lower row) was observed in areas of the plaque with foam cell accumulation than in control tissue (surrounding tissue of the plaque, the upper row). **b** Quantification of S1P1 positive cell counts. S1P1 positive cell numbers in plaques and controls are counted manually in 2–3 representative images from individual femoral plaque specimen ($n = 4$ patients). The graph presents the average mean of

positive cell counts in the plaque region (filled circles) or the control tissue (filled squares) in each section. The cell counts are 73.38 ± 14.35 in the plaque and 2.71 ± 1.17 in the control. Error bar indicates SEM. **c** Representative *in vitro* autoradiography image of [^{11}C]TZ3321 in human femoral plaque. The uptake of [^{11}C]TZ3321 was blocked by ~ 56% in the presence of 10 μM SEW2781. The structure of [^{11}C]TZ3321 and SEW2781 with binding affinities are also presented.

Fig. 2.

Representative co-registered microPET/CT/high-resolution CT images containing ROIs for quantitative analysis. Transverse (**a**, **d**), coronal (**b**, **e**), and sagittal images (**c**, **f**) of the same mouse were shown. Red dash lines indicate mouse aorta; orange dash lines indicate blood in the lumen of mouse aorta.

Fig. 3.

Quantitative analysis of [^{11}C]TZ3321 microPET data in AS mice. **a** The aorta-to-blood uptake ratio was used to indicate the radioactivity from vessel wall and the plaque. Dynamic analysis of aorta-to-blood uptake ratio from 0 to 60 min showed [^{11}C]TZ3321 had higher retention in AS mouse plaque than in vessel wall of healthy controls. **b** The averaged aorta-to-blood uptake ratio from 10 to 60 min revealed ~20 % increase in the aorta of AS mice compared with that of age-matched control mice ($n = 3$, $P < 0.05$).

Fig. 4.

Ex vivo autoradiography with [^{11}C]TZ3321 using AS mouse aorta. Representative images of excised **a** AS aorta and **b** control aorta. *Ex vivo* autoradiographic images of mouse aortas showing upregulated tracer uptake in **c** AS mouse aorta compared to **d** controls. **e** Quantification histogram of autoradiographic images revealed higher uptake of [^{11}C]TZ3321 in the AS mouse aorta relative to controls (7.15 ± 0.60 vs 1.30 ± 0.27 PSL/mm²). PSL/mm², photo-stimulated luminescence signals per square millimeter.

Fig. 5.

Expression of S1PR1 in aortic atherosclerotic plaque in a mouse model of AS. H&E staining showed typical atherosclerotic pathological changes in mouse aortic root and aorta of *ApoE*^{-/-} mice with Western diet (AS mice). S1PR1 was highly expressed in the atherosclerotic plaque in AS mouse aorta (brown positive staining in the lower row) and had low expression in control mouse aorta. The vessels were delineated by red dash lines.

Fig. 6.

Expression of S1PR1 in atherosclerotic plaque in a wire-injury-induced accelerated AS mouse model. S1PR1 expression was upregulated in the atherosclerotic plaque in the mouse aorta and injured femoral artery of wire-injured AopE^{-/-} mice with Western diet (brown positive staining in the lower row) compared with control mouse aorta and femoral artery, respectively. The vessels were delineated by red dash lines.