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Specific somatostatin receptor II expression in arterial plaque: ^{68}Ga-DOTATATE autoradiographic, immunohistochemical and flow cytometric studies in apoE-deficient mice

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ABSTRACT

Background: The rupture of atherosclerotic plaques is triggered by inflammation. Specific detection of inflammation is therefore the focus of many investigations. Noninvasive imaging methods, such as positron emission tomography (PET), also are suited for this purpose. ^{68}Ga-DOTATATE is a ^{68}Ga-labeled radiotracer with specific affinity to somatostatin receptor subtype-2 (SSTR-2). SSTR-2 was found specifically expressed on human macrophages/monocytes.
Objective: We aimed to confirm the distribution of SSTR-2 in inflammatory plaques, and to assess its co-localization with macrophages within the plaques. We also assessed ^{68}Ga-DOTATATE uptakes in plaques by autoradiography.
Method: Apolipoprotein E (ApoE)−/− mice on a high-cholesterol diet were injected with ^{68}Ga-DOTATATE. The animals were sacrificed and aorta sections were examined using autoradiography and immunohistochemistry. Furthermore, expression of SSTR-2 was analyzed by flow cytometry. Western blot was conducted to assess SSTR-2 regulation in basal and lipopolysaccharide (LPS)-activated state. To evaluate the specificity of the ^{68}Ga-DOTATATE, the sections were pre-incubated with monoclonal SSTR-2 antibody before autoradiography.
Result: Autoradiographic imaging showed uptake of ^{68}Ga-DOTATATE co-localized with the macrophage-rich plaques by immunohistochemical examination. A high expression of SSTR-2 on macrophages was found by flow cytometry and western blot. Stimulation with lipopolysaccharide did not alter expression of SSTR-2 in macrophages.
Conclusion: Due to its specific binding to macrophages, ^{68}Ga-DOTATATE might be a suitable radiotracer for the evaluation of inflammatory activity in unstable plaques.

1. Introduction

Inflammatory activity is characterized by a localized recruitment of macrophages, lymphocytes and neutrophils to normal tissue, resulting in the necrosis of vital tissues and contributing to increased patient morbidity and mortality [1]. It plays an important role in the pathophysiology of atherosclerosis. In addition, metalloproteinases (MMPs), which are secreted by activated macrophages, can degrade the fibrous cap leading to plaque rupture [2,3]. Therefore, atherosclerotic lesions are the results of a series of highly specific cellular and molecular responses to inflammation. The increasing density of macrophages is a crucial factor indicating plaque disruption, a frequent cause of myocardial infarction and stroke [4]. Macrophages therefore can serve as potential targets for the development for molecular imaging probes to assess the inflammatory activity in atherosclerotic plaques.

Clinical imaging techniques such as angiography, optical coherence tomography, intra-vascular coronary ultrasound and angioscopy

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are able to provide valuable information to characterize atherosclerosis [5–7]. However, all of them have the disadvantage of being invasive. Various non-invasive imaging strategies were investigated, including the multislice CT, magnetic resonance imaging and ultrasonography. Their sensitivity to detect inflammation however is very limited. Recently, positron emission tomography computed tomography (PET/CT) has been evaluated in vulnerable plaques by detecting inflammatory activity both in human and in animal studies [8–12]. During the search for new probes, many vascular inflammatory sites, which are responsible for the migration and accumulation of macrophage and monocyte have been considered [13,14]. Macrophages are the most commonly used target for noninvasive imaging in order to detect early atherosclerotic lesions [8,15–18].

Numerous metabolic or pathological biomarkers associated with plaque vulnerability were evaluated as targets for molecular imaging using positron emission tomography (PET) and single photon emission computed tomography (SPECT). Among them, one in vivo study used technetium-99m (^{99m}Tc) labeled MMP inhibitors [19] to estimate MMP activation in atherosclerosis. In addition, macrophage apoptosis may be detected using ^{99m}Tc-labeled annexin-V [20]. ^{18}F-galacto-RGD, in contrast, targets alpha(v) beta(3)integrin and visualizes angiogenesis within the plaque [21]. ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is the most commonly used tracer to evaluate inflammatory activity within the plaque by targeting the glucose metabolism. A large number of studies demonstrated that the ^{18}F-FDG accumulation in atherosclerosis correlates with the macrophage density in plaques [10,22–25].

^{68}Ga-DOTATATE, a selective radiotracer binding to somatostatin receptors subtype-2 (SSTR-2), has so far been most widely used in clinical diagnose of neuroendocrine tumors, showed a potential to detect the vascular inflammation, due to specific uptake in macrophages or monocytes [26–28]. A significant association with plaque calcification and risk factors of cardiovascular disease was already demonstrated [29,30]. Of note, the tracer is known to be safe for the use in humans; none of the many studies published so far states any side effects. Currently the tracer is in the process for approval for routine use in oncological settings.

In this study, our aim was to assess the binding of the ^{68}Ga-DOTATATE to macrophages and normal endothelium ex-vivo, as well as evaluating the uptake of ^{68}Ga-DOTATATE in inflammatory plaques in a mouse model of atherosclerosis.

2. Materials and methods

2.1. Preparation of ^{68}Ga-DOTATATE

^{68}Ga-DOTATATE was synthesized in a commercially available computer-assisted synthesis-module (Scintomics, Fürstenfeldbruck, Germany). ^{68}Ga for radiolabeling was eluted in form of $^{68}GaCl_3$ with 0.1 M HCl from a ^{68}Ge/^{68}Ga-generator (Obninsk, Russia) into a reactivial (Thermo Scientific, Langenselbold, Germany), containing 10 μg of DOTATATE and HEPES-buffer. After 10 min at 95 °C, the crude product was separated via a Waters-Sep-Pak-C18 cartridge (Waters, Eschborn, Germany), diluted with 0.9% NaCl and filtrated through a 0.22 μm sterile filter (Millipore, Cork, Ireland) into a sterile vial (IBA, Berlin, Germany) for biological evaluation.

Radiochemical purity, as assessed by gradient HPLC (eluent A: 0.1% TFA in H_2O, eluent B: 0.1% TFA in acetonitrile, flow rate: 0.7 ml/min, column: Nucleosil 100-5 C18 125 × 4.6 mm, gradient: nonlinear 0%–100% solvent B in 15 min; Scintomics, Fürstenfeldbruck, Germany) and TLC (eluent: 0.1 M citric acid, stationary phase: ITLC-SG stripes (Varian, Lake Forest, USA); measurement time: 1 min; miniGITA, raytest, Straubenhardt, Germany) was >99%. The specific activity of the injection solution amounted 50 MBq/μg.

2.2. Animal models and preparation

Animal care and experiments 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). Female apolipoprotein E (ApoE)−/− mice ($n = 8$) were studied at the age of 50 weeks. Mice were fed high-cholesterol diet from age of 8 weeks, which is known to result in severe atherosclerotic plaques. The experiments were conducted at room temperature (RT). Mice were anesthetized during all procedures (isoflurane 2%–2.5%, Baxter). Advanced atherosclerotic plaques were confirmed by histology. Non-atherosclerotic C57 mice ($n = 3$) were studied as control group.

2.3. In vitro analyse of SSTR-2

2.3.1. Purification of monocytes/macrophages

Erythrocyte-depleted spleen cells were stained in fully-supplemented RPMI medium with anti-CD11b-PE (clone M1/70, BD Biosciences), anti-Ly-6G (clone 1A8, Biolegend) and anti-CD11c-eFluor 450 (clone N418, eBioscience). After a final washing step, cells were resuspended in RPMI medium and sorted with FACSAria III cell sorter (BD Biosciences). Monocytic cells were defined as CD11b^{high}, Ly-6G$^-$, CD11c$^-$.

2.3.2. Cell culture

Murine macrophages/monocytes digested from five mice spleen and lymph node maintained in RPMI 1649 medium supplemented with 10% heat-inactivated FCS, non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml and 100 μg/ml streptomycin, 2 mM L-glutamine and 30 μM mercaptoethanol (all from Gibco). Cells were incubated over night at 37 °C and 5% CO_2 in the presence and absence of 10 ng/ml LPS (Sigma). Upregulation of IL-6 protein level in culture supernatants was confirmed by Enzyme-linked immunosorbent assay (ELISA) at time point of 50 h after LPS stimulation. In order to prove SSTR-2 expression on endothelium, the murine endothelial cell line bEnd.3 was purchased from American type Culture Collection. The adherently growing cells were cultivated under standard condition at 37 °C in a humidified 95% air/5% CO_2 incubator, maintained in the complete growth medium. It mainly including Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), supplemented with 10% fetal calf serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin. The bEnd.3 cells were used at passages 25 to 30.

2.3.3. FACS analyze of SSTR-2 expression on leukocytes, endothelial cells, platelets

We used flow cytometry to measure SSTR-2 expression within the arterial wall. Since cluster of differentiation 31 (CD31) is platelet endothelial cell adhesion molecule (PECAM), murine endothelial cells line (bEnd.3) was confirmed by flow cytometry separately. Samples of the aorta ($n = 3$) were dissected under a microscope. Digestion: collagenase type 2 and protease type XIV (0.895 mg/ml and 0.5 mg/ml respectively) in PBS the aorta was incubated for 1 h at 37 °C. Blood were collected as well. Cells were pre-blocked with anti-Fcγ receptor III/II monoclonal antibody (mAb) (clone 2.4G2) and 5% rabbit serum for 15 min at 4 °C for 15 min and subsequently stained with anti-CD41(biolegend), anti-CD3ε(biolegend), anti-CD11b (ebioscience), anti-Ly-6G (biolegend) and anti-SSTR-2 (Abcam). To examine the SSTR-2 expression on endothelial cell, bEnd.3 cells were stained with anti-CD31 (biolegend), after washing, cells were blocked with 5% goat serum and anti-SSTR-2