Comparison of $^{64}\text{Cu}$ and $^{68}\text{Ga}$ for Molecular Imaging of Atherosclerosis using the Apolipoprotein A-I Mimetic Peptide FAMP

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Abstract

**Background:** Molecular imaging for detection of the atherosclerotic plaque burden has been highlighted as a modality for the diagnosis of atherosclerosis. We recently developed a novel and noninvasive positron emission tomography (PET) that was functionalized with an apolipoprotein (Apo) A-I mimetic peptide [known as Fukuoka University Apo A-I mimetic peptide (FAMP)] radiolabeled with gallium-68 ($^{68}\text{Ga}$) - 1, 4, 7, 10-tetraazaacyclobdecene-1, 4, 7, 10-tetraacetic acid (DOTA) to specifically image the status of atherosclerotic plaque in myocardial infarction-prone Watanabe heritable hyperlipidemic rabbits (WHHL-MI).

**Methods and Results:** To achieve more sensitive molecular imaging, FAMP was modified with 4, 11 - bis (carboxymethyl) - 1, 4, 8, 11 - tetraazaacyclobdecene (6.6.2) hexadecane (CB-TE2A) and radiolabeled with copper-64 ($^{64}\text{Cu}$) for PET, and the ability of $^{64}\text{Cu}$-TE2A-FAMP to image plaque was compared with that of $^{68}\text{Ga}$-DOTA-FAMP. Japanese white normal (JW) and WHHL-MI rabbits were intravenously injected with $^{64}\text{Cu}$-CB-TE2A-FAMP for $^{64}\text{Cu}$-DOTA-FAMP, and subjected to continuous PET (25-30 MBq). Interestingly, $^{64}\text{Cu}$-CB-TE2A-FAMP was not taken up by atherosclerotic lesions in the aorta of WHHL-MI, whereas $^{68}\text{Ga}$-DOTA-FAMP was dramatically illuminated in the aorta of WHHL-MI. Moreover, $^{64}\text{Cu}$-CB-TE2A-FAMP was rapidly decomposed and $^{64}\text{Cu}$ was excreted to the intestine, liver or urinary bladder in both JW and WHHL-MI rabbits.

**Conclusions:** These results demonstrated that FAMP may be a target molecule for atherosclerotic molecular imaging with $^{68}\text{Ga}$-DOTA, but not with $^{64}\text{Cu}$-CB-TE2A. The selection of a suitable radio-nuclide and chelator might be important for HDL functioning imaging.

Keywords: Molecular imaging; Positron emission tomography; Apolipoprotein A-I mimetic peptide; Watanabe heritable hyperlipidemic rabbits

Introduction

Cardiovascular disease is the leading cause of death in the world [1]. Atherosclerosis affects arterial blood vessels due to chronic inflammation and induces cardiovascular disease. Molecular imaging aims to identify and guide the treatment of vulnerable atherosclerotic plaque. A novel and noninvasive positron emission tomography (PET)-based method for imaging inflammatory plaque with $^{18}$F-fluorodeoxyglucose (FDG) is currently of interest in the clinical setting [2]. Although atherosclerotic disease may produce no or only mild diffuse uptake along the vessel wall in FDG-PET, both the sensitivity and molecular specificity for targeting atherosclerosis by FDG-PET are relatively low [3-5].

Since the physical interactions between apolipoprotein A-I (Apo A-I) and ATP-binding cassette transporter 1 (ABCA1) modulate not only binding to Apo A-I but also internalization and transcytosis in macrophages and aortic endothelial cells, Apo A-I mimetic peptide may be a candidate for functional high-density lipoprotein (HDL) imaging in atherosclerosis. We recently synthesized a novel 24-amino acid Apo A-I mimetic peptide without phospholipids [known as Fukuoka University Apo A-I mimetic peptide (FAMP)], which potently removes cholesterol in vitro via specific ABCA1 [6]. In that study using a cholesterol-fed mouse model, we found that FAMP promoted macrophage reverse cholesterol transport (RCT).

Moreover, we demonstrated that the unique Apo A-I mimetic peptide FAMP with gallium-68 ($^{68}\text{Ga}$) - 1, 4, 7, 10-tetraazaacyclobdecene-1, 4, 7, 10-tetraacetic acid (DOTA) is a promising candidate diagnostic tracer for imaging the atherosclerotic lipid burden [7]. Nonetheless, molecular imaging using $^{68}\text{Ga}$-DOTA-FAMP may not be enough for detecting the atherosclerotic burden clearly because $^{68}\text{Ga}$ has a short physical half-life (68 min).

The aim of the present study was to achieve more sensitive molecular imaging in atherosclerotic lesions. Therefore, FAMP was modified with 4, 11 - bis (carboxymethyl) - 1, 4, 8, 11 - tetraazaacyclobdecene (6.6.2) hexadecane (CB-TE2A) and radiolabeled with copper-64 ($^{64}\text{Cu}$) for PET, since $^{64}\text{Cu}$ has a relatively long physical half-life (12.7 h), and...
molecular imaging using $^{64}$Cu-CB-TE2A-FAMP was compared with that using $^{68}$Ga-DOTA-FAMP.

**Methods**

**Synthesis of CB-TE2A-FAMP and DOTA-FAMP**

FAMP (H-ALEHLFTLYKALKDLDLKLKL-OH) was modified with CB-TE2A (Macrocyclics, Dallas, TX, USA) (Figure 1A) and DOTA (Strem Chemicals Inc., Newburyport, MA, USA). To a solution of O - (1H-benzotriazol-1-yl) - N, N', N'- tetramethyluronium hexafluorophosphate (HBTU, 50 μmol) and 1-hydroxybenzotriazole (HOBr, 50 μmol) in N, N - dimethylformamide (DMF, 2 ml) were added CB-TE2A (50 μmol) and DIEA (N, N - diisopropylethylamine) 34 μL, and the mixture was stirred at room temperature. After several minutes, this solution was added to 50 μmol/L FAMP resin and stirred at room temperature for 1 hour. The resulting CB-TE2A-FAMP resin was deprotected with a TFA mixture that contained triisopropylsilane (1.0%), 1, 2-ethanedithiol (2.5%) and water (2.5%) for 2 h. After deprotection, cold diethyl ether was added to the mixture, and the precipitate was collected by centrifugation. Obtained crude CB-TE2A-FAMP was purified by reversed-phase high-performance liquid chromatography (HPLC). Purified CB-TE2A-FAMP was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; found: m/z 3,137.2 (M+H)+, calculated for (M+H)+: 3,137.8) (Figure 1B).

**Radiolabeling of $^{64}$Cu-CB-TE2A-FAMP and $^{68}$Ga-DOTA-FAMP**

CB-TE2A-FAMP and DOTA-FAMP were radiolabeled with $^{64}$Cu and $^{68}$Ga, respectively, for use in noninvasive PET imaging. Cu-64 was produced by proton-irradiation of a $^{64}$Ni electroplated gold disc target using a HM-12S cyclotron (Sumitomo Heavy Industries, Ltd., Tokyo, Japan) [9]. The crude Cu-64 was dissolved in 6 N HCl and purified manually on an ion exchange resin (DOWEX 1X8 100-200 mesh; Muromachi Technos Co., Ltd., Tokyo, Japan) to produce ($^{64}$Cu) CuCl2. $^{68}$Ga-DOTA-FAMP was prepared using the method described previously [7,10].

**Animals**

We purchased Japanese white (JW) rabbits from Japan SLC Inc. (Shizuoka, Japan). In addition, myocardial infarction-prone
homozygous Watanabe heritable hyperlipidemic (WHHL-MI) rabbits with total cholesterol >700 mg/dl were provided by Dr Shiomi, Kobe University, Japan. The rabbits were injected with $^{64}$Cu-CB-TE2A-FAMP (2 nmol per animal) and $^{68}$Ga-DOTA-FAMP (2-5 nmol per animal). The rabbits were maintained at Fukuoka University, Japan, and experiments were performed at the RIKEN Center for Molecular Imaging Science, Japan. The experiments complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. The rabbits were housed in rooms maintained at 23 ± 1°C and 55 ± 5% relative humidity with free access to water and standard diet during acclimatization, and were fasted for 24 hr before PET imaging.

**PET imaging protocol**

Imaging was carried out using a microPETR Focus220 (Siemens, Knoxville, TN, USA) as described previously [7]. Briefly, emission data acquisition was started at the same time as the intravenous administration of $^{64}$Cu-CB-TE2A-FAMP and $^{68}$Ga-DOTA-FAMP for 6 h with an energy window of 400-650 keV and a coincidence timing window of 6 ns. The emission images were reconstructed by using filtered back projection with a Ramp filter and a cutoff at the Nyquist frequency. The time course of $^{64}$Cu activity in the region of the abdominal aortic bifurcation was obtained using ASIPro software included with the microPET system, and the results were adjusted for the background.

**Results**

**Time-dependent attenuation of $^{64}$Cu-CB-TE2A-FAMP at the abdominal aortic bifurcation**

We performed a PET analysis 6 h after the injection of $^{64}$Cu-CB-TE2A-FAMP at the abdominal aortic bifurcation, when radioactivity should be completely cleared from the circulation. PET images of the abdominal aorta revealed little accumulation of $^{64}$Cu-CB-TE2A-FAMP in either JW or WHHL-MI rabbits (Figure 2). The time-dependent attenuation of $^{64}$Cu-CB-TE2A-FAMP in the abdominal aortic bifurcation in WHHL-MI rabbits was mild compared with that in JW rabbits.

**PET imaging after injection of the tracer**

PET images clearly showed a high uptake of the $^{68}$Ga-DOTA-FAMP tracer in the aorta of WHHL-MI rabbits (Figure 3A), and little uptake in the aorta of JW rabbits (Figure 3B). On the other hand, PET images showed little uptake of $^{64}$Cu-CB-TE2A-FAMP in the aorta of either WHHL-MI or JW rabbits (Figure 3C, 3D). Furthermore, the clear uptake of $^{64}$Cu-CB-TE2A-FAMP in the intestine, liver and bladder was seen in both WHHL-MI and JW rabbits (Figures 3C, 3D and 4A). Finally, there were no uptake in the aorta and the clear uptake of $^{64}$Cu-CB-TE2A-FAMP in the intestine, liver and bladder at 24 h after the injection in both WHHL-MI and JW rabbits (Figure 4B, 4C).

**Discussion**

We previously developed a novel PET tracer, Apolipoprotein A-I Mimetic Peptide (FAMP).

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**Figure 2:** (A) Time-dependent attenuation of $^{64}$Cu-CB-TE2A-FAMP at the abdominal aortic bifurcation in Japanese white (JW) and (B) myocardial infarction-prone homozygous Watanabe heritable hyperlipidemic (WHHL-MI) rabbits. Upper panels show continuous images of the abdominal aortic bifurcation for 6 h after the injection of tracers. $^{64}$Cu-CB-TE2A, 4, 11 - bis (carboxymethyl) - 1, 4, 8, 11 - tetraazabicyclo [6.6.2] hexadecane; FAMP, Fukuoka University Apo A-I mimetic peptide.
peptide FAMP radiolabeled with ⁶⁸Ga-DOTA, to specifically image the status of atherosclerotic plaque in WHHL-MI [7]. To achieve more sensitive molecular imaging, FAMP was modified with CB-TE2A and radiolabeled with ⁶⁴Cu for PET, and the ability of ⁶⁴Cu-CB-TE2A-FAMP to image plaque was compared with that of ⁶⁸Ga-DOTA-FAMP. ⁶⁴Ga-DOTA-FAMP was interestingly superior to ⁶⁴Cu-CB-TE2A-FAMP in atherosclerotic molecular imaging. The selection of a suitable radionuclide and chelator should be important for HDL functioning imaging.

In this study, we selected ⁶⁴Cu as a tracer because it has a longer half-life than ⁶⁸Ga (12.7 h and 68 min, respectively) for more feasibility clinical use. The half-life of fluorescence-labeled FAMP in blood was about 4 h, according to data on blood clearance of FAMP in mice [6]. Although we thought that a combination of ⁶⁴Cu and FAMP could provide superior molecular imaging, PET images showed no uptake of ⁶⁴Cu-CB-TE2A-FAMP in the aorta at 6 h after injection (Figure 3C). Since we did not observe any uptake of ⁶⁴Cu-CB-TE2A-FAMP in the aorta at 1-2 h after injection (data not shown), we consider that ⁶⁴Cu-

Figure 3: Positron emission tomography (PET) imaging after injection of the tracer reveals a high uptake of the ⁶⁸Ga-DOTA-FAMP tracer at 1.5 h after injection in the aorta of Watanabe heritable hyperlipidemic (WHHL-MI) rabbits (A), and no uptake of ⁶⁸Ga-DOTA-FAMP at 2 h after injection in the aorta of Japanese white (JW) rabbits (B). PET images showed no uptake of ⁶⁴Cu-CB-TE2A-FAMP at 6 h after injection in the aorta of both WHHL-MI (C) and JW rabbits (D). ⁶⁸Ga-DOTA, gallium-68-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; ⁶⁴Cu-CB-TE2A, 4, 11-bis (carboxymethyl) - 1, 4, 8, 11-tetraazabicyclo [6.6.2] hexadecane; FAMP, Fukuoka University Apo A-I mimetic peptide.

Figure 4: Positron emission tomography (PET) imaging at 6 h after injection of the tracer reveals a high uptake of ⁶⁴Cu-CB-TE2A-FAMP tracer in the intestine, liver and bladder of Watanabe heritable hyperlipidemic (WHHL-MI) rabbits (A). PET imaging at 24 h after injection of the tracer reveals a high uptake of ⁶⁴Cu-CB-TE2A-FAMP tracer in the intestine, liver and bladder of WHHL-MI (B) and Japanese white (JW) rabbits (C).
CB-TE2A-FAMP did not provide HDL-functional imaging. Instead, 64Cu-CB-TE2A-FAMP may be rapidly decomposed, since 64Cu was excreted to the intestine, liver or urinary bladder in both JW and WHHL-MI rabbits (Figures 3CD and 4A). Previous reports have shown that 64Cu-DOTA and 64Cu-TETA complex are moderately unstable in vivo due to the release of uncoordinated 64Cu by decomposition in the blood or transchelation in the liver, which leads to high uptake in non-target tissues [11,12]. 64Cu-labeled DOTA/TETA conjugates often show poor blood and liver clearance, high uptake in non-target organs, and increased background radioactivity levels, resulting in reduced imaging sensitivity [13,14]. As for 4Cu-CB-TE2A complexes, Sprague et al. indicated that the 64Cu-CB-TE2A-Tyr3-octreotate shows improved blood, liver, and kidney clearance compared with the analogous 64Cu-TETA agent [15]. 64Cu-CB-TE2A-c is a potential candidate for imaging tumor angiogenesis [16]. In addition, 64Cu-CB-TE2A-ReCCMSH (Arg11) also showed greatly improved liver and blood clearance as well as higher tumor-to-non-target tissue ratios compared with 64Cu-DOTA-ReCCMSH (Arg11) [17]. Thus, many researchers have successfully performed molecular imaging using 64Cu-CB-TE2A. We do not know why 64Cu-CB-TE2A-FAMP may be rapidly decomposed in vivo at this stage, although we successfully synthesized and purified 64Cu-CB-TE2A-FAMP as shown in Figure 1. Further studies are needed to solve this issue.

Although 68Ga-DOTA-FAMP was interestingly superior to 64Cu-CB-TE2A-FAMP for atherosclerotic molecular imaging, the PET images obtained with 68Ga-DOTA-FAMP tracer show atherosclerotic plaques more clearly than those obtained using previously reported tracers ([18F]-FDG and 111In-low-density lipoprotein, etc.) [18-20]. FAMP has been shown to have a high capacity for cholesterol efflux from A172 cells and mouse and human macrophages in vitro, and this efflux was mainly dependent on ABCA1 transporter [6]. 68Ga-DOTA-FAMP may accumulate in atherosclerotic plaque, which requires FAMP. Miziochi et al. reported that a pioglitazone-treated group demonstrated significantly greater suppression of FDG imaging of carotid and aortic plaque inflammation compared with a glimepiride group in patients with diabetes mellitus [21]. Their study only found a decrease in inflammation after treatment, and could not assess changes in plaque volume or vulnerability. In addition, the precise mechanisms that underlie FDG uptake in atheroma are not clear. Recently, Santulli et al. proposed an innovative strategy based on a selective microRNA group in patients with diabetes mellitus [21]. Their study only found a decrease in inflammation after treatment, and could not assess changes in plaque volume or vulnerability. In addition, the precise mechanisms that underlie FDG uptake in atheroma are not clear. Recently, Santulli et al. proposed an innovative strategy based on a selective microRNA targeting system to match PET-positive lesions with the results of immunohistochemical analysis because of the difficulties associated with radioactive tissue.

In conclusion, these results demonstrated that FAMP may be a target molecule for atherosclerotic molecular imaging with 68Ga-DOTA, but not with 64Cu-CB-TE2A. The selection of a suitable radionuclide and chelator might be important for HDL functioning imaging.

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