⁶⁴Cu-Labeled Divalent Cystine Knot Peptide for Imaging Carotid Atherosclerotic Plaques

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The rupture of vulnerable atherosclerotic plaques that lead to stroke and myocardial infarction may be induced by macrophage infiltration and augmented by the expression of integrin $\alpha_v\beta_3$. Indeed, atherosclerotic angiogenesis may be a promising marker of inflammation. In this study, an engineered integrin $\alpha_v\beta_3$ -targeting PET probe, ⁶⁴Cu-NOTA-3-4A, derived from a divalent knottin miniprotein was evaluated in a mouse model for carotid atherosclerotic plaques. **Methods:** Atherosclerotic plaques in BALB/C mice, maintained on a high-fat diet, were induced with streptozotocin injection and carotid artery ligation and verified by MR imaging. Knottin 3-4A was synthesized by solid-phase peptide synthesis chemistry and coupled to 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) before radiolabeling with ⁶⁴Cu. PET probe stability in mouse serum was evaluated. Mice with carotid atherosclerotic plaques were injected via the tail vein with ⁶⁴Cu-NOTA-3-4A or ¹⁸F-FDG, followed by small-animal PET/CT imaging at different time points. Receptor targeting specificity of the probe was verified by coinjection of c(RGDyK) administered in molar excess. Subsequently, carotid artery dissection and immunofluorescence staining were performed to evaluate target expression. **Results:** ⁶⁴Cu-NOTA-3-4A was synthesized in high radiochemical purity and yield and demonstrated molecular stability in both phosphate-buffered saline and mouse serum at 4 h. Small-animal PET/CT showed that ⁶⁴Cu-NOTA-3-4A accumulated at significantly higher levels in the neovasculature of carotid atherosclerotic plaques (7.41 ± 1.44 vs. 0.67 ± 0.23 percentage injected dose/gram, $P < 0.05$) than healthy or normal vessels at 1 h after injection. ¹⁸F-FDG also accumulated in atherosclerotic lesions at 0.5 and 1 h after injection but at lower plaque-to-normal tissue ratios than ⁶⁴Cu-NOTA-3-4A. For example, plaque-to-normal carotid artery ratios for ¹⁸F-FDG and ⁶⁴Cu-NOTA-3-4A at 1 h after injection were 3.75 and 14.71 ($P < 0.05$), respectively. Furthermore, uptake of ⁶⁴Cu-NOTA-3-4A in atherosclerotic plaques was effectively blocked (~90% at 1 h after injection) by coinjection of c(RGDyK). Immunostaining confirmed integrin $\alpha_v\beta_3$ expression in both the infiltrating macrophages and the neovasculature of atherosclerotic plaques. **Conclusion:** ⁶⁴Cu-NOTA-3-4A demonstrates specific accumulation in carotid atherosclerotic plaques in which macrophage infiltration and angiogenesis are responsible for elevated integrin $\alpha_v\beta_3$ levels. Therefore, ⁶⁴Cu-NOTA-3-4A may demonstrate clinical utility as a PET probe for atherosclerosis imaging or for the evaluation of therapies used to treat atherosclerosis.

Key Words: knottin; atherosclerotic plaque; $\alpha_v\beta_3$ integrin; ⁶⁴Cu; PET/CT

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Cardiovascular disease induced by atherosclerosis is a leading cause of death in the Western world. Most myocardial infarctions and sudden cardiac deaths result from the rupture of vulnerable atherosclerotic plaques (1–4). Therefore, tools that detect atherosclerotic plaques are needed in the clinic. At present, several imaging modalities are used to identify atherosclerotic lesions in patients, including CT angiography, MR imaging, and intravascular ultrasound. Although these imaging modalities have been able to delineate certain features of plaques, their sensitivity and specificity are relatively low (5,6). On the other hand, nuclear imaging techniques such as PET may provide a more sensitive and quantitative route to determine the extent of cardiovascular disease (7).

Inflammation is a well-studied feature of atherosclerotic plaques that are at high risk of rupture (1,2). Therefore, molecular imaging of inflammation-associated markers may be a promising tool to quantify rupture risk of plaques and warn against further adverse cardiovascular events. The most widely studied PET probe for detection of plaque-based inflammation is ¹⁸F-FDG (8,9), which is taken up by active macrophages so that it provides information about the extent of inflammation in atherosclerotic plaques (10–12). However, ¹⁸F-FDG is a nonspecific imaging probe for inflammation, because it can also accumulate in calcified structures of vessel walls, muscle tissues adjacent to the vessels, and tissues with high metabolic rates such as the myocardium and brain. Therefore, new PET probes to image plaque-related inflammation and their propensity to rupture are needed in treating heart disease (13).

Recently, integrin $\alpha_v\beta_3$ expression in atherosclerosis has been reported to be a promising marker of vulnerable plaques (14–16). Integrin $\alpha_v\beta_3$ receptors are transmembrane glycoproteins, which are highly expressed by not only activated endothelial cells of new vessels in the atherosclerotic plaques, but also activated macrophages that mediate the inflammatory process. Therefore, radio-labeled peptides such as Arg-Gly-Aspartic acid (RGD) peptide that specifically bind to $\alpha_v\beta_3$ integrin have been studied by PET imaging of atherosclerotic plaques (13,17–20). For example, ¹⁸F-galacto-RGD (13) and ⁶⁸Ga-DOTA-RGD (17) have shown specific targeting to integrin $\alpha_v\beta_3$. However, their accumulation in atherosclerotic lesions and their plaque-to-normal tissue ratios are low

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in preclinical models. The uptake of ^{18}F -galacto-RGD by the aorta plaque was only 0.24 percentage injected dose per gram (%ID/g) at 2 h after injection, and the aorta-to-normal vessel wall uptake ratio was 1.3 (13). The aorta plaque uptake of ^{68}Ga -DOTA-RGD was 0.90 %ID/g at 1 h after injection, and aorta-to-heart and aorta-to-blood uptake ratios were 1.8 and 1.1 at 1 h after injection, respectively (17). Therefore, PET probes with higher integrin $\alpha_v\beta_3$ receptor binding affinity and specificity are needed to be of any clinical utility in management of cardiovascular disease.

Cystine knot peptides, also known as knottins, are small polypeptides that are characterized by a stable core motif formed by multiple disulfide bonds (3 or 4) that are interwoven into a knotted conformation, which endows these peptides with high thermal and proteolytic stability. They are relatively small (30–50 amino acids) and often made by 9-fluorenylmethoxycarbonyl (Fmoc) or t-butyloxy carbonyl solid-phase peptide synthesis. Importantly, knottins demonstrate fast blood clearance, high and specific integrin $\alpha_v\beta_3$ -targeting ability, and biocompatibility. Together, these biologic, chemical, and physical properties bode well for clinical imaging applications. In our previous studies, monovalent and bivalent integrin $\alpha_v\beta_3$ knottin binders have been coupled to a variety of multimodal imaging labels (radionuclides or fluorescent dyes) and successfully used to image $\alpha_v\beta_3$ -positive tumors in small-animal models (21–28).

In this work, a bivalent integrin $\alpha_v\beta_3$ knottin binder 3-4A was conjugated at its N terminus to 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and radiolabeled with ^{64}Cu for PET imaging. The resulting probe was successfully used to image integrin $\alpha_v\beta_3$ expression associated with carotid atherosclerotic plaques.

MATERIALS AND METHODS

General and Animal Model

All Fmoc-protected amino acids were purchased from Novabiochem/EMD Chemicals Inc. or CS Bio. c(RGDyK) peptide was purchased from Peptides International. Phosphate-buffered saline (PBS, 0.01 M, pH 7.4) was obtained from Gibco/Invitrogen. All other chemicals were purchased from Fisher Scientific unless otherwise specified.

Male BALB/C mice were purchased from Charles River Laboratory. As shown in Figure 1A, carotid atherosclerotic plaques were induced in male BALB/C mice by the following protocol. Mice ($n = 20$) were fed with a high-fat diet containing 40% kcal fat, 1.25% (by weight) cholesterol, and 0.5% (by weight) sodium cholate (D12109; Research Diets, Inc.). After 4 wk of high-fat diet, mice were rendered diabetic

by administration of 7 daily intraperitoneal injections of streptozotocin (40 mg/kg in citrate buffers [0.05 mol/L, pH 4.5, Sigma Aldrich]). At day 7 of the streptozotocin injections, serum glucose level was measured by sampling tail vein blood and using a glucometer. If the glucose level was below 200 mg/L, animals were injected with additional streptozotocin for 3 consecutive days. At day 14 after initiation of streptozotocin injection, the left common carotid artery was ligated below the bifurcation with 6-0 silk ligature (Ethicon) under 2% inhaled isoflurane. In sham-operated animals, the suture was put around the exposed left carotid artery but not tightened. The wound was closed by suture, and the mice were allowed to recover on a warming blanket. All procedures were approved by the Administrative Panel on Laboratory Animal Care at Stanford University.

Small-Animal MR Imaging

Mice ($n = 20$) from the treatment described above were sequentially anesthetized using 2% isoflurane and placed supine in a specially designed stage with the top of their necks centered in a small, circular MR imaging coil. The small-animal MR imaging scanner consisted of a superconducting magnet (Magnex Scientific) with 7.0-T field strength, a gradient (Resonance Research, Inc.) with a clear bore size of 9 cm, a maximum gradient amplitude of 770 mT/m and a maximum slew rate of 2,500 T/m/s, and a console and Copley 266 amplifiers (GE Healthcare). A 15-s localizer scan was conducted first, followed by a prescan to allow for manual gradient shimming. Then, a T1-weighted fast spin echo sequence was obtained from the bottom of the head to the top of the chest. No contrast agents were given. This scan was approximately 5 min long for 30 slices and had an isotropic resolution of 20 mm. The mouse was then removed from the scanner and allowed to recover in its cage. The small-animal MR imaging data were analyzed using the commercially available DICOM image viewer OsiriX (<http://www.osirix-viewer.com/>).

Peptide Synthesis and Folding, NOTA Conjugation, and ^{64}Cu Labeling

The linear knottin 3-4A was synthesized with a CS BioCS336 instrument using Fmoc-based solid-phase peptide synthesis, and the crude peptide was deprotected and cleaved from resin as reported previously (25). Linear 3-4A peptide was oxidized and folded in 4 M guanidinium chloride, 10 mM reduced glutathione, 2 mM oxidized glutathione, and 3.5% (v/v) dimethylsulfoxide at pH 8.0 in ammonium bicarbonate buffer at room temperature for 1 d with gentle mixing. Folded peptide was purified on a Vydac C18 preparatory scale column and lyophilized. Purified peptide was dissolved in water, and concentration was determined by amino acid analysis (AAA Service Laboratory). Similarly, peptide purity and molecular mass were determined by analytic-scale reversed-phase high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), respectively.

NOTA, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and *N*-hydroxysulfono-succinimide at a molar ratio of 1:1:0.8 were mixed in water and incubated at 4°C for 30 min (pH 5.5). 3-4A was then added to the in situ-prepared sulfosuccinimidyl ester of NOTA in a theoretic stoichiometry of 1:5 in sodium phosphate buffer (pH 8.5–9.0). The solution was reacted at 4°C overnight, and the resulting NOTA-3-4 conjugate was purified by reversed-phase HPLC and characterized by MALDI-TOF-MS as described above. NOTA-3-4A (25 μg) was radiolabeled with ^{64}Cu by the addition of 74–111 MBq (2–3 mCi) of $^{64}\text{CuCl}_2$ (University of Wisconsin) in 0.1N sodium acetate (pH 5.5), followed by 1-h incubation at 37°C. The radiolabeled complex was purified by a PD-10 column (GE Healthcare Life Sciences) and eluted with PBS.

In Vitro Stability

^{64}Cu -NOTA-3-4A (1.85–3.7 MBq [50–100 μCi]) was incubated in 0.5 mL of mouse serum for 4 h at 37°C. The mixture was then treated

FIGURE 1. (A) Study design diagram. (B and C) MR images of vessel of carotid atherosclerotic plaques (red arrow) and control (blue arrow). (D) Bright-field anatomic image of vessel with carotid atherosclerotic plaques (red arrow) and control (blue arrow). STZ = streptozotocin.

FIGURE 2. (A) Peptide sequences of EETI mutant 3-4A. Black lines represent disulfide bonds between Cys¹-Cys⁴, Cys²-Cys⁵, and Cys³-Cys⁶. (B) Schematic of 3-4A. Yellow lines represent disulfide bonds between cysteines (I–VI). Purple loop and light blue loop (red arrows) are engineered to contain an RGD motif.

with 0.5 mL of acetonitrile to precipitate the serum protein and centrifuged at 16,000g for 2 min. The supernatant containing greater than 95% of the radioactivity was filtered using a 0.22- μ m nylon SpinX column (Corning Inc.). Greater than 99% of the radioactivity passed through this filter. In addition, ⁶⁴Cu-NOTA-3-4A (1.85–3.7 MBq [50–100 μ Ci]) was also incubated in PBS for 4 h at room temperature. The samples were analyzed by radio-HPLC, and the percentage of intact peptide was determined by quantifying peaks corresponding to the intact peptide and degradation products.

Small-Animal PET/CT

⁶⁴Cu-NOTA-3-4A. PET/CT imaging of BALB/C mice with carotid atherosclerotic plaques was performed using a small-animal PET/CT scanner (Inveon; Siemens). Mice ($n = 4$ for each group) were injected via the tail vein with approximately 3.7–5.55 MBq (100–150 μ Ci) of ⁶⁴Cu-NOTA-3-4A. At 1, 2, 4, and 24 h after injection, mice were anesthetized for imaging experiments. On the second day, the same mice were injected through the tail vein with 2.96–3.7 MBq (80–100 μ Ci) of ⁶⁴Cu-NOTA-3-4A with or without approximately 330 μ g of c(RGDyK) as blocking agent. At 1, 2, 4, and 24 h after injection, mice were kept in a space consistent throughout (5% isoflurane for induction and 2% for maintenance in 100% O₂) for the imaging experiment.

The images were reconstructed with a 2-dimensional ordered-subset expectation maximization algorithm with CT-based attenuation correction. Image files were analyzed using the vendor-supplied software Inveon Research Workspace (Preclinical Solutions; Siemens Healthcare Molecular Imaging). For each small-animal PET scan, 3-dimensional regions of interest (ROIs) were drawn over the organs and tissues on decay-corrected whole-body images. The average radioactivity concen-

tration in the ROI was obtained from the mean pixel values within the ROI volume. These data were converted to counts per milliliter per minute using a predetermined conversion factor. The results were divided by the injected dose to obtain an image ROI-derived %ID/g of tissue (29).

¹⁸F-FDG. Similar to the ⁶⁴Cu-NOTA-3-4A PET/CT scan, mice models ($n = 4$) were also injected via the tail vein with approximately 3.7–5.55 MBq (100–150 μ Ci) of ¹⁸F-FDG. At 0.5 and 1 h after injection, mice were anesthetized for imaging experiments.

Immunofluorescence Staining

Air-dried frozen slides (10- μ m thickness) of carotid tissues were fixed in ice-cold acetone and blocked by incubation with 1% PBS/bovine serum albumin for 30 min to prevent unspecific binding. Immunofluorescence staining was performed using antiintegrin $\alpha_v + \beta_3$ (CD51+CD61) polyclonal antibody (Bioss) for $\alpha_v\beta_3$ integrin with anti-CD68 antibody (Abcam) for macrophages and with anti-CD31 antibody (Abcam) for endothelial cells, respectively. Finally, sections were counterstained with 4,6-diamidino-2-phenylindole for visualization of cell nuclei. The slides were observed under a microscope (Axiovert 200 M), and images were acquired under the same conditions and displayed at the same scale for comparison.

Statistical Analysis

The quantitative data were expressed as mean $\pm$ SD. Means were compared using the Student *t* test. A 95% confidence level was chosen to determine the significance between groups, with *P* values of less than 0.05 indicating significant differences.

RESULTS

Establishment of Carotid Atherosclerotic Plaques

MR imaging was used to evaluate whether the carotid atherosclerotic plaques were successfully established in mouse models subjected to PET imaging protocols. Compared with the normal contralateral carotid artery, MR imaging revealed stenosis within the affected artery as shown on an axial MR image (Fig. 1B). Moreover, a longitudinal plane of the 3-dimensional fast low-angle shot MR images shows that blood flow in the affected (left) artery was significantly decreased, compared with the normal (right) carotid artery (Fig. 1C). After acquisition of MR imaging and PET data, mice were euthanized so that the injured artery could be examined by bright-field magnification. These anatomic images clearly show the damaged vessel wall (solid white structure) of the left artery (Fig. 1D).

Preparation of ⁶⁴Cu-NOTA-3-4A

Linear 3-4A was synthesized by solid-phase peptide synthesis, folded in an oxidative buffer, purified by reversed-phase HPLC, and characterized by MALDI-TOF-MS (sequence and schematic structure are shown in Fig. 2). Folded peptide 3-4A recorded an *m/z* value of 3,940.2, which corresponded to the [M+H]⁺ (calculated *m/z* = 3,938.6). The knottin peptide 3-4A was then site-specifically conjugated to NOTA through its N-terminal amino group using an *N*-hydroxysuccinimide ester NOTA derivative. The resulting NOTA-3-4A showed an *m/z* of 4,225.2 for [MH]⁺ (calculated *m/z* = 4,226.0). The NOTA-3-4A peptide was then radiolabeled with ⁶⁴Cu. The radiochemical yield of ⁶⁴Cu-NOTA-3-4A peptide was greater than 70%, and radiochemical purity was greater than 95% as

FIGURE 3. Stability analysis of ⁶⁴Cu-NOTA-3-4A (A) in PBS buffer (B) at room temperature and mouse serum (C) at 37°C for 4 h.

analyzed by radio-HPLC. A modest specific activity of 4.3 MBq/nmol of ^{64}Cu -NOTA-3-4A was obtained at the end of synthesis (decay-corrected). The stability of ^{64}Cu -NOTA-3-4A was further evaluated in PBS and mouse serum. As shown by the radio-HPLC analysis, ^{64}Cu -NOTA-3-4A was highly stable, and there was no degradation observed in PBS or 4-h incubation in mouse serum at 37°C (Fig. 3).

^{64}Cu -NOTA-3-4A PET/CT Imaging Study

The atherosclerotic lesion in the left carotid artery was clearly visible at 1 h (Fig. 4A) after injection and demonstrated high contrast with low contralateral background, which persisted from

1 to 4 h after injection (Fig. 4B). PET/CT imaging also showed relatively high kidney signal at all the imaging time points, indicating the renal clearance of the probe. Meanwhile, most of the normal organs demonstrated relatively low signal. Further small-animal PET quantification analysis showed that the atherosclerotic plaque uptake was 7.41 ± 1.44 %ID/g at 1 h after injection, whereas normal tissues such as the common contralateral artery, brain, heart, liver, and muscle exhibited much lower uptake than the atherosclerotic lesion (Fig. 4C). Because of the renal clearance of the PET probe, high accumulation of ^{64}Cu -NOTA-3-4A in the kidneys was observed from 1 to 4 h, which decreased to 4.63 ± 0.54 %ID/g at 24 h after injection. Importantly, ^{64}Cu -NOTA-3-4A displayed high plaque-to-background tissue (common artery, brain, heart, muscle) ratios (Fig. 4D). For example, at 1 h after injection, the plaque-to-common artery, plaque-to-brain, plaque-to-heart, and plaque-to-muscle ratios of ^{64}Cu -NOTA-3-4A were 17.47 ± 3.50 , 27.76 ± 4.31 , 21.31 ± 3.69 , and 42.99 ± 5.48 , respectively. Moreover, the $\alpha_v\beta_3$ integrin-targeting specificity of ^{64}Cu -NOTA-3-4A was evaluated by the coinjection of a large amount of c(RGDyK). Significantly reduced uptake of ^{64}Cu -NOTA-3-4A by the left carotid atherosclerotic lesion was observed on PET images from 1 to 24 h after injection (Fig. 4B).

Comparative ^{18}F -FDG PET/CT Imaging Study

The atherosclerotic lesion in the left carotid artery was visible at 0.5 h after injection and demonstrated high contrast with low contralateral background from 0.5 to 1 h after injection (supplemental materials [available at <http://jnm.snmjournals.org>]). ^{18}F -FDG exhibited relatively high kidney uptake. Interestingly, despite fasting and consistent experimental protocols for all mice, the myocardial and brain uptake of ^{18}F -FDG were extremely variable. Quantification analysis showed that the accumulation of ^{18}F -FDG in the left atherosclerotic plaques was much higher than the common right carotid artery (10.47 ± 0.60 vs. 2.79 ± 0.40 %ID/g at 0.5 h after injection and 10.66 ± 1.24 %ID/g vs. 1.91 ± 0.45 %ID/g at 1 h after injection). The myocardial uptake of ^{18}F -FDG ranged from 3.65 to 26.08 %ID/g, and the brain uptake was from 4.13 to 32.45 %ID/g 0.5 h after injection. The kidney uptake of ^{18}F -FDG was found to be 10.02 ± 1.67 %ID/g at 0.5 h after injection and 22.88 ± 4.67 %ID/g at 1 h after injection, respectively. The plaque-to-common artery and plaque-to-muscle ratios of ^{18}F -FDG at 1 h were 5.56 ± 1.05 and 58.67 ± 3.69 , respectively.

Immunofluorescence Staining

To determine whether $\alpha_v\beta_3$ integrin expression in the carotid atherosclerotic plaques was associated with macrophage infiltration and/or angiogenesis, immunohistochemical staining was performed. CD68 and CD31 staining confirmed the presence of both macrophages and angiogenesis in atherosclerotic plaques, respectively. Moreover, CD51 and CD61 staining identified the expression integrin $\alpha_v\beta_3$ in carotid atherosclerotic plaques. The results revealed the distinct colocalization of integrin $\alpha_v\beta_3$ markers with CD68-positive macrophages (Fig. 5A) and $\alpha_v\beta_3$ integrin with vessel endothelial cells (Fig. 5B).

DISCUSSION

In the clinic, ^{18}F -FDG PET has been used to detect inflammation in vulnerable atherosclerotic plaques and to select patients for vascular surgery (12). However, the lack of specificity hampers the clinical utility of ^{18}F -FDG for cardiovascular disease. Integrin $\alpha_v\beta_3$ has been reported to be an imaging marker that is highly

FIGURE 4. Small-animal PET images and quantification analysis results of ^{64}Cu -NOTA-3-4A. (A) PET, CT, and PET/CT images of mice models with carotid atherosclerotic plaques at 1 h after injection of ^{64}Cu -NOTA-3-4A. (B) Coronal PET images of mice models with carotid atherosclerotic plaques at 1, 2, 4, and 24 h after injection of ^{64}Cu -NOTA-3-4A without or with c(RGDyK). (C) Quantification analysis of radioactivity accumulation in selected organs at different time points after injection of ^{64}Cu -NOTA-3-4A, reported as %ID/g. (D) Quantification analysis of plaque-to-normal tissue ratios at different time points. B = blood; H = heart; K = kidney; Li = liver; M = muscle; N = normal vessel wall; P = plaque.

FIGURE 5. Immunofluorescent result of carotid atherosclerotic plaques. DAPI = 4,6-diamidino-2-phenylindole; N = normal vessel wall; P = plaque.

expressed by both plaque-associated macrophages and activated endothelial cells, which form plaque neovasculature. Imaging integrin $\alpha_v\beta_3$ expression could therefore be a usefully noninvasive tool to predict the unstable plaques.

Radiolabeled RGD peptides are of particular interest because they specifically bind integrin $\alpha_v\beta_3$. ^{18}F -galacto-RGD was developed to detect atherosclerotic plaques in the aorta of hypercholesterolemic mice (13). Although the %ID/g in the aorta was more than 2 times higher than the residual activity in the blood of the same mice, the uptake of ^{18}F -galacto-RGD in the aorta was only 0.24 ± 0.07 %ID/g at 2 h after injection, and the aorta-to-normal vessel wall ratio was 1.3. Further evaluation of ^{18}F -galacto-RGD in human carotid plaques revealed a positive correlation between ^{18}F -galacto-RGD uptake and $\alpha_v\beta_3$ expression of carotid plaques (7). Moreover, ^{68}Ga -DOTA-RGD was also evaluated in the aorta of hypercholesterolemic mice and showed aorta uptake of 0.90 ± 0.21 %ID/g at 1 h after injection, and the aorta-to-heart and aorta-to-blood uptake ratios were 1.8 and 1.1 at 1 h after injection, respectively (17). ^{18}F -flotegatide was also reported in a mouse model of aortic plaques. It showed aorta uptake 2.8 ± 0.4 %ID/g at 1 h after injection, and the aorta-to-heart ratio and aorta-to-muscle ratio were 14 and 18, respectively (18).

For the current study, to the best of our knowledge, this is the first report to describe the PET-labeled knottin for imaging carotid atherosclerotic lesions. Several cystine-knot peptides, including Ecballium elaterium trypsin inhibitor (EETI-II) (21) and Agouti-related protein (30), have been used as scaffolds to engineer peptides that bind integrin receptors with low nanomolar affinities. EETI-II and Agouti-related protein-based cystine peptides are small (3–4 kDa); are amenable to amino acid substitutions; and have high chemical, thermal, and proteolytic stability. In our previous studies, divalent knottin 3-4A was successfully radiolabeled with ^{64}Cu (25) and ^{18}F (31) for imaging integrin $\alpha_v\beta_3$ -positive U87MG tumor in living animals. In this study, PET/CT imaging and quantification results demonstrated the atherosclerotic lesion in the left carotid artery to be clearly visible at 1 h after injection. Compared

with the uninjured contralateral common carotid artery (0.67 ± 0.23 %ID/g at 1 h after injection), the uptake of the left carotid atherosclerotic lesion was 7.41 ± 1.44 %ID/g, which was significantly higher than uptake values of ^{18}F -galacto-RGD, ^{68}Ga -DOTA-RGD, and ^{18}F -flotegatide ($P < 0.05$). Plaque-to-background tissue ratios, especially the plaque-to-heart ratio, are clinically important because of the potentially debilitating effects of myocardial infarctions. Compared with the previously reported RGD-based PET probes, ^{64}Cu -NOTA-3-4A also displayed much higher plaque-to-heart ratio (27.8 at 1 h after injection). Considering there is no huge difference for the $\alpha_v\beta_3$ in vitro affinity of the ^{18}F -galacto-RGD, DOTA-RGD, and DOTA-3-4A (5, 3.2, and 8 nM, respectively) (13,17,25), the different imaging results among these probes are likely caused by their different in vivo pharmacokinetic properties of the probes.

Comparisons to ^{18}F -FDG PET are also presented here. The atherosclerotic lesion in the left carotid artery was readily visible at 0.5 h after injection of ^{18}F -FDG. Although the accumulation of ^{18}F -FDG by plaques was higher than that of ^{64}Cu -NOTA-3-4A at 1 h after injection (10.66 ± 1.24 vs. 7.41 ± 1.44 %ID/g, $P < 0.05$), the plaque-to-common carotid artery ratio of ^{18}F -FDG was significantly lower than that of ^{64}Cu -NOTA-3-4A at 1 h after injection (3.75 vs. 14.71, $P < 0.05$). The normal myocardial and brain uptake of ^{18}F -FDG in mice was extremely variable, and the ratios of plaque-to-brain and plaque-to-heart were difficult to be exactly evaluated. Thus, ^{18}F -FDG imaging of atherosclerotic plaques in brain and heart arteries may be of limited clinical utility. Compared with ^{18}F -FDG, ^{64}Cu -NOTA-3-4A did not accumulate in normal myocardium and brain, which could be beneficial for imaging coronary and brain vessels. In addition, successful use of ^{64}Cu -DOTATATE in human neuroendocrine tumors highlights the promise of ^{64}Cu -based probes in clinical settings (32). Similarly, ^{64}Cu -NOTA-3-4A may potentially be used to image a variety of integrin $\alpha_v\beta_3$ -positive lesions in human subjects. A variety of engineered cystine-knot peptides coupled to different PET labels are undergoing extensive testing and validation (22,26,31).

Finally, the immunofluorescent staining displayed a correlation between integrin $\alpha_v\beta_3$ expression and macrophage infiltration as well as formation of the neovasculature. These data indicate that the uptake mechanism of ^{64}Cu -NOTA-3-4A by atherosclerotic plaques is through binding to integrin $\alpha_v\beta_3$ expressed on the surface of macrophages and endothelial cells.

CONCLUSION

^{64}Cu -NOTA-3-4A displayed significantly high and specific accumulation in carotid atherosclerotic plaques, which correlated by immunofluorescence staining to overexpression of $\alpha_v\beta_3$ integrin by both macrophages and angiogenic endothelial cells. Moreover, lower normal-tissue uptake of ^{64}Cu -NOTA-3-4A and a high plaque-to-normal tissue ratios were observed. Collectively, these results suggest that ^{64}Cu -NOTA-3-4A may be a promising PET probe for clinical imaging of carotid atherosclerotic plaques.

DISCLOSURE

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REFERENCES

- Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874.
- Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002;105:1135–1143.
- Narula J, Garg P, Achenbach S, Motoyama S, Virmani R, Strauss HW. Arithmetic of vulnerable plaques for noninvasive imaging. *Nat Clin Pract Cardiovasc Med*. 2008;5:S2–S10.
- Saraste A, Nekolla SG, Schwaiger M. Cardiovascular molecular imaging: an overview. *Cardiovasc Res*. 2009;83:643–652.
- Gerretsen S, Kessels AG, Nelemans PJ, et al. Detection of coronary plaques using MR coronary vessel wall imaging: validation of findings with intravascular ultrasound. *Eur Radiol*. 2013;23:115–124.
- Huang PT, Huang FG, Zou CP, et al. Contrast-enhanced sonographic characteristics of neovascularization in carotid atherosclerotic plaques. *J Clin Ultrasound*. 2008;36:346–351.
- Beer AJ, Pelisek J, Heider P, et al. PET/CT imaging of integrin $\alpha_v\beta_3$ expression in human carotid atherosclerosis. *JACC Cardiovasc Imaging*. 2014;7:178–187.
- Ogawa M, Magata Y, Kato T, et al. Application of ^{18}F -FDG PET for monitoring the therapeutic effect of antiinflammatory drugs on stabilization of vulnerable atherosclerotic plaques. *J Nucl Med*. 2006;47:1845–1850.
- Tahara N, Kai H, Ishibashi M, et al. Simvastatin attenuates plaque inflammation: evaluation by fluorodeoxyglucose positron emission tomography. *J Am Coll Cardiol*. 2006;48:1825–1831.
- Rudd JH, Warburton EA, Fryer TD, et al. Imaging atherosclerotic plaque inflammation with [^{18}F]-fluorodeoxyglucose positron emission tomography. *Circulation*. 2002;105:2708–2711.
- Tawakol A, Migrino RQ, Bashian GG, et al. In vivo ^{18}F -fluorodeoxyglucose positron emission tomography imaging provides a noninvasive measure of carotid plaque inflammation in patients. *J Am Coll Cardiol*. 2006;48:1818–1824.
- Pedersen SF, Graebe M, Hag AM, Hoejgaard L, Sillesen H, Kjaer A. Microvessel density but not neoangiogenesis is associated with ^{18}F -FDG uptake in human atherosclerotic carotid plaques. *Mol Imaging Biol*. 2012;14:384–392.
- Laitinen I, Saraste A, Weidl E, et al. Evaluation of $\alpha_v\beta_3$ integrin-targeted positron emission tomography tracer ^{18}F -galacto-RGD for imaging of vascular inflammation in atherosclerotic mice. *Circ Cardiovasc Imaging*. 2009;2:331–338.
- Yao Y, Jiang Y, Sheng Z, et al. Analysis of in situ and ex vivo $\alpha_v\beta_3$ integrin expression during experimental carotid atherogenesis. *Int J Nanomedicine*. 2012;7:641–649.
- Waldeck J, Hager F, Holke C, et al. Fluorescence reflectance imaging of macrophage-rich atherosclerotic plaques using an $\alpha_v\beta_3$ integrin-targeted fluorochrome. *J Nucl Med*. 2008;49:1845–1851.
- Wu W, Wang Y, Shen S, et al. In vivo ultrasound molecular imaging of inflammatory thrombosis in arteries with cyclic Arg-Gly-Asp-modified microbubbles targeted to glycoprotein IIb/IIIa. *Invest Radiol*. 2013;48:803–812.
- Haukka J, Laitinen I, Luoto P, et al. ^{68}Ga -DOTA-RGD peptide: biodistribution and binding into atherosclerotic plaques in mice. *Eur J Nucl Med Mol Imaging*. 2009;36:2058–2067.
- Su H, Gorodny N, Gomez LF, et al. Atherosclerotic plaque uptake of a novel integrin tracer F-flotegatide in a mouse model of atherosclerosis. *J Nucl Cardiol*. 2014;21:553–562.
- Burtea C, Laurent S, Murariu O, et al. Molecular imaging of $\alpha_v\beta_3$ integrin expression in atherosclerotic plaques with a mimetic of RGD peptide grafted to Gd-DTPA. *Cardiovasc Res*. 2008;78:148–157.
- Saraste A, Laitinen I, Weidl E, et al. Diet intervention reduces uptake of $\alpha_v\beta_3$ integrin-targeted PET tracer ^{18}F -galacto-RGD in mouse atherosclerotic plaques. *J Nucl Cardiol*. 2012;19:775–784.
- Kimura RH, Cheng Z, Gambhir SS, Cochran JR. Engineered knottin peptides: a new class of agents for imaging integrin expression in living subjects. *Cancer Res*. 2009;69:2435–2442.
- Miao Z, Ren G, Liu H, et al. An engineered knottin peptide labeled with ^{18}F for PET imaging of integrin expression. *Bioconjug Chem*. 2009;20:2342–2347.
- Jiang L, Kimura RH, Miao Z, et al. Evaluation of a ^{64}Cu -labeled cystine-knot peptide based on agouti-related protein for PET of tumors expressing $\alpha_v\beta_3$ integrin. *J Nucl Med*. 2010;51:251–258.
- Jiang L, Miao Z, Kimura RH, et al. Preliminary evaluation of ^{177}Lu -labeled knottin peptides for integrin receptor-targeted radionuclide therapy. *Eur J Nucl Med Mol Imaging*. 2011;38:613–622.
- Kimura RH, Jones DS, Jiang L, Miao Z, Cheng Z, Cochran JR. Functional mutation of multiple solvent-exposed loops in the Ecballium elaterium trypsin inhibitor-II cystine knot miniprotein. *PLoS ONE*. 2011;6:e16112.
- Liu S, Liu H, Ren G, Kimura RH, Cochran JR, Cheng Z. PET imaging of integrin positive tumors using F labeled knottin peptides. *Theranostics*. 2011;1:403–412.
- Jiang L, Miao Z, Kimura RH, et al. ^{111}In -labeled cystine-knot peptides based on the Agouti-related protein for targeting tumor angiogenesis. *J Biomed Biotechnol*. 2012;2012:368075.
- Jiang H, Moore SJ, Liu S, et al. A novel radiofluorinated agouti-related protein for tumor angiogenesis imaging. *Amino Acids*. 2013;44:673–681.
- Natarajan A, Habte F, Liu H, et al. Evaluation of Zr-rituximab tracer by cerenkov luminescence imaging and correlation with PET in a humanized transgenic mouse model to image NHL. *Mol Imaging Biol*. 2013;15:468–475.
- Silverman AP, Levin AM, Lahti JL, Cochran JR. Engineered cystine-knot peptides that bind $\alpha_v\beta_3$ integrin with antibody-like affinities. *J Mol Biol*. 2009;385:1064–1075.
- Jiang L, Kimura RH, Ma X, et al. A radiofluorinated divalent cystine knot peptide for tumor PET imaging. *Mol Pharm*. 2014;11:3885–3892.
- Pfeifer A, Knigge U, Mortensen J, et al. Clinical PET of neuroendocrine tumors using ^{64}Cu -DOTATATE: first-in-humans study. *J Nucl Med*. 2012;53:1207–1215.