PET Imaging of Abdominal Aortic Aneurysm with $^{64}$Cu-Labeled Anti-CD105 Antibody Fab Fragment

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ABSTRACT

The critical challenge in abdominal aortic aneurysm (AAA) research is the accurate diagnosis and assessment of AAA progression. Angiogenesis is a pathological hallmark of AAA, and CD105 is highly expressed on newly formed vessels. Our goal was to use $^{64}$Cu-labeled anti-CD105 antibody Fab fragment for noninvasive assessment of angiogenesis in the aortic wall in a murine model of AAA. Methods: Fab fragment of TRC105, a mAb that specifically binds to CD105, was generated by enzymatic papain digestion and conjugated to NOTA for $^{64}$Cu-labeling. Binding affinity/specificity of NOTA-TRC105-Fab was evaluated by flow cytometry and various ex vivo studies. BALB/c mice were anesthetized and treated with calcium phosphate to induce AAA, which underwent weekly PET scans using $^{64}$Cu-NOTA-TRC105-Fab. Biodistribution and autoradiography studies were also performed to confirm the accuracy of PET results. Results: NOTA-TRC105-Fab exhibited high purity and specifically bound to CD105 in vitro. Uptake of $^{64}$Cu-NOTA-TRC105-Fab increased from a control level of $3.4 \pm 0.1$ to $9.5 \pm 0.4$ %ID/g at 6 h p.i. on Day 5, and decreased to $7.2 \pm 1.4$ %ID/g on Day 12 which correlated well with biodistribution and autoradiography studies (i.e. much higher tracer uptake in AAA than normal aorta). Of note, enhanced AAA contrast was achieved, due to the minimal background in the abdominal area of mice. Degradation of elastic fibers and highly expressed CD105 were observed in ex vivo studies. Conclusion: $^{64}$Cu-NOTA-TRC105-Fab cleared rapidly through kidneys, which enabled noninvasive PET imaging of the aorta with enhanced contrast and showed increased angiogenesis (CD105 expression) during AAA. $^{64}$Cu-NOTA-TRC105-Fab PET may potentially be used for future diagnosis and prognosis of AAA.

Key words: abdominal aortic aneurysm (AAA); positron emission tomography (PET); antibody fragment; Fab; CD105 (endoglin)
INTRODUCTION

Abdominal aortic aneurysm (AAA) is a common and potentially lethal vascular disease more prevalent in men over the age of 65. It is characterized by progressive expansion and weakening of abdominal aortic wall (1-3). Advanced AAA usually leads to rupture, which represents a leading cause of death in the Western world. In the United States, there are approximately 30,000 deaths every year because of AAA, and 15,000 of these are related to rupture of AAA (3, 4). Although the techniques for effective diagnosis and treatment of AAA are urgently needed, non-invasive imaging of AAA at molecular/cellular level is still underexplored, which has attracted tremendous interest from the research community. Several tracers, e.g. $^{18}$F-fluoro-deoxy-glucose ($^{18}$F-FDG) (5-10), have been reported for positron emission tomography (PET) imaging of AAA in previous studies. However, the reliability of conventional tracers is still under debate (11), and there is need for new and more reliable molecular targets and tracers for PET imaging of AAA.

Angiogenesis has been proven to be a pathological hallmark of AAA in both human and animal models, and it plays an important role in the development and progression of AAA (12-15). Higher levels of neovascularization are detected at the rupture edge in aortic aneurysm, suggesting that imaging of molecules and processes involved in angiogenesis can potentially depict high-risk aneurysms (16). One important advantage of targeting angiogenesis is that rapid, persistent and specific targeting can be achieved with different probes, which endows an excellent efficiency and imaging contrast. The endothelial cells of newly formed vessels express high levels of CD105, also known as endoglin, on their surfaces (17). We have previously demonstrated the efficacy of a CD105 targeted antibody, TRC105, for in vivo imaging of angiogenesis (18-21). Therefore, we hypothesize that CD105 could act as a promising target for imaging and even for treatment of AAA.
Our goal is to investigate a novel probe for effective targeting and imaging of AAA with excellent targeting specificity and imaging contrast. To achieve this goal, we have employed a $^{64}\text{Cu}$-labeled TRC105 Fab fragment (i.e. $^{64}\text{Cu}$-NOTA-TRC105-Fab) as a probe for PET imaging of AAA. Compared with TRC105 full antibody, TRC105-Fab could be rapidly cleared by kidneys with shortened circulation half-life (22), which might result in superior imaging contrast in the abdominal area. To the best of our knowledge, this is the first report of AAA PET imaging with an antibody fragment-based probe.

MATERIALS AND METHODS

Chemicals

TRC105 was provided by TRACON Pharmaceuticals, Inc. Rat anti-mouse CD31 primary antibody was purchased from BD Biosciences. AlexaFluor488- and Cy3-labeled secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. S-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) was purchased from Macrocyclics, Inc. Fluorescein isothiocyanate (FITC) and chelex 100 resin (50-100 mesh) were acquired from Sigma-Aldrich. PD-10 desalting columns were purchased from GE Healthcare. All other reaction buffers and chemicals were from Thermo Fisher Scientific.

Generation, Characterization and NOTA/ FITC Conjugation of TRC105-Fab

TRC105-Fab was obtained by digestion of TRC105 antibody (5 mg/mL) using immobilized papain (weight ratio: papain/TRC105 = 1:40) in a reaction buffer [10 mM disodium ethylenediaminetetraacetic acid (EDTA), 20 mM sodium phosphate dibasic, and 80 mM L-Cysteine hydrochloride] for 4 h at 37 °C under constant stirring (22, 23). Subsequently, the
supernatant was collected and purified by a Sephadex G-75 size exclusion column (fractionation range 3,000-80,000 Da) using phosphate-buffered saline (PBS) as the mobile phase. The elution from Sephadex G-75 size exclusion column was tested by UV absorbance at 280 nm. The purity of TRC105-Fab was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking gel and 8% resolving gel) (24).

As-prepared TRC105-Fab was conjugated with p-SCN-Bn-NOTA or FITC at a molar ratio of 1:10 at pH 9.0 for 2 h (22). The final products (NOTA-TRC105-Fab or FITC-TRC105-Fab) were purified by PD-10 size exclusion columns using PBS as the mobile phase to remove unbound NOTA/FITC.

**Cell Lines and Flow Cytometry**

Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (ATCC) and cultured as previously described (25-27). When the cells reached ~80% confluency, they were harvested and suspended in cold PBS with 2% bovine serum albumin at a concentration of $5 \times 10^6$ cells/mL, incubated with FITC-TRC105-Fab or FITC-TRC105-Fab-NOTA for 30 min at room temperature, and washed three times with cold PBS. Subsequently, the cells were analyzed using a BD FACSCalibur 4-color analysis cytometer equipped with 488 nm and 633 nm lasers (Becton-Dickinson) and FlowJo analysis software (Tree Star, Inc.).

**AAA Animal Models**

AAA was induced by topical application of calcium phosphate in four- to five-week-old female BALB/c mice (Harlan) under anesthesia (2-4% isofluorane) as described previously (28).
Briefly, the abdominal cavity was opened with a midline abdominal incision. The infrarenal region of the abdominal aorta was freed from the surrounding adipofascial tissue by gentle dissection, and a small piece of 0.5 mol/L CaCl₂-soaked gauze was applied for 10 min to aorta followed by application of another piece of PBS-soaked gauze for 5 min. Mice in sham group were only treated with PBS for 15 min. All animal studies were conducted under a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee.

**Radiolabeling, PET Imaging and Biodistribution Studies of NOTA-TRC105-Fab**

⁶⁴Cu was produced by a GE PETrace cyclotron using the ⁶⁴Ni(p,n)⁶⁴Cu reaction. ⁶⁴Cu was diluted in 300 µL of 0.1 M sodium acetate buffer (pH 5.0) and mixed with 200 µL of NOTA-TRC105-Fab (0.4 mg/mL). The reaction was carried out at 37 °C for 45 min with constant shaking. The resulting ⁶⁴Cu-NOTA-TRC105-Fab was purified by PD-10 size exclusion column chromatography, using PBS as the mobile phase. The radioactive fraction containing ⁶⁴Cu-NOTA-TRC105-Fab was collected for further in vitro and in vivo studies.

PET imaging was performed on days 5 and 12 after the induction of AAA using a microPET/microCT Inveon rodent model scanner (Siemens Medical Solutions USA, Inc.). The animals were injected with 5-10 MBq of ⁶⁴Cu-NOTA-TRC105-Fab via tail vein and serial images were obtained at 0.5, 3, 6, 14, and 24 hours post-injection (p.i.). Data acquisition, image reconstruction, and region-of-interest (ROI) analysis of the PET images were carried out as previously described (22, 29). After the last scan at 24 h p.i. on Day 12, biodistribution studies were performed to corroborate PET data. Aorta, blood and major organs/tissues were collected and wet-weighed. The radioactivity in the tissue was measured using a γ counter (PerkinElmer) and presented as %ID/g (mean ± SD).
**Ex Vivo Imaging and Histology**

The infrarenal portion of the abdominal aorta was collected from the animals following euthanasia. Autoradiographic images of the collected aortas were obtained using a gamma camera. The aortas were frozen and cryo-sectioned for histological analysis. Haematoxylin/eosin (HE) staining was performed to observe the microscopic changes in the abdominal aortic wall, acquired by Nikon Eclipse Ti microscope (18). Frozen tissue slices of 7 μm thickness were double stained for endothelial marker CD31 and CD105 as previously reported (20). The slides were coverslipped with Vectashield® mounting media for fluorescence with DAPI (Vector Laboratories, Inc.). Fluorescent images were acquired by Nikon Digital Eclipse C1 plus confocal microscope (20).

**RESULTS**

**Generation and Characterization of TRC105-Fab**

The elution profile of the TRC105-Fab showed a narrow peak in UV intensity between 16-18 mL, which indicated a high purity (Fig. 1A). This fraction was used for further studies. A narrow band corresponding to a molecular weight of 50-55 kDa was observed in SDS-PAGE (Fig. 1B), further confirmed the purity of TRC105-Fab.

**Flow Cytometry**

CD105 is highly expressed on human umbilical vein endothelial cells (HUVECs) (27). Our previous studies have documented that TRC105 possesses superior binding affinity and specificity to CD105 (20, 27, 30). Flow cytometry was used to examine the targeting efficiency
of TRC105-Fab in vitro. After the treatment with 5 ug/mL FITC-TRC105-Fab, the fluorescence signal from the HUVECs exhibited ~ 4-fold increase over the negative control (PBS treated HUVECs) (Fig 1C), indicating that TRC105-Fab could efficiently bind to CD105, although with a lower binding affinity than the intact antibody. In addition, a similar increase was observed after incubating the cells with 5 ug/mL FITC-TRC105-Fab-NOTA, suggesting that NOTA conjugation does not compromise the binding affinity of FITC-TRC105-Fab.

**PET Imaging and Biodistribution Studies**

On Day 5 following AAA induction, a significantly higher tracer uptake was observed in the aortic region of the animals from AAA group as early as 0.5 h p.i. which remained relatively stable up to 24 h p.i. (9.4 ± 0.1, 9.3 ± 0.7, 9.5 ± 0.4, 7.9 ± 0.5, 7.9 ± 0.4 %ID/g at 0.5, 3, 6, 16, and 24 h p.i., respectively; n = 3; Fig. 2 and 4A). On Day 12, the uptake in aortas of the animals from AAA group was still high (8.0 ± 1.5, 7.9 ± 1.2, 7.2 ± 1.4, 5.8 ± 1.0, 5.4 ± 1.5 %ID/g at 0.5, 3, 6, 16, and 24 h p.i.; n = 3; Fig. 3 and 4B), although a little lower in comparison to the first week. This result suggests that 

$^{64}$Cu-NOTA-TRC105-Fab could efficiently target AAA, and the extend of angiogenesis in AAA is stronger on Day 5 than Day 12, as shown by higher tracer uptake on Day 5.

The in vivo targeting specificity of $^{64}$Cu-NOTA-TRC105-Fab was confirmed with blocking studies on both Day 5 and Day 12. The signal from aorta in the blocking group was much lower than that in the AAA group, and decreased significantly from 0.5 h p.i to 24 h p.i. (Day 5: 7.1 ± 0.9, 6.3 ± 0.8, 5.5 ± 1.0, 4.2 ± 0.4, 3.4 ± 0.2 %ID/g; Day 12: 5.7 ± 0.8, 4.5 ± 0.2, 4.5 ± 0.5, 3.1 ± 0.3, 3.0 ± 0.1 %ID/g at 0.5, 3, 6, 16, and 24 h p.i.; n = 3; Fig. 2, 3, 4A and B). The signal from normal aorta was also lower throughout the scans on both Day 5 and Day 12 in sham group (Day
5: $4.8 \pm 0.6$, $4.2 \pm 1.1$, $3.4 \pm 0.1$, $3.0 \pm 0.5$, $2.5 \pm 0.3 \text{ %ID/g}$; Day 12: $4.8 \pm 0.4$, $4.3 \pm 0.2$, $3.7 \pm 0.2$, $3.2 \pm 0.1$, $3.0 \pm 0.5 \text{ %ID/g}$ at 0.5, 3, 6, 16, and 24 h p.i.; n = 3; Fig. 2, 3, 4A and B). Taken together, a great enhancement in aortic uptake was achieved in an AAA animal model using $^{64}$Cu-NOTA-TRC105-Fab, suggesting its superior efficiency and specificity in CD105 targeting. The signals from normal tissues were similar in AAA, blocking and sham groups at all time points (Fig. S1), indicating consistent pharmacokinetics of $^{64}$Cu-NOTA-TRC105-Fab and accuracy of ROI analysis.

$^{64}$Cu-NOTA-TRC105-Fab yielded low levels of background signal in abdominal area probably because it was mainly cleared through the kidneys (Fig. 2 and 3). Aorta/muscle ratio was measured as a standard of imaging contrast. On Day 5 and Day 12, the aorta/muscle ratio was much higher in AAA group (Day 5: $7.3 \pm 0.6$, $6.6 \pm 0.8$, $7.3 \pm 0.9$, $6.0 \pm 1.0$, $5.4 \pm 1.0$; Day 12: $8.0 \pm 0.5$, $6.5 \pm 0.7$, $5.6 \pm 0.6$, $5.1 \pm 0.7$, $4.1 \pm 0.7$ at 0.5, 3, 6, 16, and 24 h p.i.; n = 3; Fig. 4C and D), compared to the blocking (Day 5: $5.4 \pm 0.4$, $4.9 \pm 0.5$, $4.3 \pm 0.5$, $3.8 \pm 0.5$, $2.7 \pm 0.4$; Day 12: $4.5 \pm 0.3$, $3.5 \pm 0.3$, $3.2 \pm 0.3$, $2.4 \pm 0.4$, $2.2 \pm 0.4$ at 0.5, 3, 6, 16, and 24 h p.i.; n = 3; Fig. 4C and D) and sham groups (Day 5: $3.9 \pm 0.3$, $3.3 \pm 0.3$, $2.7 \pm 0.3$, $2.6 \pm 0.4$, $2.1 \pm 0.3$; Day 12: $3.4 \pm 0.3$, $3.1 \pm 0.3$, $2.7 \pm 0.4$, $2.5 \pm 0.4$, $2.4 \pm 0.4$ at 0.5, 3, 6, 16, and 24 h p.i.; n = 3; Fig. 4C and D). The data clearly demonstrates that excellent imaging contrast can be achieved in AAA with $^{64}$Cu-NOTA-TRC105-Fab probe.

To confirm the accuracy of PET images and ROI analysis, mice in all three groups were sacrificed at 24 h p.i. on Day 12. Aorta and other major organs were collected and the radioactivity signals were measured with a gamma counter. The quantitative data from biodistribution and ROI analysis matched well (Fig. 5). Of note, kidney uptake was significantly higher than the liver uptake in biodistribution study, which further validates the renal clearance
of $^{64}$Cu-NOTA-TRC105-Fab. More importantly, very low uptake observed in the intestine and muscles explains the low background signal and hence superior imaging contrast of $^{64}$Cu-NOTA-TRC105-Fab in AAA.

**Ex Vivo Imaging and Histology**

A macroscopically visible aneurysmal dilation was observed in the abdominal aortas of mice from AAA group 5 days after induction (Fig S2). Ex vivo autoradiography revealed a higher signal in the aortas from AAA group than the aortas from blocking and sham groups (Fig. 6A), further confirming the accuracy of the results from PET imaging, ROI analysis and biodistribution studies. Flattening and degradation of the elastic lamina due to increased diameter was obvious in HE staining of dilated aorta (Fig. 6B). The external diameter of aorta was $0.9 \pm 0.1$ mm in AAA group on Day 5, whereas it was only $0.4 \pm 0.01$ mm in normal aorta.

The intensity of CD105 immunofluorescence was highest on Day 5 suggesting active angiogenesis after AAA induction (Fig. 6C). The fluorescence intensity was similar to normal aorta on Day 12. These results correlated well with PET imaging results. Taken together, CD105 was highly expressed in AAA at Day 5 after induction, accompanied with structural change of aortic wall, matching well with in vivo and ex vivo studies.

**DISCUSSION**

In management of AAA clinicians are often faced with the important decision of whether to perform invasive repair or to manage the condition conservatively. This obstacle arises from the lack of methods that enables non-invasive acquisition of molecular/cellular information in the developing AAA. Current guidelines recommend endovascular repair or surgery if the aortic
diameter exceeds 5.5 cm and follow-up with anatomic imaging every 3 to 6 months for aneurysms above 4 cm. However, the size of AAA is neither the sole nor the most accurate determinant for the risk of rupture (31). Individual risk is also influenced by gender, age, smoking, and comorbidities, but its assessment is rarely easy because reliable methods for the optimal evaluation of these variables are not readily available. This situation often leads to difficulties in decision making, and many patients are unnecessarily exposed to the risks of reparative surgery when their aneurysm might never have ruptured if left untreated (32). Serial monitoring of the biological activity of AAA would be ideal to identify high-risk patients and prompt earlier therapy to prevent rupture while also sparing low-risk patients from expensive and morbid procedures (33).

To date, PET imaging of AAA has been mostly performed with $^{18}$F-fluoro-deoxy-glucose ($^{18}$F-FDG) (5-10, 34-37). One disadvantage of $^{18}$F-FDG is non-specific uptake by inflammatory tissues (38). This may lead to false positive results if there is an inflammatory disease in the artery wall other than AAA. Additionally, the correlation between $^{18}$F-FDG uptake in AAA and the chronic inflammation observed in the wall of asymptomatic AAAs is controversial, so as metabolic activity of inflammatory cells in asymptomatic AAAs was not sufficient to result in an increased glucose metabolism detectable by $^{18}$F-FDG PET (10, 39), which may lead to false negative results. We also tested the imaging capacity of $^{18}$F-FDG in AAA in this study, but we could not detect a significant difference between aortic signals in normal mice and mice with AAA (Fig. S3 and S4). In the contrary, the increased expression of CD 105 in AAA could be detected with high sensitivity and specificity using TRC105-Fab. CD105 is expressed on inflamed and some normal tissues to a certain extent, but the expression on endothelial cells is significantly higher (40). Therefore, CD105 has been accepted as an important angiogenesis
marker (20). $^{64}$Cu-NOTA-TRC105-Fab possesses excellent binding efficiency and high targeting specificity towards CD105 as validated by in vitro flow cytometry and in vivo PET imaging in our study. The selective binding ability of the probe may facilitate the differential diagnosis of inflammatory arterial diseases and AAA, therefore preventing false results in PET imaging of AAA.

Antibody-based PET tracers are generally not suitable for AAA imaging because of the hepatic clearance and relatively high intestine uptake, yielding a high background in abdominal area. However, due to its small size (50-55 kDa), TRC105-Fab was cleared mainly through renal pathway and uptaken minimally by intestines, as we documented with ex vivo biodistribution studies, providing a superior imaging contrast. TRC105-Fab also had a shorter blood half-life ($t_{1/2} = 2.1$ h; Fig. S5), in comparison with TRC105 ($t_{1/2} = 3.5$ h) (30). However, the blood half-life is still longer than that of small molecules, which possibly leads to inaccuracy of AAA uptake. Therefore, more comparative imaging experiments with different targeting probes and imaging modalities are needed in future studies to future confirm the potential of TRC105-Fab for PET imaging of AAA.

The limitation to current preliminary study is the experimental AAA model. Although murine model has biological features found in human AAA (28), it does involve aortic dissection as part of the AAA expansion process and may not be ideal to study AAA rupture. Moreover, murine AAA develops over weeks, rather than decades, so the model might not fully represent human AAA in terms of biology and progression (33). Prospective outcome studies are needed first with more clinically relevant animal models of AAA and eventually in patients. These trials will determine whether an increased PET signal, despite the limited spatial resolution of PET, can indeed predict aneurysm rupture.
CONCLUSION

In this study, we demonstrated an increased expression of CD105 in AAA and defined a novel, high specificity, high sensitivity probe for the in vivo PET imaging of AAA with TRC105-Fab. More importantly, we achieved an exquisite imaging contrast due to the low background in the abdominal area. The as-designed imaging probe might open new avenues into AAA research, which will eventually benefit the healthcare of whole AAA patient population.

DISCLOSURE STATEMENT

Charles P. Theuer is an employee of TRACON Pharmaceuticals, Inc. The other authors declare that they have no conflict of interest.

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FIGURE 1. Purification and characterization of TRC105-Fab. (A) Elution profile of TRC105-Fab from a Sephadex G-75 column (arrowhead: the single fraction used for further in vitro and in vivo studies). (B) SDS-PAGE of TRC0105 (left lane) and TRC105-Fab (right lane). (C) Flow cytometry analysis of FITC-TRC105-Fab and FITC-TRC105-Fab-NOTA (5 ug/mL) in HUVECs.
FIGURE 2. In vivo PET imaging on Day 5. Serial coronal and sagittal PET images were acquired at different time points after injection of $^{64}$Cu-NOTA-TRC105-Fab in AAA-induced mice, AAA-induced mice pre-injected with 2 mg TRC105 (blocking), and sham group without AAA induction on Day 5 post-induction.
FIGURE 3. In vivo PET imaging on Day 12. Serial coronal and sagittal PET images were acquired at different time points after injection of $^{64}$Cu-NOTA-TRC105-Fab in AAA-induced mice, AAA-induced mice pre-injected with 2 mg TRC105 (blocking), and sham group without AAA induction on Day 12 post-induction.
FIGURE 4. Region of interest (ROI) analysis of the PET images. (A) $^{64}$Cu-NOTA-TRC105-Fab uptake (%ID/g) in AAA, blocking, and sham groups, on Day 5 post-induction. (B) $^{64}$Cu-NOTA-TRC105-Fab uptake (%ID/g) in AAA, blocking, and sham groups, on Day 12 post-induction. (C) Comparison of aorta/muscle ratios in AAA, blocking and sham groups on Day 5 post-induction. (D) Comparison of aorta/muscle ratios in AAA, blocking and sham groups on Day 12 post-induction. The differences of the aorta uptake and aorta/muscle ratio in three groups were statistically significant ($P < 0.05$) at all time points ($n=3$) tested by one-way analysis of variance (ANOVA).
FIGURE 5. Biodistribution of $^{64}$Cu-NOTA-TRC105-Fab in AAA, blocking, and sham groups 24 h after injection on Day 12 post-induction (n = 3).
FIGURE 6. Ex vivo analysis. (A) Autoradiography of abdominal aorta from AAA, blocking, and sham groups with $^{64}$Cu-NOTA-TRC105-Fab. (B) Hematoxylin and eosin (H&E) staining of AAA and normal aorta. (C) Immunofluorescence histology of AAA samples collected from AAA group on Day 5 and Day 12 post-induction, and normal aorta collected from normal mouse. The red fluorescence indicates CD31 expression, and the green fluorescence indicates CD105 expression. The nuclei were counterstained with DAPI.