Molecular Imaging

Integrin-Targeted Molecular Imaging of Experimental Abdominal Aortic Aneurysms by $^{18}$F-labeled Arg-Gly-Asp Positron-Emission Tomography

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Background—Both inflammation and neoangiogenesis contribute to abdominal aortic aneurysm (AAA) disease. Arg-Gly-Asp–based molecular imaging has been shown to detect the integrin $\alpha_v\beta_3$. We studied a clinical dimeric $^{18}$F-labeled Arg-Gly-Asp positron-emission tomography (PET) agent ($^{18}$F-FPPRGD$_2$) for molecular imaging of experimental AAAs.

Methods and Results—Murine AAAs were induced in Apo-E–deficient mice by angiotensin II infusion, with monitoring of aortic diameter on ultrasound. AAA (n=10) and saline-infused control mice (n=7) were injected intravenously with $^{18}$F-FPPRGD$_2$, as well as an intravascular computed tomography contrast agent, then scanned using a small-animal PET/computed tomography scanner. Aortic uptake of $^{18}$F-FPPRGD$_2$ was quantified by percentage-injected dose per gram and target-to-background ratio. Focal increased PET signal was found in AAA lesions, but not in normal control aortae, confirmed by quantitative analysis (median percentage-injected dose per gram [interquartile range], 2.05 [1.05–2.85] versus 0.63 [0.43–0.83], $P=0.003$; median target-to-background ratio [interquartile range], 2.72 [2.31–3.49] versus 1.44 [1.10–1.52], $P=0.0008$). Ex vivo autoradiography demonstrated high uptake of $^{18}$F-FPPRGD$_2$ into the AAA wall, with immunohistochemistry showing substantial cluster of differentiation (CD)-11b$^+$ macrophages and CD-31$^+$ neovessels. Target-to-background ratio of AAAs on PET did not correlate with AAA diameter ($r=0.29$, $P=0.41$) but did strongly correlate with both mural macrophage density ($r=0.79$, $P=0.007$) and neovessel counts ($r=0.87$, $P=0.001$) on immunohistochemistry.

Conclusions—PET imaging of experimental AAAs using $^{18}$F-FPPRGD$_2$ detects biologically active disease, correlating to the degree of vascular inflammation and neoangiogenesis. This may provide a clinically translatable molecular imaging approach to characterize AAA biology to predict risk beyond size alone. (Circ Cardiovasc Imaging. 2013;6:950-956.)

Key Words: aneurysm • arginyl-glycyl-aspartic acid • macrophages • positron-emission tomography

Abdominal aortic aneurysm (AAA) disease is a common and highly lethal age–associated vascular degenerative disease, which affects 6% of men and 1% of women >60 years old. Characteristic pathological features present in both clinical and experimental aneurysms include marked elastin degradation and reduced medial vascular smooth muscle cells. Although much emphasis has been placed on medial smooth muscle cell loss and matrix degeneration during aneurysm pathogenesis, the contribution of vascular inflammation and angiogenesis to AAA progression increasingly seems significant. Macrophages play a key role in vascular inflammation, producing proteases such as matrix metalloproteinases that can degrade the extracellular matrix of the vessel wall, contributing to the development and rupture of AAAs. We and others have shown that vascular angiogenesis is present in animal models of AAA and is associated with AAA progression. Clinical studies also demonstrate that AAA tissue has increased mural neovascularization compared with nonaneurysmal aorta, and that rupture foci show increased neovascularity compared with intact aneurysm segments. Therefore, both vascular inflammation and angiogenesis have the potential to be major biological markers for targeted imaging and therapy of AAA disease.

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The integrin $\alpha_v\beta_3$, a cell surface glycoprotein receptor, has been well validated as an indicator of angiogenesis because of its upregulation on vascular endothelial cells in angiogenesis. It has also been shown that vascular macrophages express high levels of $\alpha_v\beta_3$. Arg-Gly-Asp (RGD) is an extensively studied short amino acid sequence binder to $\alpha_v\beta_3$. Radiolabeled RGD peptides have been developed for clinical $\alpha_v\beta_3$-targeted tumor imaging and therapy of AAA disease.
image using single photon emission computed tomography (PET), and positron-emission tomography (PET).\textsuperscript{11,12}

We have previously developed the dimeric RGD PET tracer, \(^{18}F\)-labeled Arg-Gly-Asp \((^{18}F\text{-FPFRGD}_2)\),\textsuperscript{14,16} which has enhanced target-tissue binding and reduced background signal for tumor imaging\textsuperscript{15} and is undergoing initial human testing.\textsuperscript{15,17} In the current study, we evaluated \(^{18}F\text{-FPFRGD}_2\) for \(\alpha\beta\)-targeted PET imaging of experimental AAA disease.

**Methods**

Radiotracer Preparation

Clinical-grade \(^{18}F\)-FPFRGD\(_2\) was synthesized as previously described.\textsuperscript{15,17,18} In brief, the dimeric RGD peptide precursor PEG 2-c (RGDyK)\(_2\) was purchased from Peptides International (Louisville, KY) and coupled to 4-nitrophenyl-2-\(^{18}F\)-fluoropropionate (\(^{18}F\)-NPE). No-carrier–added \(^{18}F\)-fluoride ion was prepared on a PET trace cyclotron (GE Medical Systems, Waukesha, WI) via the \(^{18}O(p,n){\ ^{18}}F\) nuclear reaction by irradiating \(^{18}O\)-enriched water (1.6 mL, >95\% isotopic enrichment, Rotem Industries Ltd, Beer Sheva, Israel) with 16 MeV protons. Crude radiolabeled products were purified and analyzed via reversed phase high-performance liquid chromatography.\textsuperscript{15,17,18} Quality control was performed based on the criteria set for human clinical use.

Experimental AAs

Murine AAs were induced in apolipoprotein E–deficient (apo-E \(-/-\)) mice, as described previously.\textsuperscript{1,6-19} A total of 17 adult mice, 17 to 20 weeks old, 31±1 g, were studied after continuous angiotensin II infusion via subcutaneous implanted osmotic minipumps (n=10) or after receiving minipumps loaded with saline (n=7). Transabdominal 40-MHz B-mode ultrasound imaging (Vevo 770 Imaging System and RMV 704 microvisualization scan head, Visualsonics Inc, Toronto, Canada) was performed to monitor aortic diameter in vivo (in the longitudinal and transverse scan plane) for up to 28 days, as previously described,\textsuperscript{1} with >25\% diameter increase from baseline (1.0–1.2 mm) defined as AAA.

The Administrative Panel on Laboratory Animal Care at Stanford University approved all animal procedures. All animals were anesthetized with inhaled 2\% isoflurane for surgical and imaging procedures and recovered with free access to food and water.

Small-Animal PET or Computed Tomography

At 21 to 28 days after minipump implantation, 10 AAA and 7 control mice were imaged with an Inveon small-animal PET/computed tomography (CT) scanner (Siemens, Knoxville, TN). A 5-minute prone PET acquisition scan followed by a 10-minute CT scan was performed without moving the animal \(\approx\)60 minutes after a tail vein injection of \(^{18}F\text{-FPFRGD}_2\) (174±3 \(\mu\)Ci \([6.4±0.1 \text{ MBq}])\), range 155–205 \(\mu\)Ci \([5.7–7.6 \text{ MBq}]) in 120 \(\mu\)L of PBS. To observe vascular CT contrast, 0.3 to 0.4 mL of the iodine-based intravascular contrast agent Fenestra (Advanced Research Technologies, Montreal, Canada) was injected. CT acquisition consisted of 120 projections acquired with an exposure time of 240 ms, an x-ray voltage of 80 kV, and an anode current of 500 \(\mu\)A.

PET images were reconstructed with the ordered-subsets expectation-maximization algorithm using 16 subsets and 4 iterations. No corrections were made for attenuation because an attenuation-corrected cylinder phantom study and an attenuation-correction scan performed with the body outline of a mouse using uniform attenuation both showed little change in the activity profile across the mouse.\textsuperscript{20} The resulting near-isotropic voxel size was 861×861×796 \(\mu\)m with a total of 128×128×159 voxels. CT images were reconstructed using a filtered back-projection algorithm, with an isotropic resolution of 200×200×200 \(\mu\)m.

The PET and CT images were fused and analyzed using the Inveon Research Workspace software (Siemens). Image registration was performed using automatic-weighted, mutual-information algorithm and confirmed visually on the basis of anatomic landmarks showing physiological accumulation of the tracer, such as kidneys and bladder. The angiotensin II–induced murine AAA lesions are typically suprarenal. Therefore, for measurement of tracer uptake, three-dimensional volumes of interest were drawn in the most aneurysmal segment of the suprarenal aorta as well as the noneuqymal remote aortic segment and blood pool (left ventricle), as seen on the CT images, avoiding adjacent organs. The mean activities were corrected for injected dose to calculate the percentage of injected dose per gram (%ID/g) and the target-to-background ratio (TBR) of aorta activity to blood pool. Analysis was also performed by a second observer to provide interobserver variability data.

Ex Vivo Autoradiography

The uptake of \(^{18}F\text{-FPFRGD}_2\), in the aortic wall was also studied by digital autoradiography of tissue sections in a subset of 10 mice (AAA, n=6; control, n=4). The aortae were removed \(\approx\)120 minutes after the tracer injection, frozen in optimum cutting temperature compound (Sakura Finetek USA, Inc, Torrance, CA), cut in sequential and transverse 20-\(\mu\)m sections at \(-20^\circ\text{C}\), and thaw-mounted onto microscope slides. For measurement of the accumulated \(^{18}F\) activity, the sections were exposed to \(^{18}F\)-sensitive storage phosphor screens (Perkin Elmer, Waltham, MA). After overnight exposure, the imaging plates were scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare Bio-Sciences, Piscataway, NJ).

The autoradiographic images were analyzed with Image J 1.43 software (National Institutes of Health). To quantify the accumulated \(^{18}F\) activity, mean signal intensity per pixel was measured in each section. Regions of interest were traced around the tissue sections of the most aneurysmal segment of suprarenal aorta as well as an unaffected normal infrarenal segment, which allowed normalizing the AAA value to the value of normal aorta.

Histology

The aortae were embedded and frozen in optimum cutting temperature compound, then cut in sequential and transverse 5-\(\mu\)m sections. Aortic tissue sections were stained with hematoxylin and eosin to study histological features corresponding to the autoradiographic images. For immunohistochemistry, the section of the most aneurysmal segment of suprarenal aorta in each mouse was fixed in acetone for 10 minutes, washed in PBS, and then incubated overnight at 4\(^\circ\text{C}\) with antinuiss small-cluster of differentiation (CD)-11b antibody to stain for macrophages (BD Biosciences, San Jose, CA) or antiniuss CD-31 antibody to stain for endothelial cells (BD Biosciences). Sections were then incubated for 30 minutes at room temperature with biotinylated secondary antibodies. Antigen-antibody conjugates were detected with avidin–biotin horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) according to manufacturer instructions. 3-amino-9-ethylcarbazole was used as chromogen. Sections were counterstained with hematoxylin.

ImageJ 1.43 software was used to quantify the total macrophage-rich (CD-11b\(^+\)) area per AAA section as a proportion of the medial and adventitial area (mural macrophage density). For mural neovascularization, transverse sections were divided into 4 quadrants, CD-31\(^+\)–stained neovessels in each quadrant were counted, and mean numbers of neovessels per section were reported. All sections were analyzed with original magnification 200\(\times\).

**Statistical Analysis**

Measured activities of PET and autoradiography were expressed as median value and interquartile range, and other results were expressed as mean±SEM. The Mann–Whitney test was used to compare continuous variables between AAA and control mice. The Wilcoxon signed-rank test was used to compare AAA versus noneuqymal remote segments. The Pearson correlation coefficient was used to calculate the correlation among \(^{18}F\) activity on PET (%ID/g, TBR), US-determined aortic diameter, and immunohistochemical measurements (mural macrophage density, neovessel count). Interobserver variability of measured PET activities was determined by calculating a 95\% confidence interval for mean differences. All analyses were performed

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using JMP 7.0.1 statistical software (SAS Institute Inc, Cary, NC). A probability value <0.05 was considered statistically significant.

Results

Radiochemistry
Clinical-grade \(^{18}\text{F}-\text{FPPRGD}_2\) was achieved within 3.5 hours with radiochemical yield of 16.9±2.7% (decay-corrected to end of bombardment) and specific radioactivity of 114±72 GBq/μmol (end of bombardment). Radiochemical purity was >99%, and chemical purity was >95%. All the other quality control results met the criteria for human clinical use.

In Vivo US and PET/CT With \(^{18}\text{F}-\text{FPPRGD}_2\)
On the US before administration of \(^{18}\text{F}-\text{FPPRGD}_2\), AAA diameter was 1.52 to 2.66 mm, with a mean of 2.09 mm. The suprarenal aortic diameter of control mice was 1.14±0.02 mm. There was no overlap in aortic diameter between AAA and control mice.

The PET images after administration of \(^{18}\text{F}-\text{FPPRGD}_2\) showed focal increased signal in the AAA, but not in normal control aorta (Figure 1A). Note also the uptake in the kidneys, consistent with the predominant renal clearance of \(^{18}\text{F}-\text{FPPRGD}_2\) (Figure 1A).\(^{15}\) Quantitative analysis confirmed significantly higher PET activities in AAAs than in control aortae (%ID/g, 2.05 [1.05–2.85] versus 0.63 [0.43–0.83], \(P=0.003\); TBR, 2.72 [2.31–3.49] versus 1.44 [1.10–1.52], \(P=0.0008\); Figure 1B). The nonaneurysmal remote aortic segments in AAA mice showed minimal PET activity (%ID/g, 0.004 [0.002–0.04], \(P=0.008\) versus AAAs; TBR, 0.008 [0.004–0.07], \(P=0.008\) versus AAAs). There was

Figure 1. In vivo \(^{18}\text{F}\)-labeled Arg-Gly-Asp (\(^{18}\text{F}-\text{FPPRGD}_2\)) positron-emission tomographic (PET) imaging of abdominal aortic aneurysm (AAA) and normal control aorta. A, AAA mouse with aortic diameter of 1.89 mm on ultrasound (US) showed focal increase in \(^{18}\text{F}-\text{FPPRGD}_2\) signal of the AAA lesion (yellow arrows), whereas control mouse with aortic diameter 1.09 mm on US showed no significant aortic signal. Both mice (CT: coronal) showed high signal from the kidneys (K) but low signal from liver (L), spleen (S), and remote aortic segments (A). Scale bars, 0.5 cm. A indicates anterior; L, left; P, posterior; and R, right (CT: transverse). B, By quantitative analysis, both the percentage-injected dose per gram (%ID/g) and target-to-background ratio (TBR) were significantly higher in AAAs than in normal control aortae. \(^*\)\(P=0.003\) versus control; \(^*\)\(P=0.0008\) versus control.
excellent interobserver agreement for both %ID/g (mean difference, −0.03 [95% confidence interval −0.24 to 0.17]) and TBR (mean difference, −0.07 [95% confidence interval −0.54 to 0.39]).

Additionally, we examined whether PET imaging activity simply corresponded to aortic diameter. When combining AAA and control mice, 18F-FPPRGD2 activity showed a positive moderate correlation with aortic diameter (%ID/g, r=0.52, P=0.03; TBR, r=0.56, P=0.02; Figure 2). However, within the AAA mice alone, there was no significant correlation between PET activity and AAA diameter (%ID/g, r=−0.18, P=0.62; TBR, r=−0.29, P=0.41; Figure 2). Note that between AAA and control mice, there was some overlap in %ID/g, but there was no overlap in TBR (Figure 2).

**Validation With Ex Vivo Autoradiography and Histology**

The ex vivo autoradiography showed high uptake of 18F-FPPRGD2 into the AAA wall, compared with minimal uptake into normal control aortae (Figure 3A). Quantitative analysis confirmed significantly higher signal in AAA sections than in control aorta sections (normalized mean signal intensity per pixel 2.32 [2.01−3.92] versus 1.14 [0.85−1.35], P=0.01; Figure 3B). Histological correlates of 18F-FPPRGD2 uptake were studied in serial tissue sections stained with hematoxylin and eosin and immunohistochemistry, as shown in Figure 3. Immunohistochemical staining with CD-11b and CD-31 demonstrated substantial macrophage infiltration and neovascularization colocalizing with high 18F-FPPRGD2 signal intensity area of the AAA wall (Figure 3C).

**Immunohistological Findings and Correlation to Aortic Diameter and PET Data**

CD-11b immunohistochemistry showed macrophage infiltration in the media and adventitia of the AAA lesion; CD-31 immunohistochemistry showed neovessel expression within the AAA wall (Figure 4). In contrast, there was minimal CD-11b or CD-31 positivity within the noneurysmal remote aortic wall of AAA mouse (Figure in the online-only Data Supplement). Based on the immunohistochemical quantification of AAA sections, there was a significant correlation between mural macrophage density and neovessel counts (r=0.68, P=0.03; Figure 5A).

Additionally, we examined whether anatomic AAA dilation and 18F-FPPRGD2 PET activity corresponded to the immunohistochemical markers of AAA biological activity. There was no significant correlation between US-determined AAA diameter and mural macrophage density (r=−0.08, P=0.83; Figure 5B) or neovessel counts (r=0.06, P=0.87; Figure 5C). In contrast, the TBR of 18F-FPPRGD2 activity...
showed a positive strong correlation with both mural macrophage density (\(r=0.79, P=0.007\); Figure 5D) and neovessel counts (\(r=0.87, P=0.001\); Figure 5E). Note that Figure 4 shows 2 examples of AAA mice representing different levels of anatomic AAA dilation, PET activity, and histological macrophage infiltration and neovessel expression (with corresponding data points highlighted in Figure 5).

**Discussion**

We demonstrate that PET imaging with the dimeric RGD tracer, \(^{18}\text{F}-\text{FPPRGD}_2\), can assess murine AAAs, with AAA PET activity correlating significantly to vascular inflammation and neoangiogenesis but not to AAA size. To the best of our knowledge, this is the first use of RGD-based PET imaging of AAA disease, and also the first application of \(^{18}\text{F}-\text{FPPRGD}_2\) to cardiovascular disease. Our results suggest that PET imaging using \(^{18}\text{F}-\text{FPPRGD}_2\) PET is a potential strategy for in vivo visualization of vascular inflammation and neoangiogenesis in AAAs, with the ultimate goal of detecting high-risk AAA disease more effectively than relying on AAA size alone to guide care.

RGD-based probes have been used previously for in vivo fluorescence, magnetic resonance, and PET imaging in animal models of atherosclerosis,\(^6\),\(^21\),\(^22\) which highlights the potential of molecular imaging techniques with the RGD peptide to assess vascular inflammation and angiogenesis. As for AAA disease, we have previously reported the targeting abilities of RGD-conjugated human ferritin nanoparticles toward macrophages and angiogenic endothelial cells in experimental AAA using fluorescence,\(^6\) but the penetration depth of fluorescence greatly limits clinical translation for deep structures. In contrast, PET is a highly sensitive noninvasive molecular imaging technique in humans, with many examples of clinical translation, including RGD-based agents.\(^16\),\(^23\)

A previous study by Nahrendorf et al\(^{24}\) used a nanoparticle-based PET agent targeted to macrophages and showed noninvasive imaging of inflammation in experimental AAAs. They also showed a modest correlation of PET activity with aortic size when AAA and control animals were combined, but no data on the correlation for AAA mice alone were shown. A key finding in our study was the lack of correlation between \(^{18}\text{F}-\text{FPPRGD}_2\) PET activity and aortic size within the AAA mice, in contrast to the positive and strong correlation of \(^{18}\text{F}-\text{FPPRGD}_2\) PET with both vascular inflammation (macrophage density) and neoangiogenesis (neovessel counts). These results indicate that size alone is not a reliable indicator of biological activity. Although vascular inflammation and neoangiogenesis likely contribute to AAA dilatation, the timing and interrelationship of these factors is not well understood. Serial monitoring of AAA biological activity and AAA growth/rupture would be ideal to understand this complex pathobiology.

The current clinical standard of care for AAA disease is to perform serial noninvasive imaging, predominantly using US, with surgical repair or stent grafting based on AAA size. However, this approach does not eliminate the risk of rupture and typically necessitates operation earlier than may be needed with more precise risk prediction. Thus, assessing AAA biological activity could identify high-risk patients and prompt earlier therapy to prevent rupture while also sparing low-risk patients from expensive and morbid procedures.

Recent clinical studies have reported on the potential of \(^{18}\text{F}-\text{fluorodeoxyglucose (FDG)}\) PET in AAA disease\(^\text{2},\text{26}\) because increased FDG uptake has been shown in vascular inflammation. However, in contrast to the nanoparticle-based PET imaging in experimental AAAs,\(^\text{24}\) the initial clinical study with \(^{18}\text{F}-\text{FDG}\) PET showed a negative correlation between PET activity and AAA growth.\(^\text{27}\) Although \(^{18}\text{F}-\text{FPPRGD}_2\) has
vascular macrophages and neovessels. RGD-based probes cannot distinguish inflammation from angiogenesis. However, both are indicators of AAA biological activity, so RGD offers an approach to detect both processes with a single probe. Clinical studies on the ability of 18F-FPPRGD to detect AAA and predict AAA growth are needed, including comparisons with 18F-FDG PET, particularly given the mixed clinical results of the latter.

In conclusion, PET imaging with the dimeric 18F-FPPRGD can detect experimental AAAs, with activity correlating to the degree of vascular inflammation and neoangiogenesis. 18F-FPPRGD PET has potential for clinical molecular imaging of AAA, to assess AAA biological activity and perform prospective trials on predicting AAA growth and outcomes beyond AAA size alone.

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References

Abdominal aortic aneurysm (AAA) disease is a common and highly lethal age–associated vascular degenerative disease, with both vascular inflammation and angiogenesis as major AAA biological markers. We have developed a clinical dimeric 18F-labeled Arg-Gly-Asp (RGD) positron-emission tomography (PET) agent ([18F]FPPRGD2) for enhanced targeting of the integrin αvβ3, which is expressed highly on both activated macrophages and angiogenic endothelial cells. In the current study, we performed PET/computed tomography of murine AAs and normal control aortae after injection of [18F]FPPRGD2, and found focal increased PET signal in AAs, but not in normal control aortae. Ex vivo autoradiography confirmed high uptake of [18F]FPPRGD2 into the AAs, with immunohistochemistry showing substantial macrophage infiltration and neovessel expression. A key finding in our study was the lack of correlation between [18F]FPPRGD2 PET imaging activity and AAA diameter, in contrast to the positive and strong correlation of 18F-FPPRGD2 PET with both vascular inflammation (macrophage density) and neangiogenesis (neovessel counts). Our results indicate that [18F]FPPRGD2 PET has the potential for clinical molecular imaging of AAs, to help guide care beyond AAA size alone. Clinical studies on the ability of [18F]FPPRGD2 PET to detect human AAA biological activity and predict AAA growth and outcomes are needed.
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SUPPLEMENTAL MATERIAL

Supplemental Figure

CD-11b (x200)          CD-31 (x200)
Supplemental Figure Legends

Supplemental figure. Immunohistochemistry of the non-aneurysmal remote aortic wall of abdominal aortic aneurysm (AAA) mouse

Immunohistochemistry demonstrated minimal macrophages (stained with CD-11b) and neovessels (stained with CD-31) in the media and adventitia of the non-aneurysmal remote aortic wall of AAA mouse. Note that CD-31 showed positive staining, as expected, in the endothelial cell lining of the vessel lumen.