MATERIAL AND METHODS

Fibrin-binding probe ^{64}Cu-FBP8

^{64}Cu-FBP8 was synthesized in quantitative yield (purity >99% by HPLC) as previously reported[1], with a specific activity of 6-12 GBq/μmol. Briefly, the cyclic disulfide peptide precursor FHCHypY(3-Cl)DLCHIL-PXD (Hyp=*L*-4-hydroxyproline, Y(3-Cl)=*L*-3-chlorotyrosine, PXD=*para*-xylenediamine) was conjugated with the chelator NODAGA (1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid), followed by labeling with $^{64}CuCl_2$. The peptide in ^{64}Cu-FBP8 comes from a family of peptides that have been widely studied in the past, and we previously showed that these peptides have high specificity for fibrin and the DD(E) fragment when compared to either fibrinogen or serum albumin, and similar affinity to human, rat, mouse, pig, and dog fibrin[2, 3]. ^{64}Cu-FBP8 has high affinity for the soluble fibrin fragment DD(E) (K_i = 430 nM) and binds to fibrin immobilized in a well plate. Moreover, ^{64}Cu-FBP8 is remarkably stable in blood after intravenous administration (>90% intact probe up to 4 hours post injection), and clears prevalently by the renal pathway (plasma half-life 14 min)[1].

Experimental model of arterial and venous thrombosis

All animal experiments were performed in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" and were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital. Adult male Sprague-Dawley rats (n = 32, 200-250 g, Charles River Laboratories) were anesthetized with isoflurane (4% induction, 2-2.5% maintenance, in medical air), and arterial and venous thrombosis was induced by using the ferric chloride model[4]. A small piece of filter paper was soaked for 1 min in a solution of ferric chloride (Sigma, 25% w/v, in sterile saline), and then applied for 5 min on the common carotid artery and the femoral vein of each animal to induce thrombosis. At the end of the procedure, the surgical site was rinsed with sterile saline to remove the excess of ferric chloride, and the formation of the clot was confirmed by visual inspection. Thrombus location (right vs. left) was kept undisclosed until the end of the study to assess in a blinded fashion the accuracy of ^{64}Cu-FBP8-PET imaging. Contralateral incisions were performed to create a sham surgical wound, hiding the actual location of the clot.

Probe administration

1, 3, or 7 days after thrombosis induction, rats were anesthetized with isoflurane (4% induction, 2-2.5% maintenance) and then positioned in a small-animal PET/SPECT/CT scanner (Triumph; TriFoil Imaging) equipped with respiratory monitoring, heating pad system and inhalation anesthesia. Each rat was injected via tail vein with ~10 MBq in a volume of ~300 μL, followed by saline flush. The injected dose was calculated by the difference of the radioactivity in the syringe before and after the administration, as measured by a dose calibrator (CRC-25PET, Capintec)[1, 5, 6].

Micro-PET/CT imaging

^{64}Cu-FBP8 was allowed to clear from the blood for 60 min before starting the PET acquisition. Since the field of view of the PET covered just 6-7 cm of the animal, we first imaged the hindlimbs (isocenter: femoral vein), followed by the neck (isocenter: carotid artery), with each scan lasting 60 min. At the end of each PET imaging session, a CT scan was performed over

4.27 min with 512 projections and 2 frames per projection (peak tube voltage, 70 kV; tube current, 177 mA). A polyethylene glycol-coated gold nanoparticle contrast agent (300 mg/Kg, i.v.) was injected before the CT scan for angiography[7]. This gold nanoparticle contrast agent was used as it is long-circulating and provides good contrast for both arterial and venous trees. PET and CT images were reconstructed using the LabPET software package (TriFoil Imaging). The PET data were corrected for decay, scatters, randoms and dead time, and reconstructed using a maximum-likelihood expectation-maximization algorithm run over 30 iterations. CT data were used to provide attenuation correction. Reconstructed data were quantitatively evaluated using AMIDE[8], by drawing volumes of interest (VOIs) on thrombosed and contralateral arteries and veins (4.2 mm^3), muscle (acromiotrapezius and calf, 65.4 mm^3), and bone (spinous process of cervical vertebrae and tibia, 4.2 mm^3). All VOIs were placed on standardized anatomical locations based on the CT-only image. However, since the thrombus location showed some variability among the animals, and the actual location of the clot was indistinguishable in CT images, VOIs were first placed on the vessel using CT-only images and then centered on the "hot spot" using fused PET/CT images, as we previously reported[1, 5]. PET data are expressed as percentage of injected dose per cubic centimeter (%ID/cc).

Whole-body PET/MR imaging

A small cohort of rats (n = 2) was scanned 1 day after thrombus induction in a clinical PET/MR scanner, as previously described[9, 10]. Rats were imaged for 60 min starting 1 hour after ^{64}Cu-FBP8 injection. PET data were acquired using the BrainPET, a MR compatible PET scanner that operates inside of a Magnetom TIM Trio 3T MRI (Siemens) and provides an axial field of view of ~20 cm, covering the whole rat body length. The list-mode emission data were rebinned in the sinogram space for fast reconstruction. The uncorrected PET volume was first reconstructed and binary segmented based on an empirically determined threshold in soft tissue and air. A uniform linear attenuation coefficient (0.096 cm^{-1}, corresponding to water at 511 keV) was assigned to all soft tissue voxels and the resulting attenuation map (combined with the coil attenuation map) was forward projected to derive the attenuation correction factors in sinogram space. The scatter sinogram is obtained using a fully 3D scatter calculation method based on the single scatter estimation method. First the normalization and attenuation corrected emission volume is reconstructed. Second, a scatter estimate is obtained by Monte Carlo simulations from this volume and the attenuation map. Third, this scatter estimate is scaled axially to fit the tails of the normalized true data which accounts for the out of field of view scatter. The normalization was calculated from a 64 hr scan of a plane-source rotated in the FOV. The images were reconstructed with the ordinary Poisson ordered subsets expectation maximization (OP-OSEM) algorithm using 16 subsets and 6 iterations. The reconstructed volume consists of 153 slices with 256×256 pixels (1.25×1.25×1.25 mm^3). The spatial resolution at the center of the field of view is approximately 2.5 mm. MR imaging was performed simultaneously to the PET acquisition. 3D gradient echo T1-weighted time-of-flight (TOF) MR angiography sequences were performed with an echo time of 3.86 ms, repetition time of 21 ms, flip angle of 18°, field of view = 9 × 18 cm, bandwidth = 178 Hz/pixel, 2 averages, and an acquisition time of 15.3 min. TOF data was reconstructed into 256 256 × 128 matrices with an in-plane pixel spacing of 0.703 × 0.703 mm^2, and a slice thickness of 0.7 mm.

Ex vivo studies

Animals were euthanized at the end of the imaging experiments, and tissues harvested and processed for biodistribution, autoradiography and histopathology.

Biodistribution

For biodistribution studies (n = 7/group), the radioactivity of the thrombosed carotid artery and femoral vein, the contralateral vessels, muscle and bone (calf and tibia, obtained from the contralateral paw) were quantified with a gamma-counter (Wizard^2, PerkinElmer) to determine the percentage of injected dose per gram of tissue (%ID/g)[1, 5, 6].

Autoradiography

Ipsilateral and contralateral carotid arteries and femoral veins were further analyzed by autoradiography (n = 5/group) using a multipurpose film and phosphor imaging system (Cyclone Plus, PerkinElmer). A subset of vessels was fresh-frozen, cryosectioned and then exposed on a multipurpose film for 4 hours. Adjacent slices were stained with Hematoxylin and Eosin to detect the intraluminal thrombus. Regions of interest were drawn around thrombosed and contralateral vessels (OptiQuant, PerkinElmer), and ipsi:contra activity ratios were obtained by dividing matched ipsilateral and contralateral raw values from each animal[1, 5, 6].

Histopathology

Histopathology (n = 5/group) was performed to evaluate thrombus morphology and composition. Preliminary experiments showed that the morphology of the vessels was not preserved after autoradiography. Therefore, 2 out of 7 animals per group were randomly assigned to the histopathological analysis, and 3 additional rats were added to each group only for histology. Thrombus size and composition were comparable between the animals that were imaged and those that were not. Arteries and veins were rinsed in phosphate buffer, fixed in neutral buffered formalin, embedded in optimal cutting temperature mounting medium (OCT, Tissue-Tek), and then snap-frozen. Vessels were cryosectioned to sample the entire length of the thrombus, and stained using the trichrome method Martius Scarlet Blue (MSB) which allows to differentiate between erythrocytes (yellow), fibrin (purple-red) and collagen (blue)[11]. Color segmentation was performed using the ImageJ software (NIH), and the sum of the areas occupied by fibrin was integrated with the interval between adjacent sections (500 µm) to obtain the total volume (mm^3) [12-14]. This method has shown high correlation with fibrin quantification obtained with western blot analysis[12-14] and, differently from western blot, provides volumetric information about the thrombus size. To confirm the specificity of MSB to detect fibrin, adjacent slices were stained with H&E, MSB and an anti-fibrin antibody (Abcam, U45, 10 µg/mL)[5]. Negative control experiments were performed by omitting the primary antibody. Images were acquired using a microscope equipped with epifluorescence illumination (TE-2000, Nikon).

Statistics

Data were expressed as mean ± SEM. Differences between groups were analyzed using 1-way ANOVA followed by Tukey post-hoc test. The Pearson correlation coefficient was computed to assess quality of linear correlations and a t-statistic was calculated based on the null hypothesis that the correlation coefficient was zero. A p-value <0.05 was considered significant.

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