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Molecular MRI of Thrombosis

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Abstract

This review focuses on recent approaches in using targeted MRI probes for noninvasive molecular imaging of thrombosis. Probe design strategies are discussed: choice of molecular target; nanoparticle versus small-molecule probe; and gadolinium versus iron oxide imaging reporter. Examples of these different design strategies are chosen from the recent literature. Novel contrast agents used to image direct and indirect binding to fibrin have been described as well as direct binding to activated platelets. Emphasis is placed on probes where utility has been demonstrated in animal models or in human clinical trials.

Keywords

Thrombus; MRI; molecular imaging; fibrin; factor XIII; activated platelets; gadolinium; iron oxide

Introduction

Thromboembolic diseases such as myocardial infarction, stroke, and pulmonary embolism (PE) are major causes of morbidity and mortality worldwide [1]. Imaging is forefront in identifying thrombus. Currently, thrombus imaging relies on different modalities depending on the vascular territory. Carotid ultrasound is used to search for carotid thrombus, transesophageal echocardiography (TEE) searches for cardiac chamber clot, ultrasound searches for deep vein thrombosis (DVT), and CT has become the gold standard for PE detection. Despite the success of these techniques, there continues to be a strong push for a molecular imaging solution for thrombus detection and monitoring.

Molecular imaging of thrombosis has several motivations. First, there are certain vascular territories that are underserved. For instance, despite best imaging efforts some 30% to 40% of ischemic strokes are “cryptogenic,” that is, of indefinite cause, or in other words, the source of the thromboembolism is never identified [2]. Underlying sources of cryptogenic stroke include atherosclerosis in the aortic arch [3], intracranial arteries (eg, moderate stenosis) [4], or vertebral artery stenosis thought to be unrelated to the stroke [5]; or patent foramen ovale, which allows venous thrombus to embolize to the brain [6]. Plaque rupture in the arch or other major vessels, in particular, is thought to be a major source of cryptogenic strokes [7–15] but can be difficult to detect with routine methods.

There is also the added molecular specificity. Current clinical imaging methods detect the thrombus indirectly. For instance, in the ascending aorta, in the absence of mobile thrombus,

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conventional imaging identifies gross vessel wall thickening and cannot distinguish stable atherosclerotic plaque from plaque with associated thrombus. Recent TEE clinical trial data showed that the presence of thickened vessel wall in the aortic arch was not predictive of ischemic stroke [16,17], although *ulcerated* aortic arch plaques are associated with cryptogenic stroke [7]. A thrombus-targeted molecular imaging approach could potentially identify clot in the presence of atherosclerotic plaque. Depending on the choice of imaging target, it may be possible to distinguish active, forming clots from older constituted thrombi. By targeting activated platelets or enzymes upregulated in the clotting cascade (eg, thrombin, factor XIII), it may be possible to visualize fresh thrombus.

Finally, there is a desire for a “one stop shopping” approach where a single modality could be used to identify thrombus throughout the body. For instance, in stroke follow-up, multiple examinations are required to search for the source of the embolus.

Molecular Imaging

Thrombus molecular imaging dates to the 1970s with gamma scintigraphy approaches: technetium-99m-labeled fibrinogen and iodine-131-labeled anti-fibrin antibody [18,19]. The various nuclear medicine approaches have failed to gain clinical adoption due to low clot:blood background, unfavorable pharmacokinetics, and ultimately low sensitivity and specificity. However, the different targeting approaches used by the nuclear medicine researchers continue to be applied to other modalities.

Compared to other modalities, molecular MRI has great potential for thrombus characterization. There is no ionizing radiation like nuclear or CT methods; there is deep tissue penetration unlike ultrasound or optical methods; spatial resolution is much higher (sub-millimeter) than nuclear imaging. Moreover, the molecular information from a contrast agent can be overlaid onto the inherent anatomical image to provide context. The different image weightings in MR can also provide information on the composition of other components in complex plaque [20-22]. The drawback of MR is the lower sensitivity of contrast agent detection compared to nuclear techniques. As a result, choice of target in the design of the molecular probe is paramount.

Probe Design Considerations

The two most robust choices for changing the MR signal are metal-based T1 or T2-weighted imaging agents. T1-agents are gadolinium (and occasionally manganese) based and provide positive image contrast (increased signal). T2 agents are typically based on iron oxide nanoparticles and give negative image contrast (destroy signal). Bright spot imaging is preferred, but a benefit of iron oxides is their increased sensitivity and in many instances the target concentration is too low for effective imaging with a gadolinium-based agent. A second consideration is whether to use a discrete molecule or to use a nanoparticle. Relative to small molecules, the benefits of nanoparticles include a high payload of contrast-enhancing ions, the ability to use multiple targeting vectors to improve affinity, and ease of conjugation of fluorophores to make a bimodal probe. The latter can be very useful to correlate probe distribution with target distribution at the microscopic level using immunohistochemical and optical imaging methods. Drawbacks of nanoparticles can include slow targeting kinetics, slow blood clearance, and poor penetration into the target (slow diffusion).

MR agents are characterized by their relaxivity (r_1). This is the ability of an agent to increase the relaxation rate (R_1 or R_2) of tissue water normalized to the concentration of the metal ion ($r_1 = \Delta R_1/[Gd \text{ or } Fe]$) and has units of $s^{-1}mM^{-1}$. For targeted agents at 1.5 T, r_1 is in the 5 to 30 $s^{-1}mM^{-1}$ range for Gd-based probes. The T1 of thrombus can vary but in the

absence of met-hemoglobin is on the order of 1 second ($R1 = 1 \text{ s}^{-1}$). For a 10% change in $R1$, one requires gadolinium on the order of 3 to 20 μM . For strong signal enhancement where the thrombus is as bright as fat on T1-weighted imaging ($\Delta R1 = 2 \text{ s}^{-1}$), then the gadolinium requirements increase to 70 to 400 μM . In order to achieve such signal enhancement, one needs to bind to a target present at similar high micromolar concentrations, use a probe with very high relaxivity, and/or use a probe with multiple copies of gadolinium (or iron) per probe.

When a blood vessel is damaged or an atherosclerotic plaque ruptures, prothrombotic factors come in contact with the blood, platelets begin aggregating, and the coagulation cascade is activated. The enzyme thrombin catalyzes the cleavage of short fibrinopeptides from circulating fibrinogen and results in the polymerization of fibrin monomers. The growing fibrin polymer is stabilized by factor XIII, which crosslinks the fibrin strands. The product of this cascade of enzymatic reactions is a fibrin mesh, which aids in stopping blood flow. All of the players in the clotting cascade represent potential targets for detecting thrombi. From a molecular imaging perspective, two general approaches have been taken (Fig. 1). One is to focus on direct binding to fibrin or activated platelets. The second is to create a signal amplification strategy by having the imaging probe be a substrate for an enzyme like factor XIII.

Fibrin as a Target

Fibrin is a good target for imaging both forming and constituted clots. It is only present in thrombi and not in circulating blood, suggesting high specificity for thrombus imaging. Fibrin is also found in all thrombi—venous and arterial, fresh and aged thrombi in high concentrations—suggesting high sensitivity for thrombus imaging. In a recent study, fibrin was detected in all thrombi retrieved from acute stroke patients [23]. Circulating fibrinogen is about 7 μM (2.5 g/L), so a clot would have a minimum of 7 μM fibrin monomer. Fibrin concentrations in clots are likely much higher since the clot contracts and fresh fibrinogen is delivered from in-flowing blood. Based on probe binding to excised chronic human thrombi, fibrin concentrations are likely in excess of 30 μM [24,25].

Small molecules targeting fibrin

Botnar et al. [26] reported EP-1873, a short fibrin-specific peptide derivatized with four Gd-DTPA units and demonstrated the selective enhancement of ruptured atherosclerotic plaques in a rabbit model. An improved version of this probe, EP-2104R, replaced the Gd-DTPA groups with much more stable Gd-DOTA chelates for MR signal enhancement. EP-2104R binds to two equivalent sites on fibrin ($K_d = 1.7 \mu\text{M}$) with high specificity for fibrin over fibrinogen (over 100 times) and for fibrin over serum albumin (over 1000 times) [27]. When bound to fibrin, the relaxivity of EP-2104R at 37° C and 1.4 T is $71.4 \text{ mM}^{-1}\text{s}^{-1}$ per molecule of EP-2104R ($17.4 \text{ mM}^{-1}\text{s}^{-1}$ per molecule of Gd), which is about 25 times higher as compared to Gd-DOTA measured under the same conditions [27]. After extensive evaluation in arterial [25,28,29], venous [30,31], and chamber clot models employing both fresh [28,30,32] and aged [24,25,31,33] thrombi, EP-2104R advanced to human clinical trials.

In a phase 2 feasibility study, EP-2104R was administered to 52 patients with known or suspected thrombus in the deep veins of the legs, pulmonary arteries, carotid arteries, aortic arch, left ventricle, or the atria of the heart [34••]. The compound was well tolerated and there were no serious adverse events considered related to the study drug. Patients were either imaged immediately and for as long as 1 hour after EP-2104R injection ($n = 16$) or imaged 2 to 6 hours after injection ($n = 36$). It was noted that thrombi were more conspicuous at the later time points as the blood background signal cleared. EP-2104R

appeared more effective in enhancing arterial and chamber clots (32/38 or 84% sensitivity) than thrombi in the venous circulation (4/14, 29% sensitivity). However, the overall sample size is small. Thirty-one of the patients were imaged again the next day (20–36 hours post-injection) and the thrombi remained enhanced. Thrombus:muscle signal intensity ratios were enhanced twofold ($P < 0.01$) at either 2–6 hour or 20–36 hour post-injection compared to the signal intensity ratio prior to EP-2104R injection.

An example of EP-2104R enhanced thrombus imaging is shown in Figure 2. Inversion recovery black blood gradient echo imaging was performed to provide T1 weighting with the blood signal suppressed [35•]. Two adjacent slices showing clear enhancement of left ventricular thrombus are shown.

Recently, Uppal et al. [36•] showed that EP-2104R could be used in small animal models and at high fields. The contrast agent was evaluated in a rat embolic stroke model and occlusive embolic thrombus was readily apparent with high contrast-to-noise ratios [36•]. The animal model involves delivering a 24-hour aged thrombus via catheter through the carotid artery to the level of the middle cerebral artery (MCA). Interestingly, the maximum-intensity projection image following EP-2104R injection showed a line of enhancement extending along the craniocaudal axis (Fig. 3C). Inspection of the source images showed that this was partially due to enhancement of the occlusive thrombus (Fig. 3D–3F). More caudal was vessel wall enhancement that extended along the right internal carotid (Fig. 3A, 3B). Endothelial damage induced by catheter delivery of the aged clot likely caused fresh mural thrombus (confirmed by histology) that then enhanced with EP-2104R.

Nanoparticles targeting fibrin

Lanza, Wickline, and coworkers have worked with nanoparticles targeted to fibrin via an anti-fibrin antibody [37,38]. Recently they have extended this work to use manganese-based MR reporters, either Mn(II) oxide or Mn(II) oleate encapsulated by a phospholipid shell or with Mn(III)-porphyrin encapsulated in an inverted micelle [39,40]. Mn-nanoparticles were targeted to fibrin via a fibrin-specific monoclonal antibody coupled using classic biotin-avidin interactions. In both cases, T1-weighted MR images of in vitro clots showed a significant contrast enhancement with the fibrin-targeted manganese nanoparticles and no contrast improvement was seen from control nanoparticles. The efficacy of these contrast agents in vivo has yet to be determined.

Factor XIII as a Target

The transglutaminase factor XIII is responsible for cross-linking fibrin to stabilize thrombi. In addition to crosslinking fibrin polymers, activated factor XIII (FXIIIa) also covalently cross-links α_2 -antiplasmin (α_2 AP) to fibrin during the early stages of thrombus formation. The presence of α_2 AP, a serine protease inhibitor, suppresses fibrinolysis by inactivating plasmin, the enzyme responsible for degrading fibrin. Because factor XIII is present at very low concentrations, it is difficult to image using direct targeting. For imaging, various groups have relied on its enzymatic activity as a signal amplification mechanism. One approach is to include a substrate based on α_2 AP into the contrast agent. Factor XIII then covalently links the contrast agent to fibrin. This nanomolar enzyme can amplify the MR signal by linking many multiples of contrast agent to fibrin, present at micromolar levels. However, the ability of FXIIIa to incorporate α_2 AP into clots is limited to the stage of forming thrombus. Therefore, FXIII and α_2 AP may be targets for diagnosing early thrombus formation.

Gd-based probes targeting factor XIII

Miserus et al. [41•] reported a bimodal contrast agent containing Gd-DTPA and rhodamine attached to an α_2 AP-based peptide (GNQEQVSPLTLL) sequence. Two-photon laser-scanning microscopy demonstrated that this α_2 AP contrast agent binds to fibrin in vitro. Immunohistochemistry was used to analyze fresh (< 1 day), lytic (1–5 days), and organized (> 5 days) human pulmonary thromboemboli for the presence of factor XIII and α_2 AP. Staining results showed significantly more α_2 AP in fresh thrombi than lytic and organized thrombi, and FXIII was present at higher levels in fresh and lytic thrombi than in organized thrombi. Figure 4 shows in vivo MR images and corresponding contrast-to-noise ratios (CNRs) of fresh and 24 to 48-hour-old thrombi post-injection of bimodal α_2 -antiplasmin-based contrast agent (Bi- α_2 AP-CA) or bimodal control contrast agent (Bi-con-CA) in a mouse model [41•]. Immediately after FeCl₃-induced thrombus formation, administration of Bi- α_2 AP-CA causes a hyperintense magnetic signal at the site of the clot (Fig. 4A). No hyperintense signal was observed when Bi-con-CA was used to visualize a fresh clot (Fig. 4B), and no signal enhancement was observed when Bi- α_2 AP-CA was used for 24 to 48-hour-old thrombus visualization. Figure 4D shows that CNRs are highest in fresh thrombi after administration of Bi- α_2 AP-CA as compared to Bi-con-CA or when Bi- α_2 AP-CA is administered to mice with 24 to 48-hour-old thrombi.

Nanoparticles targeting factor XIII

McCarthy and coworkers [42•] targeted FXIII and fibrin by conjugating peptide-targeted ligands to the surface of fluorescently labeled cross-linked iron oxide (CLIO) nanoparticles. The nanoparticles were functionalized with α_2 AP-based peptide (GNQEQVSPLTLKLC) to target FXIII or with a fibrin(ogen) targeting peptide (GPRPPGGSKGC), and further fluorescently labeled with dyes VT680 and Cy7, respectively, to allow detection by both MR and optical imaging modalities. The utilization of fluorescent dyes with distinct excitation and emission enables simultaneous fluorescence imaging of FXIII and fibrin via multichannel fluorescence imaging approaches. Near-infrared fluorescence and MRI demonstrate that the nanoparticles bind to clots in vitro. These nanoparticles were tested in vivo in a FeCl₃-induced thrombosis mouse model, and ex vivo analysis showed that both nanoagents accumulate within vascular thrombi as compared to analogously synthesized control agents [42•].

Targeting Activated Platelets

Platelet activation is a highly regulated process and is critical for maintaining normal homeostasis. Injury to endothelial cells such as the rupture or erosion of atherosclerotic plaques exposes matrix proteins, which induces platelets to adhere to the vessel wall. Platelets then spread and become activated. A key feature of activated platelets is the externalization of integrin $\alpha_{IIb}\beta_{IIIa}$ (also referred to as GP2B3A). Its change in conformation upon platelet activation makes it a suitable target for imaging.

Gd-based probes targeting activated platelets

Klink et al. [43] reported a contrast agent termed P975, composed of a cyclic peptide (cyclo[CRGDC] targeted to the $\alpha_{IIb}\beta_{IIIa}$ receptor) conjugated to Gd-DOTA to visualize activated platelets. P975 showed a K_i of 1.5 μ M for binding to activated platelets, and its relaxivity at 1.4 T, 40° C was 9 mM⁻¹s⁻¹. In an arachidonic acid-induced arterial thrombosis model in mice, P975 showed sustained enhancement of the thrombus compared to Gd-DOTA control. This is quite remarkable given the relatively low affinity of P975 for the receptor and its relatively low relaxivity, and implies an extremely high concentration (10s of μ M) of the $\alpha_{IIb}\beta_{IIIa}$ in the thrombus or a considerable nonspecific component to the thrombus enhancement.

Nanoparticles targeting activated platelets

von zur Muhlen et al. [44••] employed microparticles of iron oxide (MPIO) attached to a single chain antibody to selectively bind to ligand-induced binding sites (LIBS) on activated platelet integrin $\alpha_{IIb}\beta_{IIIa}$ to image carotid thrombi using a mouse model of carotid thrombosis. Figure 5 shows in vivo MR images of an injured right carotid artery pre and post-contrast agent injection. Figure 5A shows the vessel prior to injection, with a significant and progressive signal void 12 and 48 minutes after LIBS MPIO injection (Fig. 5B). After 48 minutes, thrombolysis was initiated with human or mouse urokinase restoring the vessel signal (Fig. 5C). Immunohistochemistry confirmed the reduction in size of thrombus after thrombolysis. In addition, LIBS MPIO were investigated for ex vivo binding to symptomatic human endarterectomy specimens. In that study, a LIBS MPIO-induced signal void reported reliably on platelet adhesion/aggregation.

Conclusions

Molecular MRI of thrombosis is slowly becoming reality. Several probes have been synthesized and tested that interrogate different aspects of thrombus. There is no one ideal strategy for molecular MR probes. The choices of small molecule versus nanoparticle, and gadolinium versus iron oxide, will largely depend on the molecular target and the application. Preclinically, there are probes available that can identify both forming and constituted thrombus by targeting fibrin, factor XIII, and activated platelets. The clinical trial data with the fibrin-specific probe EP-2104R show that molecular MR of thrombosis is feasible in patients as well.

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- 44 ••. von zur Muhlen C, von Elverfeldt D, Moeller JA, et al. Magnetic resonance imaging contrast agent targeted toward activated platelets allows in vivo detection of thrombosis and monitoring of thrombolysis. *Circulation* 2008;118:258–267. [PubMed: 18574047] This is an antibody–iron oxide approach to imaging activated platelets. Binding to activated platelets was demonstrated ex vivo to the surface of symptomatic human carotid plaques and in mouse models of thrombosis and thrombolysis.

Figure 1.

Schematic representation of the clotting cascade. Two pathways, contact activation (intrinsic) and tissue factor (extrinsic), which lead to thrombus formation are shown. Inactive precursors, represented in *green*, become activated coagulation factors, represented in *blue*, and catalyze the next reaction in the cascade, ultimately leading to cross-linked fibrin and thrombus formation. The targets for detecting thrombi discussed in this review are highlighted in *yellow*.

Figure 2. Molecular MRI of thrombus in the left ventricle of an 80-year-old man pre and post-EP-2104 injection. Two adjacent slices (1 and 2) from a three-dimensional dataset using an inversion recovery black-blood gradient-echo sequence are shown. The high local signal amplification allows for definitive localization of thrombus in the left ventricle (*arrow*). Note there is a slightly different view with respect to the main cardiac axis in the two MR sessions (pre-contrast and post-EP-2104R). LV—left ventricle; RV—right ventricle. (*From Spuentrup et al. [35••]; with permission.*)

Figure 3.

Occlusive aged thrombus and vessel wall enhancement (fresh thrombus) using EP-2104R-enhanced MRI in a rat embolic stroke model. Center panel (C) coronal maximum-intensity projection showing a region of enhancement extending the length of the right carotid artery to the MCA origin depicted by *arrowheads*. Left panel: (A) Source image and (B) enlargement of boxed area in (A) anterior to the carotid bifurcation showing vessel wall enhancement, likely mural thrombus (*arrow*), in the right internal carotid artery and enhanced clotted side branches (*arrowhead*) while contralateral carotid (*arrowhead*) shows no vessel wall enhancement. Right panel: (D) source image and (E) enlargement of boxed area in (D) at level of MCA origin revealing the presence of an occlusive thrombus; (F) axial reformat demonstrating occlusive thrombus (*arrow*) and patent contralateral artery (*arrowhead*). (From Uppal et al. [36•]; with permission.)

Figure 4.

A–C, Molecular MRI of fresh (*Panel A*) and 24 to 48-hour-old thrombi (*Panel C*) in murine carotid arteries after injection of bimodal α_2 -antiplasmin–based contrast agent (Bi- α_2 AP-CA) or bimodal control contrast agent (Bi-con-CA, *Panel B*). The *boxes* at the *lower left* of the images are expansions around the carotid, and the *arrows* denote the location of the thrombus. *Panel D* shows the corresponding contrast-to-noise ratios (CNRs), which demonstrate a significant enhancement of the early thrombus with the targeted probe. (From Miserus et al. [41•]; with permission.)

Figure 5.

MRIs of an injured right carotid artery pre and post-injection of LIB MPIO in a mouse model of carotid thrombosis. *Panel A* shows the vessel pre-injection, which appears bright in this image. *Panel B* shows a time course after injection of the platelet-targeted iron oxide probe, which results in a signal decrease between 12 and 48 minutes. *Panel C* shows the effect of treatment with intravenous mouse urokinase beginning after the 48-minute scan and shows the reappearance of the vessel lumen signal over time. (From von zur Muhlen et al. [44••]; with permission.)