Chronic Thrombus Detection With In Vivo Magnetic Resonance Imaging and a Fibrin-Targeted Contrast Agent

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Background—Arterial thrombosis plays a critical role in acute coronary syndromes and stroke. Therefore, the ability to detect thrombus in vivo has a significant clinical implication. Magnetic resonance imaging (MRI) has shown promise in noninvasive thrombus detection. However, thrombus characterization and age definition remain difficult. We sought to evaluate the use of a fibrin-targeted peptide (EP-2104R) for MR thrombus detection and to compare this modality with non–contrast-enhanced (NCE) MRI and with Gd-DTPA injection at various ages and time points after thrombus generation.

Methods and Results—Carotid artery thrombosis was induced by external injury and stasis in 18 rabbits. T1-weighted MRI was performed before and after contrast agent injection, within 6 hours of thrombus induction, at 48 hours, at 1 week, and every week up to 8 weeks after injury. Correlation with histopathology was performed. The fibrin-targeted contrast agent accurately detected all thrombi, regardless of their size, location, and age. Although thrombus signal intensity after injection decreased with thrombus age (P<0.001), enhancement at 8 weeks was still present. Gd-DTPA injection was not associated with an improvement of thrombus detection. EP-2104R was superior to both NCE and Gd-DTPA injection (P<0.001). Histopathologic examination showed thrombus organization over time. Fibrin was gradually replaced by fibrous tissue. A strong correlation was found between thrombus enhancement and collagen content of the organizing thrombus with time (R=−0.89; P<0.001).

Conclusions—In an experimental animal model of carotid thrombosis, we have demonstrated the superiority of a fibrin-targeted MR contrast agent for in vivo detection of chronic or organized thrombus, compared with NCE MRI and Gd-DTPA injection. (Circulation. 2005;112:1594-1600.)

Key Words: atherosclerosis ■ contrast media ■ fibrin ■ magnetic resonance imaging ■ thrombosis

Thrombus formation after erosion or rupture of atherosclerotic plaque is critical to the onset of acute coronary syndromes and stroke. Platelets and fibrin are the major components of all thrombi but may also be involved in the development and progression of atherosclerotic disease.1 Recent studies suggest that plaque rupture and microthrombus formation precede acute myocardial infarction by days to months,2 providing an opportunity to intervene and prevent the clinical manifestations associated with plaque rupture. Early detection of thrombus formation and age definition may be clinically helpful for both diagnosis and therapy. This may improve our ability to stratify the risk of potential clinical complications.

Although various imaging modalities, either invasive3 or noninvasive,4 have been used for detection of high-risk or vulnerable plaques, magnetic resonance imaging (MRI) has emerged as one of the most promising noninvasive imaging techniques for in vivo detection and characterization of atherosclerotic lesions.5–7 Non–contrast-enhanced (NCE) MRI has been reported to identify thrombi in vitro8 and in vivo in the aorta9 and in carotid arteries.10,11 Contrast agents such as conventional gadolinium (Gd) chelates have been shown to improve plaque characterization by enhancing the MR signal.12,13 Moreover, the recent development of novel, targeted MR contrast agents has led to molecular imaging of specific atherosclerotic plaque components.14 Paramagnetic and superparamagnetic MR contrast agents targeted to platelets, angiogenesis markers, or fibrin have been used successfully for thrombus imaging.15–20 Nevertheless, complete thrombus characterization with MRI remains difficult,21 and accurate age definition and chronic thrombus detection have not been possible so far.

We sought to evaluate the use of a fibrin-targeted peptide and compare it with NCE MRI and conventional Gd chelate...
injection (Gd-DTPA) for detection of occlusive and nonocclusive thrombi of different size, age, and time points after their generation in an experimental animal model of acute to chronic carotid artery thrombosis.

Methods

Animal Protocol

New Zealand White rabbits maintained on a normal diet (n=18; age 3 to 4 months; 3.5 to 4 kg body weight; Covance, Princeton, NJ) were used for this study. Arterial thrombosis was induced by carotid artery crush injury and blood stasis. The left common carotid artery was exposed after midline neck incision, under anesthesia (35 mg/kg IM ketamine and 7 mg/kg IM xylazine). A deep external crush injury was performed on the carotid artery by using a circular hemostatic clamp capable of distributing a homogeneous circumferential pressure on the vessel wall (7-62 Lahey hemostatic forceps, curved; Miltex Instrument Co) as previously described.22,23 Injury was performed 1 cm below the carotid bifurcation with a variable length of 0.5 to 1.5 cm in a random manner. Approximately 3 minutes after repeated serial vessel damage, flow was reestablished. Then a stepwise stenosis was produced distally by constricting the artery with a 4-0 silk suture to reduce the flow by 40% (stenotic flow). Injury was always performed with the same hemostat and by the same operator (M.S.) to minimize variability. The Mount Sinai School of Medicine Institute Animal Care and Use Committee approved all experiments.

Contrast Agent

EP-2104R (Epix Pharmaceuticals) is a Gd-based contrast agent. EP-2104R is a small molecule that selectively and reversibly binds to fibrin. No interactions and competitive binding with other proteins such as fibrinogen or collagen have been noted in both in vitro and in vivo experiments (authors’ unpublished data). An earlier but very similar generation of the compound has been recently described in detail.20

Magnetic Resonance Imaging

Rabbits were sedated with ketamine/xylazine (as described earlier) and imaged in a supine position with a 1.5-T MRI system (Siemens). The injured artery and the contralateral normal carotid artery were imaged shortly after thrombus induction (≤6 hours), at 48 hours, and at weeks 1, 2, 3, 4, 6, and 8 after thrombus induction (Figure 1). After an initial rapid localization of the carotid arteries, a 3-dimensional (3D), time-of-flight MR angiogram was obtained from the aortic arch to the carotid bifurcation. Imaging parameters were as follows: repetition time/echo time/time of excitations =255/5/1 ms, and flip angle =20°. Five contiguous slabs, each containing 16 slices per slab, were imaged. Slice thickness was 0.8 mm. A field-of-view of 200 mm and matrix of 256×256 were used. The angiographic images were then reconstructed with a maximum-intensity projection (MIP) algorithm. Using the carotid bifurcation as an anatomic landmark, we obtained contiguous cross-sectional images perpendicular to the long axis of the neck with T1-weighted (T1W) and T2-weighted (T2W) double-inversion recovery, 22D turbo-spin echo sequences. The T1W and T2W images were acquired with a repetition time and echo time of 400/7 ms and 2300/62 ms, respectively. MRI parameters included a field of view of 8×8 cm; acquisition matrix of 256×256; 4 signal average; a receiver bandwidth of ±42 kHz; turbo factor of 16; and a slice thickness of 2 mm. No interslice gap was used. The inversion time after the double-inversion recovery pulses was 350 ms. In addition, a chemical shift–selective, fat-suppression pulse was used. A 2D phase-contrast MRI was then used to differentiate slow and absent flow from normal flow to confirm vessel patency. Two different expert observers examined the images to confirm absence of flow. Complementary imaging was performed, when necessary, to eliminate slow-flow artifacts by using a T1W 2D segmented gradient-echo sequence with a combination of inversion recovery and a diffusion-based, flow-suppression prepulse, as previously reported by our group.24

Images were acquired immediately before contrast agent administration (Figure 1) and 30 minutes after bolus injection (5 μmol/kg IV), based on preliminary data.23,25 The injection was immediately followed by a 10-mL saline flush (0.9% NaCl) in the same lateral ear vein.

Four additional New Zealand White rabbits were used to compare EP-2104R and a conventional Gd chelite (Gd-DTPA) for thrombus detection at different time points: acute (≤6 hours), subacute (≤1 week), 2-week-old, and 4-week-old thrombi. A protocol similar to that described previously was used. Gd-DTPA injection (50 μmol Gd/mL IV) was followed next day by EP-2104R. A custom-made, 2-element (10×5.5 cm²) phased-array receive-only coil was used for signal reception.

Image and Data Analyses

An experienced observer, blinded to the experimental design (ie, to the time point after thrombus induction and to the side and size of carotid injury), performed the image data analysis. Images were analyzed with ImagePro Plus (Media Cybernetics). Because thrombus composition changes over time, we assessed the intrinsic MR properties of the thrombus by measuring the relative signal intensity (SI) to the reference muscle [SI (%)=100×(SI thrombus/SI muscle)] on T1W and T2W images (ie, in NCE images). The immediately adjacent muscle tissue equidistant from the surface phased-array coil was selected as a standard reference. For NCE MRI, the contrast-to-noise ratio (CNR) was determined 30 minutes after EP-2104R or Gd-DTPA injection [CNR=(SI thrombus−SI muscle)/SD noise] on MR images and was normalized to precontrast CNR. The SD of noise was determined within a region of interest drawn outside the animal. The individual values of SI measurements from 3 contiguous MR images were averaged for both precontrast and postcontrast images, and the mean SI at each time point was plotted over time. Thrombus SI was assessed in its lengthwise central portion, excluding the proximal and distal edges, because this central segment may better reflect thrombus age.10 Thrombus length was determined in MR images after EP-2104R injection by manual tracing in reconstructed MIP images.

Histopathology

To correlate the MRI findings with the histopathologic findings, 2 randomly selected animals were humanely killed immediately after
MRI (100 mg/kg IV sodium pentobarbital) at each imaging time point. At the acute time point (≤6 hours) and the 1-week time point, 3 animals were killed. To avoid postmortem thrombus formation, euthanization was performed at the exact time of carotid excision. Because of artery length shrinkage after removal, carotid arteries were first stretched to the same length. The length of the carotid artery was then determined, and a linear correction factor was calculated, as previously described. Thereafter, carotid arteries were transferred to 4% paraformaldehyde solution for overnight fixation and then embedded in paraffin. Serial cross sections of selected carotid segments were taken (5-μm thickness) at 2-mm intervals to match the corresponding MR images. Specimens were then stained with the combined Masson’s elastin technique. The histopathologic sections of thrombotic carotid artery specimens were matched with corresponding MR images. Coregistration was performed carefully by using one or more anatomic landmarks, including the carotid bifurcation and the origin of the artery. An independent experienced pathologist, blinded to thrombus age and the MR findings, performed the histopathologic analysis and determined thrombus composition.

**Statistical Analysis**

Changes in SI and CNR over time were modeled in SAS with a mixed model approach with the PROC MIXED procedure for repeated measurements (SAS Institute Inc). Because both SI and CNR were measured during an 8-week period and analyzed with repeated measurements, the probability of correlation within animals is increased. The specific approach of repeated measurements used for this analysis allows for an accurate and improved estimate of the SEs of measurement and therefore, a more powerful test. To compare CNR before and after Gd-DTPA and EP-2104R before and after injection, differences among the 3 contrast techniques were explored and modeled with a 2-factor repeated-measures ANOVA, with repeated measures for both factors, ie, the change in contrast agent used and 4 replicate measurements per animal. A Pearson’s correlation coefficient was used to compare lengths of the thrombi obtained between MR images and histopathologic results. All measurements are expressed as mean±SE. *P*<0.05 was considered statistically significant.

**Results**

Arterial thrombosis was successfully induced in all animals (n=18). Thrombi were totally obstructive in all cases but 4 (77.7%).

**NCE MRI for Thrombus Detection**

MR angiography with the 3D time-of-flight technique was not useful for occlusive thrombus detection because of flow signal dropout secondary to turbulent flow in the obstructed vascular segments. Thrombus transverse MR images on T1W and T2W turbo spin-echo sequences (ie, NCE MRI) showed time-dependent SI changes (Figure 2). In acute (ie, ≤6 hours) and subacute (ie, ≤1 week after induction) thrombi as well as in 2-week-old thrombi, relative SI was significantly higher in T2W than in T1W images. In chronic thrombi (ie, ≥4 weeks after induction), relative SI was no longer different in T1W and T2W images.

**Fibrin-Targeted Contrast Agent for Thrombus Detection**

EP-2104R localized and accurately detected occlusive and nonocclusive thrombi, regardless of their location, size, and organizational stage (Figure 3). Relative SI and normalized CNR were significantly increased at each imaging time point (*P*<0.01) compared with precontrast T1W images. Over time, the CNR decreased significantly (*P*<0.001) with thrombus age (Figure 4). Acute and subacute thrombi were readily detected by EP-2104R. In chronic thrombus (ie, ≥4 weeks after induction), CNR was 2- to 4-fold higher (*P*<0.001) than on the T1W precontrast images. No difference was found between 6- and 8-week-old thrombi (*P*=0.3272), whereas CNR at 4 weeks after induction (4.28±1.21) was significantly higher (*P*<0.001) than at 6 weeks (3.12±1.06) after induction (Figure 4).

**Figure 2.** Transverse MR images of a rabbit carotid artery, 1 week after thrombus induction, with a double-inversion recovery, turbo spin-echo sequence. T1W (A) and T2W (B) images were obtained without any injection of contrast agent. The white arrow indicates the location of the thrombus. C, The T1W images obtained 30 minutes after EP-2104R injection. D, The thrombus relative SI changes (mean±SE) over time are shown for T1W (open circles), T2W (gray squares), and after EP-2104R injection (black triangles). The Gd-based, fibrin-targeted MR contrast agent (EP-2104R) demonstrated significant enhancement of the thrombus compared with T1W images (*P*<0.001).

**Figure 3.** Transverse T1W MR images of a 1-week-old thrombus, before (left, A and C) and after (right, B and D) EP-2104R injection. Note the benefit of using the fibrin-targeted MR contrast agent for thrombus detection. Accurate detection and localization of the thrombus were successful in both nonocclusive (upper row, A and B) and occlusive (lower row, C and D) thrombi. White arrows denote the thrombus location in the NCE MRI study.
Figure 4. Time dependence of thrombus enhancement at different imaging time points after injection of the fibrin-targeted contrast agent (EP-2104R). Gray bars depict the normalized thrombus CNR according to the time after thrombus induction. Although a significant decrease of CNR was noted over time (P<0.001), CNR was significantly increased (P<0.001) in acute (≤6 hours), subacute (≤1 week), and chronic (≥4 weeks) thrombi, compared with precontrast (Pre) imaging (black bar).

Because of the high MRI SI of EP-2104R–enhanced thrombi, targeted MIPs of carotid arteries could be reconstructed, and this readily allowed depiction of focal thrombi (Figure 5). Average thrombus length on MIP images was 9.2±4.5 mm (range, 4.9 to 13.7) and was correlated highly with histopathologic measurements (R=0.90, P<0.001). No difference in SI or CNR was noted between occlusive and nonocclusive thrombi. As expected, no changes were seen in the uninjured arteries (controls).

Gd-DTPA for Thrombus Detection

The use of Gd-DTPA was not associated with a significant improvement in thrombus detection, as confirmed by CNR values after Gd-DTPA injection in all animals (n=4) and at each time point. In 4-week-old thrombi (MIP images) thrombus CNR was 1.18±0.72 at baseline and decreased to 1.08±0.87 after Gd-DTPA injection (P=NS). Gd-DTPA did not enhance the thrombus and provided only nonspecific enhancement of perivascular muscle. The same trend was observed at each imaging time point (ie, acute, subacute, or chronic thrombus). In contrast, thrombus CNR increased after EP-2104R injection to 4.62±0.97 (P=0.006) without any perivascular muscle enhancement (Figure 6).

Histopathologic Analysis of Thrombi

Thrombus composition reflected distinctive characteristics, as described in human studies. Histological study demonstrated chronological organization of the thrombi. Progressively, platelets and fibrin deposits were replaced by revascularized fibrocollagenous tissue, as shown in Figure 7. The acute thrombus showed histopathologic characteristics of heterogeneous, unorganized early thrombus, mostly red blood cells and fibrin-rich areas (Figure 7A). Platelet-rich conglomerates were densely packed and surrounded by layers of fibrin and red blood cells. Fibrin-rich areas were present mostly at the periphery of the artery, in particular, where the thrombus was attached to the areas of exposed media. A similar histological pattern was seen 48 hours after thrombus induction. At 1 week (Figure 7B), fibrin masses still appeared compacted, with the beginning of organization due to infiltration by macrophages, neointima, and fibroblast ingrowth from the periphery to the center of the thrombus. At 2 weeks, initial fibrotic replacement was detected inside the thrombus, with formation of layers of immature, collagenized, connective tissue, whereas unresorbed fibrin and cellular debris were still present. At 4 weeks, thrombus organization appeared more evident, with fibrous tissue occupying half of the area and the appearance of neovessels and smooth muscle cells. At 6 weeks (Figure 7C), the thrombus appeared completely organized, showing a dense collagen matrix, whereas the cellular content was reduced. At 8 weeks, fibrous tissue was no longer forming a dense matrix but was separated by numerous neovessels of various shapes and sizes, associated with adipocyte infiltration (Figure 7D). A strong, linear correlation (R=−0.89, P<0.001) was found between CNR as measured on MR images and thrombus collagen content (mm²) as measured by planimetry in histopathologic sections.

Discussion

In this study, we have demonstrated the superiority of contrast-enhanced MRI with a fibrin-targeted contrast agent...
for noninvasive thrombus detection in vivo. We report that EP-2104R plays a critical role in the discrimination not only between occlusive and nonocclusive arterial thrombi but also between thrombi of different sizes and ages (ie, recent and old thrombi). We have demonstrated the benefit of fibrin contrast-enhanced MRI for chronic thrombi detection compared with conventional Gd chelates and NCE MRI.

The MR appearance of arterial thrombi and the changes detected over time on NCE MRI (ie, T1W and T2W images) resulted from the combination of different oxygenation states of hemoglobin, changes in the intracellular and matrix content of proteins, and hydration of the cellular components, as already reported by our group. However, despite these temporal changes on T2W and T1W images, NCE MRI did not provide any information on the composition of the thrombus and did not allow reliable thrombus detection compared with EP-2104R, in particular, in chronic or organized thrombi.

Although these findings are considered preliminary and are limited by the relative small number of animals assessed by histopathology (2 to 3 per time point), they support the fact that EP-2104R improved detection of old and organized thrombi in vivo compared with NCE MRI and a conventional Gd chelate. Because fibrin is present in all types of thrombus (arterial and venous, acute and chronic) and in a low concentration in flowing blood, thus diminishing the background signal, fibrin is a favorable molecular imaging target. Therefore, EP-2104R enhances acute and chronic thrombi and permits imaging of large thrombi as well as submillimeter mural thrombi (Figure 3B). EP-2104R provided high signal enhancement of the entire thrombus, not just the thrombus surface, with good differentiation from thrombus-free segments of the vessel wall. EP-2104R yielded information on thrombus composition, highlighting the fibrin-rich areas, even in organized, old thrombi. Thrombus main composition (ie, presence of a large or small amount of fibrin within the thrombus) is important when considering the alternatives for efficient treatment of thrombotic phenomena.

The mechanism by which EP-2104R seems to penetrate the thrombus is thought to be passive diffusion. However, according to the results of our study, although the mechanism of delivery might be passive diffusion, retention of the contrast agent is selective. EP-2104R, unlike Gd-DTPA, accumulates within the thrombus after binding to fibrin. Because of its high affinity for fibrin with tight binding and the apparent slow excretion kinetics relative to blood clearance of the compound, EP-2104R remains within the thrombus for 1.5 to 2 hours, as supported by preliminary work (M. Sirol et al, 2004, unpublished data).

Related Work
Although MRI has shown promise in thrombus detection in both experimental studies and humans, accurate thrombus age definition and detection of old and organized thrombi remain difficult. NCE MRI with the T1-shortening properties of methemoglobin (direct thrombus imaging) cannot be widely applied because of the relatively low content of red blood cells in arterial thrombi and uncertain thrombus time-course enhancement. NCE MRI with conventional Gd chelates has been reported to improve tissue characterization in human carotid arteries and enhance the contrast between the myocardium and intracardiac thrombi. However, Gd-DTPA is a nonspecific contrast agent that distributes to the intravascular and interstitial spaces by diffusion and does not allow for thrombus enhancement. Interestingly, no uptake of Gd-DTPA within the thrombus was noted at any imaging time in our study, in contrast to the perivascular muscle enhancement due to Gd-DTPA diffusion. Still, the exact identification and definition of thrombus age with Gd-DTPA remain difficult.

Recent technical development has led to paramagnetic and superparamagnetic contrast agents that are targeted to thrombus components such as fibrin. The feasibility of in vivo acute thrombus detection with a fibrin-binding, MR contrast agent has been reported by our group and others. Nevertheless, no study had demonstrated the ability of chronic or organized thrombus detection in vivo with a targeted contrast agent with MRI.

Biological Assessment of Thrombus In Vivo With MRI
We have demonstrated that MRI SI decreases with thrombus age and is related to thrombus composition and organization. Thrombus enhancement due to the presence of fibrin deposits within it decreased in older thrombi in our experimental model of carotid thrombosis. The ability to image fibrin within thrombi in vivo may have significant clinical implications. Imaging fibrin may not only improve our knowledge of the kinetics and mechanism of thrombus formation but may...
also facilitate the design of safer and more effective therapeutic regimens based on fibrin inhibition and lysis. The administration of a fibrin-specific contrast agent may facilitate the understanding of thrombus behavior in vivo.

Clinical Relevance

Because thrombosis is a dynamic process, with thrombus material of different ages forming a layered structure due to successive mural deposition,29 fibrin-targeted, contrast-enhanced MRI may be used to differentiate between layers of fibrin deposits in all types of thrombi and in superimposed thrombosis associated with plaque rupture. Acute coronary syndromes, stroke, deep vein thrombosis complicated by pulmonary embolism, and atrial fibrillation with a high incidence of various cardiac manifestations result from thrombus formation in different vascular beds after either plaque rupture/erosion or increased blood thrombogenicity.33,34 Fibrin deposits in old, organized thrombi have recently been confirmed by histopathologic study after thrombectomy in percutaneous coronary intervention after acute myocardial infarction.35 Therefore, a noninvasive modality with a fibrin-targeted contrast agent might be ideal for in vivo detection of all thrombi. Potential applications include assessment of thrombus burden in patients with acute coronary syndromes and detection of coronary in-stent thrombosis.27 In addition, this technique may benefit patients with suspected deep venous thrombosis or pulmonary embolism36 and facilitate detection of atrial thrombus in patients with atrial fibrillation. Early-phase clinical studies (phases I and II) are currently testing the safety of EP-2104R in humans.

Conclusion

In this study, we demonstrate the benefit of contrast-enhanced MRI with a fibrin-targeted contrast agent for noninvasive chronic or organized thrombus detection in an experimental animal model of carotid artery thrombosis. We report the ability of EP-2104R to discriminate not only between occlusive and nonocclusive arterial thrombi in vivo but also between recent and old thrombi with respect to thrombus composition. We demonstrate the superiority of a fibrin-targeted MR contrast agent for thrombus detection compared with both Gd-DTPA and NCE MRI.

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References

CLINICAL PERSPECTIVE

Atherosclerotic plaque rupture or erosion and its thrombotic complications are responsible for substantial death and disability; thus, the ability to detect thrombus in vivo with a noninvasive imaging modality may allow improved risk stratification and therapy in humans. Sirol et al in this issue of Circulation describe the use of magnetic resonance imaging (MRI) at a clinically relevant magnetic field (1.5 T) with a contrast agent targeted to fibrin (EP-2104R) to accurately identify thrombus. MRI is a robust imaging modality for plaque detection and characterization with very high reproducibility. The combined use of MRI and EP-2104R would potentially provide a powerful tool to identify thrombus not only in atherosclerotic, vulnerable plaque in coronary arteries or other vascular beds but also in deep venous thrombosis and pulmonary embolism, as well as thrombi in the left atrium in patients with atrial fibrillation. Because this technique also can identify plaque composition, these data provide the starting point for giving clinicians the opportunity to choose appropriate therapy based on a comprehensive evaluation of plaque composition and the presence of thrombus. The present study, by assessing thrombus detection in vivo in an acute and chronic setting, provides novel information on the potential clinical impact of this technique. EP-2104R is currently being tested in human subjects in phase I and phase II clinical trials.

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