Molecular MRI of Intracranial Thrombus in a Rat Ischemic Stroke Model

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Background and Purpose—Intracranial thrombus is a principal feature in most ischemic stroke, and thrombus location and size may correlate with outcome and response to thrombolytic therapy. EP-2104R is a fibrin-specific molecular MR agent that has previously been shown to enhance extracranial and venous sinus thrombi in animal models and, recently, in clinical trials. In this study, we examined whether this fibrin-specific MR probe could noninvasively characterize intracranial arterial thrombi.

Methods—Embolic stroke was induced in adult rats by occlusion of the right internal carotid artery with an aged thrombus. We used diffusion-weighted imaging, time of flight angiography, and high-resolution 3-dimensional T1-weighted MRI at 4.7 T before and after use of contrast agents EP-2104R (n = 6) and gadopentetate dimeglumine (n = 5).

Results—In all animals, MR angiography revealed a flow deficit and diffusion-weighted imaging showed hyperintensity consistent with ischemia. Using EP-2104R-enhanced MRI, we saw occlusive thrombi and vessel wall enhancement in all 6 animals with high contrast to noise relative to blood, whereas gadopentetate dimeglumine-injected animals showed no occlusive thrombus or vessel wall enhancement. The concentration of gadolinium in the thrombus after EP-2104R was 18 times that in the blood pool.

Conclusions—EP-2104R-enhanced MRI successfully identifies intracranial thrombus in a rat embolic stroke model. (Stroke. 2010;41:1271-1277.)

Key Words: fibrin • gadolinium • ischemic stroke • molecular imaging • thrombus

Stroke is the second leading cause of mortality worldwide, and ischemic stroke represents 85% of strokes in the Western world. Imaging with CT or MRI is well established in stroke workup to distinguish ischemic from hemorrhagic stroke, to select patients for thrombolysis, and for assessment of prognosis. The culprit thrombus is sometimes observed as a hyperdense sign on noncontrast CT. The presence of either a hyperdense sign in the middle cerebral artery (MCA),2-3 basilar artery,4 or posterior cerebral artery4,5 or the hyperdense “dot” sign in the MCA6 all demonstrate high specificity (95% to 100%) for thrombus. However, the sensitivity of this sign ranges from 30% to 70%.2-6 Hyperdense MCA is associated with poor outcome,7 but patients may benefit from intravenous or intra-arterial tissue plasminogen activator.7-9 The hyperdense sign is believed to result from increased red cell content in the thrombus. Given that the action of tissue plasminogen activator ultimately results in fibrinolysis, a direct molecular imaging technique to assess thrombus size and fibrin content may be useful in predicting response to thrombolysis and/or in estimating the dose of tissue plasminogen activator required for lysis. Visualization of thrombus could also contribute to understanding the pathophysiology of stroke in a given patient, particularly posttherapy; patterns of intracranial thrombus distribution could aid determination of the stroke etiology; and visualization of any residual source thrombus outside the brain may aid in secondary stroke prevention. Because visualization of clot could have such potential, many groups have worked to create targeted agents. Nuclear medicine-based approaches have not been widely accepted to date, largely due to limited spatial resolution and inadequate target to background performance.

Advances in molecular imaging have led to the identification of several thrombus-specific MR contrast agents that target either fibrin10,11 or activated platelets.12 Recent clinical trial data with a fibrin-targeted gadolinium (Gd)-based probe, EP-2104R, indicated that this probe can identify thrombi in the heart chambers, carotid arteries, or aortic arch and that the positive image contrast persists for hours.13,14 These clinical studies followed a series of molecular MRI studies of thrombosis in swine and rabbits,11,15-23 but surprisingly, there has not been either a study characterizing intracranial throm-
bus after ischemic stroke or literature on the efficacy of EP-2104R in rodent models of thrombosis, most likely because of the complexity of the surgical model as well as the requirement for high-field MRI. We therefore sought to evaluate the efficacy of EP-2104R for molecular imaging of intracranial thrombus at high field in a rat model of embolic stroke. For this, pre- and postcontrast images with EP-2104R were compared with gadopentetate dimeglumine (Gd-DPTA; Magnevist; Bayer Healthcare, Montville, NJ) -enhanced images. Because thrombus molecular imaging may be incorporated into the stroke imaging workup, the impact of EP-2104R on diffusion-weighted imaging (DWI), perfusion-weighted imaging, and time-of-flight (TOF) angiography is also assessed.

Methods

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Surgical Procedures

Focal embolic cerebral ischemia was produced in adult male Wistar rats (350 to 400 g, n = 13; Charles River Laboratories, Wilmington, Mass) using a previously described protocol. Briefly, 24 hours before the ischemia surgery, animals were anesthetized with isoflurane (4% to 5% for induction, 1% to 2% for maintenance; in 30% oxygen–70% nitrous oxide) and rectal temperature was maintained at 37.5°C. Femoral arterial blood was collected into 20-cm-long PE50 tubing and allowed to clot first for 2 hours at room temperature and then for 22 hours at 4°C. The next day, rats were reanesthetized, rectal temperature was maintained at 37.5°C, and the right femoral vein was canulated for contrast agent injection. A midline incision was made in the neck and a 25-mm single thrombus that was thoroughly washed with saline and transferred to a modified PE50 catheter was injected into the right internal carotid artery at the level of the MCA. Skin incisions were closed and animal was transferred to the MR-compatible stereotaxic frame.

Contrast Agents

EP-2104R (Epix Pharmaceuticals, Lexington, Mass) comprises a fibrin-targeting peptide with 2 Gd-DOTA chelates on each of the C- and N-termini of the peptide (4 Gd in total). EP-2104R binds equally to 2 sites on human or rat fibrin (Kd = 1.7 or 1.8 μmol/L, respectively) and has high specificity for fibrin over fibrinogen or other plasma proteins. The relaxivity of EP-2104R bound to fibrin is 40.0 Lmmol−1 s−1 (10.0 per Gd) at 4.7 T. The commercial extracellular agent, Gd-DTPA was used as a control. Gd-DTPA does not bind fibrin or plasma proteins and has a relaxivity of 3.2 Lmmol−1 s−1 at 4.7 T in water.

MRI Protocols

All MRI was performed on a 4.7-T small animal scanner (Bruker Biospin, Billerica, Mass). Immediately after the ischemia surgery, rats were placed in the prone position in the scanner and the head was secured with a stereotactic frame. Anesthesia was maintained with isoflurane (1% to 2% in 70% N2O and 30% O2) and body temperature was kept at 37.5°C by a heating pad during imaging. Animals were imaged using a homemade 50×39-mm oval surface coil with the following MR sequences: 3-dimensional TOF angiography; 2-dimensional DWI; 2-dimensional T1-weighted multislice multiecho; 3-dimensional T1-weighted gradient echo (molecular MRI sequence). The field of view scanned for both 3-dimensional MRI sequences was 58×29×29 mm. The repetition time for the TOF sequence was 40 ms, echo time was 4.08 ms, flip angle was 75°, and number of excitations was 1 with a matrix size of 192×96×96 giving 0.30 mm/pixel resolution in a scan time of 6 minutes. The molecular MR sequence (repetition time/echo time/flip angle) was 405/8/75°, giving a scan time of 405 × 405 × 54 000/8/75°, number of excitations = 4, matrix = 300×150×150, resolution = 0.193 mm/pixel and scan time = 1 hour) was performed with inferior saturation to null inflowing arterial blood. For DWI, 9.1-mm axial slices were imaged with repetition time/echo time = 5000/33.4, number of excitations = 4, field of view = 22×22 mm, matrix = 64×64, resolution = 0.344 mm/pixel using 5 b values = 0.4, 574, 1147, 1720, 2335 s/mm2, giving a scan time = 1 minute 30 seconds. In some instances, up to 64 averages were collected. For the T1-weighted rapid acquisition with relaxation enhancement sequence, 9.1-mm axial slices were imaged with repetition time/echo time = 650/6.3, number of excitations = 1, field of view = 22×22 mm, matrix = 128×128, resolution = 0.172 mm/pixel giving a scan time = 2 minutes 50 seconds.

Tissue and Blood Gd Analysis

The ipsilateral internal carotid artery and MCA containing the thrombus, contralateral internal carotid artery, and MCA as well as ipsilateral and contralateral cerebral hemispheres and blood were collected from all the animals. The tissues were homogenized in nitric acid and analyzed for Gd concentration by inductively coupled plasma–mass spectrometry using added lutetium as an internal standard.

Fibrin Immunostaining

After perfusion with 4% paraformaldehyde, the brain was removed and processed after an overnight postfixation at 4°C. Five-micron-thick paraffin sections were mounted on slides and immunohistochemistry was performed using an antihuman monoclonal antibody to fibrin (1:25 dilution, 1 hour at room temperature; product NYB T2G1; Accurate Chemical & Scientific Corp, Westbury, NY). Sections were counterstained with hematoxylin.

Experimental Protocols

Imaging began approximately 20 minutes after delivery of the thrombus. Subsequent to collection of localizer images, a series of baseline imaging studies were performed in the following order: TOF angiography, T1-weighted multislice multiecho, 3-dimensional molecular MRI sequence, and DWI. After the baseline scans were completed, 0.4 mL of the contrast agent, either EP-2104R (10 μmol/kg, n = 6) or Gd-DTPA (control; 200 μmol/kg, n = 5), was injected as a bolus through the femoral vein. Postcontrast agent, the same imaging sequences as baseline were repeated.

After the last scan, the animal was removed from the scanner and euthanized. The vascular and brain tissues and an aliquot of blood were collected, weighed, and processed for inductively coupled plasma–mass spectrometry analysis.

Two additional studies were performed in which the animal received both contrast agents in succession. Briefly, after surgery and baseline scanning as described previously, Gd-DTPA (200 μmol/kg intravenously) was given as a bolus and the postcontrast imaging sequence repeated. Then, 80 minutes after Gd-DTPA was administered, EP-2104R (10 μmol/kg intravenously) was injected and the postcontrast scans repeated.

Data Analysis

The images were analyzed using the program OsiriX (www.osirix-viewer.com) by drawing regions of interest in the thrombus, contralateral artery, and adjacent brain or muscle tissue and signal intensity (SI) was quantified for the same slice. Noise was quantified as the SD of the signal measured in the air outside the animal. Apparent diffusion coefficient maps were calculated using an in-house Matlab program by fitting the natural log of the SI as a function of b value. Contrast-to-noise ratios (CNR) were calculated for the difference between tissue A and tissue B using the following equation:

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\text{CNR}_{\text{Tissue A vs. Tissue B}} = \frac{\text{SI}_{\text{Tissue A}} - \text{SI}_{\text{Tissue B}}}{\text{SD}_{\text{air}}} \]

Gd concentration was determined by comparing the Gd-lutetium ratio with a standard curve. Concentrations are reported as nmol Gd/g tissue. Because the Gd-DTPA and EP-2104R were adminin-
tered at different doses, concentrations were also expressed as percent of initial dose per gram tissue. Differences between 2 groups (pre- versus postcontrast, Gd-DTPA versus EP-2104R) were compared using repeated-measures analysis of variance followed by Student-Newman-Keuls post hoc test when needed. Comparison of MR signal or Gd levels between the 2 brain hemispheres was tested with a 1-sample t test with the test value (ratio between the 2 hemispheres) set at 1. Probability value \( P < 0.05 \) was considered significant. Uncertainties are expressed as 1 SD.

**Results**

In all 13 animals, the acute embolic stroke model was reproduced successfully. The TOF angiograms demonstrated restricted flow to the right side of the brain in all animals, suggesting an occlusive thrombus (Figure 1A–B). DWI was performed pre- and postinjection and both showed hyperintensity in the right MCA territory suggesting an ischemic lesion in all animals imaged (Figure 1C). Likewise, the corresponding apparent diffusion coefficient maps show a lesion at the same localization (Figure 1D). In the EP-2104R-injected animals, the lesion size of the preinjection DWI was 5.17\( \pm 1.8\% \) of the contralateral hemisphere and postinjection DWI was 9.28\( \pm 3.1\% \) of the contralateral hemisphere. In the Gd-DTPA-injected animals, the lesion size of the preinjection DWI was 4.9\( \pm 1.6\% \) of the contralateral hemisphere and postinjection DWI was 7.12\( \pm 3.5\% \) of the contralateral hemisphere. There was no difference in lesion size between EP-2104R and Gd-DTPA groups at either pre- or postinjection DWI scans (repeated-measures analysis of variance: \( F = 0.840 \), \( P = 0.3832 \)).

The thrombus was not directly visible on baseline scans. After injection of EP-2104R, the occlusive thrombus was clearly evident in the right internal carotid artery, near the origin of MCA, using the high-resolution molecular MRI sequence (Figure 2). In all EP-2104R-injected animals, there was considerable enhancement of the thrombus compared with the pre-EP-2104R

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**Figure 1.** A, Coronal maximum intensity projection of TOF angiogram shows right-sided flow deficit. B, Axial slice from the 3-dimensional TOF angiogram shows no flow in the right MCA (arrow). C, DWI at the same level shows a hyperintense lesion in the MCA territory that is also obvious on the corresponding apparent diffusion coefficient map (D) with a calculated lesion apparent diffusion coefficient of 0.324\( \pm 0.043 \times 10^{-3} \) mm\(^2\)/s.

**Figure 2.** Coronal slice from 3-dimensional T1-weighted images at level of the MCA origin: the EP-2104R-enhanced image (B) clearly identifies the thrombus (arrow) that was not visible on the image acquired before EP-2104R injection (A). C, Pictorial representation of the location of the thrombus within the cerebral arterial tree. BA indicates basilar artery; PCA, posterior cerebral artery; ICA, internal carotid artery; OA, olfactory artery; ACA, anterior cerebral artery.
The source images reveal the presence of an occlusive thrombus at the level of the MCA (Figure 4D–4F). Proximal to this occlusive thrombus is vessel wall enhancement (Figure 4A–B) that is likely fresh mural thrombus caused by endothelial damage induced during catheter delivery of the aged clot typical for this model. The vessel wall enhancement was observed in all 6 animals post-EP-2104R injection but was not observed before EP-2104R injection or observed in the contralateral vessel. Thrombus CNR was high post-EP-2104R and highly significant compared with pre-EP-2104R images CNR (Figure 3B; $\text{CNR}_{\text{thrombus/blood}} = 22.3 \pm 3.2$ post versus $3.3 \pm 0.6$ pre; $\text{CNR}_{\text{thrombus/muscle}} = 17.7 \pm 2.8$ versus $1.2 \pm 0.4$ pre; $P < 0.005$). No significant difference in vessel wall CNR was observed after Gd-DTPA administration.

The ex vivo tissue analyses mirrored the imaging findings (Figure 3C). The Gd thrombus to blood ratio was $18.5 \pm 8.7$ at 80 minutes after EP-2104R injection, which was significantly higher than that measured after Gd-DTPA administration ($2.18 \pm 0.66; P < 0.05$). The concentration of Gd in the thrombus was 5.8 times higher in the EP-2104R group than the control ($P < 0.05$). To determine if the blood–brain barrier was compromised at this hyperacute phase of ischemia, T1-weighted imaging was performed before and after (15 minutes) contrast agent administration at the level of the lesion. No difference in signal intensity was observed between the 2 hemispheres of the brain after either EP-2104R or Gd-DTPA administration. Ex vivo analysis of the left and right cortex indicated a small but significant ($P < 0.025$) 2-fold increase in Gd concentration in the injured right cortex compared with the left side. The 2-fold increase was seen with both Gd-DTPA and EP-2104R. However, the absolute concentration of Gd in the brain was quite low ($0.47 \pm 0.14 \text{ nmol/g for EP-2104R and 1.16} \pm 0.42 \text{ nmol/g for Gd-DTPA}$) and is below the limit detectable by MRI.

Two additional animals that were imaged received Gd-DTPA and then EP-2104R 80 minutes later. In these animals, the thrombus was only visible postinjection of EP-2104R and was not apparent on the precontrast images or on the images obtained up to 80 minutes postinjection of Gd-DTPA.

In an additional animal, immunohistochemistry with monoclonal antibody to fibrin revealed the presence of fibrin along the internal carotid artery wall (data not shown).

Discussion

This study demonstrates direct, positive contrast detection of intracranial thrombi in a rat model of ischemic stroke. This model is known from ex vivo analyses to produce occlusive thrombi, which result in reduced cerebral blood flow and hyperintense lesions on diffusion-weighted MR images. The thrombus was readily visible in all animals after EP-2104R but was not visualized before contrast agent or if conventional Gd-DTPA was given. Because fibrin is present in most thrombi, fibrin-targeted molecular MRI may prove more sensitive than techniques like the hyperdense sign on CT2 or direct detection by MRI due to methemoglobin. As the thrombus ages, hemoglobin oxidizes to methemoglobin, which has a strong T1-shortening effect. This phenomenon has been exploited in direct detection of thrombi by MRI and used to identify complex plaque and venous thromboem-
The sensitivity of the direct thrombus detection method relies in part on the presence of methemoglobin at the time of imaging, which depends on the age of the thrombus. The thrombus preparation in this model was not T1-bright indicating the absence of methemoglobin. Given the success of EP-2104R in detecting thrombi outside the brain in humans and in large animal models, it is likely that the present results are translatable to clinical imaging.

EP-2104R is a Gd-based contrast agent with high affinity and selectivity for fibrin. It allows visualization of fresh as well as aged, pulmonary, coronary, cardiac, and deep vein thrombi in swine. Phase II clinical studies in patients with venous, arterial, and cardiac thrombi showed that EP-2104R enhanced thrombi and that the signal enhancement persisted up to 24 hours after the contrast agent injection. Despite these extensive studies on molecular imaging of peripheral thrombus, however, there is only one published study on intracranial thrombus in which enhancement of cerebral sinus thrombus in swine was demonstrated. The present study builds on this literature of direct thrombus imaging and expands the use of EP-2104R into the detection of intracranial arterial thrombi in rats and into thrombus imaging at high field.

The animal model used is well established in stroke research. The thrombus was aged ex vivo for 24 hours and imaging was performed within 1 hour of the thrombus being introduced into the internal carotid artery. The facile characterization of the thrombus in this study suggests that EP-2104R-enhanced MRI may be a powerful tool to investigate the temporal evolution of the thrombus and characterize microemboli as the primary lesion undergoes lysis or to investigate the effect of thrombolytic interventions.

DWI, perfusion-weighted imaging, and TOF MR angiography are widely used in stroke workup. To assess the potential impact of EP-2104R on these studies, DWI and TOF imaging were performed before and after EP-2104R injection. The presence of EP-2104R had no impact on the diffusion images. The MR angiographic images were obtained approximately 5 minutes postinjection and at the relatively low dose used, there was little to no venous enhancement compared with the pre-EP-2104R MR angiography. Attempts to measure perfusion with EP-2104R were unsuccessful, presumably because of the low dose of Gd used. In 2 studies, rats were imaged at baseline, then after Gd-DTPA (200 μmol/kg) injection, and then, 80 minutes later, after EP-2104R injection (10 μmol/kg). The thrombus was only visible after EP-2104R administration, and the Gd-DTPA did not interfere with the EP-2104R detection of the thrombus. This finding suggests that a high dose of Gd-DTPA could be used for the perfusion study followed by EP-2104R to identify the thrombus. Alternately, the 2 contrast agents could be mixed together and administered at once to first acquire a perfusion map and then to identify thrombi or, simply, a higher dose of EP-2104R could be given. It is important to note in here that to compensate for the 45% lower relaxivity of EP-2104R at 4.7 T compared with 1.5 T, the dose was increased to 10 μmol/kg compared with doses of 4 and 7.5 μmol/kg used previously. Previous studies mainly used either a heavily T1-weighted 3-dimensional gradient echo sequence or a black blood inversion recovery sequence to detect the thrombus. In this study, a 3-dimensional gradient recalled echo sequence with in-flow saturation was used and this approach is readily adapted to clinical imaging. The inversion recovery sequence would also likely result in high thrombus CNR.

Interestingly, EP-2104R also caused a signal enhancement along the wall of the internal carotid artery. The signal enhancement was only apparent in the vessel that was catheterized and no enhancement was observed in the contralateral artery. Vessel trauma from the catheterization procedure is known to result in fresh thrombus along the vessel wall and in the present study, immunohistochemistry re-
revealed heavy staining for fibrin along the wall. The vessel itself remained patent and such mural thrombi would not be visible on angiograms. This finding further highlights the potential of molecular MRI to provide additional layers of biochemical information to the anatomic image. It also reveals the potential acute vessel damage associated with catheterization; given the widespread use of this animal model, further characterization of endothelial impact may be warranted.

Fibrin is detected in every thrombus retrieved from patients with acute stroke, although its amount in each thrombus varies not only by the age of the thrombus, but also by its nature. Fibrin is the predominant component of acute and organized thrombi but with aging, the thrombus undergoes degradation. The critical question is, how much aging? Radioimmunoimaging with a fibrin-specific monoclonal antibody showed that the concentration in fresh and up to 5-day-old thrombi were comparable. Molecular MRI of fibrin provides more detailed information on this; signal enhancement occurs in fresh thrombus and declines as the age of thrombus past 4 weeks. Previous work showed EP-2104R-enhanced MRI could detect chronic human thrombectomy samples (4 weeks to 1.5 years old) that were implanted in the atria or lungs of swine as well as freshly prepared thrombi. Based on this body of prior work, it is highly likely that EP-2104R will cause a similar degree of signal enhancement on fresh and aged thrombi in the brain. Indeed, our auxiliary finding of carotid vessel wall enhancement supports this assumption. However, it is important to demonstrate the time window of efficacy of EP-2104R in fresh and aged intracranial thrombi in future studies.

There are some limitations to the present study, notably the small sample size and the arbitrary time window for imaging. Animals were imaged immediately after thrombus injection, and the effective time window of imaging after thromboembolic stroke was not determined. The model used an occlusive, aged thrombus and also resulted in fresh thrombus along the arterial wall; however, like most animal models, this does not completely mimic the clinical setting. Further studies are warranted to determine the efficacy of EP-2104R in identifying intracranial thrombi in a clinical setting and to establish its sensitivity and specificity. Further imaging studies should also include a perfusion component, which may be enabled by an additional dose of Gd-DTPA or a higher dose of EP-2104R.

In the management of stroke, identification of the culprit thrombotic lesion, any downstream microemboli, and the source of the embolus are all critical. The properties of EP-2104R such as its ability to bind to fibrin in thrombi for long periods of time, the high CNR obtained for clot to blood pool, its relatively low dose, and its lack of interference with DWI, perfusion-weighted imaging, and angiography sequences, make it a potentially valuable tool in the stroke imaging workup. In previous studies including recent work in humans, EP-2104R was shown to enhance cardiac, aortic, and carotid thrombi and that the enhancement persists for hours. One potential application of this probe would be a single injection followed by a multistation imaging study to characterize the primary lesion, search for intracranial microemboli, and then search for the source of the thrombus in the carotid artery, thoracic aorta, and cardiac chambers.

Conclusion
Fibrin-specific molecular MRI allows visualization of occlusive and mural thrombi with high positive image contrast.

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Disclosures
A full listing of A.G.S.’s competing interests is available at www.biomarkers.org.

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