Magnetic Resonance Imaging Contrast Agent Targeted Toward Activated Platelets Allows In Vivo Detection of Thrombosis and Monitoring of Thrombolysis

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Background—Platelets are the key to thrombus formation and play a role in the development of atherosclerosis. Noninvasive imaging of activated platelets would be of great clinical interest. Here, we evaluate the ability of a magnetic resonance imaging (MRI) contrast agent consisting of microparticles of iron oxide (MPIOs) and a single-chain antibody targeting ligand-induced binding sites (LIBS) on activated glycoprotein IIb/IIIa to image carotid artery thrombi and atherosclerotic plaques.

Methods and Results—Anti-LIBS antibody or control antibody was conjugated to 1-μm MPIOs (LIBS MPIO/control MPIO). Nonocclusive mural thrombi were induced in mice with 6% ferric chloride. MRI (at 9.4 T) was performed once before and repeatedly in 12-minute-long sequences after LIBS MPIO/control MPIO injection. After 36 minutes, a significant signal void, corresponding to MPIO accumulation, was observed with LIBS MPIOs but not control MPIOs (P<0.05). After thrombolysis, in LIBS MPIO–injected mice, the signal void subsided, indicating successful thrombolysis. On histology, the MPIO content of the thrombus, as well as thrombus size, correlated significantly with LIBS MPIO–induced signal void (both P<0.01). After ex vivo incubation of symptomatic human carotid plaques, MRI and histology confirmed binding to areas of platelet adhesion/aggregation for LIBS MPIOs but not for control MPIOs.

Conclusions—LIBS MPIOs allow in vivo MRI of activated platelets with excellent contrast properties and monitoring of thrombolytic therapy. Furthermore, activated platelets were detected on the surface of symptomatic human carotid plaques by ex vivo MRI. This approach represents a novel noninvasive technique allowing the detection and quantification of platelet-containing thrombi. (Circulation. 2008;118:258-267.)

Key Words: magnetic resonance imaging || plaque || platelets || thrombolysis || thrombus

The rupture or erosion of atherosclerotic plaques is regarded as the precipitating event for thrombus formation in myocardial infarction and, to a certain extent, in stroke. Platelets play a pivotal role in this process through both the formation of platelet aggregates and activation of the coagulation cascade. Accordingly, clinical guidelines recommend early and aggressive antiplatelet therapy in patients with acute coronary syndromes, including aspirin, clopidogrel, and in selected patients, glycoprotein IIb/IIIa (GPIIb/IIIa) inhibitors. However, platelets are involved not only in the final events leading to thrombotic vessel occlusion but also in the earlier phases of plaque development. Thus, imaging of aggregated or adhering platelets may offer a unique opportunity to identify thrombi and to characterize atherosclerotic plaques.

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Recent advances in magnetic resonance technology allow imaging of vessels and atherosclerotic plaques at a submillimeter level. However, the identification of small thrombi and characterization of atherosclerotic plaques remain major challenges. Promising pilot studies have used functional imaging with gadolinium contrast agents targeting fibrin as the end product of the activated coagulation system. These studies mostly use large occlusive thrombi; thus, fibrin-targeted magnetic resonance imaging (MRI) seems to be highly suitable for the detection of strong fibrin accumulation. Imaging of platelets promises the sensitive detection of small thrombi and the identification of the role of platelets in plaque development. Recent MRI studies have used micro-
particles of iron oxide (MPIOs), which cause an intensely negative contrast effect in T2*-weighted MRI for in vivo detection of single cells by MRI\textsuperscript{13,14} and identification of vascular cell adhesion molecule-1 expression in an animal model of brain inflammation\textsuperscript{15} and atherosclerosis.\textsuperscript{16}

Here, we report the generation and application of an MRI contrast agent consisting of MPIOs and a single-chain antibody that selectively binds to ligand-induced binding sites (LIBS) on the activated platelet integrin GPIIb/IIIa.\textsuperscript{17} We show the utility of this contrast agent in rapid identification of mural platelet-containing thrombi in vivo using a mouse model of wall-adherent carotid thrombosis. Pharmacological thrombolysis was used to further evaluate whether LIBS MPIO–induced signal void intensity reports reliably on thrombus size, particularly size reduction. Furthermore, we applied LIBS MPIOs in symptomatic human eversion endarterectomy specimens to show the detection of clinically relevant platelet adhesion/aggregation in humans.

\textbf{Methods}

\textbf{In Vitro MPIO Phantoms and MPIO Relaxivity}

To estimate MPIO-induced susceptibility properties for the establishment of the in vivo experimental protocols, we prepared in vitro phantoms using different MPIO concentrations and quantified the MPIO-induced signal extinction in MRI via relaxometry according to the literature.\textsuperscript{18} A detailed description of the experimental setup and the MRI data acquisition and quantification can be found in Figure 1 and in the Methods section of the online Data Supplement.

\textbf{Construction of the MRI Contrast Agent Specific for Activated Platelets}

For construction of the contrast agent, autofluorescent cobalt-functionalized MPIOs (size, 1\textmu m) were conjugated to the histidine tag of the LIBS/control single-chain antibody following the protocol of the manufacturer (Dynal Biotech, Oslo, Norway). The detailed protocol is described in the supplementary Methods section.

\textbf{Cloning of Truncated Mouse scuPALMW and Expression/Purification of Mouse scuPALMW in Drosophila S2 Cells}

A detailed description of cloning, expression, and purification of low-molecular-weight single-chain urokinase-type plasminogen activator (scuPALMW) is given in the supplementary Methods section.

\textbf{Adoption of the Carotid Artery Model for Reproducible Wall-Adherent Thrombosis}

To obtain a semioclusive platelet-derived thrombosis of the carotid artery, we first had to adopt the well-established model of ferric chloride–induced carotid artery thrombosis to obtain a reproducible semioclusive thrombosis.\textsuperscript{19,20} This was important to guarantee blood circulation and therefore contrast agent flow over the surface of the wall-adherent thrombus. Sample size was chosen according to our previous experience with the mouse carotid thrombosis model.\textsuperscript{21,22} Ten- to 11-week-old male C57BL/6 mice weighing 24\pm0.2 g (Charles River, Sulzfeld, Germany) were used. Care and

\begin{figure}
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\includegraphics[width=\textwidth]{figure1}
\caption{A, MPIO relaxivity demonstrates T2 rapid acquisition with relaxation enhancement images of the MPIO dilution series in tubes acquired at a TE of 15 ms. B, A gradient-echo image series used for \(r_2^*\) determination with a TE of 3.5 ms; MPIOs typically cause a negative contrast, which can be recognized in a dose-dependent manner. The number of MPIOs and the concentration diluted are given, as well as below the images of the relative signal intensities compared with the tube containing no MPIOs. The relaxation rates \(R_2^\text{C}\) and \(R_2^\text{D}\), dependent on the contrast agent concentration, were fitted linearly (solid line) to gain the independent relaxivities \(r_2\) and \(r_2^*\). The dashed lines depict the upper and lower 95% confidence limits.}
\end{figure}
use of laboratory animals followed the national guidelines and were approved by the institutional animal care and ethics committees of the University of Freiburg (Freiburg, Germany). Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight) and were then placed under a dissecting microscope. An incision of the skin was made superficially to the right common carotid artery. The fascia was bluntly dissected, and a segment of the right common carotid artery was exposed. Thrombosis was induced by applying a piece of filter paper (1×2 mm, GB003, Schleicher & Schuell, Dassel, Germany) saturated with ferric chloride (3% to 10% solution, Sigma Chemical Co, Munich, Germany) under the right carotid artery, which was removed after 3 minutes. After 1 hour, animals were terminally anesthetized with ketamine and xylazine and flushed with saline via the left ventricle; then, the injured carotid artery was removed.

### In Vivo Experimental and MRI Protocol of Contrast Agent Injection and Thrombolysis

Mice were consecutively and randomly assigned to either the LIBS MPIO or control MPIO group before the MRI was performed. After the wall-adherent thrombosis was induced with ferric chloride as described above, animals were transferred to the MRI system (94/20 Bruker Biospec, Bruker, Germany). A venous catheter, connected with a 1-mL syringe containing 0.9% saline via a 120-cm-long tube (Portex, Smiths Medical International, Walford, UK), was placed in the tail vein. The animals were connected with an ECG and breathing-rate monitor and placed in the animal bed. Vital signs were monitored throughout the entire experiment, and body temperature was supported by a warm-water tube integrated in the animal bed. Anesthesia was continuously switched from ketamine to 1% to 1.6% isoflurane in O2 starting with a heart rate rising above 190 bpm and maintaining a breathing rate of 60±20 breaths per minute. During the complete measurement, mice were placed in the center of a quadrature whole-body birdcage resonator (35-mm inner diameter). Imaging consisted of a pilot scan with 3 orthogonal slices centered on a quadrature whole-body birdcage resonator (35-mm inner diameter). Imaging consisted of a pilot scan with 3 orthogonal slices centered on the exophagous (Figure 2A) with an echo time (TE) of 4.4 ms, a repetition time (TR) of 250 ms, a flip angle of 40°, and a field of view of 25×25 mm. Via the coronal images, a volume (27×27×7.5 mm), including the bifurcation and the upstream carotid arteries beyond the lesion, was planned for the 3-dimensional (3D) gradient-echo sequence, monitoring the wall-adherent thrombosis. This 3D fast low-angle shot (FLASH) sequence had a TE of 2.8 ms, a TR of 20 ms, a bandwidth of 55 kHz, and an asymmetric echo with an echo position of 25%; a flip angle of 15° was chosen to gain good contrast between blood and surrounding tissue. With a matrix of 256×256×96, a resolution of 105×105×78 μm per pixel was achieved at a total acquisition time of 12 minutes 17 seconds.

After a baseline 3D volume was acquired, mice were injected via the tail vein catheter with either the LIBS MPIO or control MPIO contrast agent (4×10⁶ MPIOs) in a total volume of 200 μL saline; we previously used this dose and volume of MPIOs successfully in a study of in vivo vascular cell adhesion molecule-1 imaging.15 The 3D FLASH sequence was then repeated with equal parameters and geometry. For the LIBS MPIO and control MPIO groups, MRI scans were performed continuously for up to 108±5 minutes.

To observe MPIO-induced signal void and to track platelet dynamics in vivo, we performed thrombolysis in a second cohort of LIBS MPIO-injected animals by injecting either human urokinase (Medac, Wedel, Germany) or recombinant mouse urokinase. Therefore, after the strongest LIBS MPIO–induced signal extinction was observed by MRI, either 50 000 IU human urokinase or 1200 IU mouse urokinase was injected in a total volume of 250 μL via the tail vein catheter, and repetitive MRI sequences were performed.

### Quantification of MPIO-Induced Signal Void

Image evaluation was Matlab based. For each time point, we calculated the quotient of mean signal intensity of the ROI in the thrombotic vessel divided by the mean of the region of interest in the contralateral, nonthrombotic vessel. These quotients were then normalized on the baseline 3D FLASH starting value, gaining a time series of the fractional amount of remaining signal after contrast agent injection. Further descriptions are given in the supplementary Methods section.

### LIBS MPIO Binding to Old Thrombi

To evaluate whether LIBS MPIO can be used to detect old platelet-containing thrombi, we induced wall-adherent thrombi in the carotid artery of mice using the above protocol and injected LIBS MPIO either 24 hours, 48 hours, or 7 days after the injury (n=3 per group). Two hours after injection, animals were killed, carotid arteries were removed, and tissue was stained for platelets as described later. Thrombus size as a percentage of total vessel lumen was quantified for each group, as well as the number of MPIOs averaged per section.

### Tissue Harvesting and Histology

After in vivo MRI was performed, animals were deeply anesthetized with ketamine and xylazine. Transcardiac perfusions via the left ventricle were carried out with saline. The injured carotid was removed, embedded in optical coherence tomography TissueTec (Sakura Finetec, Loeterwoude, the Netherlands), and frozen for histology. Although thrombus weight would be a direct measure of thrombus size, the small size and the difficulty of reliably removing the thrombus in toto did not allow accurate quantification. For the...
Relaxivities and their errors are derived via linear regression on the relaxation enhancement protocol. Imaging parameters included a 45-ms TEeff, 6000-ms TR, 45° flip angle, TurboFactor of 1.17, and a high-resolution T2-weighted rapid acquisition with relaxation enhancement protocol. Imaging parameters were considered significant at values of 0.05.

Statistical Methods
Relaxivities and their errors are derived via linear regression on the exponentially fitted relaxation rates. The impact of LIBS MPIOs on MRI signal void and histological MPIO counts and the influence of thrombolysis on histologically measured vessel occlusion were tested with an exact, unpaired, 2-sided permutation test. Associations between variables were investigated by correlation analyses. Test results were considered significant at values of P<0.05.

Results
Relaxivity Measurements With MPIO Dilution Series
Relaxivity (r) measurements yield information on the contrast agent characteristics of the MPIOs. In this context, r2* is especially of interest because it is directly responsible for the observed signal attenuation. The longitudinal relaxivity measurements of the MPIOs revealed a negligible r1 of 0.05±0.01 mmol⁻¹ · s⁻¹. The T2-weighted dilution series images acquired at a TE of 15 ms with a spin-echo sequence (Figure 1A) and the T2* gradient-echo images acquired at a TE of 3.5 ms (Figure 1B) depict examples of the signal void induced through evenly distributed MPIOs. The spin-echo sequence yields an r1 of 121.2±6.81 mmol⁻¹ · s⁻¹ at an R of 0.992 (Figure 1C). The relaxivity r2* (494.4±25.01 mmol⁻¹ · s⁻¹; R=0.994) is, as expected for the susceptibility effect induced by MPIOs, higher than r1 (Figure 1D). Calculated with the equation above, we would expect a 30% signal void in a FLASH sequence with a TE of 3.5 ms at a concentration of 0.21 mmol/L Fe, which corresponds well with the signal values displayed in Figure 1.

Results of Establishment of Wall-Adherent Thrombosis
After performing serial histology of carotid arteries exposed to ferric chloride concentrations of 3% to 10%, we observed reproducible platelet-containing wall-adherent thrombosis partially occluding (15% to 45%) the total vessel lumen in animals exposed with 6% ferric chloride (Figure 2A). Importantly, this semiocclusive thrombosis remained unchanged 1 hour after surgery as detected by a time course in histology (data not shown), which is an important prerequisite because in vivo MRI and contrast agent injection were started about this time point. Thus, we assured blood and contrast agent flow over the surface of the thrombosis.

In Vivo MR Platelet Imaging of Thrombosis and Thrombolysis
Carotid artery injury using 6% ferric chloride exposure for 3 minutes was performed without any side effects in mice. After the mouse was placed in the MRI scanner, a first MRI sequence was performed to obtain images before contrast agent injection. Thereafter, either LIBS MPIOs or control MPIOs were injected via the tail vein catheter contrast agent injection were started about this time point. Thus, we assured blood and contrast agent flow over the surface of the thrombosis.

Pharmacological thrombolysis was used to further evaluate whether LIBS MPIO–induced signal void intensity reports reliably on thrombus size, particularly size reduction. Furthermore, pharmacological thrombolysis is a good experimental model for one of the potential applications of the presented platelet-targeted imaging, which is the monitoring of the therapeutic success or failure of therapeutic approaches in thrombotic disease. Because the decrease in signal intensity in this first cohort of LIBS MPIO animals was clearly visible after 48 minutes (Figure 3), we decided to use this time point for thrombolytic therapy in the second cohort.
Therefore, 48 minutes after the injection of LIBS MPIOs, urokinase injection was performed. For the MRI data evaluation, only animals with an initial LIBS MPIO–induced signal drop comparable to the first cohort of LIBS MPIO animals were included in the thrombolysis group (Figures 4 and 5). Thus, we guaranteed that these animals had a degree of wall-adherent thrombosis comparable to the first cohort. After MRI data quantification in those animals, we observed a significant signal increase in the animals injected with human urokinase after 24 minutes \(n=5\) and in animals with mouse urokinase 36 minutes after injection of urokinase \(n=5;\) Figures 4C and 5) compared with the LIBS MPIO signal curve of the first cohort. Immunohistochemistry of animals confirmed an expected and significantly smaller thrombus after thrombolysis in such animals (Figure 2B and 2C; \(P<0.05\)). However, there was minor residual wall-adherent platelet aggregation in this group (Figure 2B), possibly explaining why the contrast effects did not dissipate completely and return to the level of the control MPIO group immediately after thrombolysis. Because signal intensity almost reached baseline values at the time point of thrombolysis in the control MPIO group and therefore the effect of thrombolysis would not have been detectable, we did not perform thrombolysis in control MPIO animals.

When animals first showed signs of hemodynamic instability, the running scan was interrupted and not used for data evaluation. For later time points \((t+96\) minutes and \(t+108\) minutes), we therefore have smaller numbers of animals \((n=3\) for control MPIO, LIBS MPIO, and human urokinase; \(n=4\) for mouse urokinase). Although limited by the maximal time of animals in the magnetic resonance scanner, the latest time point \((t+108\) minutes; Figure 5) demonstrated adaption of the signal effect of animals with LIBS MPIO injection toward control MPIOs and toward a steady state.

Figure 3. In vivo MRI after carotid artery injury. A, Localizer sequence with labeled anatomic landmarks: the noninjured left carotid artery (a), trachea (b), and injured right carotid artery (c). B and C, Transverse sections demonstrate the injured right carotid artery (red circle), which is well perfused despite wall-adherent thrombosis (negative control: noninjured left carotid artery [green circle]). After injection of control MPIOs, signal intensity in both vessels is similar at 12, 48, and 72 minutes. After LIBS MPIO injection, there is increasing signal drop at 12, 24, and 72 minutes compared with preinjection images and the noninjured left carotid artery. This represents the typical susceptibility artifact caused by MPIOs in T2*-weighted MRI and therefore indicates LIBS MPIO binding.

Figure 4. Treatment with intravenous mouse urokinase. Enlarged transverse sections of the injured right carotid artery. A, The vessel before injection, with a marked and progressive diminution in signal after LIBS MPIO injection between 12 and 48 minutes (B). Thrombolysis was performed after the 48-minute scan. Images obtained after intravenous application of urokinase show the reappearance of the vessel lumen signal over time indicated by (C).
MPIO Quantification in Carotid Artery

Thrombosis by Histology and Correlation to MRI Signal

Quantifying the number of MPIOs detected in the ferric chloride–induced thrombus (Figure 6A; arrows in the inlet depict the typical appearance of MPIOs in histology of frozen sections), we found a significantly higher number in the LIBS MPIO group compared with control MPIO and the thrombolysed groups of animals (P<0.01; Figure 6B). For the control MPIO group, we observed only random, probably nonspecifically attached MPIOs in the superficial, reticular thrombus network without penetration of MPIOs in deeper layers of the thrombus. Thrombus size in a representative selection of animals was measured in percent (Figure 6C) or micrometers squared (Figure 6D) of the total vessel lumen, and correlation with MRI signal extinction was highly significant. However, measurements of the percentage of thrombus occlusion yielded clearer differences compared with thrombus area because the first were not influenced as much by deformed vessel lumen or vessel shrinkage during processing of the histology specimens. When correlating these 2 measures of thrombosis in animals representing a wide range of thrombus size (2% to 40% of total vessel lumen) with the MRI signal void, we observed a strong correlation between those 2 parameters (Figure 6C and 6D). Although the luminal thrombus surface, which determines the number of adherent targeted LIBS MPIOs, may not perfectly reflect thrombus size, it seems to provide a sufficient correlation with thrombus size. In addition, volume average errors have to be taken into account in MRI studies validated by histology. However, the numbers of MPIOs detected in the thrombus correlated significantly with MRI-induced signal void (Figure 6E). These results indicate that the MRI signal void reflects the number of bound MPIOs and the extent of the wall-adherent platelet-derived thrombosis.

Detection of Activated Platelets in Old Thrombi

Thrombus size was small after 24 and 48 hours, and platelet-containing thrombus almost completely disappeared after 7 days (Figure 1 of the online Data Supplement; thrombus size after 24 hours, 3.5±0.6%; after 48 hours, 2.2±1.0%; and after 7 days, 0.2±0.1%; P<0.05 for 24 and 48 hours versus...
7 days). Despite this limitation, however, comparison of thrombus size and numbers of MPIOs in histology again revealed a significant correlation between those 2 parameters (Data Supplement Figure, B). Thus, LIBS MPIOs seem to be able to attach to older thrombi. However, further systematic large-scale time studies need to determine a potential cutoff in thrombus age in regard to LIBS MPIO binding.

Confirmation of LIBS MPIO Binding to Symptomatic Carotid Atherosclerosis in Humans

As initial evidence of the potential for LIBS MPIO imaging in humans, we performed ex vivo MRI of human endarterectomy specimens from patients with symptomatic carotid atherosclerosis. Typical MPIO-induced signal void could be imaged on the surface of an atherosclerotic plaque after incubation with LIBS MPIOs (Figure 7A) but not after incubation with control MPIOs (Figure 7B). LIBS MPIO binding as detected by MRI can be further confirmed by immunohistochemistry for platelets, which shows dense binding of LIBS MPIOs to superficial platelet adhesion/aggregation on the surface of the atherosclerotic plaque (Figure 7C).

Discussion

In the present study, we demonstrate in vivo MRI of activated platelets in wall-adherent thrombosis in the mouse carotid artery and the monitoring of thrombus dissolution in response to treatment. We also demonstrate ex vivo MRI of activated platelets on symptomatic human endarterectomy specimens. Importantly, the MRI contrast agent was directed against an activation-specific epitope on the platelet fibrinogen receptor GPlIb/IIIa (αIIb, β3, CD41/CD61). The activation of this integrin is the final common pathway of platelet activation, regardless of the platelet-activating stimulus, and GPlIb/IIIa is a major therapeutic target in acute coronary syndromes. The abundance of GPlIb/IIIa and its change in conformation on platelet activation make this receptor a unique, highly specific target for imaging. The magnitude of the contrast effects in detecting small platelet thrombi was striking and was derived from the use of MPIOs coupled to a platelet-targeting single-chain antibody. Two important technical features should be emphasized. First, the MPIOs used here are in the micron size range and are considerably larger than the ultrasmall particles of iron oxide previously used for similar applications.23 MPIOs create MRI signal voids far exceeding their own diameter, thus providing potent contrast that is readily resolved. Second, the single-chain antibody used confers function-specific binding to activated platelets via its specific binding to the ligand-induced binding site of GPlIb/IIIa that is exposed only on platelet activation and ligand (typically fibrinogen) binding.

MRI protocols and experimental setups were optimized to image exactly the same position of the vessel in repetitive measurements with high resolution and accuracy, which provides the opportunity for functional imaging of a specific and localized vascular pathology and its response to treatment. LIBS MPIO-induced signal extinction correlated well with the number of MPIOs counted in histology and with thrombus size. LIBS MPIOs bind to activated platelets early...
after application, providing a strong and early contrast effect, exceeding the background signal of circulating unbound contrast agent. These are important prerequisites for the transfer of this technology into clinical settings for exact localization and size determination of thrombi.

Recently, localization and quantification of platelet-rich thrombi in large blood vessels were performed with fluorescence-labeled platelets, which allowed imaging of clot formation and real-time imaging of thrombolysis. In this approach, $3.6 \times 10^{10}$ in vitro–labeled platelets were used to achieve good contrast properties. Conversely, the in vivo MR approach reported here uses only an exogenous reagent and does not require subject-specific preparation, which would not be practicable in the context of acute thrombotic syndromes.

Pharmacological thrombolysis was used as a model for therapeutic approaches in thrombotic disease. There are 2 important justifications for these experiments. First, for potential therapeutic applications, it is important not only to locate and quantify thrombosis but also to monitor the response to therapies such as fibrinolysis, platelet inhibition with GPIIb/IIIa antagonists, or dissolution through mechanical means. Such a capability would be a great asset in the management of acute vascular syndromes. Second, from an experimental point of view, the abolition of signal (because of the accumulation of targeted iron oxide in MPIOs) from the vessel lumen and the subsequent restoration of signal after thrombus dispersal provide important confirmation on the specificity of the contrast effect.

For the thrombolysis experiments, we used commercially available human urokinase and newly generated recombinant mouse urokinase (uPA) in our study. It is well known that murine plasminogen is more resistant to activation than human plasminogen. Therefore, differences in efficiency as seen in our study were to be expected. However, most interesting is our finding that differences in the timing of activation of both forms of urokinase are detectable with MRI. The clinically used preparation of human urokinase used in this study contains a mixture of nonactivated single-chain uPA and the activated form 2-chain uPA. In contrast, the newly generated recombinant mouse urokinase was a nonactive proenzyme single-chain uPA that needs to be converted to the active 2-chain form after injection. This difference explains the delayed thrombolysis seen in the comparison between mouse and human urokinase. The final extent of fibrinolysis was comparable to both forms of urokinase.

Sensitive in vivo detection of activated platelets may help to define the role of platelet activation in the pathogenesis of thromboembolism, atherothrombosis, and plaque rupture. Binding of this contrast agent to mouse and human platelets could facilitate transfer of data obtained in mice to humans. Besides the role of platelets in thrombosis, the potential role of platelets in inflammatory reactions has recently attracted major interest, and LIBS MPIOs may provide a means to investigate the role of platelets in inflammation. As a proof-of-principle study, we imaged fresh carotid end-arterectomy specimens from patients with acutely symptomatic carotid artery disease before and after incubation with LIBS MPIOs. LIBS MPIO binding was detected by MRI and further confirmed by immunohistochemistry for platelets. Further characterization in serial studies involving larger numbers of patient specimens is ongoing. However, the data presented here demonstrate in an exemplary manner the potential of LIBS MPIOs to recognize platelet adhesion/aggregation in acute and symptomatic atherothrombosis in humans. Prophylactic assessment of larger vessels by MRI will be technically challenging but clinically highly rewarding. A combination of clinical symptoms with MRI sequences obtaining anatomic information and additional functional imaging of activated platelets could help to identify patients at risk.

Another area of interest for MRI is the diagnosis and monitoring of the therapeutic success or failure of venous thrombotic disease and pulmonary artery embolism. However, the role of platelets and platelet activation in venous thrombosis...
is not as relevant as in arterial thrombosis. Therefore, whether platelet-targeted imaging is suitable for imaging of venous thrombi/emboli has yet to be determined. However, fibrin is another target that provides clot specificity and seems to be of particular importance in venous clots but also may be suitable for targeting of arterial clots. We have previously used a fibrin-specific single-chain antibody for therapeutic targeting, but this system is adaptable to MPIO targeting.27

A possible limitation of MPIO thrombus imaging is the size of the particle itself. Thrombus formation and existence is a dynamic process; MPIOs are randomly caught nonspecifically in the superficial, reticular thrombus network observed in the histology of control MPIO–injected animals. However, the numbers seem to be too small to induce detectable signal voids because the signal curve of control MPIO animals goes back to baseline over time (Figure 5). Even when larger (4.5-μm) MPIOs are used, there do not appear to be issues of capillary plugging, infarction, or inflammation, and MPIOs are rapidly sequestered by the spleen and liver.16 Shear stress is an important determinant of the binding characteristics of micrometer-sized particles. Using 4.5-μm MPIOs, we previously adopted a multiple ligand technique to address this challenge in the context of vascular inflammation.16 We took an alternative approach, downsizing the MPIOs to 1 μm, anticipating that such particles would be less “buoyant” and have attained sufficient MPIO binding under flow conditions to obtain significant contrast effects.

The MPIOs used in our study have not been used in human in vivo applications so far. However, no toxicity after MPIO injection was observed in any animal in our study, and organ histology did not indicate clotting or vascular microembolism (data not shown). The development of micrometer-sized particles for human applications is ongoing.28,29 and the available animal data are highly promising.13 In addition, for eventual clinical applications, the use of biodegradable iron–rich particles is envisaged.30 Furthermore, single-chain antibodies are highly versatile as targeting agents. They can be tailored in size and coupling moieties for various applications using molecular biology tools.31 Antigenicity may be avoided with the use of purely human sequence–derived antibodies.23–25 Bifunctional or multifunctional single-chain antibodies targeting ≥2 epitopes also can be designed, allowing more stable targeting, as shown previously.16

The reflection of our histological findings by MRI measurements is remarkable. A transverse relaxivity r₂* of 494.4 L · mmol⁻¹ · s⁻¹ results in a concentration of 0.16 mmol iron required for a 20% signal attenuation with the 3D FLASH sequence. Assuming an arterial vessel diameter of 0.42 mm and a histological slice thickness of 10 μm, this corresponds to an approximate number of 46.5 MPIOs in the relevant volume. Histology confirmed the number of MPIOs in the LIBS MPIO–injected animals to range from 20 to 40 MPIOs per slice. These slightly lower numbers can possibly be explained through the nonphysiological high-shear-stress conditions generated through the flushing procedure before tissue harvesting, washing away some amount of micrometer-sized particles. Numbers of histologically confirmed MPIOs in thrombi of control MPIO and thrombolysed animals do not differ significantly, although the signal void in urokinase-treated animals after thrombolysis does not go back to control MPIO baseline. This might be explained by a number of MPIOs bound to the surface of the remaining thrombus in thrombolysed animals, which are removed during histology preparation by the effect described above. Therefore, the numbers of MPIOs on the thrombus surface in LIBS MPIO–injected animals might be even higher than observed in our histology studies.

Conclusions

We are able to image in vivo wall-adherent platelets and small platelet-containing thrombi using a contrast agent that specifically binds to the activated GPIIb/IIIa on platelets. Going a step further, we also demonstrate the feasibility of direct monitoring of the success or failure of thrombolytic treatment. LIBS MPIO–induced signal void quantitatively reflected the numbers of MPIOs bound and the thrombus size. Together with the excellent contrast properties, these characteristics allow high-resolution, sensitive, and quantitative in vivo platelet-derived thrombus imaging. Thus, the described targeted MRI contrast agent represents a novel and unique noninvasive imaging technique that allows the detection and quantification of thrombi and can be used to monitor the success or failure of thrombolytic therapy. Notably, pilot data with ex vivo MRI of carotid endarterectomy specimens suggest that LIBS MPIOs are suitable for diagnostic procedures in humans. Overall, these properties are a promising basis for further development and application of LIBS MPIO–based contrast agents for detection of arterial thrombi and monitoring of therapeutic interventions. Furthermore, studies that investigate the potential of LIBS targeting to identify the role of platelets in the development of atherosclerotic plaques in humans are warranted.

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Disclosures

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