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_Circulation_. 2013;128:729-736; originally published online July 2, 2013;
doi: 10.1161/CIRCULATIONAHA.113.001371

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/128/7/729

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/07/02/CIRCULATIONAHA.113.001371.DC1.html

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Deep venous thrombosis (DVT) affects 1 in 500 of the elderly population per year.¹ Its sequelae include pulmonary embolism, which may be fatal, and the postthrombotic syndrome, which causes chronic morbidity such as leg ulceration that is both debilitating and expensive to treat.² The diagnosis of DVT is challenging because the symptoms and signs are nonspecific.³ Confirmation of thrombosis relies on imaging, but current methods provide little information on thrombus structure or composition, and thrombus burden is generally assessed by the presence or absence of a filling defect in the vein.

Clinical Perspective on p 736

The speed of thrombus resolution is the main determinant of subsequent outcome following DVT, both in terms of valve preservation and long-term complications.⁴–⁶ Catheter-directed thrombolysis can reduce postthrombotic syndrome in patients with acute proximal DVT,⁷ and, with the increasing use of pharmacomechanical delivery systems that have potential to improve the efficiency of thrombolysis, the paradigm for the management of patients with DVT is changing.⁸ Currently,

Background—The magnetic resonance longitudinal relaxation time (T₁) changes with thrombus age in humans. In this study, we investigate the possible mechanisms that give rise to the T₁ signal in venous thrombi and whether changes in T₁ relaxation time are informative of the susceptibility to lysis.

Methods and Results—Venous thrombosis was induced in the vena cava of BALB/C mice, and temporal changes in T₁ relaxation time correlated with thrombus composition. The mean T₁ relaxation time of thrombus was shortest at 7 days following thrombus induction and returned to that of blood as the thrombus resolved. T₁ relaxation time was related to thrombus methemoglobin formation and further processing. Studies in inducible nitric oxide synthase (iNOS⁺−)–deficient mice revealed that inducible nitric oxide synthase mediates oxidation of erythrocyte lysis–derived iron to paramagnetic Fe³⁺, which causes thrombus T₁ relaxation time shortening. Studies using chemokine receptor-2–deficient mice (Ccr2−−) revealed that the return of the T₁ signal to that of blood is regulated by removal of Fe³⁺ by macrophages that accumulate in the thrombus during its resolution. Quantification of T₁ relaxation time was a good predictor of successful thrombolysis with a cutoff point of <747 ms having a sensitivity and specificity to predict successful lysis of 83% and 94%, respectively.

Conclusions—The source of the T₁ signal in the thrombus results from the oxidation of iron (released from the lysis of trapped erythrocytes in the thrombus) to its paramagnetic Fe³⁺ form. Quantification of T₁ relaxation time appears to be a good predictor of the success of thrombolysis. (Circulation. 2013;128:729-736.)

Key Words: macrophages ■ magnetic resonance imaging ■ therapeutic thrombolysis ■ venous thrombosis

Magnetic Resonance T₁ Relaxation Time of Venous Thrombus Is Determined by Iron Processing and Predicts Susceptibility to Lysis

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only fresh thrombi are considered for lysis, because it is thought that the collagenous, older thrombi are less responsive to this treatment. The use of clinical history recalled by the patient and signs at presentation are subjective, however, and an unreliable method for estimating thrombus age, and there is no consensus defining the exact timing for intervention. Not all thrombi defined as fresh respond to lysis, exposing the patient to an unnecessary risk of hemorrhage associated with this treatment; whereas some older thrombi have an effective lytic response. An objective method to identify thrombi susceptible to lysis would, therefore, improve the care of patients presenting with thrombosis.

Advances in MRI have made it possible to image venous thrombi with high sensitivity (MR direct thrombus imaging). This method relies on the shorter longitudinal T1 relaxation time of the thrombus in comparison with blood. Spatial quantification of the longitudinal relaxation time is made possible by using a T1-mapping technique that we have previously employed in humans. The biological correlate of the T1 relaxation time is not known, however. In vitro studies suggest that changes in T1 are caused by the accumulation of methemoglobin (metHb) in the thrombus, but this has not been validated in vivo because venous thrombi are not routinely removed from patients. In this study, we use an experimental murine model of venous thrombosis to investigate the mechanisms that give rise to the T1 signal in vivo and examine whether T1 is informative of the susceptibility of thrombus to lysis.

**Methods**

**Study Approval**

Mouse procedures were performed under the UK Animals (Scientific Procedures) Act, 1986. Ethical approval for the collection of human blood samples was obtained from the local research ethics committee at Guy’s & St Thomas’ NHS Foundation Trust.

**Generation of metHb In Vitro**

Five milliliters of blood was collected from 8- to 10-week-old (22–30 g) male BALB/C mice and human volunteers into 3.2% sodium citrate by using standard venipuncture techniques. Ten milligrams of diethylamine NONOate (Merck) was dissolved into 1.25 mL of 0.9% saline to make 4 mmol/L solution. Several concentrations (400, 300, 200, 100, and 0 μmol/L) of NONOate were prepared by dilution with normal saline, and 0.1 mL of each solution added to separate aliquots of human and mouse blood. Containers were incubated in a water bath at 37°C for 15 minutes. Samples were imaged by the use of MRI before analysis for metHb with the use of a blood gas analyzer (Roche). metHb measurements for human and murine blood were validated in 20 separate samples by the use of an established colorimetric method.

**Animal Model**

Venous thrombosis was induced in the inferior vena cava (IVC) of 8- to 10-week-old (22–30 g) male mice in a surgical procedure that involved a combination of reduced blood flow and endothelial disturbance. The infrarenal portion of the IVC was exposed through a midline laparotomy, and a 5-mm segment of the IVC just below the left renal vein was dissected out. A length of 5-0 polypropylene suture was placed alongside the IVC, and a 4-0 silk ligature (Ethicon Ltd) was passed around the IVC, incorporating the polypropylene suture just below the left renal vein. The ligature was tightened and tied, and the polypropylene was withdrawn, leaving a stenosis in the IVC causing an ≈80% to 90% reduction in blood flow. Endothelial damage to this portion of the IVC was then induced by the application of a neurosurgical vascular clip (Braun Medical) for 15 s on 2 occasions, 30 s apart.

MRI Methods

Mice were imaged with the use of a clinical 3-Tesla Achieva Gyroscan MR scanner (Philips Healthcare, Best, The Netherlands) with a clinical gradient system (30 mT/m, 200 mT/m per ms). The rationale for use of a clinical scanner instead of a preclinical high-field system was to facilitate the translation of the results into clinical practice. All the images were acquired by using a dedicated 47-mm, single-loop, small-animal surface receiver coil to maximize the MR signal. Mice were anesthetized with 1.5% to 2% isoflurane and 100% oxygen delivered through a nose cone and scanned in prone position. Mice were monitored with the use of an MRI-compatible video camera system (Philips Healthcare, Best, The Netherlands). To quickly identify mouse anatomy, an initial survey scan was acquired. Scout imaging was performed by using low-resolution, multiplanar images with a multislice, segmented, fast gradient echo pulse sequence. The field of view of the scout images encompassed the full dimensions of the mouse body. A flow-sensitive time-of-flight sequence was used to identify the IVC and the abdominal aorta, which was used as a fixed anatomic landmark. The filling defect attributable to the presence of thrombus in the IVC was used as a measure of thrombus volume. The imaging parameters of this spoiled gradient echo sequence were as follows: echo time/repitition time=6.2/40 ms, flip angle=60°, a field of view=20×33 mm, slice thickness=80, slice thickness=0.3 mm, acquired matrix=68×110, acquired resolution=0.3×0.3×0.5 mm3 and reconstructed resolution=0.13×0.13×0.5 mm3, averages=2. The average velocity of blood was measured across the IVC to confirm the presence of a thrombus and to measure the changes in recanalization over time. A phase-contrast sequence was used to quantify blood flow with the following imaging parameters: spatial resolution=100×100 μm, slice thickness=2 mm, repetition time/echo time=17.5/7.2 ms, flip angle=30°, averages=6, and velocity encoding=15 cm/s.

After the acquisition of the venous and arterial time of flight, the data sets were used to plan the location of a 3-dimensional T1-mapping sequence on the thrombus. The fast T1-mapping sequence consisted of a modified Look-Locker sequence. A 3-dimensional volume covering the infrarenal IVC was planned to acquire 16 time points for each pixel. A long relaxation delay of 3 s after each acquisition was used to ensure magnetization recovery. The other sequence parameters were repetition time/echo time 9.0/4.6 ms and flip angle 10°, field of view=36×22 mm, acquired matrix=180×102, measured slice thickness=0.5 mm, acquired resolution=0.2×0.2 mm, reconstructed resolution=0.1×0.1 mm, slice thickness=30, averages=1. Total scan time for this sequence was 28 minutes. T1 maps were calculated for each slice by the use of custom-made software implemented in Matlab (Mathworks, Natick, MA).

**Imaging Schedules**

The T1 relaxation time in the region of the IVC without thrombus was measured in 6 mice to provide a baseline value for blood. Initial longitudinal imaging experiments were performed to determine the temporal changes in thrombus T1 relaxation time during resolution. Natural thrombus resolution in our mouse model of thrombosis occurs over a period of 28 days, with the majority of the cellular changes occurring between days 1 and 14. We therefore chose to consecutively image at 1, 4, 7, 10, 14, 21, and 28 days after induction. This sequential imaging was performed by using 6 mice, because we have previously found that this number is sufficient to show changes in other MRI parameters in venous thrombosis. At 28 days, all thrombi were processed for histological analysis (Figure IA in the online-only Data Supplement).

Further experiments were then performed to examine the relationship between T1 relaxation time, thrombus structure, and thrombus iron content. In a cross-sectional study design, T1 relaxation times of thrombi were measured at 1, 4, 7, 10, 14, and 21 days after induction (n=14 per time point). At each time point, thrombi from 8 mice were harvested and used to measure the total iron (4 mice) and Fe²⁺ concentration (4 mice), whereas thrombi from the remaining 6 mice were processed for histological analysis of fibrin and collagen content (Figure IB in the online-only Data Supplement).
Thrombi were also induced in the IVC of iNOS−/− and Ccr2−/− mice (n=6 per group) to investigate the iron-processing mechanisms that give rise to the T₁ magnetic signal. In these strains, thrombus was consecutively imaged at 1, 4, 7, 10, 14, and 21 days after induction. A schematic of the imaging protocols is shown in Figure IA in the online-only Data Supplement.

**Histology**

The position of the thrombus in relation to anatomic landmarks that were imaged (renal and iliac vessels) was measured before harvest by using a Vernier caliper. All samples were taken en bloc to include the portion of IVC containing thrombus and the aorta from the top of the suture to the confluence of the iliac veins. IVC containing thrombus and aorta were pinned on to cork mats to the same length as that measured before harvest and placed in 10% formalin for 24 hours before being embedded in wax. Thrombus was remeasured before embedding to determine whether any shrinkage had taken place. Paraffin sections (5 µm) were taken at 500-µm intervals throughout the length of the IVC to correspond with MR slices. Sections were stained by using hematoxylin and eosin for anatomic detail and Martius Scarlet Blue for red blood cell, fibrin, and collagen content. Digital images of stained sections were captured and processed by using image analysis software (Image Pro-plus 7, MediaCybernetics) with the percentage area of thrombus containing each stain calculated (Figure II in the online-only Data Supplement). Thrombus fibrin and Martius Scarlet Blue content was validated by the use of immunohistochemistry and Western blotting20 (Figure III in the online-only Data Supplement).

**Image Analysis**

Data sets were exported from the MRI scanner and anonymized. T₁ maps were then calculated and imported into OSIRIX for analysis by measuring the value of signal intensities and standard deviations on T₁ maps in user-specified regions of interest placed over thrombus by using a threshold of 1300 ms. Independent observers (n=5) were oriented to MRI murine anatomy before analyzing thrombus T₁ relaxation time to show the interobserver variability (Figure IV in the online-only Data Supplement).

**Iron Quantification**

Inductively coupled mass spectroscopy (PE 200 LC system linked to PerkinElmer Sciex Elan 6100 DRC, PerkinElmer, Boston, MA) was performed on a subset of thrombus samples for total iron concentration quantification. Thrombus was digested in 70% nitric acid at 37°C for 24 hours before being embedded in wax. Thrombus was remeasured before embedding to determine whether any shrinkage had taken place. Paraffin sections (5 µm) were taken at 500-µm intervals throughout the length of the IVC to correspond with MR slices. Sections were stained by using hematoxylin and eosin for anatomic detail and Martius Scarlet Blue for red blood cell, fibrin, and collagen content. Digital images of stained sections were captured and processed by using image analysis software (Image Pro-plus 7, MediaCybernetics) with the percentage area of thrombus containing each stain calculated (Figure II in the online-only Data Supplement). Thrombus fibrin and Martius Scarlet Blue content was validated by the use of immunohistochemistry and Western blotting20 (Figure III in the online-only Data Supplement).

**Thrombolysis**

IVC thrombi were induced in 36 mice. Mice were imaged between day 2 and day 21 following thrombus induction by the use of the T₁-mapping protocol described. Immediately following MRI scanning, 200 µL of tissue plasminogen activator solution (10 mg/kg, Actilyse) was administered via a tail vein injection over 5 minutes. Phase-contrast images were used to estimate blood flow in the IVC before and 24 hours after thrombolytic therapy. Thrombolysis was considered successful if there was at least an increase of 50% in IVC blood flow in comparison with prelysis blood flow. The imaging protocol is shown in Figure IC in the online-only Data Supplement.

**Statistical Analysis**

Data were inserted into PRISM version 5 (GraphPad) for analysis. Continuous data are expressed as mean±standard error of the mean. One-way analysis of variance (ANOVA) was used to measure changes in T₁ relaxation time, thrombus size and composition, IVC flow, and thrombus iron content over time, and to determine the relationship between T₁ relaxation time and susceptibility to lyse. Two-way ANOVA was used to analyze the difference between red blood cell and iron content over time, changes in T₁ relaxation times in individual mice over time, and all comparisons between iNOS−/− and Ccr2−/− mice and their respective wild-type controls. Post hoc test with appropriate Bonferroni correction was made, and the values after the correction were given.

**Results**

**Imaging Murine Venous Thrombi by the Use of MRI**

Murine metHb was comparable to human metHb in shortening T₁ relaxation time in vitro with T₁ shortening dependent on concentration of metHb in both species (Figure V in the online-only Data Supplement, murine blood, R²=0.69, P<0.0001, n=25; and human blood, R²=0.74, P<0.0001, n=25). An experimental model of venous thrombosis was therefore used in subsequent experiments. Thrombi in our model of thrombosis naturally resolved over a 4-week period, with thrombus volumes (mm³) decreasing gradually over this time (day 1, 17.6±0.9; day 4, 12.6±0.8; day 7, 10.6±0; day 10, 7.6±0.6; day 14, 4.1±0.4; day 21, 0.9±0.2; day 28, 0.3±0.1, 1-way ANOVA, P<0.0001; Figure VI in the online-only Data Supplement).

Phase-contrast flow measurements across the IVC confirmed the presence of thrombus. The average blood flow in the IVC, midway between the confluence of the iliac veins and the renal vessels, was 0.428±0.04 mL/min in the steady state. After almost complete occlusion at day 1, an increase in blood flow (mL/min) was detected in the IVC following thrombosis, and this increased progressively as the thrombus resolved (day 1, 0.007±0.002; day 4, 0.031±0.008; day 7, 0.072±0.015; day 10, 0.193±0.013; day 14, 0.246±0.017; day 21, 0.423±0.019; day 28, 0.448±0.041, 1-way ANOVA, P<0.0001; Figure VI in the online-only Data Supplement). There was a significant reduction in flow at days 1, 4, 7, 10, and 14 in comparison with IVC without thrombus (Bonferroni multiple comparison test, P<0.001). There was no difference in flow at days 21 and 28 in comparison with flow across the IVC without thrombus (Bonferroni multiple comparison test, P>0.05).

**T₁ Relaxation Time of the Thrombus**

The mean T₁, relaxation time of venous blood in mice without thrombus was 1557±25 ms. Thrombus was defined as a volume of tissue within the infrarenal IVC (in which there was a filling defect and reduction in blood flow) with a T₁ relaxation time of <1300 ms. This arbitrary cutoff was used, because it was >2 standard deviations from the mean T₁, relaxation time of blood. Mean T₁ relaxation times (ms) of the thrombus changed following thrombus induction (day 1, 866±19; day 4, 758±12; day 7, 631±18; day 10, 671±21; day 14, 741±23; day 21, 970±29; day 28, 1196±42, P<0.0001, 1-way ANOVA; Figure I and Figure VII in the online-only Data Supplement). As the thrombus resolved, the T₁, relaxation time returned to that of
blood. The relationship was similar between individual mice over time (Figure VII in the online-only Data Supplement).

Red Blood Cells and Iron Content of the Thrombus During Its Resolution

The total thrombus iron content (μg), measured by mass spectrometry, was highest 1 day after induction (day 1, 10.2±2.3; day 4, 6.3±0.6; day 7, 6.2±1.0; day 10, 5.1±0.3; day 14, 4.0±0.1; day 21, 0.5±0.1; Figure 2A), when the red blood cell content was greatest. As the thrombus resolved, iron could still be detected in the thrombus microenvironment despite an absence of red blood cell staining (Figure 2A). The concentration of Fe³⁺ in the thrombus (μg/dL per mg of thrombus) changed with time, and was highest at day 7, when T1...
relaxation times were shortest (day 1, 3.1±0.3; day 4, 4.6±0.5; day 7, 6.6±0.8; day 10, 4.6±0.8; day 14, 2.1±0.9; day 21, 0.2±0.1, 1-way ANOVA, P<0.0001; Figure 2B).

Thrombus $T_1$ Relaxation Times in $\text{iNOS}^{-/-}$ Mice
Thrombus in $\text{iNOS}^{-/-}$ mice had longer $T_1$ relaxation times (ms) (day 1, 1094±16; day 4, 1048±21; day 7, 962±25; day 10, 882±49; day 14, 950±56; day 21, 1108±50) than $\text{iNOS}^{+/+}$ controls (day 1, 1094±16; day 4, 1048±21; day 7, 962±25; day 10, 882±49; day 14, 950±56; day 21, 1108±50) and then increased during resolution (day 1, 871±25; day 4, 712±23; day 7, 693±25; day 10, 688±29; day 14, 770±35; and day 21, 1127±40, 2-way ANOVA, P<0.0001; Figure VIII in the online-only Data Supplement), after which the collagen content (the percentage of thrombus area stained for collagen) appeared to predominately decrease after day 1, with the percentage of thrombus area stained for red blood cells as follows: day 1, 93±1%; day 4, 71±2%; day 7, 28±1%; day 10, 8±1%; day 14, 2±0.2%; day 21, 1±0.2%; day 28, 0.2±0.2% (1-way ANOVA, P<0.0001; Figure XC in the online-only Data Supplement). The percentage of thrombus area stained for fibrin increased between days 1 and 10 (day 1, 0±0%; day 4, 0±0.1%; day 7, 2±0.2%; day 10, 70±2%; day 14, 54±3%; day 21, 5±1%; day 28, 2±0.5%, 1-way ANOVA, P<0.0001; Figure XB in the online-only Data Supplement), after which the collagen content (the percentage of thrombus area stained for collagen) appeared to predominate (day 1, 0±0%; day 4, 0.1±0.03%; day 7, 2±0.2%; day 10, 10±1%; day 14, 23±1%; day 21, 47±2%; day 28, 51±2%, 1-way ANOVA, P<0.0001, Figure XC in the online-only Data Supplement). Fibrin staining was greatest in thrombi with the shortest $T_1$ relaxation times, irrespective of thrombus age (Figure 3A).

Figure 2. Mechanisms governing the generation of $T_1$ signal in venous thrombi. A, The total iron content of the thrombus (black bars) quantified by using mass spectrometry (n=4/gp) is greatest immediately after thrombus induction when red blood cell content (red line) is high (n=6/gp). During thrombus resolution, the iron content falls, but it remains relatively higher than the red blood cell content of the thrombus (mean±SEM shown, P<0.0001, 2-way ANOVA). B, Fe$^{3+}$ concentration (μg/Dl per mg of thrombus) analyzed with the use of a colorimetric iron assay kit at different time points mirrors the $T_1$ signal during thrombus resolution (mean±SEM shown, n=7/gp, P<0.0001, 1-way ANOVA with Bonferroni post test analysis, P<0.05, **P<0.01, ***P<0.001). C, $T_1$ relaxation times (ms) of the thrombus are significantly longer during thrombus resolution in $\text{iNOS}^{-/-}$ in comparison with wild-type controls (mean±SEM, n=6/gp, P<0.0001, 2-way ANOVA. Bonferroni post test: "*P<0.01, "**P<0.001"). D, $T_1$ relaxation times (ms) of the thrombus remain persistently short in $\text{CCR2}^{-/-}$ mice during resolution (mean±SEM, n=7/gp, P<0.0001, 2-way ANOVA, Bonferroni post test: "*P<0.05, "**P<0.001"). Mouse procedures were performed under the Animals (Scientific Procedures) Act, 1986, UK. ANOVA indicates analysis of variance; gp, group; and SEM, standard error of the mean.

Structure of Experimental Venous Thrombi During Resolution
The composition of the thrombus varied spatially and temporally during its resolution (Figure 1B and Figure X in the online-only Data Supplement). Red blood cell content progressively decreased after day 1, with the percentage of thrombus area stained for red blood cells as follows: day 1, 93±1%; day 4, 71±2%; day 7, 28±1%; day 10, 8±1%; day 14, 2±0.2%; day 21, 1±0.2%; day 28, 0.2±0.2% (1-way ANOVA, P<0.0001; Figure XC in the online-only Data Supplement), after which the collagen content (the percentage of thrombus area stained for collagen) appeared to predominate (day 1, 0±0%; day 4, 0.1±0.03%; day 7, 2±0.2%; day 10, 10±1%; day 14, 23±1%; day 21, 47±2%; day 28, 51±2%, 1-way ANOVA, P<0.0001, Figure XC in the online-only Data Supplement). Fibrin staining was greatest in thrombi with the shortest $T_1$ relaxation times, irrespective of thrombus age (Figure 3A).

Thrombolysis of Experimental Venous Thrombi
Fibrinolysis had the greatest effect in thrombi with the shortest $T_1$ relaxation time, regardless of thrombus age (Figure 3B). Receiver operator curve analysis demonstrated that $T_1$ relaxation time was a good predictor of successful thrombolysis (area under the curve, 0.954, P<0.0001; 95% confidence interval, 0.894–1.00). A cutoff point of $T_1$ relaxation time <747 ms had a sensitivity and specificity to predict successful lysis of 83% and 94%, respectively.
Discussion

In vitro studies suggest that shortening of \( T_1 \) relaxation time of venous thrombi in humans is caused by the paramagnetic properties of Fe\(^{3+}\) in the metHb that accumulates.\(^{12}\) This, however, has not been validated in vivo. The shortening of \( T_1 \) relaxation time by murine metHb is comparable to that of human metHb ex vivo, and we therefore optimized a \( T_1 \)-mapping protocol, previously used to image DVT in humans,\(^{11}\) to analyze thrombus in an established murine model of venous thrombosis.\(^{16,17}\)

The mean \( T_1 \) relaxation time of thrombus was shortest at 7 days following thrombus induction and returned to that of blood as the thrombus resolved. The total thrombus iron content, measured by mass spectrometry, was highest 1 day after induction when the red blood cell content was greatest. These heme-containing cells, which are the source of iron in the thrombus, are trapped by the cross-linked fibrin that is generated during thrombogenesis. We speculate that red blood cell lysis, previously described at sites of vascular injury,\(^{21}\) releases iron into the thrombus microenvironment, and this could explain why relatively higher levels of iron are found despite decreasing red blood cell content.

Paramagnetic Fe\(^{3+}\) concentrations increased in the thrombus until day 7, when \( T_1 \) relaxation time was shortest. Nitric oxide is a powerful inducer of metHb in vitro, whereas inflammatory cell accumulation is a characteristic of thrombus resolution.\(^{16,22}\) This led us to hypothesize that inducible nitric oxide synthase (iNOS) could be a source of nitric oxide that gave rise to the iron oxidation and \( T_1 \) shortening observed in this study. Thrombus \( T_1 \) relaxation times were consistently longer in \( iNOS^{-/-} \) mice than in wild-type controls, confirming that nitric oxide production is important for the generation of a \( T_1 \) signal following induction of venous thrombosis. Other oxidative mechanisms are, however, also likely to be important, because there was moderate shortening in \( T_1 \) relaxation times observed from thrombi in these mice.

Inflammatory monocytes, characterized by Ly6C expression in the mouse, use iNOS for antimicrobial defense against disease.\(^{23}\) Macrophage accumulation is a hallmark of venous thrombus resolution,\(^{18,22}\) and, although their function in thrombosis remains largely speculative, we hypothesized that these were the source of iNOS responsible for nitric oxide production and the oxidation of iron. The CC chemokine receptor 2 is required for the emigration of inflammatory monocytes from the bone marrow and their recruitment to inflammatory tissues.\(^{24}\) Previous studies show that thrombus resolution is impaired in \( Ccr2^{-/-} \) mice, and this is associated with a reduced accumulation of macrophages in the thrombus.\(^{25,26}\) Shortening of \( T_1 \) relaxation time in thrombi formed in \( Ccr2^{-/-} \) mice was, however, similar to that in wild-type controls suggesting that inflammatory monocyte/macrophages were not the source of nitric oxide in the thrombus as we had hypothesized. Neutrophils are also capable of producing iNOS and accumulate during early thrombogenesis.\(^{27,28}\) We therefore speculate that these cells could be the effectors of the thrombus \( T_1 \) shortening that we observed between days 1 and 7 after induction.

Although the deletion of CC chemokine receptor 2 had no effect on thrombus \( T_1 \) shortening, an absence of thrombus macrophages in \( Ccr2^{-/-} \) mice affected \( T_1 \) relaxation time during the latter phase of thrombus resolution. Hemosiderin-laden macrophages (containing Fe\(^{3+}\)) are known to accumulate in venous thrombi from both wild-type mice and in humans. Iron in these cells is unable to exert a \( T_1 \) effect because of the large cluster size and the water insolubility of hemosiderin.\(^{30}\) Without macrophages, Fe\(^{3+}\) remains in the thrombus environment in the latter phases of resolution and exerts a \( T_1 \)
relaxation time-shortening effect. Recent evidence suggests that following intraplaque hemorrhage, the accumulation of heme in macrophages leads to an atheroprotective phenotype in which genes central to cholesterol efflux are induced via activating transcription factor-1. It would be interesting to investigate whether macrophage iron uptake in venous thrombi is important to stimulate thrombus resolution by phagocytosis or through the production of angiogenic, proteolytic, and fibronolytic factors that influence remodeling.

Thrombolytic treatments are designed to lyse fibrin in venous thrombi, with conventional wisdom suggesting that young thrombi, defined by clinical history, are most readily lysed. In this study, thrombus fibrin content was greatest between days 7 and 10 following induction, the point at which thrombus T1 relaxation time was shortest. Administration of tissue plasminogen activator had the greatest effect in experimental thrombi with the shortest T1, regardless of thrombus age. Older thrombi with high collagen content did not respond well to lysis, but unexpectedly very young thrombi had an equally poor response. There is evidence from studies in humans to show that not all young venous thrombi can be lysed, while early preclinical studies of thrombolysis using tissue plasminogen activator show that this agent more readily lysed 7-day-old thrombi in comparison with 1- or 3-day-old thrombi in a rabbit model of this condition. The results from our study are therefore not all together surprising. One explanation for our finding of impaired lysis in 1- or 4-day-old thrombi could be the result of a shielding effect by red blood cells. Recent in vitro studies show that the presence of red blood cells confers resistance to fibrinolysis through the modification of fibrin structure and impairment of plasminogen activation. Whether a similar mechanism exists in vivo requires further investigation.

Conclusion

These data shed light on the biological mechanisms that affect T1 relaxation during thrombus resolution in vivo. Iron, brought into the thrombus by fibrin-trapped red blood cells, is oxidized through iNOS, resulting in the accumulation of paramagnetic Fe3+, which shortens T1. Removal of Fe3+ by action of macrophages returns T1 relaxation time to that of blood. Quantification of T1 relaxation time was informative of the susceptibility of a thrombus to lysis, whereas our use of a clinical 3-T field strength MRI scanner without a contrast agent facilitates immediate translation of this technique to the clinic.

Acknowledgments

We thank Prof Beverly Hunt for supplying the method for the generation of methemoglobin in vitro and Prof Frederic Geissmann for supplying the knockout mice used in this study. We also thank Roshini Joseph for her help with the fibrin quantification and Dr Victoria Cornelius for support with statistical analysis.

Sources of Funding

This work was supported by the British Heart Foundation Project grant (PG/08/039/24436, to Dr Schaeffter, Waltham, and Smith), Wellcome Trest (WT090252MA, to Dr Saha), and the Chilean Agency for Research in Science and Technology (CONICYT, to Dr Andia).

Disclosures

The MRI scanner is supported in part by Philips Healthcare, Best. Dr Wiethoff is an employee of Philips Healthcare. All other authors were not consultants or employees of Philips Healthcare and had control of inclusion of any data that might present a conflict of interest for Dr Wiethoff. The other authors report no conflicts.

References


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The paradigm of deep venous thrombosis management is changing with a greater focus on restoring vein patency by the use of catheter-directed thrombolysis and pharmaco-mechanical adjuncts with the aim of reducing the incidence of the post-thrombotic syndrome. Selection criteria used to identify thrombi that are most susceptible to lysis remain controversial. Thrombus age is currently the main determinant for thrombolysis, but clinical history and signs at presentation are subjective and unreliable. Not all young thrombi can be successfully lysed, whereas some old thrombi may be amenable to lysis. In this study, we show that fast T1 mapping can be used as a surrogate measure of the organization of experimental venous thrombi and that this technique identifies those thrombi most suitable for lysis. Experiments in this study were performed with the use of a clinical 3-T field strength MRI scanner without contrast agent, which facilitates the immediate translation of T1 mapping of thrombosis to the clinic.
SUPPLEMENTAL MATERIAL
**Imaging protocols used in this study**

a) Longitudinal protocol used to image BALB/C, iNOS<sup>−/−</sup>, CCR2<sup>−/−</sup> and wild-type controls mice. Each mouse was imaged consecutively between day 1 and 28 before thrombus was harvested and processed for histology; b) Cross-sectional protocol used to image BALB/C mice. Thrombus was induced at day 0 and imaged between days 1 and 21 before being harvested for analysis at each time point; c) Thrombolysis protocol used to image BALB/C mice. Thrombus was induced as before and scanned between day 2 and 21. After each scan, tissue plasminogen activator (t-PA) was infused into the tail vein. Mice were scanned 24 hours after lysis to check for a response and the thrombus was harvested for analysis.
Image analysis of MSB sections of venous thrombi

A) Bitmap images of histological sections of experimental venous thrombi were imported into Image Pro-plus 7. B) A region of interest (ROI) was drawn around the thrombus (green line) as shown with black arrow heads to calculate the area of thrombus. C) Images were segmented based on their colour intensity histogram to identify the fibrin stain (red). D) The area of the segmented region within the ROI was then calculated and used for analysis. The same segmentation parameters of colour intensity was applied to all histological sections.
Spatial and temporal distribution of fibrin in experimental venous thrombi

A) The spatial distribution of fibrin appears similar with MSB sections and immunohistochemistry (5F3 clone) during thrombus resolution (bar=200µm). High magnification images are shown in (i). Black arrows indicate platelets, white arrows show fibrin (bar=25µm). B) Representative western blot analysis of fibrin during thrombus resolution (anti fibrin Ab, 59D8 clone). C) Comparison of fibrin quantification using western blot and MSB sections (n=6 per group).
T1 quantification and inter-observer analysis

Acquired images were imported into MATLAB as individual slices and analysed using a bespoke programme to quantify T1 relaxation time on a pixel by pixel basis. An example of a region with a long T1 relaxation time is shown in A) and short T1 relaxation time in B). T1 relaxation across the whole cross sectional area of thrombus were averaged for each slice and mean thrombus T1 relaxation time calculated across the length of the thrombus. C) 5 independent observers were asked to analyse 40 slices to identify thrombus and calculate mean T1 relaxation time. Correlation between observers and the authors' measured T1 relaxation time is shown in D).

\[ M(TI) = M_0 \left( 1 - e^{-\frac{T1}{T1}} \right) \]
$T_1$ relaxation time of blood *in vitro* with different percentages of Methaemoglobin

Human blood (squares) and murine blood (circles) exert a similar shortening of $T_1$ relaxation time *in vitro* as percentage of methaemoglobin (metHb) increases (n=25/gp).
Natural resolution of murine venous thrombosis

The velocity of blood was measured across the IVC. (A) In the image white indicates normal blood flow, which is disturbed by the presence of a thrombus (B). Time of flight images demonstrate a filling defect in the IVC (blue) where thrombus is present (C). The aorta (red) represents a fixed anatomical landmark for reference. (D) Volume (mm$^3$) of the thrombus as it resolves with time (E) Flow (ml/min) across the IVC during thrombus resolution. Mean±SEM shown. One way ANOVA with Bonferroni post test analysis. (*=P<0.05, **=P<0.01, ***=P<0.001, n=18/gp)
Longitudinal changes in T1 relaxation times (ms) during resolution

T1 maps were quantified of individual mice that were sequentially scanned between day 1 and 28. Mean T1 relaxation time shown. Two way ANOVA over time (P<0.0001). 2 mice which died during this experiment have been excluded from analysis.
Venous thrombi in *iNos<sup>−/−</sup>* mice

a) Venogram of the murine IVC in *iNos<sup>−/−</sup>* mice following thrombus induction. b) Corresponding T<sub>1</sub> maps of thrombi in *iNos<sup>−/−</sup>* mice. c) Volume of thrombus (mm<sup>3</sup>) in *iNos<sup>−/−</sup>* and *iNos<sup>+/+</sup>* mice over time measured using TOF sequences. Mean±SEM of thrombus volume (mm<sup>3</sup>) of *iNos<sup>−/−</sup>* (grey bar) and *iNos<sup>+/+</sup>* (black bar) mice are shown over time (n=6/gp). Two way ANOVA comparing differences in groups over time is used for analysis with Bonferroni post test for each time point.
Venous thrombi in Ccr2−/− mice

a) Venogram of the murine IVC in Ccr2−/− mice following thrombus induction. b) Corresponding T₁ maps of thrombi in Ccr2−/− mice. c) Volume of thrombus (mm³) in Ccr2−/− and Ccr2+/+ mice over time measured using TOF sequences in during resolution. Mean±SEM of thrombus volume (mm³) of Ccr2−/− (black bar) and Ccr2+/+ (grey bar) mice are shown over time (n=6/gp). Two way ANOVA comparing differences in groups over time is used for analysis with Bonferroni post test for each time point (*=P<0.05, **=P<0.01, ***=P<0.001).
**MSB sections of experimental venous thrombi during its resolution**

A) Examples of histological sections of venous thrombi during its resolution are shown (yellow=red cells, red=fibrin, blue=collagen, bar=200µm). High magnification images of the boxes are shown in i) and ii), bar=25µm. White arrows indicate red cells, black arrows indicate fibrin, white arrow heads indicate collagen and black arrow heads regions of neovascular channels. Box and whisker quantification of MSB sections for red cell (B), fibrin (C) and collagen (D) is shown for each level of individual sections across the whole thrombus at each time point (n=6 per group, range: minimum to maximum, median line is shown and mean represented by ‘+’, ***=P<0.001).