Fibrin-Targeted Magnetic Resonance Imaging Allows In Vivo Quantification of Thrombus Fibrin Content and Identifies Thrombi Amenable for Thrombolysis

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Objective—Deep venous thrombosis is a major health problem. Thrombolytic therapies are effective in recanalizing the veins and preventing post-thrombotic complications, but there is no consensus on selection criteria. The aim of this study was to investigate a fibrin-specific MRI contrast agent (EP-2104R) for the accurate quantification of thrombus’ fibrin content in vivo and for the identification of thrombus suitable for thrombolysis.

Approach and Results—Venous thrombosis was induced in the inferior vena cava of 8- to 10-week-old male BALB/C mice and MRI performed 2, 4, 7, 10, 14, and 21 days later. Eighteen mice were scanned at each time point pre and 2 hours post injection of EP-2104R (8.0 μmol/kg) with 12 mice at each time point used to correlate fibrin contrast uptake with thrombosis’ histological stage and fibrin content. Six mice at each time point were immediately subjected to intravascular thrombolytic therapy (10 mg/kg of tissue-type plasminogen activator). Mice were imaged to assess response to lytic therapy 24 hours after thrombolytic treatment. Two mice at each time point were scanned post injection of 0.2 mmol/kg of Gd-DTPA (gadolinium with diethylenetriaminepentaacetate, Magnevist, Schering AG, Berlin, Germany) for control purpose. Contrast uptake was correlated positively with the fibrin content of the thrombus measured by Western blotting (R²=0.889; P<0.001). Thrombus relaxation rate (R₂) post contrast and the change in visualized thrombus size on late gadolinium enhancement inversion recovery MRI pre–EP-2104R and post–EP-2104R injection were the best predictors for successful thrombolysis (area under the curve, 0.989 [95% confidence interval, 0.97–1.00] and 0.994 [95% confidence interval, 0.98–1.00] respectively).

Conclusions—MRI with a fibrin-specific contrast agent accurately estimates thrombus fibrin content in vivo and identifies thrombi that are amenable for thrombolysis. (Arterioscler Thromb Vasc Biol. 2014;34:1193-1198.)

Key Words: fibrin ■ magnetic resonance imaging ■ molecular imaging ■ thrombolytic therapy ■ venous thrombosis

Deep vein thrombosis has an annual incidence of ≈1 to 2 per 1000 people.1-3 This condition can give rise to the post-thrombotic syndrome, which is a major cause of morbidity,4,5 and pulmonary emboli, which may be fatal.6-8 The mainstay of current treatment involves anticoagulation to prevent thrombus propagation,2 but this has little effect on the resolution of a thrombus.4,8 A recent randomized control trial has shown that early vein recanalization by thrombolysis reduces post-thrombotic complications in patients with acute proximal deep venous thrombosis.9 The advent of pharmacomechanical systems to deliver thrombolytics efficiently has also prompted a more aggressive approach to the treatment of this condition.10-13

Thrombolysis is indicated for young thrombi, with older thrombi thought to be less responsive to this treatment. Clinical history and signs at presentation used to determine thrombus age are, however, subjective and unreliable.12,14 Thrombus age is also not always an informative determinant of the susceptibility to lysis. Not all fresh thrombi respond to this treatment, which unnecessarily exposes some patients to hemorrhagic complications, whereas some patients with older thrombi seem to respond well.12,15,16 An objective method capable of identifying thrombi susceptible to lysis is therefore needed.16-18

See cover image

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.302931/-/DC1. Correspondence to Marcelo E. Andia, MD, PhD, Division of Imaging Sciences and Biomedical Engineering, The Rayne Institute, St Thomas’ Hospital, King’s College London, 4th Floor, Lambeth Wing, London SE1 7EH, United Kingdom. E-mail mandia@med.puc.cl

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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.113.302931

Received on: May 3, 2013; final version accepted on: April 1, 2014.
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Venous thrombi resolve by a process of organization that leads to vein recanalization.19–21 During this process, the structure of the thrombus changes with cross-linked fibrin being gradually replaced by collagen.99–24 Experimental data suggest that thrombi, rich in fibrin, are more susceptible to thrombolysis than organized, collagen-rich thrombi.25 As thrombolytic therapies target fibrin within a thrombus, we hypothesized that this molecule represents an imaging target that could better direct lytic therapy.17,24,26 A gadolinium-based fibrin-specific MRI (FSMRI) contrast agent has been used to detect arterial and venous thrombi in both animal models and man.27–32

In this study, we sought to investigate whether this fibrin-specific contrast agent allows estimation of the fibrin content of resolving venous thrombi and indicates thrombi that are susceptible fibrinolysis.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

$R_1$ Relaxivity After Administration of EP-2104R During Thrombus Resolution

In precontrast images the average blood relaxation rate ($R_1$) was $0.68±0.04$ s$^{-1}$, whereas in post–EP-2104R images the average blood $R_1$, 2 hours after contrast agent, was $0.74±0.03$ s$^{-1}$ ($P<0.01$). Thrombus signal intensity and $R_1$ were greater after administration of EP-2104R, compared with precontrast images and Gd-DTPA controls (Figure 1). The difference between $R_1$ of thrombus post and pre contrast ($ΔR_1$) changed during thrombus resolution ($P<0.001$, 1-way ANOVA) and was greatest at days 7 ($ΔR_1=0.945±0.049$ s$^{-1}$; $P<0.01$; Bonferroni post hoc test compared with day 2) and 10 ($ΔR_1=1.005±0.054$ s$^{-1}$; $P<0.01$; Bonferroni post hoc test compared with day 2; Figure 1A and 1C).

The mean gadolinium concentration in thrombus of mice injected with EP-2104R mirrored the changes in $R_1$ over time, reaching a maximum gadolinium concentration at days 7 ($886±131$ μmol/L) and 10 ($1116±155$ μmol/L; $P<0.001$; 1-way ANOVA; Figure 1B). Traces of gadolinium (<10 μmol/L) were detected in thrombus post injection of Gd-DTPA, but did not change during thrombus organization.

Fibrin Content of the Thrombus During Its Resolution

The fibrin content of the thrombus changed during its resolution (Figure 2A and 2B). Fibrin content was greatest at days 7 and 10 after induction. Fibrin content at days 2, 4, 7, 10, 14, and 21 was $1.7±1.1$, $5.2±1.4$, $9.3±1.9$, $9.6±1.4$, $6.6±2.4$, and $0.9±1.0$, respectively ($P<0.01$; 1-way ANOVA; Figure II in the online-only Data Supplement). The fibrin content of the thrombus correlated with thrombus $R_1$ ($R^2=0.89$; $P<0.001$; Figure 2B), and the average fibrin content at each time point correlated with the average thrombus gadolinium concentration after the administration of EP-2104R ($R^2=0.95$; $P<0.001$; Figure 2C).

Change in $R_1$ Relaxation Rate After Thrombolysis

The inferior vena cava (IVC) blood flow in normal mice, without thrombus, was $0.55±0.05$ mL/min. Blood flow in the IVC was not detected 2 days after the induction of thrombosis, and no significant differences were observed in the IVC blood flow at days 2, 4, and 7. After day 7 of thrombus induction, the IVC flow was seen to increase (Figure IIIB in the online-only Data Supplement). Thrombolysis was most effective between days 7 and 14 (Figure 3A; Figure IVA in the online-only Data Supplement), when $ΔR_1$ was greatest after the administration of EP-2104R (Figure 3B). The $R_1$ relaxation rate before the administration of the contrast agent resulted in a sensitivity of 72% and specificity of 93% to predict successful lysis when $R_1>1.30$ s$^{-1}$. The thrombus $R_1$ relaxation rate after the administration of EP-2104R improved the sensitivity and specificity to predict successful thrombolysis to 94% and 99%, respectively, when $R_1$ thrombus relaxation rate was $>1.59$ s$^{-1}$ post–EP-2104R injection.

Change in Visualized Thrombus After Administration of EP-2104R

Administration of EP-2104R increased the visualized thrombus volume. The difference between visualized thrombus volume pre and post contrast ($ΔV$) is largest between days 7 and 14 on late gadolinium enhancement-FSMRI (Figure 4C; Figure IVB in the online-only Data Supplement). Mice with the greatest change in visualized thrombus volume, $ΔV$, after the administration of EP-2104R were most susceptible to lysis (Figure 4; Figure IVB and IVC in the online-only Data Supplement). Receiver operator curve analysis demonstrated that visualized thrombus volume before the administration of EP-2104R, $V_t$, was not a good predictor of successful lysis (area under the curve=0.748; 95% confidence interval, 0.58–0.92), but after the administration of EP-2104R, the visualized difference in thrombus volume, $ΔV$, on late gadolinium enhancement-FSMRI had an area under the curve of $0.972$ (95% confidence interval, 0.95–1.00), and an increase in visualized thrombus volume larger than $ΔV=3.5$ mm$^3$ had a sensitivity and specificity to determine successful lysis of 99% and 95%, respectively.

Multivariate regression analysis showed that the change in the visualized thrombus volume pre and post contrast ($ΔV$) and the $R_1$ relaxation rate postcontrast administration were independent variables that significantly correlated with the change in the IVC blood flow pre and post contrast, $β$ coefficients of 0.087 ($P=0.001$) and 2.383 ($P=0.003$), respectively.

Discussion

Tissue-type plasminogen activator, the main lytic agent used in clinical practice, specifically targets fibrin found in thrombus.23 In vivo quantification of the thrombus fibrin content has, therefore, the potential to direct therapy, both in terms of identifying patients suitable for treatment and in titering the dose of tissue-type plasminogen activator required. We have demonstrated previously that an FSMRI contrast agent, EP-2104R, can visualize acute thrombi in both experimental models and man.27–32
but have never quantified previously the changes in contrast agent uptake during thrombus resolution. In this study, we show that uptake of the fibrin-specific contrast agent, EP-2104R, correlates with the fibrin content of a murine thrombus during its resolution and that thrombi with high EP-2104R uptake are more susceptible to lysis by tissue-type plasminogen activator.

The change in $R_1$ relaxation rate and gadolinium content of murine thrombi after administration of EP-2104R was greatest between days 7 and 10 after thrombus induction. Although conventional wisdom suggests that fibrin content is greatest in the early thrombus, in this and our previous studies, we have shown that the fibrin content of thrombus in our model varies over time, with the highest content found at 7 to 10 days.

Here, we also showed that the changes in fibrin content of the thrombus correlate well with the spatial and temporal uptake of EP-2104R. These data suggest, therefore, that quantification of EP-2104R uptake could be used as an in vivo measure of thrombus fibrin content.

Venous thrombus resolution in our murine model of thrombosis naturally occurs during a period of 4 weeks in a process reminiscent of wound healing. Accumulation of inflammatory cells and their mediators orchestrates resolution through processes involving fibrinolysis, proteolysis, and neovascularization. In the early thrombus, red blood cells are trapped within strands of fibrin and form the main body of thrombus. As the thrombus resolves, thicker mature fibrin strands predominate and are subsequently replaced by other extracellular matrix proteins, including collagen. As this process progresses, the thrombus gradually becomes incorporated into the vein wall.

**Figure 1.** A, Changes in thrombus relaxation rate $\Delta R_1$ ($s^{-1}$) pre–EP-2104R and post–EP-2104R administration at different time point during thrombus organization. Greatest differences in $\Delta R_1$ pre and post contrast are observed at days 7 and 10. B, Thrombus gadolinium concentration ($\mu$mol/L) mirrored the changes in $R_1$ over time. C, $R_1$ mapping images pre–EP-2104R and post–EP-2104R injection and Martius Scarlet Blue (MSB) histology sections at the corresponding levels and fibrin selective segmentation. Gd-DTPA indicates gadolinium with diethylenetriaminepentacetate (Magnevist, Schering AG, Berlin, Germany).
resolution is therefore a dynamic process during which its structure changes over time. We have demonstrated previously that iron-processing mechanisms after the lysis of
trapped red blood cells in thrombus determine its longitudinal ($T_1$) relaxation time and that quantification of the relaxation rate ($R_1$) can be used as a surrogate measure of thrombus fibrin content. Direct thrombus $R_1$ estimation can therefore be used to identify thrombi that are suitable for lysis. However, the data in this study show that by specifically targeting of fibrin using the novel contrast agent, EP-2104R, it is possible to significantly improve the sensitivity and specificity of this selection.

$R_1$ rate without contrast agent cannot be used to determine the age of a thrombus. Short thrombus $R_1$ rates indicate early or mature thrombus, which limits its clinical use if used alone. In addition, despite the development of fast $T_1$-mapping protocols, long scan times are still required to accurately quantify thrombus $R_1$ in vivo, whereas postprocessing software needs careful analysis. Faster late gadolinium enhancement sequences are readily available on commercial MRI scanners and do not require sophisticated postprocessing tools for analysis as needed for $R_1$ maps. In this study, we used late gadolinium enhancement sequences with our fibrin-specific contrast agent and showed that an increase in the volume of visualized thrombus after the administration of EP-2104R was associated with thrombi that were most amenable to lysis. Although this combination has improved significantly the speed of analysis, a limitation is that a pre- and postcontrast agent scan is required.

**Conclusions**

These data show that the uptake of an FSMRI contrast agent, EP-2104R, changes during the resolution of an experimental venous thrombus. These changes strongly correlate with the fibrin content of the thrombus and uptake of the contrast agent can be used to identify thrombi that are susceptible to fibrinolysis. Although this study was conducted in an experimental model of thrombosis, it was performed using a clinical 3-Tesla field strength MRI scanner. Given that we used sequences that are readily available on commercial MRI scanners, and the safety of this contrast agent is already proven in man, EP-2104R should now be tested in the clinical setting.

**Acknowledgments**

We thank David Eastham for his technical assistance and useful comments during the preparation of this article.

**Sources of Funding**

This study was partly supported by the British Heart Foundation Centre of Excellence at King’s College London and Wellcome Trust, the Engineering and Physical Sciences Research Council Medical Engineering Centre at King’s College London (grant no. WT 088641/Z/09/Z), and the National Institute for Health Research Biomedical Research Centre of Guy’s and St Thomas’ Trust and King’s College London. M.E. Andia was funded by the Chilean Agency for Research in Science and Technology (FONDECYT 1130379). P. Saha was funded by a Wellcome Trust Clinical Research Training Fellowship (WT 090252/Z/09/Z).

**Disclosures**

None.

**References**

Deep venous thrombosis is a major health problem. Our results show that the quantification of the fibrin-specific MRI contrast agent, EP-2104R, uptake changes during the resolution of an experimental venous thrombus. These changes strongly correlate with the fibrin content of the thrombus and uptake of the contrast agent can be used to identify thrombi that are susceptible to fibrinolysis. This approach has great potential to increasing the effectiveness of current venous thrombolytic therapy and decreasing the incidence of acute and chronic complications.
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Arterioscler Thromb Vasc Biol. 2014;34:1193-1198; originally published online April 10, 2014;
doi: 10.1161/ATVBAHA.113.302931
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/6/1193

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Supplementary Figure I- Study design. (A) Twelve mice were scanned at each time point (day 2, 4, 7, 10, 14 and 21) after IVC thrombosis induction pre and 2 hours post injection of 8 µmol/kg of a fibrin-specific MRI contrast agent, EP-2104R. After the imaging session those mice were culled to quantify the fibrin content with Western Blotting, visualize fibrin with Martius, Scarlet and Blue (MSB) staining by histology and quantify gadolinium uptake by ICP-MS. Two additional mice were scanned at each time point post injection of 0.2mmol/kg of Gd-DTPA to quantify non-specific contrast agent uptake. (B) In the thrombolytic experiment, six mice were scanned at each time point pre and post EP-2104R administration. Immediately after the imaging session the mice were subjected to thrombolysis with tissue plasminogen activator. 24 hours later the mice were re-injected with EP-2104R and scanned two hours later to evaluate the success of lysis. (ICP-MS: inductively coupled plasma mass spectroscopy, IVC: inferior vena cava).
Supplementary Figure II-  (A) Scan planning in a normal mouse. Maximum intensity projection (MIP) images of the abdominal aorta and inferior vena cava from the time of flight (TOF) acquisition showing schematically the scan planning. 3D inversion recovery (IR) fast gradient echo and T1 mapping sequence covering the inferior vena cava (IVC) and the abdominal aorta (30 slices) was performed in all animals. Immediately after the post-contrast scans the abdominal cava vein was harvested for histological analysis. (B) Mouse with a venous thrombus in the IVC showing compromised luminal area in the IVC MIP images. Anatomical and blood velocity images acquired in the central portion of the thrombus location using Phase contrast MRI in: a normal mouse (C) and mouse with a venous thrombus in the IVC (D) showing a flow defect due to the intravascular thrombus. Ao: Aorta, Th: Thrombus, IVC: inferior vena cava, and scale bar for blood velocity quantification (cm/s).
Supplementary Figure III- (A) Relative quantification of thrombus fibrin content by Western blot at different time points during thrombus organisation (n=4 mice per time point). (B) IVC blood flow (ml/min) measured by phase contrast MRI in normal mice and mice at different time points during thrombus organisation. One-way ANOVA analysis with Bonferroni post-hoc test (&=P<0.01, §=P<0.001, ns: non significant) (IVC: inferior vena cava).
Supplementary Figure IV- Inferior Vena Cava (IVC) blood flow and fibrin contrast uptake pre and post thrombolytic treatment. (A) IVC blood flow measured by phase contrast MRI pre and post thrombolytic treatment at different time point of thrombus organisation. (B) Visualized difference in thrombus enhanced volume [mm³] estimated from pre and post contrast LGE-FSMRI images showed different contrast uptake at different time point of thrombus organisation. (C) Visualized enhanced thrombus volume before and after thrombolytic therapy after EP-2104R injection at different time point of thrombus organisation. (FSMRI: fibrin specific MRI).
MATERIAL AND METHODS

Venous thrombosis mouse model

Venous thrombosis was induced in the inferior vena cava (IVC) of 8-10wk (25-30g) male BALB/C mice (n=120) using an established technique that involves reduced blood flow and mechanical endothelial disruption\textsuperscript{1-3}. All procedures used in these studies were licensed under the United Kingdom Animal (Scientific Procedures) Act 1986.

Characterisation of fibrin-specific contrast uptake during resolution

MRI was carried out at 2, 4, 7, 10, 14 and 21 days after thrombus induction (n=12/time-point, Supplementary Figure 1A). Mice were imaged before and 2hrs after tail vein injection of a gadolinium based, fibrin-specific contrast agent: EP-2104R (EPIX Pharmaceuticals, Lexington, MA)\textsuperscript{4-6} and a dose of 8µmol/kg of EP-2104R dissolved in 20µl of saline was used\textsuperscript{4,5,7}. EP-2104R is a Gd-based, fibrin-binding contrast agent with four Gd chelates per molecule\textsuperscript{4,5,8}. This agent has a specificity for fibrin 100-fold larger than the specificity for fibrinogen and 1000-fold larger than the specificity for serum albumin\textsuperscript{4}. The fibrin scans were carried out 2hrs after EP-2104R injection to allow for contrast agent blood clearance and to minimize background signal\textsuperscript{5,8,9}. Immediately after scanning, the IVC and thrombus were harvested for: gadolinium quantification using inductively coupled plasma mass spectrometry (ICP-MS) (n=4/time-point); western blot analysis of fibrin content (n=4/time-point) and histological analysis (n=4/time-point). Additional mice (n=2/time-point) were scanned at each time point post injection of 0.2mmol/kg of Gd-DTPA (Magnevist, Schering AG, Berlin, Germany) and the thrombi were analysed with ICP-MS to quantify non-specific contrast uptake.

Lysis

In further groups of animals, thrombus was imaged with EP-2104R at 2, 4, 7, 10, 14 and 21 days after thrombus induction (n=6/time-point, Supplementary Figure 1B), before undergoing thrombolysis with 10mg/kg of tissue plasminogen activator (Actilyse, Boehringer Ingelheim, Germany), infused via the tail vein over 5mins. 24hrs after thrombolysis, mice were imaged again after EP-2104R injection to examine the size of the remaining thrombus and the blood flow in the IVC calculated.

In vivo MRI protocol

Mice were anesthetized with 1.5-2% isofluorane and 100% oxygen delivered through a nose cone and scanned in prone position on a 3T Philips Achieva Gyroscan scanner (Philips Healthcare, Best, The Netherlands) equipped with a clinical gradient system (30mT m\textsuperscript{-1}, 200mT/m/ms) and a single-loop receiver-only surface coil (diameter=47mm). Following a 3D gradient echo (GRE) scout scan, arterial and venous time-of-flight angiography (TOF) was performed as followed: arterial TOF with TR=40ms, TE=6.2ms, flip angle=60°, FOV=20x33x17mm, acquired matrix=68x110, slice thickness=0.3mm, resolution=0.3x0.3mm, reconstructed resolution=0.1x0.1mm, slices=50, averages=2, duration=7.5min, and a venous TOF with TR=50ms resulting in a duration of 9min with all other parameters maintained. The maximum intensity projection (MIP) images were used to visualize the abdominal aorta, the renal and iliac bifurcations, the vena cava and the region of flow obstruction corresponding to the thrombus (Supplementary Figure 2A&B). These images were used for planning the subsequent scans and for reference to guide the correlation between MRI images and histology. A late gadolinium enhancement inversion recovery 3D segmented gradient echo (TFE) sequence was performed to selectively visualise thrombus pre and post-contrast (late gadolinium enhancement fibrin selective magnetic resonance imaging: LGE-FSMRI). Slices (n=30) were acquired starting just above the renal veins and extending down to the union of the two common iliac veins (Supplementary Figure 2A&B). Imaging parameters included matrix...
The visualised thrombus volume enhancement was calculated in the pre and post-contrast images. On LGE-FSMRI images, thrombus was defined as intravascular pixels with signal intensities greater than 2 standard deviations of the mean blood signal measured in normal vessel segments. On R1 maps thrombus size was estimated as intraluminal pixels with a R1 relaxation rate higher than the measured blood R1 in normal vessel segments plus two standard deviations. This criterion was used to estimate the thrombus volume and thrombus average R1 value. Blood R1 value was estimated in the pre-renal portion of the IVC. Phase contrast images were used to estimate blood flow in the IVC pre and 24hrs post thrombolytic therapy.

Thrombolysis was considered successful if an increase in IVC blood flow greater than 50% was observed on 24hrs post-lysis scans. We estimated the percentage of lysis (%Lysis) for each treated mouse, as the actual change in the IVC blood flow after treatment, compare with the maximum expected IVC blood flow change if 100% of the lumen were successfully cleared of thrombus, therefore:

\[
\%Lysis = \frac{B - A}{C - A} \times 100\%
\]

**Histology**

The length of the IVC, including the thrombus, from the renal branches down to the union of the two iliac veins was measured in situ and then was harvested en-bloc. The harvested IVC was pinned onto cork mats and stretched to its original length in vivo. The tissue was placed in 10% formalin for 24hrs before processing for impregnation and paraffin wax embedding. Individual animals (n=4/time-point) were analysed. Sections (5μm) of the entire IVC were taken at 500μm intervals. Renal and iliac veins were used as anatomical reference to allow comparison with the corresponding MRI slices. Sections were stained using haematoxylin and eosin (H&E) and Martius Scarlet Blue (MSB) to provide morphological and compositional (fibrin) detail of the thrombus. Computer-assisted colour image analysis (Colour Threshold plug in, ImageJ, NIH, Bethesda, MD, US) was used to selective visualized the fibrin area on MSB-stained sections.

**Analysis of fibrin content**
Western blotting for fibrin was carried out using a fibrin-specific antibody (5F3 clone)\textsuperscript{11}. Fibrin was extracted from thrombi by homogenisation in sodium phosphate buffer (10mmol/l, pH 7.5), with a cocktail of protease inhibitors that included, ε-amino-n-caproic acid (0.1mol/l), trisodium EDTA (5mmol/L), aprotinin (10U/ml), heparin (10U/ml), and polymethylsulfonyl fluoride (PMSF, 2mmol/l). The homogenate was agitated for 14hrs at 4°C and sedimented by centrifugation at 10000g for 10mins. The sediment was resuspended in extraction buffer without PMSF, sedimented again, and dispersed in urea (3mol/l). The suspension was further agitated for 2hrs at 37°C, vigorously vortexed, and centrifuged at 14000g for 15mins. The supernatant was aspirated and the sediment dissolved at 65°C in reducing SDS buffer, subjected to SDS–polyacrylamide gel (8%) electrophoresis, followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Corp) by electroblotting. Densitometric values were obtained using Image Lab 4.1 software (Biorad, UK). Values were normalised to thrombus weight and values expressed relative to a mouse plasma clot.

**Inductively coupled plasma mass spectrometry**

Gadolinium (Gd) concentrations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for a subset of thrombi (n=4/time-point). Thrombus was digested in 70% nitric acid at 37°C overnight followed by dilution with deionised water for ICP-MS analysis. A standard curve was run with each sample set for Gd concentration determination.

**Statistical Analysis**

Data was inserted into PRISM version 5 (GraphPad, USA) for analysis. Continuous data are expressed as mean±SEM. One-way ANOVA was used to measure changes between groups over time followed by the Bonferroni post-hoc test. Paired Student's t-test was used to compare pre and post contrast parameters in the same group of mice.
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