Assessment of Venous Thrombosis in Animal Models

Steven P. Grover,* Colin E. Evans,* Ashish S. Patel, Bijan Modarai, Prakash Saha, Alberto Smith

Abstract—Deep vein thrombosis and common complications, including pulmonary embolism and post-thrombotic syndrome, represent a major source of morbidity and mortality worldwide. Experimental models of venous thrombosis have provided considerable insight into the cellular and molecular mechanisms that regulate thrombus formation and subsequent resolution. Here, we critically appraise the ex vivo and in vivo techniques used to assess venous thrombosis in these models. Particular attention is paid to imaging modalities, including magnetic resonance imaging, microcomputed tomography, and high-frequency ultrasound that facilitate longitudinal assessment of thrombus size and composition. 

Key Words: histological techniques ■ models, animal ■ molecular imaging ■ venous thrombosis

Deep vein thrombosis (DVT) is a common condition with an annual incidence of ≈1 in 1000 in the general population and can lead to fatal pulmonary embolism. Together these conditions account for a greater number of deaths in the United Kingdom than those caused by breast cancer, road traffic accidents, and AIDS combined.1,2 Approximately one third of patients with DVT develop post-thrombotic syndrome, a chronic condition characterized by persistent limb pain, swelling, and ulceration, which carries a significant health and economic burden and is associated with a reduced quality of life.3

Treatment of DVT currently involves systemic anticoagulation, aimed at preventing secondary thrombotic events, and catheter-directed thrombolysis, which in the acute setting has proven effective in the removal of the thrombus. However, both of these therapeutic strategies give rise to pathological bleeding in a significant number of cases and may be contraindicated in specific patient subpopulations. Novel treatments that either prevent thrombus formation or hasten resolution without these side effects are desirable and are likely to arise from a better understanding of the molecular and cellular mechanisms that control venous thrombosis.

Experimental models of DVT have been developed in a variety of animals, including the mouse,4 rat,5 rabbit,6 dog,7 pig,8 and non-human primates.9 Spontaneous, symptomatic DVT is not observed in these animals with researchers instead relying on a number of physical or chemical interventions on a given vessel (such as ligation or ferric chloride) to induce thrombosis. Although the coagulation and fibrinolytic systems in non-human primates most closely resemble those in man, the use of these species present researchers with both financial and ethical dilemmas. Other large species such as pigs have a similar fibrinolytic responses to man, lending themselves to assessment of thrombolytics, whereas the close resemblance of the coagulation system in sheep to that of man may be of particular use when assessing novel antithrombotic agents.10 However, murine models particularly those involving the infrarenal vena cava (IVC) currently predominate; owing to their technical simplicity, compatibility with available imaging platforms and the availability of transgenic strains. These murine models have proven especially useful in elucidating the molecular and cellular determinants of venous thrombosis.11

Comparison of data from different studies is complicated by the variety of models used (both animal species and mechanisms of induction) in conjunction with the wide array of analytical techniques employed. Although the relative merits of respective models have been the source of critical review,12 there remains no consensus on the best method(s) to accurately quantify venous thrombosis in the preclinical setting. In this review, we critically evaluate ex vivo and in vivo methods currently used in the assessment of venous thrombosis and describe emerging imaging techniques that may prove useful in studying this dynamic condition.

Ex Vivo Assessment of Venous Thrombosis Weight

Thrombus weight is a simple, quantitative, and inexpensive measure of thrombus formation and subsequent resolution, with weight decreasing as the thrombus resolves (Figure 1A). Thrombus weight can be obtained with or without excision from the surrounding vessel.13,14 Measurements of thrombus weight without excision from the vessel may introduce...
variability associated with the inclusion of the vein wall and extraneous adherent tissue. Excision of the thrombus provides a more direct measure of weight, but during later stages of thrombus resolution, thrombus and vein wall become difficult to separate. Importantly, measurement of thrombus weight permits further biochemical and cellular analysis of the thrombus. It has been proposed that adjusting for thrombus length reduces apparent intragroup variability when measuring thrombus weight. In our experience, such adjustments are not always sensitive to changes in thrombus size; for example, when thrombus weight decreases proportionally to thrombus length, similar values of length:weight ratio will be obtained.

Histological Analysis

Histological techniques are commonly used to provide estimates of both thrombus size and composition. Thrombus is excised in situ with surrounding vein wall and prepared for histological sectioning. Estimation of thrombus size is performed by analysis of thrombus cross-sectional area in sections taken at set intervals (300–500 μm) along the entire length of the thrombus. The summed area multiplied by the distance between sections provides an estimate of thrombus volume. We believe that reconstitution of thrombus volume provides the best estimate of overall thrombus burden. Measurements of representative cross-sectional area or scoring for the presence of thrombus at set intervals may not adequately account for changes in thrombus length or area, respectively. Histological analysis does, however, present many technical challenges. Sectioning of thrombus, particularly at early time points, can prove difficult because of the friable nature of this tissue. Sectioning individual thrombi at defined intervals along their length, with subsequent staining and analysis of sections at each level, is also time consuming. A further limitation of this technique is that processing of thrombus for wax embedding results in significant shrinkage that may distort differences between groups.

Histological preparation of venous thrombi also enables parallel analysis of thrombus composition by either tinctorial

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<tr>
<td>CT</td>
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<td>DVT</td>
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<td>FMT</td>
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<td>HFUS</td>
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<td>IVC</td>
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<td>IVM</td>
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<td>MRI</td>
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Figure 1. Weight and morphology of resolving murine venous thrombi. A, Thrombus weight in the St Thomas’ model of infrarenal vena cava stenosis measured at days 1, 4, 7, 10, and 14 post induction; bars represent mean±standard error. B, Representative micrographs of transverse thrombus sections stained with Martius scarlet blue (MSB) at days 1, 7, 14, 21, and 28 post induction. MSB detects collagen (blue), fibrin (red), and erythrocytes (yellow); scale bars, 200 μm (low power) and 25 μm (high power). Adapted from Saha et al.
or immunohistochemical staining. Thrombus resolution is characterized by extensive remodeling of the extracellular matrix by cells that infiltrate the thrombus. Extracellular matrix deposition can be readily detected with many tinctorial stains such as Picrosirius Red (collagen), Van Giessen (elastin), Alcian Blue (proteoglycans), and Martius Scarlet Blue (fibrin and collagen; Figure 1B). Although these techniques are well established, conditions must be tightly controlled to ensure tissue structures are appropriately and consistently stained. Immunohistochemical localization of the cellular infiltrate (eg, leukocytes, endothelial cells, and myofibroblasts) can also be used to assess thrombus organization (Table 1). Careful consideration of cell specific markers and extensive optimization of antigen binding is required.

### In Vivo Assessment of Venous Thrombosis

The terminal nature of ex vivo assessment is a significant limitation in the study of thrombus resolution as it precludes longitudinal measurements of changes in thrombus size in the same animal. Imaging techniques that facilitate longitudinal and reproducible quantification of thrombus burden overcome this limitation and provide powerful analysis through the generation of paired data. This approach can also limit the number of animals required for experimentation consistent with the principles laid out by the national center for the replacement, reduction, and refinement of animals in research.

### Magnetic Resonance Imaging

#### Noncontrast Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) can be used to diagnose DVT in man without the need for contrast agents. MRI time-of-flight venography is a contrast-free technique for assessing thrombus volume in preclinical studies. Phased sequences are used to allow specific visualization of the venous system, where thrombus seems as a flow deficit that can be segmented to allow quantification of thrombus volume (Figure 2A). MR longitudinal relaxation time (T1) mapping can also be used to image thrombus, which has a shorter T1 than surrounding blood and tissue. Shortened T1 times are likely caused by the accumulation of paramagnetic iron (Fe^{3+}) in the thrombus. Temporal changes in thrombus T1 have been observed during murine venous thrombus resolution and are associated with increasing thrombus organization (Figure 2B). Magnetization transfer and diffusion-weighted MR sequences have also been developed to characterize the age and protein composition of the thrombus. Combining the T1, magnetization transfer and diffusion-weighted sequences, while time consuming to acquire, could provide valuable information of both thrombus size and organization. A major benefit of noncontrast MRI is that newly developed sequences are rapidly translatable from the laboratory to the clinic without the need of regulatory approval. The time intensive nature of MRI, and multisequence imaging in particular, limit the use of this

#### Table 1. Cell Types Present in the Venous Thrombus

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Marker (Antibody Clone)</th>
<th>Infiltration</th>
<th>References</th>
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<tbody>
<tr>
<td>Platelet</td>
<td>CD41 (MWReg30)</td>
<td>Early</td>
<td>21</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Ly6G (NIMP-R14 or 1A8)</td>
<td>Early</td>
<td>18, 21</td>
</tr>
<tr>
<td>Macrophage</td>
<td>Mac2 (M3/38), F4/80 (Cl:A3-1 or BM8)</td>
<td>Mid-late 17, 21, 22</td>
<td></td>
</tr>
<tr>
<td>Endothelial</td>
<td>vWF (F8/86), CD31 (JC70A), Isolectin-B4 (GSL I-B4)</td>
<td>Mid-late 17, 23, 24</td>
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In murine models of vena cava thrombosis, early, and mid-late stages of thrombus resolution refer to days 1 to 7 and days 7 to 28 post induction, respectively.

Figure 2. Imaging of the resolving venous thrombus by magnetic resonance imaging. A, Venous phase time-of-flight scans used for imaging of the infrarenal vena cava, presence of thrombus results in a filling defect in the vessel, which can be used to estimate thrombus volume. B, Generation of T1 maps demonstrates a temporal shortening in T1 relaxivity (red shift) as the thrombus resolves. Adapted from Saha et al.26
Technique to assess formation of thrombus resolution that occurs over a period of weeks, rather than formation in which the thrombus forms in the order of minutes.

**Contrast MRI**

Agents, such as gadolinium, have been used to image thrombi in experimental models. In a baboon model of IVC thrombosis, gadolinium-enhanced MR venography was effective in identifying the thrombosed segment.\(^9\) Several peptide-conjugated contrast agents have been developed that target matrix components present in the acute and resolving venous thrombus, such as fibrin and collagen.\(^{30,31}\) A fibrin-targeted contrast agent, EP-2104R, has been used to visualize intracranial thrombosis in preclinical models of stroke\(^32\) and is currently under evaluation for the detection of thrombi in man.\(^33\) Furthermore, EP-2104R allows visualization of the component of the venous thrombus that is susceptible to lysis in a low-flow mouse model and could find clinical utility in stratifying patients for thrombolysis.\(^34\) A significant limitation of contrast-based approaches is the need for extensive validation of target specificity and the need for regulatory approval before use in human subjects, requiring costly clinical trials. The use of MRI in the preclinical setting has many limitations that include the availability of scan time on much in demand clinical 3-Tesla scanners, the cost of scan time, and the extended length of time required for an individual scan.

**Micro-CT**

Technological advances in the field of computed tomography (CT) have facilitated the development of high-resolution micro-CT imaging platforms suitable for preclinical use. Contrast-enhanced micro-CT has been used extensively in the study of murine models of cardiovascular pathologies, including critical limb ischemia, abdominal aortic aneurysms, and myocardial infarction.\(^{35-37}\)

Visualization of the vasculature by micro-CT requires intravenous administration of high molecular-weight blood-pool contrast agents such as Iopremol, ExiTron nano 12000, or Aurovisit.\(^{38}\) Contrast-enhanced micro-CT enables longitudinal measurements of thrombus resolution as demonstrated in a murine model of IVC stenosis.\(^{39}\) This technique requires segmentation of thrombus from surrounding tissue, allowing 3-dimensional (3D) reconstruction and extraction of volumetric data. A strength of contrast-enhanced micro-CT is the ability to obtain high-resolution images with reconstructed voxel dimensions in the range of 20 to 60 μm.\(^{39}\) Limitations of this technique for thrombus imaging include the high doses of ionizing radiation required, the use of expensive and potentially nephrotoxic contrast agents, and the cost of the imaging time. In addition, contrast-enhanced micro-CT provides only anatomical data and not information on thrombus composition as can be obtained by MRI.

The use of fusion imaging modalities, such as CT fluorescence molecular tomography, enables the concurrent collection of anatomical and biological data in a noninvasive manner. CT fluorescence molecular tomography provides imaging of cells, proteins, and enzymatic activity through the use of targeted and activatable near-infrared probes. This technique has been used to evaluate thrombus composition with respect to macrophage and fibrin content, and matrix metalloproteinase activity localized to the thrombus.\(^{40,41}\) Angiosense a near-infrared blood-pool contrast agent used for quantification of tumor vasculature may also be relevant to studies of venous thrombus neovascularization.\(^{42}\) Fluorescence molecular tomography allows for fast acquisition of data with scan times in the range of 5 to 8 minutes but affords limited spatial resolution in the sub-millimeter to millimeter range dependent on object depth.\(^{40,43}\)

**Radionucleotide Imaging**

Many targeted radionucleotide probes have been developed to localize components of the thrombus in vivo. Initial efforts to visualize fibrin used labeled components of the fibrinolytic system including \(^{99m}\text{Tc}-\text{tissue-type plasminogen activator}, ^{67}\text{Ga}-\text{urokinase-type plasminogen activator}, \) and \(^{99m}\text{Tc}-\text{fibrin fragment E1}, imaged by single photon emission CT in a rabbit model of venous thrombosis.\(^{44,45}\) Integrin αIIbβ3 has also proven to be an attractive target for the generation of single photon emission CT probes with labeled disintegrin-like peptides localizing to thrombus in a canine femoral vein model.\(^{46}\) Alternatively, positron emission tomography CT (PET-CT) can also be used to image venous thrombi. The PET tracer 18F-fluorodeoxyglucose localizes to the acute thrombus in a novel model of recurrent DVT.\(^{47}\) Other targeted PET probes developed for imaging of arterial thrombi and atherosclerosis, which bind fibrin and the platelet surface receptor glycoprotein VI, may be directly applicable to the study of venous thrombosis.\(^{48,49}\) Advantages of single photon emission CT over PET include simpler probe generation, longer probe half-lives, and higher-resolution images (approximate voxel dimensions of 350 and 850 μm, respectively). However, PET provides higher sensitivity than single photon emission CT that may be of particular importance when quantifying fibrin in mature venous thrombi.

**Ultrasonography**

This technique is commonly used in the assessment of venous thrombosis in the clinical setting and can be used to assess thrombus formation and its resolution in the experimental setting.

**High-Frequency Ultrasound**

Duplex ultrasonography is a commonly used noncontrast imaging modality for the diagnosis of venous thrombosis in man. Preclinical high-frequency ultrasound systems have been developed that allow 2D high-resolution imaging of murine venous thrombi with pixel dimensions in range of 40 to 70 μm.\(^{50,51}\) IVC thrombus can be identified in transverse and longitudinal planes because of the hyperechoic nature of the thrombus when compared with surrounding blood (Figure 3). The echogenicity of the thrombus periphery is, however, similar to that of blood making accurate segmentation of the thrombus difficult. The absence of flow in the thrombus can be used to improve thrombus segmentation through the use of the color Doppler modality.\(^{50,51}\) To further improve contrast between the thrombus and the blood transpulmonary circulating microbubble contrast agents, such as Sonovue, can be administered intravenously with
the thrombus presenting as a hypoechoic structure within the vessel lumen.\textsuperscript{52}

Ultrasonic imaging techniques can also be used to provide surrogate measures of thrombus composition. Ultrasound elastography of venous thrombi shows a consistent increase in strain measurements over time indicative of thrombus hardening and is taken as thrombus organization. This technique has been used to accurately estimate clot age in a rat IVC stasis model.\textsuperscript{53} Measurements of thrombus size using high-frequency ultrasound have to date been 2D limiting data acquisition to either length or cross-sectional area. Available 3D acquisition systems commonly used to measure subcutaneous tumor development could be used to reconstruct the thrombus and provide a better measure of thrombus burden.\textsuperscript{54}

Antibody-targeted microbubble contrast agents have been developed to identify fibrin and platelet integrin \(\alpha_{IIb}\beta3\) at sites of arterial thrombus formation, and these agents may also be applicable to the study of venous thrombosis.\textsuperscript{55,56} Alternative agents that provide measures of endothelial cell activation at the sites of atherogenesis through binding cell surface markers such as vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 have also been developed.\textsuperscript{57,58} These agents may be of use when studying venous thrombus formation given the role of the endothelium during initiation.\textsuperscript{11}

**Ultrasonic Flow Probes**

Measurements of blood flow in the vessel lumen have been used in the assessment of venous thrombus formation. After induction, blood flow in the thrombosed segment can be monitored using an ultrasonic flow probe over a period of 30 to 40 minutes.\textsuperscript{59,60} Time to occlusion of the vessel can be used as a measure of thrombus formation. Calculation of time to occlusion may, however, be complicated by residual flow through the thrombosed vessel. Alternatively, flow can be monitored at fixed time points to observe the degree of vessel stenosis, which may be a more clinically relevant end point than measurement of thrombus size. The invasive and terminal nature of this procedure precludes the use of ultrasonic flow measures in longitudinal studies of the same animal.

**Figure 3.** Visualization of venous thrombi by high-frequency ultrasound. Transverse view of (A) sham-operated mouse with a patent infrarenal vena cava (IVC) and (B) after IVC ligation with hypoechoic thrombus present in the lumen. Adapted from Aghourian et al\textsuperscript{62} with permission of the publisher. Copyright \(\textcopyright2012\), John Wiley and Sons.

| Table 2. Methods of Assessing Venous Thrombus Formation and Resolution |
|-------------------|---|---|---|---|---|---|---|
|                   | W | H | MRI | CT | RI | HFUS | IVM |
| Real-time         |   |   |     |    |    |      | ● |
| Cell tracking     | ● | ● |     |    |    |      | ● |
| Colocalization    | ● | ● |     |    |    |      | ● |
| Large vessels*    | ● | ● |     |    |    |      | ● |
| Small vessels†    | ● | ● |     |    |    |      | ● |
| Quantitative      | ● | ● |     |    |    |      | ● |
| Noninvasive       |   |   |     |    |    |      | ● |
| Rapid             |   |   |     |    |    |      | ● |
| Longitudinal      |   |   |     |    |    |      | ● |
| Clinically relevant|     |  |    |    |    |      | ● |
| Reported in mice  |   |   |     |    |    |      | ● |
| Resolution, \(\mu\)m | N/A | N/A | 100–500 | 20–60 | 350–850 | 40–70 | <1 |

CT indicates computed tomography; H, histology; HFUS, high-frequency ultrasound; IVM, intravital microscopy; MRI, magnetic resonance imaging; RI, radionuclide imaging; and W, weight.

*Large vessels >1 mm in diameter.
†Small vessels <100 \(\mu\)m in diameter.

**Intravital Microscopy**

A variety of intravital microscopy (IVM) techniques have been used to image experimental thrombus formation in vivo including wide-field video and confocal microscopy. The relatively small depth of field achieved by these techniques has limited the majority of studies to imaging of thrombosis in the mesenteric and cremaster muscle microvasculature. Thrombi, formed in venules, can be visualized by fluorescently labeled antibodies to thrombus constituents such as fibrin and platelets, the accumulation of which allows temporal quantification of thrombus formation.\textsuperscript{62,63} Accurate estimation of the spatial resolution of current wide-field IVM systems is complicated by movement and light scatter of tissue during capture; however, the ability of this technique to resolve single platelets suggests that observed resolutions are in the submicrometer range.\textsuperscript{64} This intravital approach has been adapted to study the dynamics of thrombus formation in the femoral vein after localized electrolytic injury of the vessel wall.\textsuperscript{65,66} One of the major benefits of IVM-based studies of the microvasculature is that multiple thrombotic events can be initiated in a single animal (\(\approx10\) per mouse), greatly reducing the numbers needed for experimentation. Although flow rate and leukocyte rolling differ between the micro- and macrovasculature findings have been largely complementary with respect to thrombosis.\textsuperscript{52,63}

IVM has also been used to study the cooperation between neutrophils and monocytes and platelets during thrombus initiation in large vessels such as the femoral vein and IVC.\textsuperscript{21} IVM of the macrovasculature allows imaging of the luminal vein surface and the thrombus periphery, but because of limitations in tissue penetration, it is not possible to image the thrombus in its entirety. Near-infrared probes have distinct advantages for imaging of macrovascular thrombosis by IVM owing to the greater penetration of light through biological tissues in the range of 700 to 900 nm. Confocal IVM with a novel near-infrared probe
has been used to localize fibrin deposition in femoral vein thrombi, demonstrating the potential use of this technique in informing thrombus structure. The terminal nature of the procedure, however, prevents longitudinal measurements of thrombus size and composition. These techniques are limited to studies that are cross-sectional in design because of their terminal nature. Developments in imaging modalities (such as MRI, micro-CT, and high-frequency ultrasound) have enabled longitudinal analysis of not only thrombus burden but also thrombus composition that is beginning to rival the information provided by histology. Further developments in these imaging techniques will facilitate concomitant physical, cellular, and molecular analysis that could significantly enhance our capacity to investigate the mechanisms regulating formation and resolution of venous thrombi.

Conclusions

The most commonly used methods of analyzing venous thrombosis in experimental models remain physical measurement of thrombus weight and histological estimates of thrombus size and composition. These techniques are limited to studies that are cross-sectional in design because of their terminal nature. Developments in imaging modalities (such as MRI, micro-CT, and high-frequency ultrasound) have enabled longitudinal analysis of not only thrombus burden but also thrombus composition that is beginning to rival the information provided by histology. Further developments in these imaging techniques will facilitate concomitant physical, cellular, and molecular analysis that could significantly enhance our capacity to investigate the mechanisms regulating formation and resolution of venous thrombi.

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Disclosures

None.

References


Significance

Animal models have contributed significantly to our understanding of the mechanisms that govern formation and subsequent resolution of venous thrombi. Here, we critically appraise the techniques commonly used to assess thrombosis in these models. Particular attention is paid to imaging modalities that enable longitudinal measurements of thrombus burden and that provide data on composition in a noninvasive manner.
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