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Antiangiogenic Therapy Inhibits Venous Thrombus Resolution

Colin E. Evans,* Steven P. Grover,* J. Humphries, Prakash Saha, Anant P. Patel, Ashish S. Patel, Oliver T. Lyons, Bijan Modarai,† Alberto Smith†

Objective—Venous thromboembolism is a common complication in patients with cancer, resulting in significant morbidity and mortality. Clinical studies suggest that the incidence of venous thromboembolic events increases after treatment of these patients with antiangiogenic agents. Thrombi resolve through a process of remodeling, involving the formation of microvascular channels within the thrombus. Our aim was to determine whether inhibiting angiogenesis affects venous thrombus resolution.

Approach and Results—Thrombus was induced in the inferior vena cava of mice. These mice were treated with axitinib (50 mg/kg per day), 2-methoxyestradiol (2ME, 150 mg/kg per day), or vehicle control. Thrombus size, recanalization, neovascularization, inflammatory cell content, and collagen content were assessed after axitinib (days 3, 10, 17) and 2ME (day 10 only) treatment (n=6/group). Axitinib treatment resulted in reduced thrombus resolution (P<0.002) and vein recanalization (P<0.001) compared with vehicle-treated controls. This was associated with inhibition of organization as seen through reduced thrombus neovascularization (P<0.0001) and collagen (P<0.0001) content, as well as reduced macrophage accumulation in the thrombus (P<0.001). Treatment with a second antiangiogenic agent, 2ME, mirrored these findings, with a similar order of magnitude of effect of treatment over vehicle control in all of the parameters measured, with the exception of neutrophil content, which was significantly reduced after 2ME treatment but not affected by axitinib.

Conclusions—Antiangiogenic therapy (using axitinib and 2ME) inhibits the resolution of venous thrombi, which could lead to persistent venous obstruction and the possibility of thrombus extension. This potential prolongation of venous occlusion by antiangiogenic agents should therefore be taken into consideration in trials of these agents and when managing the complications of venous thromboembolic events in patients with cancer. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: 2-methoxyestradiol ■ antiangiogenesis inhibitors ■ axitinib ■ thrombosis

Venous thromboembolism (VTE) is a common complication and leading cause of mortality in patients with cancer.12 Management is usually by anticoagulation, but this only prevents thrombus extension and has little effect on thrombus resolution. Venous thrombi resolve through a natural process of organization and the formation of neovascular channels within the thrombus, which, together, ultimately lead to recanalization of the vein.3,4 Poor resolution is associated with post-thrombotic syndrome, symptoms of which include leg pain and swelling, and increases the likelihood of rethrombosis.5,6 Enhancing the angiogenic response that occurs during natural resolution can accelerate thrombus removal.7–10

There are a growing number of antiangiogenic agents under investigation for the prevention of tumor growth.11 Vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) signaling through cognate VEGF receptors (VEGFR) 1, 2, and 3 has been a major target, resulting in the development of axitinib that is a potent and selective small-molecule pan-VEGFR inhibitor12 now in phase I to III trials for multiple tumor types13–16 and approved for treatment of metastatic renal cell carcinoma.17 Axitinib inhibits angiogenesis by blocking ligand-induced VEGFR phosphorylation and affects downstream VEGF-mediated processes, including vascular permeability, endothelial cell survival, and tubule formation.12,18–21

Previous studies have demonstrated increasing levels of VEGF in venous thrombosi as they resolve.22 Elevation of VEGF in the thrombus, achieved through either direct injection or gene-mediated overexpression,8,10 results in accelerated thrombus neovascularization and vein recanalization. The newly formed venous thrombus is hypoxic, leading to increased hypoxia-inducible factor (HIF) 1α expression during natural resolution.23 Alternative antiangiogenic agents

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such as 2-methoxyestradiol (2ME)\textsuperscript{24,25} prevent the transcription of several angiogenic and chemotactic factors such as VEGF and PlGF\textsuperscript{26,27} by blocking nuclear accumulation of HIF1\textalpha.\textsuperscript{28} Conversely, preventing the degradation of HIF1\textalpha in the thrombus leads to increased expression of a variety of angiogenic factors and results in enhanced thrombus neovascularization and vein recanalization.\textsuperscript{23}

The use of antiangiogenic agents to treat cancers has been associated with an increased incidence of VTE,\textsuperscript{29,30} but any direct effect of these agents on the processes that govern thrombus formation and resolution is yet to be established. The aim of this study was to investigate the effect of antiangiogenic therapy on venous thrombus resolution using an experimental model of this condition.

### Materials and Methods

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

### Results

Thrombus resolution was significantly impaired by axitinib treatment, with a maintained thrombus burden ($P<0.002$; 2-way ANOVA; Figure 1A and 1B) and reduced vein recanalization ($P<0.001$, 2-way ANOVA; Figure 1A and 1C) compared with controls. At day 3, there were no significant differences in either thrombus volume (6.4±0.4 versus 5.8±0.3 mm\textsuperscript{3}; $P>0.05$) or vein recanalization (5.5±1.3\% versus 6.6±1.2\%; $P>0.05$) between axitinib-treated mice and controls, respectively, suggesting that this treatment did not affect the processes that govern thrombus propagation that occurs in this model between 1 and 3 days after induction.\textsuperscript{31,32}

Axitinib treatment also resulted in reduced thrombus organization, as measured by thrombus neovascularization ($P<0.0001$; 2-way ANOVA; Figure 2A and Figure I in the online-only Data Supplement) and fibrillar collagen content ($P<0.0001$; 2-way ANOVA; Figure 2B). Again at day 3, there were no initial effects of treatment on either the collagen content (1.3±0.09\% versus 1.4±0.06\% in controls; $P>0.05$) or the number of neovascular channels that developed within the thrombus (0.17±0.02 versus 0.33±0.05 channels in controls; $P>0.05$).

The progressive accumulation of macrophages within the thrombus was also significantly impaired by axitinib treatment ($P<0.0001$; 2-way ANOVA; Figure 2C and Figure IIA in the online-only Data Supplement). Few Mac-2–positive

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>2ME</td>
<td>2-methoxyestradiol</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>PlGF</td>
<td>placental growth factor</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VTE</td>
<td>venous thromboembolism</td>
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**Figure 1.** Thrombus volume and vein recanalization in mice treated with axitinib. **A,** Representative hematoxylin and eosin–stained sections of thrombi in mice treated with either axitinib or vehicle control. **B,** Thrombus volume was greater, and **C** vein recanalization was reduced in mice treated with axitinib compared with vehicle.
cells were located in the 3-day-old thrombus in both control and axitinib-treated mice (0.06±0.01% versus 0.05±0.01%, respectively). By day 10, the macrophage content of axitinib-treated mice was approximately half of that found in vehicle-treated controls. Thrombus neutrophil content was not affected by axitinib treatment (P>0.05; 2-way ANOVA; Figure 2D).

Treatment with 2ME was associated with an increase in thrombus size (2.9±0.3 versus 1.8±0.2 mm³ in controls; P<0.02; Figure 3A) and 2-fold decreases in vein recanalization (6.5±0.6% versus 16.5±1.7% in controls; P<0.005; Figure 3B) and thrombus neovascularization (2.0±0.2 versus 4.2±0.8 channels in controls; P<0.005; Figure 3C) at day 10. As with axitinib, thrombi also contained significantly less collagen (1.4±0.5% versus 9.3±3.3%; P<0.05; Figure 3D) and macrophage staining (4.9±0.6% versus 7.1±0.7%; P<0.05; Figure 3E and Figure IIB in the online-only Data Supplement) after 2ME treatment compared with vehicle, respectively, at this time point while neutrophil content was also reduced (0.6±0.1% versus 1.5±0.2% in controls; P<0.005; Figure 3F). To confirm an antiangiogenic phenotype after treatment with 2ME, we measured thrombus levels of HIF1α, HIF2α, VEGF, and PLGF at day 10. With the exception of HIF2α, all of these factors were significantly reduced in 2ME-treated mice compared with vehicle-treated controls (P<0.001 to P<0.02; Figure IIIA–IIID in the online-only Data Supplement).

**Discussion**

Treatment with the pan-VEGF receptor inhibitor, axitinib, and a second antiangiogenic agent, 2ME, significantly impaired venous thrombus organization, resolution (maintaining thrombus burden for longer), and vein recanalization. This was associated with a marked reduction (halving) in thrombus macrophage content.

Macrophage accumulation in the thrombus is a hallmark of venous thrombus resolution. Inhibition of the ingress of these cells into thrombus through deletion of the gene encoding CC chemokine receptor 2 or through inhibition of the CC chemokine system as a whole results in impaired resolution. Conversely, directly increasing macrophage numbers has been found to enhance thrombus resolution. VEGFR1, present on the surface of primary human monocytes, stimulates their migration into tissues. Inhibition of VEGFR1 activity on macrophages by axitinib may account for the reduced number of these cells accumulating in thrombus in this study. The comparable effects of axitinib and 2ME on thrombus macrophage content and association, in both instances, with reduced
resolution demonstrate the important contribution of this cell type toward this process.

Axitinib treatment did not affect the content of the other predominant inflammatory cell type normally found in thrombus, the neutrophil, during the time course of these experiments. The majority of neutrophils resident in the thrombus are thought to be included at the time of formation.33 There is little evidence to suggest that VEGF signaling affects neutrophil survival, which may go some way to explaining the lack of effect of axitinib treatment on thrombus neutrophil content. In contrast, however, treatment with 2ME significantly reduced thrombus neutrophil content, suggesting that neutrophil survival may be dependent on HIF1α but independent of VEGF/PIGF.

Fibrillar collagen deposition and neovascularity were quantified as measures of thrombus organization. The low levels of collagen observed at day 3 after induction are consistent with previous observations that demonstrate that fibrin and red cells predominate the early thrombus.32 Over time, treatment with axitinib resulted in reduced collagen deposition in thrombi, but it remains unclear which cell types contribute to this deposition and extracellular matrix remodeling in the thrombus. Macrophages have been found to express a wide range of collagen isoforms,39 and therefore reduction in their levels could have contributed to the observed decrease in fibrillar collagen content after axitinib treatment. Inhibition of VEGFRs by axitinib also resulted in a sustained reduction of another marker of organization, thrombus neovascularization. This is consistent with the role of this agent as a potent inhibitor of tumor microvessel growth.18,19,40 Inhibition of VEGFR2 and VEGFR3 signaling using monoclonal antibodies reduces vascular network development and endothelial sprouting.41

The impairment of vein recanalization seen after axitinib treatment may be the result of increased thrombus volume in this group. Alternatively, it is possible that axitinib acts on the thrombosed vessel affecting either vessel tone or pathological vein wall remodeling. Antiangiogenic agents such as axitinib may have vasoactive properties because hypertension is a common side effect of these drugs.17 For example, in humans, treatment with bevacizumab, a monoclonal VEGF-A blocking antibody, significantly reduced vasodilatation.42

Treatment with a second agent with antiangiogenic properties, 2ME, confirmed that inhibiting angiogenesis in the thrombus impairs its resolution because many of the differences observed after treatment with axitinib were also evident in experiments using 2ME. This agent disrupts angiogenesis through a variety of pathways. It can do this directly by inhibiting endothelial cell proliferation,43 migration,44 survival,45 and inflammation or indirectly by blocking HIF1α nuclear accumulation, which prevents transcription of a large number of angiogenic growth factors with hypoxia response elements in their genes (including VEGF and PlGF).27,28 HIF1α and HIF1α-mediated angiogenic factors are expressed in a temporal pattern during thrombus resolution, and their expression is strongly correlated.31 Treatment with 2ME results in impaired (50% to 60%) neovascularization in a murine breast cancer model,27 the magnitude of which is similar to that observed in the current study (≈60%). The 2ME-induced reduction in the levels of potent angiogenic factors (eg, VEGF and PIGF) in the thrombus could account for this reduced neovascularization. 2ME could have also affected thrombus organization and resolution by inhibiting monocyte adhesion and subsequent infiltration.45,46 Monocyte infiltration may be mediated by the monocyte chemoattractants VEGF and PIGF under the regulation of HIF1α.47,48 The lack of any extra impairment of thrombus resolution by 2ME over that caused by axitinib was somewhat unexpected and suggests that a significant proportion of the observed changes seen after these treatments may be the result of inhibition of the interaction between VEGF and its receptors. This notion is supported by studies that show that upregulating thrombus VEGF levels alone promote resolution and recanalization of the vein.5–10

Patients with cancer have a higher incidence of venous thrombosis than is found in the normal population.1 There is evidence to suggest that treatment with antitumorigenic agents increases the incidence of venous thrombosis, possibly
by reducing endothelial antithrombotic activity and increasing platelet prothrombotic activity.\textsuperscript{12,13} Treatment of patients with cancer with the anti–VEGF-A monoclonal antibody, bevacizumab, has been found to increase the incidence of VTE.\textsuperscript{49} Treatment with axitinib resulted in a significant increase in the incidence of VTE compared with sorafenib in a phase III trial for the treatment of metastatic renal cell carcinoma.\textsuperscript{17} In this trial, patients with a recent history of VTE were excluded because licensed axitinib is available for these patients. It remains to be seen what effect axitinib has on this subpopulation of patients.

The present study provides, to the best of our knowledge, the first evidence that clinically used antiangiogenic agents could have a detrimental effect on venous thrombus resolution. In doing so, these agents may increase the incidence of clinically significant thrombosis by maintaining a persistent venous obstruction. Although priority is rightly given to strategies that delay disease progression and increase survival in cancer treatment, the results of this study suggest that the potentially prothrombotic effect of antiangiogenic agents should be taken into consideration when managing the complications of VTE in these patients. Further studies are warranted to investigate the effect of antiangiogenic agents on VTE incidence in humans and the effect this has on survival and quality of life.

Sources of Funding

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Disclosures

None.

References

Significance

Venous thrombosis is a common healthcare problem and is particularly prevalent in patients with cancer, causing significant morbidity and mortality, although antiangiogenic therapy is increasingly being viewed as an effective weapon in the fight against cancer. Given that antiangiogenic therapy inhibits the resolution of venous thrombi and is associated with reduced inflammatory cell numbers in the thrombus, consideration should be given to the effect of antiangiogenic therapy on the incidence of venous thromboembolic complications in patients with cancer. Angiogenic hypoxia-inducible factor 1/vascular endothelial growth factor signaling seems to be an important mechanism of venous thrombus resolution.
Materials and Methods

Experimental venous thrombi were induced in the inferior vena cava of 8-week-old male BALB/C mice using a combination of blood flow restriction and endothelial disturbance as previously described\(^1\). Occlusive thrombi form within 4hrs in this model and resolve naturally over a period of ~30days\(^1\). All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Axitinib (LC Laboratories, USA) or vehicle was administered twice daily by intraperitoneal injection at a dose of 25mg/kg prepared in 30% (v/v) PEG400 70% (v/v) acidified water\(^2\), with treatment commencing 24hrs after thrombus induction. The effect of axitinib treatment on thrombus resolution was compared with vehicle control at 3, 10 and 17 days post-induction by histological analysis as described below (n=6/group).

2-methoxyestradiol (2ME, Enzo Life Sciences, UK) or vehicle was administered to a second cohort of thrombosed mice by daily intraperitoneal injection at a dose of 150mg/kg prepared in 1% DMSO. The effect of 2ME treatment on thrombus resolution was also compared with vehicle control at day 10 post-induction by histological analysis as described below (n=6/group). To confirm the anti-angiogenic effect of 2ME, thrombi from a third cohort of drug treated and control mice were analysed at day 10 using biochemical analysis as described in the supplementary methods (n=7/group).

Transverse paraffin sections (5µm) were taken at 300µm intervals along the entire length of the thrombus and stained with haematoxylin and eosin (H&E). Images of whole tissue sections were obtained in a blinded fashion using Image Pro Plus (Media Cybernetics, USA). Estimates of thrombus volume (mm\(^3\)), vein recanalisation (% area of lumen) and neovascularisation (average number of vascular channels per level) were obtained as previously described\(^3\).

Neutrophils are recruited during thrombus formation\(^4\), but their levels may be maintained if HIF1\(\alpha\) levels are upregulated during resolution\(^3\). Macrophages are recruited during thrombus resolution and their levels in the thrombus are associated with enhanced resolution\(^5\). Additional contiguous sections, taken at intervals throughout the thrombus from groups used to estimate thrombus resolution, were immunostained using the mouse macrophage marker, Mac2 (BioLegend, UK), or the mouse neutrophil marker, Gr1 (NIMPR14, Abcam, UK) as previously described\(^3\). Picrosirius red staining was used to localise collagen fibrils in the thrombus. Images of whole tissue sections were obtained in a blinded fashion and macrophage, neutrophil and collagen content estimated by measuring the percentage area of each thrombus containing Mac2, Gr1 or picrosirius positive staining respectively using Image Pro Plus software (Media Cybernetics, USA) as previously described\(^3,\;6\).

Statistical analysis

Thrombus resolution, collagen and inflammatory cell content, and vein recanalisation after axitinib treatment were compared with those of vehicle controls using 2way ANOVA. Differences following 2ME treatment were compared with vehicle controls using unpaired student t-tests. Data are expressed as means ± standard error.
Supplementary methods
2ME has been used in experimental models at doses of 100-150mg/kg and inhibits HIF1α nuclear accumulation\(^7\)\(^8\). Thrombi from 2ME and vehicle-treated mice were harvested at day 10, immediately snap frozen, and stored at -80°C for biochemical analysis (n=7/group). The anti-angiogenic activity of 2ME was confirmed by measurement of HIF1α, HIF2α, VEGF, and PIGF expression as described\(^3\). The rate-limiting factor for HIF activation is accumulation and translocation of HIFα to the nucleus, given that its heterodimerisation partner, HIFβ, is constitutively expressed. Nuclear and cytoplasmic fractions of thrombus homogenates were therefore obtained using the NE-PER Extraction Kit (Pierce, UK) according to manufacturer’s instructions. HIF1α expression was measured in nuclear fractions using a human/mouse HIF1α immunoassay (R&D Systems, UK). The cytoplasmic fraction was used to measure VEGF and PIGF expression, also by immunoassay (R&D Systems, UK). All values were normalised to the soluble protein content of the extract measured using the Coomassie Plus modified Bradford assay (Pierce, UK).

References
Supplementary Figure I: Neovascular channels in the resolving thrombus
Representative immunostaining of CD31+ (brown) endothelial cell-lined neovascular channels within the resolving thrombus. Contiguous sections were exposed to IgG isotype control.
Supplementary Figure II: Macrophage staining in resolving thrombus of mice treated with 2ME and Axitinib

Macrophages (black) were observed in the 10-day-old thrombus of mice treated with (A) Axitinib and (B) 2ME or their respective vehicle control. These Mac2 positive cells were particularly abundant at the thrombus periphery and in vascularising regions.
Supplementary Figure III: HIF1α, HIF2α, VEGF, and PIGF expression in the thrombus of mice treated with 2ME
At day 10 post-thrombus induction, (A) HIF1α, (C) VEGF, and (D) PIGF expression were reduced in the thrombus of mice treated with 2ME compared with control. There was no significant difference in (B) HIF2α expression in the 10 day-old thrombus of 2ME- versus vehicle-treated mice. *P<0.001 versus control.
COLIN EVANS: BIOGRAPHICAL SKETCH

**Previous and current research**

My previous studies have investigated the role of hypoxia in the vascular remodelling of skeletal muscle, venous thrombus, and mammary tumour. During my undergraduate studies at the University of Birmingham, I showed in the *Journal of Sports Science* that blood flow restriction during short-term resistance exercise enhances capillarisation of human calf muscles (Evans et al., 2010, 28, 999-1007).

The British Heart Foundation then awarded me a 4-year PhD studentship in Cardiovascular Medicine. During my studies in the laboratory of Professor Alberto Smith at King’s College London (2007-2010), I investigated the role of hypoxia (low oxygenation) in the resolution of venous thrombi using rodent models of venous thrombosis. My work published in *Arteriosclerosis, Thrombosis, and Vascular Biology* (Evans et al., 2010, 30, 2443-51) and *Thrombosis Research* (Evans et al., 2011, 128, 346-51; Evans et al., 2012, 129, 812-4) showed that conditions of hypoxia stimulate venous thrombus resolution via stabilisation of hypoxia-inducible factor (HIF) 1 in neutrophils and endothelial cells within the thrombus and surrounding vein. I also showed that pharmacological upregulation of the HIF1 response in thrombus and surrounding vein enhances thrombus remodelling and resolution, while pharmacological downregulation has the opposite effect (Evans et al., submitted to *Arteriosclerosis, Thrombosis, and Vascular Biology*). These studies identified HIF1 as a potential therapeutic target for treatments that accelerate thrombus resolution. In other related work, I contributed to the development of novel prognostic imaging methods to detect tissue hypoxia and predict successful thrombolysis in patients with deep vein thrombosis (Saha et al., *Circulation*, 2013, 128, 729-36).

I am currently a Postdoctoral Research Associate in the laboratory of Professor Randall Johnson at the University of Cambridge, studying the association between thrombosis and cancer progression. My current aim is to investigate mechanisms that regulate the positive correlations between hypoxia, hypercoagulation/thrombosis, and tumour vascularisation. We recently showed in *Cancer Cell* and *Cancer Research* that tumour vascularisation and metastasis are controlled by the hypoxic response derived from fibroblasts and endothelial cells respectively. These findings showed that regulation of cancer progression is not limited to tumour cells, and I therefore aim to determine whether thrombus formation triggers a hypoxic response that stimulates tumour growth and metastasis, and conversely, whether hypoxia within the tumour stimulates hypercoagulation and thrombosis (Evans et al., 2012, *International Journal of Hematology*, 95, 471-7).
Key publications

Original articles


Reviews


Book chapter

Future research plans
My future research will include the elucidation of cell type-specific HIF-mediated mechanisms that control thrombus formation and resolution using mouse models of venous thrombosis.

**Background.** Hypoxia and endothelial activation are strongly associated with venous thrombosis (1, 2), but the mechanisms that regulate thrombus formation are unclear. We (and others) showed that endothelial hypoxia and activation (e.g. in venous valves and during tumourigenesis) (2, 3) stimulate a thrombotic response that is controlled by hypoxia-inducible factor (HIF) 1 and 2. Endothelial hypoxia stimulates thrombus formation (2, 4, 5), and many HIF targets enhance thrombosis, including tissue factor (TF), E-selectin, intracellular adhesion molecule (ICAM) 1, and plasminogen activator inhibitor (PAI) 1 (1). I also showed that venous thrombus propagation is HIF-dependent (6-8). I therefore speculate that endothelial hypoxia and activation lead to HIF-mediated increases in the expression of coagulants that enhance thrombus formation (9). The aim of this work is to determine the role of endothelial HIF in thrombus formation using cell type-specific HIF knockout mice, in vivo models of thrombosis, in vitro coagulation assays, and ex vivo analysis of human tissue. A better understanding of mechanisms that regulate thrombus formation could lead to the development of new therapies for people predisposed to thrombosis.

**Preliminary data.** I showed that: (i) pharmacological upregulation of HIF in thrombosed mice increases the expression of several factors that regulate coagulation; (ii) HIF1 deletion abolishes hypoxia-induced increases in coagulability in mouse embryonic fibroblasts (MEFs) and endothelial cells (MECs); and (iii) pharmacological downregulation of HIF by treatment of thrombosed mice with 2-methoxyestradiol inhibits thrombus resolution (Evans et al., submitted to *Thrombosis and Haemostasis*). These findings suggest that HIF can regulate coagulation and thrombosis.
Plan of investigation. Venous thrombosis will be induced in endothelial HIF1 knockout, endothelial HIF2 knockout, and wild-type mice as we described (3, 7). To determine the effect of endothelial HIF on thrombus formation and resolution; thrombus weight, size, and inflammatory cell infiltration will be quantified as I have described (7). To determine the effect of endothelial HIF on coagulation; tail bleeding and plasma coagulability times will be measured as described (10). To investigate mechanisms that regulate HIF-mediated thrombus formation, thrombus will be collected from endothelial HIF knockout and wild-type mice and analysed by gene microarrays, including quantification of HIF-mediated factors that regulate coagulation such as TF and PAI1. Molecular targets with therapeutic potential could be manipulated in future studies e.g. as I have described (7). Table 1 shows potential challenges and how they would be addressed.

<table>
<thead>
<tr>
<th>Challenge</th>
<th>How challenge would be addressed</th>
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<tr>
<td>Vena cava thrombus does not form in endothelial HIF knockout mice</td>
<td>Increase thrombotic stimulus by complete stenosis of vena cava or increased endothelial disturbance</td>
</tr>
<tr>
<td>Differences in thrombus formation as measured by thrombus size and weight cannot be detected</td>
<td>Decrease thrombotic stimulus by reducing or removing endothelial disturbance or by creating a less severe stenosis</td>
</tr>
<tr>
<td>Other cell types control HIF-mediated regulation of coagulation and thrombus formation</td>
<td>Mice with targeted deletion of HIF in other cells that affect thrombosis (e.g. myeloid cells and fibroblasts) are available</td>
</tr>
<tr>
<td>Effects of HIF1 deletion masked by actions of HIF2 or vice versa</td>
<td>Mice with targeted deletion of both HIF1α and HIF2 are available</td>
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References