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 increased 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 postthrombotic 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.3,10

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 thrombi as they resolve.22 Elevation of VEGF in the thrombus, achieved through either direct injection9 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 HIF1α.\textsuperscript{28} Conversely, preventing the degradation of HIF1α 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\(^3\); \(P>0.05\)) 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>
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<th>Abbreviation</th>
<th>Description</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 IIIB 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,12 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.33,34 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 system35,36 results in impaired resolution. Conversely, directly increasing macrophage numbers has been found to enhance thrombus resolution.36 VEGFR1, present on the surface of primary human monocytes,37 stimulates their migration into tissues.37,38 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.23 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 PlGF) 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 PlGF 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. Treatment of patients with cancer with the anti–VEGF-A monoclonal antibody, bevacizumab, has been found to increase the incidence of VTE. 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. 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.

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Disclosures
None.

References
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.
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Materials and Methods

Experimental venous thrombi were induced in the inferior vena cava of 8week-old male BALB/C mice using a combination of blood flow restriction and endothelial disturbance as previously described. Occlusive thrombi form within 4hrs in this model and resolve naturally over a period of ~30days. 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, 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.

Neutrophils are recruited during thrombus formation, but their levels may be maintained if HIF1α levels are upregulated during resolution. Macrophages are recruited during thrombus resolution and their levels in the thrombus are associated with enhanced resolution. 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. 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.

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 -80ºC 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.