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Objective—Angiogenic factors are expressed within thrombus during resolution, but the primary stimulus for neovascularization is unknown. Our aims were to determine whether (1) hypoxia and hypoxia-inducible factor 1α (HIF1α) are induced in resolving thrombus, (2) this stimulates angiogenic factor production, and (3) upregulating HIF1α enhances thrombus resolution and vein recanalization.

Methods and Results—Oxygen tension in the thrombus was negatively correlated with HIF1α levels (Spearman correlation [RS]=−0.77, $P<0.0001$), whereas HIF1α levels positively correlated with vascular endothelial growth factor (VEGF) expression (Pearson correlation [R]=0.85, $P<0.0005$), during resolution in a murine model. HIF1α ($P<0.005$), VEGF ($P<0.005$), and VEGF receptor 1 (VEGFR1) ($P<0.05$) expression was 2-fold greater in the thrombus of mice treated with the prolyl hydroxylase domain inhibitor L-mimosine compared with controls. The levels of 13 other HIF1-mediated angiogenic factors were also increased. Thrombus weight ($P<0.001$) and volume ($P<0.05$) were reduced by a third in L-mimosine–treated mice compared with controls, whereas vein recanalization ($P<0.005$) and thrombus neovascularization ($P<0.001$) were 2-fold greater, and this was associated with increased inflammatory cell content.

Conclusion—Hypoxia and HIF1α are induced in the naturally resolving thrombus and correlate with increased angiogenic factor expression. Upregulation of HIF1α enhances thrombus resolution and vein recanalization. HIF1α may represent a novel target for treatments that promote resolution and recanalization and reduce the incidence of post-thrombotic syndrome. **(*Arterioscler Thromb Vasc Biol*. 2010;30:2443-2451.)**

Key Words: angiogenesis ■ hypoxia ■ hypoxia-inducible factor ■ ischemia ■ resolution

Deep vein thrombosis (DVT) has an annual incidence of approximately 1 in 500,[1] and approximately 1 in 3 DVT patients develop post-thrombotic syndrome,[2] which is characterized by leg pain, swelling, and ulceration.

Venous thrombus resolution is a slow, natural process.[3] Veins that recanalize more rapidly following a DVT have preserved valve integrity and a lower incidence of venous obstruction, which is associated with improved clinical outcome.[4–6] Anticoagulation is the usual treatment for DVT, but this does not promote resolution and carries a risk of major bleeding. Thrombolysis and thrombectomy remove thrombus rapidly but may be contraindicated in many patients and carry the risks of major bleeding or rethrombosis.

The organization of venous thrombi involves several processes, including recruitment of inflammatory cells, myofibroblasts, and endothelial cells, leading to the formation of neovascular channels both throughout and around the thrombus.[7–9] Potent angiogenic and chemotactic factors are expressed within the thrombus during natural resolution,[10–12] and increasing their levels in the thrombus enhances its resolution.[12–15] The stimulus for the production of these factors remains unknown.

Hypoxia is characteristic of several pathophysiological states, including cancer, atherosclerosis, chronic obstructive pulmonary disease, pre-eclampsia, and cardiovascular ischemic disorders.[16] Low oxygen tension stimulates the transcription of a number of genes, including those encoding vascular endothelial growth factor (VEGF), which is a potent promoter of both physiological and pathological vascular remodeling.[17,18] Under hypoxic conditions, VEGF stimulates elongation and branching of endothelial cells,[19] mobilizes progenitor cells from the bone marrow, and is chemotactic for both monocytes and endothelial cells.[20–22] Increasing the expression of VEGF within the thrombus by directly injecting either VEGF recombinant protein,[14] VEGF naked plasmid,[23] or VEGF adenovirus[24] results in an acceleration in thrombus organization and resolution.

The remodeling response that follows hypoxia is controlled primarily by hypoxia-inducible factor 1 (HIF1).[17] HIF1 is a heterodimeric transcription factor composed of α and β

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subunits. HIF1β is stable and constitutively expressed, but HIF1α is regulated in an oxygen-dependent manner through the activity of prolyl hydroxylase domain (PHD) proteins 1, 2, and 3.[25] During hypoxia, HIF1α accumulates and translocates to the nucleus, where it forms the active HIF1 complex with HIF1β.[26] HIF1 binds to the hypoxia-responsive element of target genes such as VEGF and upregulates their transcription.[18]

L-Mimosine is an effective PHD inhibitor that binds to the active site of PHDs and therefore interferes with the reconstitution of the active enzyme.[27] It is structurally related to 2-oxoglutarate (a PHD substrate) and probably competes with 2-oxoglutarate at the catalytic core of PHDs.[28] L-Mimosine has been used to increase the levels of HIF1α and induce angiogenesis both in vitro[29] and in vivo.[28–30]

The recruitment of monocytes and endothelial cells, which infiltrate resolving thrombus, is also associated with hypoxia.[31] Stimulating or inhibiting macrophage recruitment has profound effects on thrombus recanalization and resolution.[13,24,32,33] We hypothesized that the formation of an occlusive thrombus causes relative hypoxia of the cells within it and that this is a stimulus for the expression of angiogenic factors that promote thrombus recanalization and resolution, possibly through the stimulation of monocyte recruitment.

The aims of this study were to determine whether hypoxia and HIF1α are induced in the resolving thrombus and whether this leads to an increase in the production of angiogenic growth factors such as VEGF. A second aim was to determine whether administration of the PHD inhibitor L-mimosine increases HIF1α expression in the thrombus and augments its recanalization and resolution.

Methods

Mouse Model of Venous Thrombosis

Experimental venous thrombi were induced in the inferior vena cava (IVC) of 8-week-old male BALB/c mice using a combination of blood flow restriction and endothelial disturbance as previously described.[7,33,34] In this model, totally occlusive thrombi form within 4 hours following thrombus induction and resolve completely by day 21.[33,34]

Assessment of Hypoxia in Thrombus During Natural Resolution

An oxygen microelectrode and tissue-monitoring system (OxyLab, Oxford Optronix) was used to measure oxygen tension (pO_2) in the thrombus. The microelectrode was inserted through the vein wall into the thrombus at 1 day (n=13), 3 days (n=10), 7 days (n=13), and 14 days (n=10) after induction. Normal venous pO_2 was measured by inserting the microelectrode into the blood of the right ventricle (n=16). Thrombus was removed from the vein, snap frozen, and stored at −80°C until measurement of HIF1α and VEGF expression by enzyme-linked immunosorbent assay (ELISA).

HIF1α and VEGF Expression in Thrombus During Natural Resolution

As HIF1β is constitutively expressed, the rate-limiting factor for HIF1 activation is accumulation and translocation of HIF1α to the nucleus. Both the nuclear and cytoplasmic fractions of thrombus homogenates were therefore extracted using an extraction kit according to the manufacturer's instructions (NE-PER Extraction Kit, Pierce).[35–37] Soluble protein concentrations in each fraction were measured using the Coomassie Plus modified Bradford assay (Pierce).

The levels of HIF1α in nuclear fractions were measured using a human/mouse HIF1α ELISA (R&D Systems). The level of VEGF in the cytoplasmic fractions was measured using a mouse $VEGF_{164}$ ELISA (R&D Systems).

Localization of HIF1α by Immunohistochemistry

Contiguous transverse paraffin sections (5 μm) of 1-day-old thrombus (n=3) were immunostained for HIF1α (anti-HIF1α, Stratech), macrophages (anti-Mac-2, BioLegend), and neutrophils (anti-NIMP R14, Abcam) following antigen retrieval by pressure cooking sections in citrate buffer (0.3% sodium citrate, pH 6). Primary antibody binding was detected using biotinylated rabbit anti-rat (Dako) and ExtrAvidin horseradish peroxidase complex (Sigma). Isotype-matched IgG was used as a negative control. Peroxidase activity was visualized using the Vector SG peroxidase substrate kit (Vector Laboratories), and sections were counterstained using nuclear fast red.

Treatment With L-Mimosine

Dosing Regimen

L-Mimosine dosage is limited by its low solubility.[29] A dosing regimen of 50 mg/kg per day given over a 2-week period upregulates HIF1α,[30] and our dosing regimen was designed to administer the maximum amount of L-mimosine possible. An initial intraperitoneal injection of 300 mg/kg L-mimosine in 10% $NaHCO_3$, pH 7.2 (Sigma), was administered 5 hours after thrombus induction. A subsequent injection of 300 mg/kg was administered 16 hours later. Additional daily injections of 120 mg/kg were administered to animals in which the effect of L-mimosine was to be evaluated over 7 and 10 days.

Effect on Thrombus Formation

Thrombus was formed in 30 mice. Mice were treated with either L-mimosine or a vehicle control (n=15 per group) 5 hours later. Thrombi were harvested 24 hours after thrombus induction and weighed.

Effect on HIF1α and Angiogenic Factor Production

Thrombus was formed in 30 mice. Mice were treated with either L-mimosine or vehicle control (n=15 per group). Thrombi were harvested at day 7, immediately snap frozen, and stored at −80°C. All thrombi were weighed, and 14 thrombi (n=7 per group) underwent nuclear and cytoplasmic extraction. HIF1α expression was measured in nuclear fractions, whereas VEGF expression was measured in cytoplasmic fractions.

The remaining 16 thrombi (n=8 per group) were analyzed using a protein array (Mouse Angiogenesis Array Kit, R&D Systems) for the expression of 15 factors whose transcription is known to be regulated by HIF1 (ie, angiopoietin 1,[38] endoglin,[39] endothelin 1,[40] insulin-like growth factor binding protein 1,[41] 2,[42] and 3[42] [IGFBP1, IGFBP2, and IGFBP3], leptin,[43,44] monocyte chemotactic protein 1 [MCP1],[45] matrix metalloproteinase 9 [MMP9],[45] platelet-derived growth factor B [PDGF-B],[38] placental growth factor [PLGF],[38] stromal cell–derived factor 1[46] [SDF1], plasminogen activator inhibitor 1,[47,48] tissue inhibitor of metalloproteinase 1[49] [TIMP1], and VEGF[50]). The mean pixel density of the duplicate spots for each factor was measured using Quantity One (BioRad) and expressed as a percentage of the mean pixel density of the duplicate positive controls on the same membrane. The levels of VEGFR1 were measured using a mouse VEGFR1 ELISA (R&D Systems).

Effect on Thrombus Resolution, Neovascularization, and Vein Recanalization

Thrombus was formed in 26 mice. Mice were treated with either L-mimosine or vehicle control (n=13 per group). The IVC containing thrombus was harvested at day 7 (n=6 per group) and 10 (n=7 per group) and fixed in 10% formalin. Transverse paraffin sections (5 μm) were taken at 300-μm intervals along the entire length of the thrombus and stained with hematoxylin and eosin. Images of whole tissue sections were obtained in a blinded fashion using Image Pro Plus (Media Cybernetics). Estimates of thrombus volume (mm^3) and

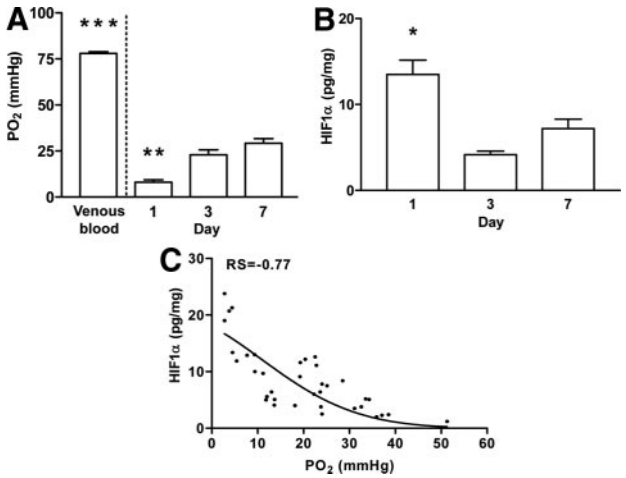

Figure 1. Oxygen tension and HIF1α expression in the naturally resolving thrombus. A, pO_2 at day 1 was lower than that at days 3 and 7. pO_2 at days 1, 3, and 7 was lower than pO_2 of venous blood. B, HIF1α expression was greater at day 1 than at days 3 and 7. *$P<0.001$ versus day 3 and $P<0.01$ versus day 7; **$P<0.001$ versus days 3 and 7; ***$P<0.0001$ versus days 1, 3, and 7. C, Negative correlation between pO_2 and HIF1α expression (RS=−0.77, n=36, $P<0.0001$).

IVC recanalization (% area of lumen) were obtained as previously described.[24]

Sections were also immunostained for CD31 (anti-CD31, Santa Cruz Biotechnology), following antigen retrieval by incubation with Proteinase K. Primary antibody binding was detected using biotinylated rabbit anti-goat (Dako) and ExtrAvidin alkaline phosphatase complex (Sigma). Isotype-matched IgG was used as a negative control. Phosphatase activity was visualized using the Vector BCIP/NBT substrate kit (Vector Laboratories), and sections were counterstained using nuclear fast red.

An estimate of neovascularization was obtained in a blinded fashion by counting the number of vascular channels within the thrombus (ie, channels that contained erythrocytes or were lined with cells that stained positive for the endothelial cell marker CD31). An average count was taken throughout the length of the thrombus.

Effect on Macrophage and Neutrophil Content

Paraffin sections (5 μm) of 7-day-old (n=6 per group) and 10-day-old (n=7 per group) thrombi and surrounding IVC of mice treated with L-mimosine or vehicle control were immunostained using the mouse macrophage marker Mac-2 (BioLegend) or the mouse neutrophil marker NIMP R14 (Abcam) as previously described. Images were obtained of sections taken at 300-μm intervals throughout the length of the thrombus. Macrophage or neutrophil content was estimated in a blinded fashion by measuring the percentage area of each thrombus or vein wall containing Mac-2 or NIMP R14 stain using Image Pro Plus software (Media Cybernetics) as previously described.[24]

Statistical Analysis

One-way analysis of variance with the Bonferroni post hoc test was used to determine whether there was a relationship between pO_2, HIF1α, or VEGF in the thrombus and time after thrombus induction. The relationship between pO_2 and HIF1α expression was tested using the Spearman correlation (RS), and the relationship between HIF1α and VEGF expression was tested using the Pearson correlation (R). To compare differences between L-mimosine–treated mice and controls, statistical significance ($P<0.05$) was determined by using parametric or nonparametric tests as appropriate. Data are expressed as means±standard error.

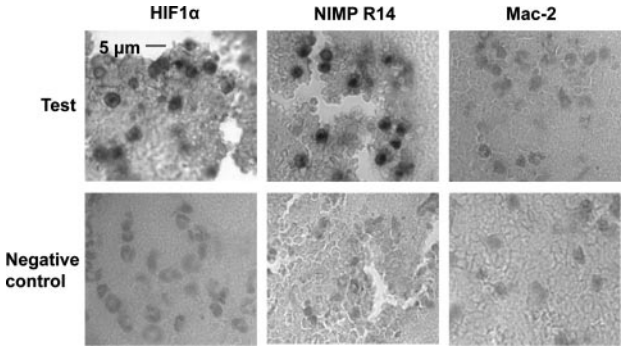

Figure 2. HIF1α, neutrophil (NIMP R14), and macrophage (Mac-2) staining of day 1 thrombus. Nucleated cells within the thrombus stained positive for HIF1α and NIMP R14 on contiguous sections (black, ×400 magnification).

Results

Oxygen Tension in the Naturally Resolving Thrombus

pO_2 in the thrombus at 1 day (8.0±1.3 mm Hg), 3 days (22.9±2.7 mm Hg), and 7 days (29.2±2.5 mm Hg) after thrombus induction was significantly lower than the pO_2 of venous blood (78.0±0.9 mm Hg, $P<0.0001$ for all 3 comparisons, Figure 1A). There was an inverse relationship between pO_2 in the thrombus and time following thrombus induction ($P<0.0001$). pO_2 in the thrombus was significantly lower at day 1 compared with days 3 ($P<0.001$) and 7 ($P<0.001$, Figure 1A). There was no difference in pO_2 in the thrombus between days 3 and 7.

HIF1α Expression and Localization in the Thrombus During Natural Resolution

HIF1α at day 1 (13.5±1.7 pg/mg) was significantly higher than at day 3 (4.2±0.4 pg/mg, $P<0.001$) and day 7 (7.2±1.1 pg/mg, $P<0.01$, Figure 1B). There was no difference between HIF1α in thrombi at day 3 compared with day 7. There was a strong negative correlation between pO_2 and HIF1α expression in the thrombi at days 1, 3, and 7 after thrombus formation (RS=−0.77, n=36, $P<0.0001$, Figure 1C). Thrombus at day 14 was too small to yield a sufficient amount of nuclear extract to allow measurement of HIF1α expression by ELISA.

Immunohistochemical analysis showed that at day 1 (the time of greatest hypoxia), HIF1α was expressed mainly in cell-dense regions within the thrombus and appeared to be almost completely located within the nuclei of cells having a polymorphonuclear morphology and staining positively for the neutrophil marker NIMP R14 (Figure 2). There were very few macrophages present in the thrombus at this time, as shown by the minimal amount of Mac-2 staining (Figure 2).

VEGF Expression in the Naturally Resolving Thrombus

VEGF in the thrombus was elevated at 3 days (32.1±4.1 pg/mg) and 7 days (26.7±3.0 pg/mg) compared with 1 day (4.4±0.3 pg/mg) and 14 days (8.5±0.7 pg/mg) after thrombus induction ($P<0.001$ for all 4 comparisons, Figure 3A). There was a strong positive relationship between HIF1α and VEGF in the thrombus at 3 days ($R=0.81$, n=10, $P<0.005$,

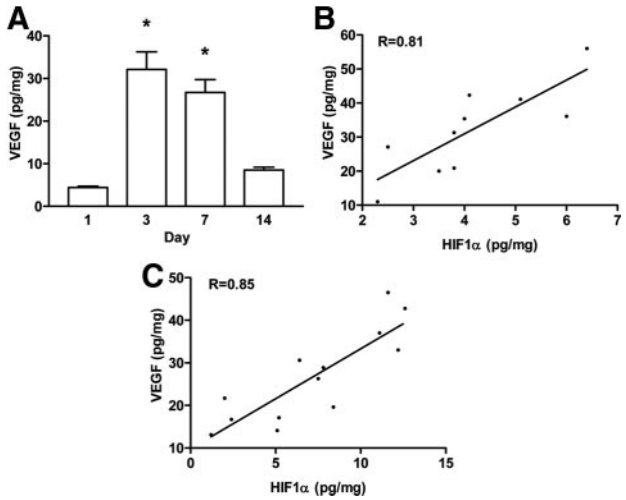

Figure 3. VEGF expression in the naturally resolving mouse thrombus. A, VEGF was elevated at days 3 and 7 compared with days 1 and 14 after thrombus induction. *$P<0.001$ versus days 1 and 14. B and C, Positive correlation between HIF1α and VEGF expression at day 3 (B) ($R=0.81$, n=10, $P<0.005$) and day 7 (C) ($R=0.85$, n=13, $P<0.0005$).

Figure 3B) and 7 days ($R=0.85$, n=13, $P<0.0005$, Figure 3C) after thrombus formation.

The Effect of L-Mimosine Treatment on HIF1α Expression and HIF1-Mediated Cytokine Expression

HIF1α (13.3±1.6 versus 5.9±1.1 pg/mg, $P<0.005$, Figure 4A), VEGF (48.5±4.3 versus 23.2±4.6 pg/mg, $P<0.005$, Figure 4B), and VEGFR1 expression (1330.1±175.8 versus 753.1±117.4 pg/mg, $P<0.05$, Figure 4C) were approximately 2-fold greater in the thrombus of L-mimosine–treated mice compared with controls.

Protein array analysis revealed that VEGF was not the only HIF1-regulated factor to be significantly overexpressed in the thrombus of mice injected with L-mimosine compared with vehicle control. The levels of angiopoietin 1 ($P<0.02$), endoglin ($P<0.05$), endothelin 1 ($P<0.02$), IGFBP1 ($P<0.005$), IGFBP2 ($P<0.05$),

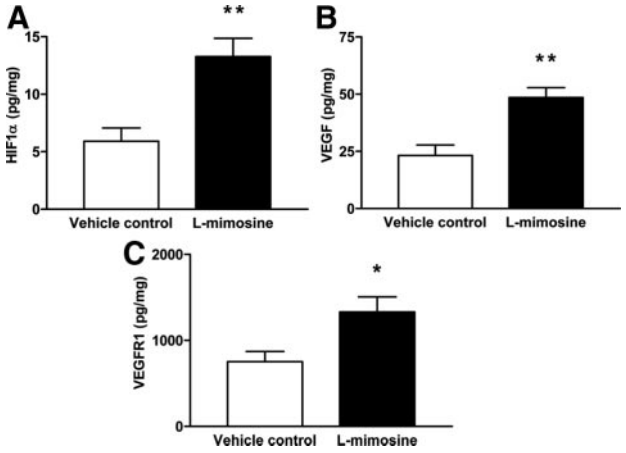

Figure 4. HIF1α, VEGF, and VEGFR1 expression at day 7 in the thrombus of mice treated with L-mimosine or vehicle control. A to C, HIF1α (A), VEGF (B), and VEGFR1 (C) expression was greater in the thrombus of mice treated with L-mimosine compared with vehicle control. *$P<0.05$ and **$P<0.005$ versus control.

Table. HIF1-Mediated Angiogenic Factors at Day 7 in the Thrombus of Mice Treated With L-Mimosine or Vehicle Control

	Mean Pixel Density (%)			
Factor	Vehicle	L-Mimosine	Fold Increase	Significance
Ang1	4.6±1.9	23±7.9	5	$P<0.02$
Endoglin	15±5.6	41±7.7	2.7	$P<0.05$
Endothelin 1	6.9±3.5	24±7.0	3.5	$P<0.02$
IGFBP1	2.0±1.0	19±6.2	9.7	$P<0.005$
IGFBP2	12±4.6	36±7.5	2.9	$P<0.05$
IGFBP3	66±8.7	96±5.6	1.5	$P<0.02$
Leptin	2.2±1.9	11±6.0	5	$P<0.05$
MCP1	11±4.4	36±5.1	3.3	$P<0.01$
MMP9	100.1±7.5	122±5.1	1.2	$P<0.05$
PDGF-B	2.9±1.0	14±4.2	4.9	$P<0.01$
PLGF	7.1±3.6	26±7.2	3.6	$P<0.05$
SDF1	1.9±1.2	14±5.7	7.6	$P<0.005$
PAI1	103±14.3	115±3.3	1.1	$P>0.5$
TIMP1	25±8.8	57±9.4	2.3	$P<0.05$
VEGF	1.1±0.8	4.4±2.5	4	$P<0.005$

Data are expressed as mean pixel density as a percentage of positive control (±SEM, n=8 per group). Ang indicates angiopoietin; PAI, plasminogen activator inhibitor.

IGFBP3 ($P<0.02$), leptin ($P<0.05$), MCP1 ($P<0.01$), MMP9 ($P<0.05$), PDGF-B ($P<0.01$), PLGF ($P<0.05$), SDF1 ($P<0.005$), and TIMP1 ($P<0.05$) were also increased (Table).

The Effect of L-Mimosine on Thrombus Weight, Volume, Neovascularization, and Vein Recanalization

There was no difference in thrombus weight 24 hours after thrombus induction in mice treated with L-mimosine (22.9±1.2 mg) compared with control (20.0±1.3 mg, Figure 5A). Thrombus weight was reduced by approximately 30% in L-mimosine–treated mice (15.0±1.0 mg) versus controls (20.5±0.9 mg, $P<0.001$, Figure 5A) at day 7. Similarly, thrombus volume was approximately 40% lower in L-mimosine–treated mice (2.9±0.4 mm^3) versus controls (4.6±0.3 mm^3, $P<0.05$) at day 7 and approximately 25% lower at day 10 (1.9±0.1 versus 2.6±0.3 mm^3, $P<0.05$, Figure 5B). Recanalization of the IVC was increased by 2-fold following L-mimosine treatment compared with control at day 7 (8.3±1.5% versus 4.2±1.0%, $P<0.05$) and also at day 10 (20.6±2.2 versus 9.0±1.2%, $P<0.005$, Figure 5C). Thrombus neovascularization was increased by 3-fold in mice treated with L-mimosine (5.8±0.9 channels) compared with control at day 7 (1.9±0.3 channels, $P<0.005$) and by 2-fold at day 10 (4.6±0.3 versus 2.3±0.2 channels, $P<0.001$, Figure 5D).

The Effect of L-Mimosine on Macrophage and Neutrophil Content

Thrombus

There was no difference in the numbers of macrophages (1.7±0.1% versus 1.5±0.3%, $P>0.1$) or neutrophils (1.7±0.2% versus 1.3±0.3%, $P>0.1$) in the thrombus of L-mimosine–treated mice compared with vehicle-treated mice at day 7. By day 10, there were greater numbers of both

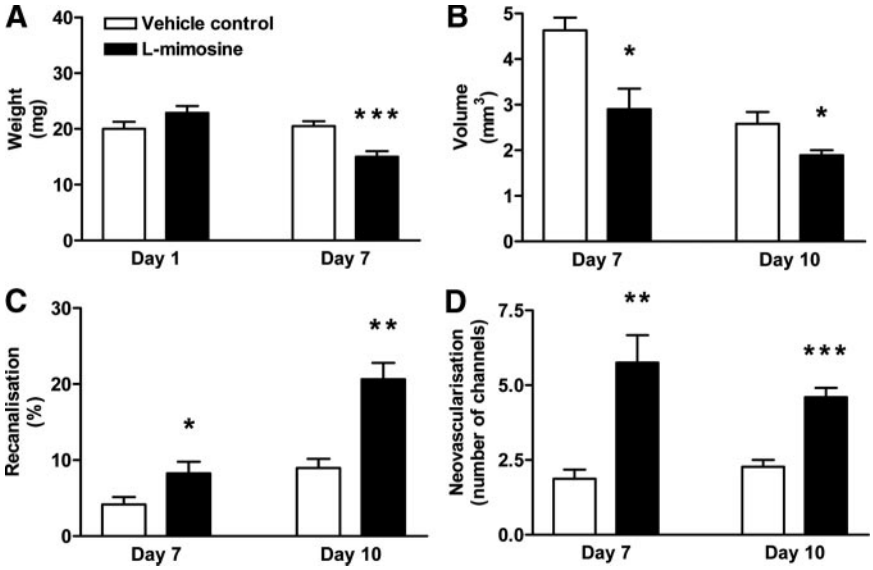

Figure 5. Thrombus weight, size, neovascularization, and vein recanalization, at days 7 and 10 in mice treated with L-mimosine or vehicle control. A and B, Thrombus weight at day 7 (A) and thrombus size at days 7 and 10 (B) were reduced in mice treated with L-mimosine compared with control. C, IVC recanalization at days 7 and 10, and D, Thrombus neovascularization at days 7 and 10 were increased in L-mimosine–treated mice compared with controls. IVC recanalization is expressed as a percentage of lumen area. *$P<0.05$, **$P<0.005$, and ***$P<0.001$ versus the respective controls.

macrophages (6.3±0.4 versus 4.4±0.4%, $P<0.01$, Figure 6A) and neutrophils (2.0±0.2 versus 1.0±0.2%, $P<0.05$, Figure 6B) in the thrombus of mice treated with L-mimosine compared with vehicle.

Vein Wall

Macrophage content was higher in the vein wall of L-mimosine–treated mice compared with controls at both day 7 (3.7±0.5 versus 1.4±0.3%, $P<0.01$) and day 10 (7.5±0.7 versus 4.7±0.6%, $P<0.05$, Figure 6C). Neutrophil content in the IVC of mice treated with L-mimosine was also increased at day 7 (12.0±0.6 versus 8.1±0.6%, $P<0.005$) and day 10 (8.2±0.4 versus 5.7±0.7%, $P<0.01$, Figure 6D).

Discussion

This study is the first to show a relative hypoxia within newly formed thrombi, with a pO_2 of approximately 10% of that in circulating venous blood. This is closely related to the presence of increased levels of HIF1α and angiogenic factors within the thrombus. Preventing HIF1α degradation with the PHD inhibitor L-mimosine led to increased angiogenic factor expression and inflammatory cell content, and it enhanced IVC recanalization and thrombus resolution.

Hypoxia within the thrombus probably results from the formation of an occlusive thrombus, the volume and cross-sectional area of which are greatest at day 1 in this model.[32] The radius of the thrombus at this time point exceeds the maximal oxygen diffusion distance of 100 to 250 μm.[51] Recanalization and contraction of the thrombus away from the IVC is seen at days 3 and 7.[8,12] This allows blood flow into and around the thrombus, which, together with the decreased oxygen diffusion distance that results from reduced thrombus size, may be the reason for the rise in pO_2 levels observed at these time points. This would be in keeping with data from tumor models, which shows increased tumor pO_2 as tumor volume decreases.[53] The finding that thrombi at days 3 and 7 remain hypoxic relative to the pO_2 of venous blood could be the result of an increase in oxygen demand in the

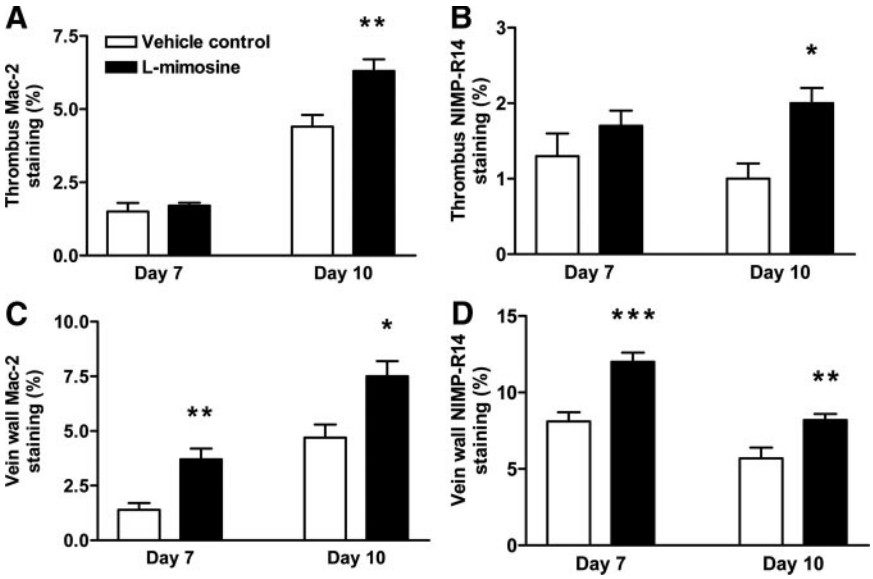

Figure 6. Macrophage and neutrophil content at days 7 and 10 in mice treated with L-mimosine or vehicle control. A and B, Macrophage (A) and neutrophil (B) content in the thrombus was greater in L-mimosine–treated compared with vehicle-treated mice at day 10 but not day 7. C and D, Macrophage (C) and neutrophil (D) content was increased in the IVC of L-mimosine–treated mice compared with controls at days 7 and 10. Macrophage or neutrophil content is expressed as a percentage of thrombus or vein wall staining positively for Mac-2 or NIMP R14 antibody, respectively. *$P<0.05$, **$P<0.01$, and ***$P<0.005$ versus the respective controls.

face of a relatively poor blood supply in these rapidly remodeling structures.

There was a strong inverse correlation between HIF1α and pO_2, suggesting that HIF1α stabilization is oxygen dependent in the naturally resolving thrombus. HIF1α expression may, however, be regulated in an oxygen-independent manner via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinase pathways. Growth factors that have been shown to stimulate HIF1α production include insulin, interleukin 1β,[54] and insulin-like growth factors (IGF) 1 and 2.[42,55] Neutrophils are the predominant nucleated cell-type present in the newly formed and 1-day-old thrombus[32,56] and are capable of expressing interleukin 1β.[57] Therefore, we cannot discount the possibility that these pathways may be partly responsible for the HIF1α levels found in this study.

HIF1α staining in day 1 thrombi was located in the nucleus of neutrophils (NIMP R14–positive polymorphonucleated cells). Staining for macrophages (Mac-2-positive cells) in contiguous sections revealed very few of these cells were present in the thrombus at this time point. These data were not unexpected, as macrophages infiltrate the thrombus much later,[32] possibly as a result of HIF1-mediated transcriptional upregulation of growth factors such as VEGF, which facilitate chemoattraction of monocytes/macrophages through VEGFR1.[21]

We have previously shown that upregulating VEGF within the thrombus enhances its resolution.[14,23,24] In this study, there was a strong positive correlation between HIF1α and VEGF expression in the thrombus during natural resolution. Upregulation of HIF1α is a more efficacious stimulus for angiogenesis than upregulation of VEGF alone.[58] We therefore hypothesized that augmenting the accumulation of HIF1α should enhance thrombus resolution by stimulating local angiogenesis through the expression of multiple growth and chemotactic factors[17,18] and in this way be more effective than upregulating a single factor, such as VEGF.[14,24] These growth and chemotactic factors could affect thrombus remodeling and subsequent resolution in a number of ways: first, by promoting endothelial cell proliferation and survival[59] and mobilizing vascular progenitors,[59,60] which lead to the development of vessels within and around the thrombus.[18] Second, they could work by recruitment and activation of monocytes,[21] which secrete growth factors and proteases important for tissue remodeling.[22,61] Third, they could work by upregulating plasminogen activator expression, which increases local fibrinolysis.[62]

Treatment with the PHD inhibitor L-mimosine resulted in an approximately 2-fold increase in HIF1α expression in the thrombus. This reflects the increase in HIF1α seen in rat medulla following a similar treatment.[30] L-Mimosine treatment led to a 2- to 3-fold increase in vein recanalization and thrombus neovascularization, which is also comparable to the increase in microvessel density following a similar treatment in a rat sponge model of angiogenesis.[29] L-Mimosine treatment was unlikely to have affected thrombus formation, as it was administered 5 hours after stenosis of the IVC, and fully occlusive thrombi form within 4 hours in this model.[34] The similar thrombus weights of 24-hour-old thrombi obtained from mice treated with L-mimosine and vehicle controls provides further evidence that this treatment did not affect thrombogenesis.

Analysis of the effects of L-mimosine on thrombus resolution and vein recanalization was carried out at 2 time points: day 7, when thrombus organization is first readily visible (this or a similar time point have been used previously),[10,12,14,15,23,24,63] and day 10, when thrombus organization is well under way in this model.[33] By day 14, thrombus resolution is highly advanced, with only small thrombi visible within the IVC.[33] L-Mimosine treatment resulted in 21% recanalization at day 10 (compared with 8% seen at day 7). The difference in recanalization between treatment groups and respective controls was greater at day 10 (12%) than at day 7 (4%), suggesting that continuous L-mimosine treatment accelerated recanalization. Although assessment at a later time point (eg, day 16) may have revealed a reduction in time to complete resolution, comparison between treatments would probably have proved less reliable, as natural resolution in this model accelerates rapidly after 7 to 10 days,[33] resulting in a small and highly variable volume of remaining thrombus.

Adenovirus construct-mediated upregulation of VEGF increased IVC recanalization 3-fold and reduced thrombus size by half.[24] In this study, there was a more modest effect, with L-mimosine treatment doubling IVC recanalization and reducing thrombus size by one-third. L-Mimosine is known to inhibit other members of the 2-oxoglutarate–dependent dioxygenases,[28] including collagen hydroxylase, which regulates collagen synthesis.[64] This action would be expected to inhibit resolution, as collagen synthesis is an important component of the organization process.[63] These opposing actions of L-mimosine may account for some of the differences in the efficacy of the treatments between this and previous studies. The relatively short half-life of L-mimosine (approximately 5 hours in the rat[65]) compared with the relatively long expression of a viral construct that continually produces VEGF for the duration of the experiment could also account for the lesser effect of L-mimosine on thrombus resolution and IVC recanalization. In this context, it is also important to note that the amount of L-mimosine that can be injected per day is limited by the solubility of the compound and the maximum permissible volume that can be injected.

VEGF levels in the thrombus induced by L-mimosine treatment were also 20-fold lower than those caused by treatment with the adenoviral VEGF construct[24] (20 versus 400 pg/mg, respectively), but L-mimosine still resulted in a 2-fold increase in IVC recanalization compared with the 3-fold increase induced by the adenoviral VEGF construct.[24] This might be explained by the increased expression of 13 other HIF1-mediated angiogenic factors in the thrombus of L-mimosine–treated mice compared with controls. All of these factors could contribute to thrombus resolution. Angiopoietin 1 activates the Tie2 receptor and mediates endothelial cell sprouting and formation of new vascular networks.[66] Endoglin is a marker of neovascular endothelium,[39] and endothelin 1 contributes to vascular remodeling by stimulating the growth and proliferation of vascular smooth muscle cells.[67] IGFBP1 modulates the effects of IGF1 and IGF2, which have important roles in vessel growth.[68] Leptin targets the vascular endothelium and induces neovascularization in

vivo.[69] MCP1 levels increase in the thrombus as it resolves, and increasing the amount of MCP1 in the thrombus enhances thrombus organization and resolution.[10] MMP9 has been found in abundance in the resolving thrombus[63] and could facilitate thrombus canalization by degrading its extracellular matrix to facilitate the migration of endothelial cells.[70] Net extracellular matrix turnover is also affected by the level of the MMP inhibitor TIMP1.[71] The higher levels of TIMP seen in this study may reflect the increased production of MMP9, as this inhibitor is expressed to prevent MMP overactivity under physiological conditions.[71,72] PDGF-B can induce VEGF expression[73] and activate the extracellular signal-regulated kinase and phosphatidylinositol 3-kinase pathways, which stimulate HIF1α synthesis.[74] PLGF may promote thrombus recanalization by recruiting monocytes, mobilizing progenitor cells from the bone marrow, and affecting endothelial cells directly via VEGFR1,[75] which was also upregulated following L-mimosine treatment. SDF1 expression in ischemic tissue is directly proportional to reduced pO_2.[46] HIF1-mediated SDF1 expression increases the migration and adhesion of circulating progenitor cells to ischemic tissue.[46] It is possible that HIF1-mediated expression of SDF1 increases bone marrow-derived progenitor cell recruitment to the resolving thrombus.[7] Under homeostatic conditions, SDF1 is also responsible for recruitment and homing of neutrophils,[76,77] which infiltrate the thrombus during the early stages of resolution.[8,12] These data suggest that the expression of other HIF1-mediated angiogenic cytokines and receptors (in addition to VEGF and VEGFR1) were induced by L-mimosine and may have helped to accelerate vein recanalization. This leads us to speculate that stimulating HIF1α in a more concerted way (eg, with a viral construct expressing stabilized HIF1α[38]) could have an even greater impact on thrombus resolution and vein recanalization.

We provide data for the efficacy of L-mimosine in promoting thrombus resolution and IVC recanalization through HIF1-mediated induction of angiogenic factors. It is possible, however, that L-mimosine stimulated this process through pathways independent of HIF1α, for example by stabilization of HIF2α.[28]

The importance of thrombus neovascularization in vein recanalization is also uncertain. Although it is likely that thrombus retraction has the greatest effect on vein recanalization (in terms of luminal cross-sectional area available for restoration of blood flow), this process may involve the coalescence of neovascular channels that form at the thrombus edge. The relative importance of each process remains to be defined.

L-Mimosine treatment led to increased numbers of inflammatory cells (neutrophils and macrophages) in the vein wall at day 7 but not in thrombi at this time point. It is possible that HIF1-mediated expression of cytokines and growth factors (such as endothelin 1 or others not measured in this study) could be responsible for the recruitment of these cells into the vein wall.[31] Treatment with L-mimosine for 10 days appeared to accelerate IVC recanalization and thrombus resolution, and this was associated with an increase of $\approx$50% in macrophage numbers in both the IVC and the thrombus. Macrophages are capable of generating a plethora of growth, chemotactic, and proteolytic agents that regulate tissue remodeling in wound healing and thrombosis, and ablating these cells has an impact on these processes.[8,13,15,33,39,78,79] There was also a small but significant increase in the numbers of neutrophils present in the thrombus and vein wall of L-mimosine–treated mice compared with controls at day 10. This was unexpected, as these cells are associated with the early thrombus, and their numbers reduce with time during natural resolution.[32,80] Neutropenia retards thrombus resolution,[56] however, and it is possible that L-mimosine exerted some of its effects on this process by stimulating recruitment of neutrophils and preventing their apoptosis.[81,82]

Thrombus organization and recanalization appears to be driven through an "outside-in" process,[8] with inflammatory cells entering the thrombus via the vein wall.[8,13,15,32,33,56] Relative hypoxia in the thrombus could provide a directional stimulus for the growth of microvessels from the vein wall into the thrombus, which, together with thrombus retraction, leads to vein recanalization and blood flow restoration. A better insight into the expression of transcription factors such as HIF1 and their target genes will lead to a greater understanding of the mechanisms responsible for natural thrombus resolution and vein recanalization. This could direct the development of novel treatments that enhance these processes and reduce the incidence of post-thrombotic complications.

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Disclosures

None.

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Correction

In the article by Evans et al which appeared in the December 2010 issue of the journal (*Arterioscler Thromb Vasc Biol*. 2010;30:2443–2451; DOI: 10.1161/ATVBAHA.110.215038), the author Patrick J. Maxwell should have appeared as "Patrick H. Maxwell."

The online version has been corrected.

The publisher sincerely regrets the error.

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