Defective Angiogenesis Delays Thrombus Resolution
A Potential Pathogenetic Mechanism Underlying Chronic Thromboembolic Pulmonary Hypertension

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Objective—Restoration of patency is a natural target of vascular remodeling after venous thrombosis that involves vascular endothelial cells and smooth muscle cells, as well as leukocytes. Acute pulmonary emboli usually resolve <6 months. However, in some instances, thrombi transform into fibrous vascular obstructions, resulting in occlusion of the deep veins, or in chronic thromboembolic pulmonary hypertension (CTEPH). We proposed that dysregulated thrombus angiogenesis may contribute to thrombus persistence.

Approach and Results—Mice with an endothelial cell–specific conditional deletion of vascular endothelial growth factor receptor 2/kinase insert domain protein receptor were used in a model of stagnant flow venous thrombosis closely resembling human deep vein thrombosis. Biochemical and functional analyses were performed on pulmonary endarterectomy specimens from patients with CTEPH, a human model of nonresolving venous thromboembolism. Endothelial cell–specific deletion of kinase insert domain protein receptor and subsequent ablation of thrombus vascularization delayed thrombus resolution. In accordance with these findings, organized human CTEPH thrombi were largely devoid of vascular structures. Several vessel-specific genes, such as kinase insert domain protein receptor, vascular endothelial cadherin, and podoplanin, were expressed at lower levels in white CTEPH thrombi than in organizing deep vein thrombi and organizing thrombi from aortic aneurysms. In addition, red CTEPH thrombi attenuated the angiogenic response induced by vascular endothelial growth factor.

Conclusions—in the present work, we propose a mechanism of thrombus nonresolution demonstrating that endothelial cell–specific deletion of kinase insert domain protein receptor abates thrombus vessel formation, misguiding thrombus resolution. Medical conditions associated with the development of CTEPH may be compromising early thrombus angiogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:810-819.)

Key Words: endothelium ■ thrombosis ■ vascular endothelial growth factor receptor-2

Restoration of vascular patency is a key target of vascular remodeling after thrombosis.1,2 Apart from plasmin-driven fibrinolysis as a major proteolytic system in venous thrombus resolution, angiogenesis3 and leukocyte recruitment4 hallmark this process, with the expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor,5 and recruitment of bone marrow–derived Tie-2–expressing cells.6,7 Acute pulmonary thromboembolism undergo almost complete resolution <6 months; however, in 0.1% to 9.1% of cases, they persist in the major pulmonary arteries and trigger a vascular remodeling process with medial fibrosis and intimal hyperplasia leading to vascular stenosis and occlusions, clinically translating into chronic thromboembolic pulmonary hypertension (CTEPH).5,10 All currently available evidence indicates that CTEPH is primarily caused by pulmonary thromboembolism, as opposed to primary pulmonary in situ vascular thrombosis.11 We speculate that pulmonary embolism may be followed by a pulmonary vascular remodeling process modified by infection,12 immune phenomena,13 inflammation,14 circulating and vascular-resident progenitor
cells, thyroid hormone replacement, and malignancy. Hypercoagulation, sticky red blood cells, high platelet count, and uncleavable fibrinogen also contribute to obliteration of large and small vessels in CTEPH. Thus, CTEPH may serve as a human model disease for venous thrombus nonresolution. CTEPH thrombus classically represents a cast of the pulmonary vascular bed, consisting of endothelium, smooth muscle cells, and fibroblasts.

Previous animal studies demonstrated that venous thrombus recanalization may occur <24 hours of thrombus formation, and several of these studies focused on the effect of angiogenesis on thrombus resolution. In one study, the VEGF gene therapy facilitated thrombus recanalization and organization. Another study found that the concentration of proangiogenic agents on thrombus resolution. Tie-2 and KDR (Figure 1C). Thus, controversy remains regarding the role of angiogenesis in venous thrombus resolution.

In this work we hypothesized that (1) angiogenesis plays a key role for thrombus resolution, and that (2) CTEPH may result from a condition of decreased thrombus vascularization leading to thrombus nonresolution. We used an experimental model resembling human deep vein thrombosis in transgenic mice conditionally deficient in kinase insert domain protein receptor (Kdr), the predominant cellular receptor for VEGF in endothelial cells, to prove that thrombus resolution is critically dependent on Kdr. Furthermore, we aimed to study thrombus angiogenesis and vascularization at different stages of thrombosis in CTEPH. We harvested red fresh and white fibrotic CTEPH thrombus material and compared those tissues with parent unthrombosed pulmonary artery and with a spectrum of venous and arterial thrombus samples. Parent, unthrombosed CTEPH pulmonary artery is a small piece of artery obtained from a proximal, apparently disease-free site (see online-only Data Supplement for a full description).

**Materials and Methods**

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

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<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>Cdh5/CDH5</td>
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<tr>
<td>CTEPH</td>
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<tr>
<td>Ctgf/CTGF</td>
</tr>
<tr>
<td>HUVEC</td>
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<td>IVC</td>
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<td>Kdr/KDR</td>
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<td>PDPN</td>
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<td>Ptprc/PTPRC</td>
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<td>TX</td>
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<tr>
<td>Vegfa/VEGF</td>
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**Results**

**Characterization of Mice With Endothelial Cell–Specific Deletion of Kdr (Kdr<sup>Δend</sup>)**

Kdr loci and the position of the probe used for Southern blot analysis are shown in Figure 1A. Total DNA was isolated from lungs, kidneys (Figure 1B, lanes 1 and 2), livers, and hearts (data not shown) of controls (n=8) and Kdr<sup>Δend</sup> (n=8; Figure 1B, lanes 3 and 4). Digestion with Spe I resulted in a 4.9-kb fragment for the floxed allele and a 15.3-kb fragment for the CRE-deleted allele (∆ allele; Figure 1B). The floxed allele contains a diagnostic Spe I site, which is absent in the ∆ allele (Figure 1A). Although only the floxed allele could be detected in the organs of controls (Figure 1B, lanes 1 and 2), both the floxed and ∆ alleles were discernible in Kdr<sup>Δend</sup> (Figure 1B, lanes 3 and 4). Kdr expression in endothelial cells isolated from lungs, kidneys, and liver of Kdr<sup>flox/flox/Tie-2CreER</sup> was significantly decreased after tamoxifen (TX) induction compared with cells from animals that did not receive TX (P<0.05; Figure 1C).

KDR expression was also examined in Tie-2–expressing monocytes of Kdr<sup>Δend</sup> and controls (n=8 each). Flow cytometry analyses demonstrated that the proportion of KDR<sup>+</sup> monocytes of total monocytes and of Tie-2<sup>+</sup> monocytes did not differ between Kdr<sup>Δend</sup> and controls (Figure 1D). Only ~0.25% monocytes expressed KDR, ~0.25% monocytes expressed Tie-2, and ~0.1% monocytes coexpressed Tie-2 and KDR (Figure 1D). Mean fluorescence intensity of KDR in KDR<sup>+</sup> monocytes and in Tie-2<sup>+</sup> KDR<sup>+</sup> monocytes was not different (mean fluorescence intensities in Kdr<sup>Δend</sup>: 1851±746 and 1686±1335; and in controls: 1780±870 and 1644±1343). Kdr mRNA gene expression level of isolated monocytes was equal in Kdr<sup>flox/flox/Tie-2CreER</sup> before and after TX treatment (Figure 1C). Kdr mRNA expression was 1000 times less in monocytes than in endothelial cells (Figure 1C), lending support to the expectation that the Kdr<sup>Δend</sup> phenotype was primarily endothelial cell–dependent.

Kdr<sup>Δend</sup> did not display a prothrombotic phenotype, because tail bleeding times did not differ from controls (n=10 each; Figure 1E). Regarding platelet function, the percentage of total and activated leukocyte/platelet aggregates and the percentage of total and activated monocyte/platelet aggregates were similar in Kdr<sup>Δend</sup> and controls (n=8 each; Figure 1F).

**Delayed Thrombus Resolution in Kdr<sup>Δend</sup>**

To study thrombus resolution, a murine stagnant flow inferior vena cava (IVC) thrombosis model was used. Thrombosis was induced by partial ligation of IVC in Kdr<sup>Δend</sup> and controls, and thrombi were harvested 3, 7, 14, and 28 days after surgery (Figure 2A–2H). We were able to harvest thrombi in 73% of Kdr<sup>Δend</sup> and in 70% of controls (n=8 or 9 per group and individual time point). Weights, cross-sectional areas, and volumes of thrombi from Kdr<sup>Δend</sup> were significantly increased at all time points compared with controls (Figure 2I–2K).

Mean relative thrombus volume changes are illustrated in Figure 2L. Thrombus volumes from Kdr<sup>Δend</sup> decreased at a slower rate between days 3 and 7 and days 7 and 14 than in control thrombi.
Endothelial Cell–Specific Deletion of Kdr Abates Thrombus Angiogenesis

Thrombus microvessels (expressed as a percentage of isolecitin B4–positive cells per total cells within a cross-sectional sample of the thrombus) were quantified by immunohistochemistry. On day 3 after ligation, microvessel density was similar in both groups. In thrombi of controls, microvessel density steadily increased until day 28. By contrast, microvessel density in KdrΔend thrombi was significantly decreased by days 7 and 14 compared with controls (Figure 3A–3E; Table I in the online-only Data Supplement). By day 28, thrombus microvessel density was similar to controls.

To verify immunohistochemical findings, the expression of angiogenic markers vascular endothelial cadherin (Cdh5), VEGF (Vegfa), and Kdr was analyzed in thrombi by quantitative real-time PCR. In controls, these 3 markers were highly expressed on days 3 and 7, and decreased significantly by days 14 and 28. By contrast, the expression of Cdh5 and Vegfa in KdrΔend remained low at all time points (Figure 3F and 3G). As expected, Kdr expression was very low on days 3, 7, and 14 in KdrΔend (Figure 3H).

Diminished Thrombus Macrophage Numbers in KdrΔend

By day 7, control thrombi had higher counts of macrophages (expressed as a percentage of F4/80-positive cells of total cells per thrombus area) than KdrΔend thrombi. No significant differences in macrophage counts were observed on days 3, 14, and 28 (Figure 3M; Table I in the online-only Data Supplement). CD68 mRNA gene expression was equal in thrombi of KdrΔend and controls at all time points, except for day 7, where control thrombi displayed more CD68 mRNA compared with KdrΔend thrombi (Figure 3N). This was in good agreement with immunohistochemistry findings. In controls, common leukocyte antigen CD45 (Ptprc, protein tyrosine phosphatase receptor type C) was highly expressed on days 3 and 7, and decreased significantly by days 14 and 28. In KdrΔend thrombi, Ptprc expression remained low on days 3, 7, and 14; however, by day 28, the expression had increased (Figure 3O). mRNA levels of connective tissue growth factor (Ctgf), a marker for organization and fibrotic transformation, was increased on days 3 and 7 in controls, and decreased by days 14 and 28. In KdrΔend thrombi, Ctgf expression was low on days 3, 7, and 14; however, by day 28, the expression had increased (Figure 3P).

Patients

Consecutive CTEPH patients were consented for tissue analyses at the General Hospital of Vienna and the Kerckhoff Clinic in Bad Nauheim. Mean age of CTEPH patients was 58±12 years; 46% were women; and general characteristics matched those of a typical European CTEPH population (Table).
Patients from whom venous and arterial thrombi, acute coronary thrombi, and carotid endarterectomy specimens had been harvested were sex- and age-matched with CTEPH patients (n=46).

**Low Expression of Angiogenic Factors in CTEPH Thrombi**

By histology, mouse IVC thrombi were similar to human CTEPH thrombi (Figure 4A–4D). Von Willebrand factor and platelet endothelial cell adhesion molecule-1 (PECAM1)-positive cell counts were lower in white and red CTEPH thrombi than in parent unthrombosed pulmonary arteries (Figure 4F–4L). Gene expression levels of angiopoietin-2 (ANGPT2), PECAM1, CDH5, KDR, podoplanin (PDPN), Tie-2 (TEK), and VEGFA were decreased in white CTEPH thrombi compared with respective parent unthrombosed pulmonary arteries as reference standards (Figure 5A). Furthermore, the expression of factors involved in proliferative pathways of vascular cells, such as bone morphogenetic protein receptor type 2 (BMPR2) or transforming growth factor-β1 (TGFβ1, TGBF1), was decreased. By contrast, the thrombogenic molecule plasminogen activator inhibitor-1 (PAI1, SERPINE1) was increased in white CTEPH thrombi (Figure 5A). There was no difference in gene expression of CTGF and leukocyte-specific genes (PTPRC, CD68, and MPO) between white CTEPH thrombi and parent unthrombosed pulmonary arteries. When other vascular thrombi (acute femoral thrombi, coronary aspirates, organizing aortic thrombi, carotid thrombendarterectomies, acute pulmonary emboli, subacute, and organizing deep vein thrombi) were analyzed, white CTEPH thrombi displayed more vessel-specific gene expression than in any acute thrombi, but significantly less than any organizing thrombi (Figure 5B and 5C).

**Low Ratio of Angiogenesis Gene Expression/CTGF Expression in White CTEPH Thrombi**

Figure 5B and 5C displays angiogenesis gene expression levels in arterial and venous thrombi at different stages of organization, in comparison with white CTEPH thrombi. CTGF expression levels were rising in venous thrombi in the following order:
in vitro clots from CTEPH patients, acute pulmonary emboli, subacute deep vein thrombi, and organizing venous thrombi occluding deep vein segments that were harvested during variceal surgeries. In arterial thrombi, CTGF gene expression levels were increasing in the following order: acute femoral thrombi, coronary aspirates from acute myocardial infarction, and organizing thrombi from aortic aneurysms. In comparator thrombus tissues, but not in white CTEPH thrombi, gene expressions of CDH5, KDR, and PDPN were increasing with increasing CTGF levels. Ratios of CDH5/CTGF, KDR/CTGF, and PDPN/CTGF expression were lowest in white CTEPH thrombi. Respective gene expressions in red CTEPH thrombi were similar to acute arterial and venous thrombi.

Angiogenic Inhibitory Activity of Red CTEPH Thrombus

After 2-dimensional gel electrophoresis of extracts of representative fresh red and organized white CTEPH thrombi (Figure 4E), as well as of pulmonary artery tissue from the same patient (Figure I in the online-only Data Supplement), protein fingerprinting revealed significant differences in protein profiles from these 3 tissues. Organized CTEPH thrombi demonstrated the least variety of protein bands (Figure IB in the online-only Data Supplement). To find out whether these lysates contain cell stimulatory factors, a BrdU assay was used to analyze DNA synthesis rates of human umbilical vein endothelial cells (HUVECs). Here, stimulation for 24 hours with lysates of fresh red and white CTEPH thrombus material, as well as from parent pulmonary artery tissue in the absence or presence of VEGF, was assessed, while DNA synthesis rates with basal medium served as control. Material from white CTEPH thrombi and CTEPH pulmonary artery tissue was found to stimulate DNA synthesis in HUVEC in a dose-dependent manner >24 hours (Figure 6A), similar to lysates from healthy pulmonary artery tissue (data not shown). Heat-induced denaturation of proteins in these lysates...
reversed their stimulatory effect (data not shown), indicating that thrombus-bound proteins were responsible for the mitogenic activity. The stimulatory activity of lysates at 40 μg/mL was equivalent to VEGF alone at 50 ng/mL, and both, lysates together with VEGF, exhibited an additive effect on DNA synthesis rates in HUVECs. By contrast, red CTEPH thrombi did not influence DNA synthesis in HUVECs.

In an in vitro 3-dimensional angiogenesis assay, isolated tissue lysates did not induce sprout formation, whereas red thrombus material at 40 μg/mL inhibited VEGF-induced sprouting by 15% (Figure 6B). Material from white CTEPH thrombi, as well as from unthrombosed pulmonary artery tissue, did not affect VEGF-induced endothelial cell sprouting.

### Discussion

Vascular endothelial growth factor receptor-2 (KDR) is a type III transmembrane kinase receptor and a predominant endothelial cell receptor to promote VEGF functions through specific intracellular signaling cascades leading to proliferation, migration, survival, and increased permeability, each of which contributes to the angiogenic response. Endothelial cell–specific deletion of this pathway allowed us to demonstrate the effects of angiogenesis in a model of vascular thrombosis. We were able to confirm the results derived from animal studies in CTEPH, which is a human model disease for thrombus nonresolution.

The Tie-2 receptor is expressed by endothelial cells and hematopoietic stem cells. Transgenic mice expressing inducible CRE recombinase under the control of a Tie2 promoter/enhancer quantitatively excise loxP-flanked (floxed) genes in endothelial cells. Tie-2–expressing monocytes and macrophages have been shown to regulate revascularization of the ischemic limb. However, we do not think that in our model monocytes and macrophages are affected by Kdr deletion, because there was no difference in gene and protein expression levels between Kdr<sup><small>floxed</small></sup>/Tie-2CreER before and after TX treatment and between Kdr<sup><small>end</small></sup> and control littermates (Figure 1C and 1D). The targeted gene deletion in this model primarily addressed angiogenesis. We found that baseline KDR expression in monocytes was extremely low in our model; values were similarly low as in a previously reported study. Only <0.25% monocytes expressed KDR. Kdr mRNA expression was 1000 times less in monocytes than in endothelial cells (Figure 1C). In addition, it has been shown that human monocytes express only vascular endothelial growth factor receptor-1 (Flt-1) and not KDR, whereas endothelial cells express both receptors.

In our study, Kdr<sup><small>end</small></sup> were used in a stagnant flow venous thrombosis model, where thrombus formation and thrombus resolution are inextricably overlapping. Kdr<sup><small>end</small></sup> did not have a prothrombotic phenotype, as tail bleeding times and the percentage of total and activated leukocyte/platelet aggregates and monocyte/platelet aggregates did not differ from controls (Figure 1E and 1F), but the lack of Kdr impacted thrombus early on, leading to larger thrombus by days 1 to 3 (Figure 2I–2K). Decreased numbers of microvessels in Kdr<sup><small>end</small></sup> were primarily apparent during days 7 to 14, whereas vessel density was increasing by day 28 (Figure 3E; Table I in the online-only Data Supplement), which may be enhanced by thrombus shrinkage in later stages of this model. Because vascular endothelial cadherin as well as VEGF and Kdr mRNA levels were significantly decreased between ligation and day 7 (Figure 3F–3H), we conclude that early compromise of angiogenesis in this model accounts for the difference in thrombus size. This concept is further supported by the analysis of changes in thrombus volumes with the largest difference between days 3 and 7 (Figure 2L) and by published data showing that recanalization may occur <24 hours of thrombus formation.

Leukocyte recruitment and especially macrophages play a central role in thrombus resolution, and impaired monocyte recruitment delayed thrombus resolution. Monocytes secrete chemotaxtractants, growth factors, and proteases, directing the chemokine/cytokine milieu to resolution. Areas of venous thrombi containing large numbers of monocytes are also rich in neovascular channels. In our model, the expression of CD68 and Ptpre was attenuated during the early phase of thrombus resolution in Kdr<sup><small>end</small></sup> (Figure 3N and 3O). There was no evidence that monocytic Kdr is targeted by the gene deletion, suggesting that angiogenesis may have to occur first to enable monocyte recruitment into resolving thrombi.

A possible intrinsic effect of TX and CRE recombinase on angiogenesis and thrombus resolution was excluded by experiments showing similar thrombus resolution rates in Kdr<sup><small>floxed</small></sup> (with and without TX administration) and in Kdr<sup><small>end</small></sup>/Tie-2CreER mice compared with Kdr<sup><small>floxed</small></sup> (both without TX administration), respectively (data now shown). Repeated injections of TX after ligation might have led to a more complete suppression of Kdr expression and might have prevented the reappearance of endothelial cells and Kdr expression by day 28 (Figure 3H). TX treatment was terminated 1 week before

### Table. Baseline Clinical and Hemodynamic Characteristics of Patients With Chronic Thromboembolic Pulmonary Hypertension in This Study (n=24; mean±SD)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<td>Age, y</td>
<td>58±12</td>
</tr>
<tr>
<td>Female sex, n</td>
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<tr>
<td>6 min walking distance, m</td>
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<tr>
<td>BDS</td>
<td>4±2</td>
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<tr>
<td>mPAWP, mmHg</td>
<td>12±5</td>
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<td>mPAP, mmHg</td>
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<tr>
<td>CO, L/min</td>
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<tr>
<td>PVR, dynes/sec per cm&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>NYHA functional class (% patients)</td>
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<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>12.5</td>
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<tr>
<td>C-reactive protein, mg/dL</td>
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<td>Fibrinogen, mg/dL</td>
<td>369±78</td>
</tr>
<tr>
<td>Leukocytes, g/L</td>
<td>7.8±4.5</td>
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BDS indicates Borg Dyspnea Score; CO, cardiac output; mPAWP, mean pulmonary artery pressure; mPAP, mean pulmonary arterial wedge pressure; NYHA, New York Heart Association; and PVR, pulmonary vascular resistance.
ligation to minimize toxicity and avoid a direct effect of the compound on venous thrombus resolution. The re-emergence of Kdr expression by day 28 in Kdr<sup>Δend</sup> may derive from other cells, such as bone marrow–derived endothelial progenitor cells (Figure 3H) that invade the late thrombus, and may be enhanced by thrombus shrinkage in later stages of this model. Many other signaling pathways beyond VEGF participate in the formation of new vessels, for example, notch/delta, ephrin/Eph receptor, roundabout/slit, and netrin/UNC receptor families, as well as intracellular proteins such as hedgehog and sprouty. It is possible that Kdr<sup>Δend</sup> compensated the loss of Kdr by 1 of these angiogenic signaling pathways, contributing to an increase in microvessels in Kdr<sup>Δend</sup> by day 28.

After a successful proof-of-concept demonstration that angiogenesis in the animal model is a key component for resolution of experimental thrombosis, angiogenesis was investigated in the vascular obstructive material of CTEPH. CTEPH is a rare and late sequela of venous thromboembolism (VTE), which is characterized by nonresolving thrombi in
the pulmonary arteries (Figure 4E) and is, therefore, an ideal model disease to study the vascular remodeling of thrombosis.

Parent pulmonary artery is a traditional comparator tissue for CTEPH thrombi because it eliminates patient-to-patient variability. In addition, pulmonary artery is characterized by the same main components, that is, endothelial cells, smooth muscle cells, fibroblasts, and occasional inflammatory cells as CTEPH vascular occlusions. Previously published work by our group has used parent pulmonary artery as control tissue for gene expression studies in CTEPH thrombus.19 Red appositional CTEPH thrombus corresponds to erythrocyte-rich nonorganized thrombus that is thought to originate from slow flow. In addition, red thrombus composition is characterized by very few vascular resident cells, such as smooth muscle cells and myofibroblasts (Figure 4), and significantly differs from white thrombus by proteomic analyses (Figure I in the online-only Data Supplement).

Lower gene expression levels of KDR were found in white CTEPH thrombi compared with pulmonary arteries from the same patients (Figure 5A). Not only KDR but also other genes important for endothelial cells (e.g., ANGPT2, PECAM1, CDH5, PDNP, TEK, VEGFA, BMPR2, and TGFBI) were expressed at lower levels in CTEPH thrombi compared with pulmonary arteries. Changes in gene expression may reflect different proportions of cell types (especially of endothelial cells) present in CTEPH vascular scars versus relatively normal pulmonary artery walls. Lymphangiogenesis seems to be...
more important in arterial thrombosis (Figure 5) and remains to be characterized in the vascular remodeling of venous thrombosis. In accord with reported data, PAI1 showed an increased expression level in CTEPH thrombi19 (Figure 5A). In addition to its role as the main fibrinolysis inhibitor in humans, PAI1 was shown to inhibit cerebral angiogenesis48 and sprout formation of HUVECs and human lung microvascular endothelial cells.39 Moreover, pharmacological inhibition of PAI1 promoted angiogenesis in a mouse model of hind limb ischemia.40

Because CTEPH is a rare disease, we may assume that acute thrombi that were harvested as controls were unlikely not to resolve. Under these assumptions, we performed relative gene expression analyses using CTGF as a marker for fibrotic transformation (ie, thrombus age) and KDR, CDH5, and PDGPN as markers for angiogenesis. The relative quantity of CTGF mRNA as a molecular marker of fibrosis has demonstrated a strong correlation with thrombus collagen content.17 The first observation was that fresh red appositional CTEPH thrombi contained more CTGF than acute pulmonary emboli. In addition, white CTEPH thrombi at the same level of CTGF expression as organizing deep vein thrombi were characterized by significantly reduced angiogenic gene expression. Comparisons of white CTEPH thrombi with vascular thrombi of different origin and age suggested discordant gene expressions, with a lower angiogenic gene/CTGF expression ratio of white CTEPH thrombi.

Despite islands of vessels particularly at the borders of thrombi,19 CTEPH thrombi were overall less vascularized compared with pulmonary arteries, which was confirmed by decreased vWf and PECAM1-positive cell counts per mm² (Figure 4I). Our observations are corroborated by recent data showing angiostatic factors, such as platelet factor 4, collagen type I, and interferon-γ-inducible 10 kDa protein, within CTEPH thrombi.41

Therefore, experiments were designed to understand the biochemical composition of pulmonary vascular thrombus in CTEPH. Thrombus fingerprinting by 2-dimensional gel analysis illustrated that red CTEPH thrombus was significantly different from white organized CTEPH thrombus and thrombus-free CTEPH pulmonary artery (Figure 1 in the online-only Data Supplement). DNA synthesis rates of HUVECs in response to incubation with pulmonary endarterectomy material served to illustrate the biological activity of homogenates. When the activity of lysates was tested in an in vitro 3-dimensional angiogenesis assay, red CTEPH thrombi inhibited VEGF-induced HUVEC spheroids sprouting, whereas white clots and pulmonary arteries were neutral (Figure 6B). There are 2 explanations: either white CTEPH thrombi and parent unthrombosed pulmonary artery tissue have a stimulatory factor not present in red CTEPH thrombi, or all 3 have a stimulatory factor and red CTEPH thrombi also carry an inhibitory factor. As previously reported,19 healthy pulmonary artery did not differ from unthrombosed CTEPH pulmonary artery either in a BrdU assay or in an in vitro 3-dimensional angiogenesis assay (data not shown). These results suggest that the paucity of vessels in white CTEPH thrombus may be enhanced by the angiostatic activity of red thrombus.

One of the limitations of the present work is that we have drawn conclusions on thrombus biology in CTEPH, which is a chronic condition that evolves over months to decades after the initiating VTE, based on experimentation in an acute thrombosis model in the mouse. In addition, despite striking histological similarities between mouse and human thrombi (Figure 4A–4D), stagnant flow venous thrombosis in the caval vein may not completely replicate pulmonary embolism. Other limitations of the study include the use of tissue lysates in the in vitro experiments and the focus on soluble proteins rather than cellular components of endarterectomy specimens. Comparisons with arterial thrombosis may be biased by a component of atherosclerotic inflammation, which may be distinct from venous thrombosis. Plasmin and matrix metalloproteinase–related mechanisms play important roles in venous thrombus resolution,42 yet those remain to be studied in Kdrfl/fl.

The results of our study suggest that angiogenesis and an intact angiogenic response during the early phase of thrombus resolution are key mediators of normal thrombus resolution. The analysis of pulmonary endarterectomy specimens illustrates a paucity of vessels and low ratio between thrombus angiogenesis and fibrosis, supporting the hypothesis that deficient angiogenesis is a key biological mechanism of occlusive vascular remodeling after VTE. Clinical conditions that have been found to be associated with CTEPH, for example, splenectomy, infection, or phospholipid antibodies, must be examined for their possible interaction with the angiogenic response during thrombus organization. Insights into these processes might be helpful in tailoring more specific and effective therapies for VTE, preventing CTEPH.

Acknowledgments

We thank M. Hammer for help with mouse experiments, and Dr G. Lochnit for help with 2-dimensional gel electrophoresis.

Sources of Funding

This research project received financial support from FWF NFN S94-B11 (Angiogenesis in Disease, to I.M.L. and E.F.W.), SFB-F54 (Zelluläre Mediatoren zwischen Entziindung und Thrombose, to I.M.L.), Rudolf-Marx-Fellowship of the Gesellschaft für Thrombose und Hämostaseforschung e.V. (to S.A.), European Respiratory Society Short Term Research Fellowship (STRF #1138, to S.A.), German Research Foundation (Bonn, Germany) within the Excellence Cluster Cardio-pulmonary System (ECCPS) and the International Graduate Program Protecting the Heart from Ischemia (PROMISE, to K.T.P.).

Disclosures

None.

References

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Arterioscler Thromb Vasc Biol. 2014;34:810-819; originally published online February 13, 2014;
doi: 10.1161/ATVBAHA.113.302991

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Materials and Methods

Generation of Kdr\textsuperscript{floxflox}/Tie-2CreER mice

All animals used were under the care and supervision of the Department and Core Unit of Biomedical Research, Medical University of Vienna (MUW). Procedures were approved by the MUW Institutional Animal Care Committee and the Austrian Ministry of Science (BMBWK-66.009/0277-BrGT/2005). A mouse strain with a tamoxifen (TX)-inducible CRE (CreER) under the control of the Tie2 promoter was generated to produce a targeted deletion of the first exon of Kdr in endothelial cells \cite{1} and showed endothelial-specific CRE activity upon TX induction. Mice backcrossed at least 6 generations into the C57/BL6J background were used. Kdr\textsuperscript{floxflox}/Tie-2CreER mice and Cre negative littermates Kdr\textsuperscript{floxflox} mice were obtained. Genomic DNA was extracted from tail biopsies with the DNA Mini Kit (Qiagen, Hilden, Germany) and transgenic mice were genotyped by using the following primers to amplify a Cre-specific product of 620 bp: 5′-CGGTCGATGCAACGAGTGATGAGG-3′ and 5′-CCAGAGACGGAAATCCATCGCTG-3′. PCR parameters were 30 cycles of 94°C for 1 min, 60°C for 2 min, 72°C for 3 min.

Deletion of Kdr in endothelial cells (Kdr\textsubscript{end})

3 weeks prior to the experiments mice were treated with 100 µl of TX (20 mg/ml in 10 % ethanol and 90 % sunflower seed oil) intraperitoneally (i.p.) once daily for 5 days, followed by once a week for two weeks until thrombus induction. Kdr\textsuperscript{floxflox}/Tie-2CreER mice and Cre negative littermates Kdr\textsuperscript{floxflox} mice after TX treatment are labeled as Kdr\textsubscript{end} and controls, respectively.

Southern blot analysis confirmed that the floxed Kdr allele was deleted in Kdr\textsubscript{end}. Total DNA was isolated from lungs, kidneys, liver and heart of Kdr\textsubscript{end} and controls (n=8 each) using the DNA Mini Kit (Qiagen). A 1.4 kb Xho I – Spe I fragment located within the first intron (but outside of the targeting vector) was used as a probe for Southern blot analysis. Approximately 15 µg of genomic DNA were digested with 20 U of Spe I (Roche Applied Science, Vienna, Austria) and separated by gel electrophoresis (0.8% (w/v) agarose/TAE). The agarose gel was incubated for 15 min in 0.25 N HCl solution, then for 30 min in 0.5 M NaOH/1.5 M NaCl solution, and for 30 min in 0.5 M Tris/1.5 M NaCl, pH 7.5. DNA fragments were transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA) by capillary tank blotting over night using 20X SSC (300 mM Sodium citrate/3 M NaCl). DNA was cross-linked to the membrane by 1200 J/m² UVC (Stratagene Crosslinker). Probes for hybridization were labeled using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). Prehybridization (1 hour) and hybridization (over night) were carried out at 42°C in DIG Easy Hyb buffer (Roche), washed in 1X SSC/0.1% SDS, then in 0.2X SSC/0.1% SDS at 65°C. The hybridized probes were immunodetected with anti-digoxigenin-AP Fab fragments and visualized with chemiluminescence substrate CSPD (Roche).

Endothelial cells were isolated as previously described \cite{2} from lungs, kidneys and liver from Kdr\textsuperscript{floxflox}/Tie-2CreER mice before and after TX induction (n=4 each), and Kdr expression was determined by quantitative Real-time PCR. Monocytes were isolated from whole blood from Kdr\textsuperscript{floxflox}/Tie-2CreER mice before and after TX induction (n=8 each) using the EasySep Mouse Monocyte Enrichment Kit (Stemcell Technologies). Purity of isolated monocytes was assessed by flow cytometry. Monocytes were characterized by forward and sideward scatter gating, and CD45 and CD11b positivity. Monocytic Kdr expression was determined by quantitative Real-time PCR.

Analysis of platelet aggregates and monocytes in murine blood

EDTA-anticoagulated whole blood samples from Kdr\textsubscript{end} and controls (n=8 each) were incubated with fluorochrome-labeled antibodies. Erythrocytes were lysed by addition of BD FACS lysing solution (Becton Dickinson). Cells were incubated with antibodies staining for CD45, CD41, CD62P and CD11b, Tie-2, KDR (BD Biosciences Pharmingen, San Jose, CA, USA; Biologend, San Diego, CA, USA). For negative controls, isotype-matched antibodies were used (Biologend). Stained cells were analyzed using a BD Canto II (Becton Dickinson) and FACSDiva Software (BD Biosciences).
For leukocyte/platelet aggregate analysis, CD45+ cells were gated and CD45+CD41+ (leukocyte/platelet aggregates) and CD45+CD11b+CD41+ (monocyte/platelet aggregates) cells were counted. Aggregates that were also positive for CD62P were regarded as activated aggregates.

Monocytes were characterized by forward and sideward scatter gating, and CD45 and CD11b positivity. Expression of Tie-2 and KDR on monocytes was analyzed.

Tail bleeding time
Adult female mice (20–30 g) were anesthetized as previously described. A 2 mm portion of distal tail was amputated from anesthetized mice using a scalpel, and the tail was immersed in isotonic saline (37°C). The time from incision to the cessation of bleeding was recorded as the bleeding time. Any continued bleeding was stopped by cauterization at 3 minutes to prevent hypovolemic shock.

Murine model of IVC thrombosis
Adult female Kdr<sup>end</sup> and controls (age: 8 weeks, weight: 18 to 20 g, Himberg, Austria) were employed in the stagnant flow venous thrombosis model. Infrarenal vena cava surgery and thrombus harvest on days 3, 7, 14 and 28 after surgery were performed as previously described. Thrombus lengths, cross-sectional areas, and volumes (lengths × cross-sectional areas) were determined. Mean relative volume changes were calculated as the differences of mean thrombus volumes between two subsequent time points.

Human tissues
Patients gave written informed consent under a protocol approved by the Ethics Committee of the MUW (EK 220/2008) and the Kerkhoff Clinic in Bad Nauheim (EK 10/06), both being national CTEPH reference centers. White fibrotic CTEPH thrombi, red fresh portions of CTEPH thrombi, and unthrombosed pulmonary arteries were collected during PEA. During pulmonary endarterectomy, a blunt dissection is performed in a plane that is separating thrombus from the pulmonary arterial media. We define parent, unthrombosed CTEPH pulmonary artery in the following manner: For the purpose of this study, the surgeon had agreed to harvest a piece of healthy proximal pulmonary artery whenever possible that was free of thrombus and had remained undissected until the end of the procedure, prior to resuturing the pulmonary arteriotomy site. Healthy pulmonary arteries were collected from lung transplant donors.

Tissues harvested in the operating room were immediately fixed in 7.5 % buffered formaldehyde and frozen in liquid nitrogen.

For the BrdU and angiogenesis assay, frozen tissues were homogenized in ice cold PBS with Precellys® 24 (Peqlab Biotechnologie GmbH, Austria) for 30 seconds at 6,000 rpm, and cooled at 4°C. The suspension was centrifuged at 10,000 rpm for 10 minutes at 4°C, and the supernatant was recovered. Protein concentrations of the supernatant were determined with the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Germany) according to the manufacturer’s instructions.

All assays were performed in triplicates with tissue lysates prepared from red and white CTEPH thrombi, unthrombosed pulmonary artery from the same patients, and healthy pulmonary artery from lung transplants.

Venous thrombi (n=15) were harvested in the course of variceal surgeries (subacute and organizing deep vein thrombi) and during thrombectomies of acute pulmonary embolism (acute venous thrombi). Arterial thrombi (n=10) were obtained during thrombectomies of acute femoral artery occlusions (acute arterial thrombi), and during aortic aneurysm surgeries (organizing arterial thrombi). Coronary thrombi (n=7) were harvested from patients suffering from ST-elevation acute coronary syndrome undergoing primary percutaneous coronary intervention. Carotid plaques (n=8) were harvested during carotid endarterectomy. In vitro whole blood clots from CTEPH patients (n=6) were obtained by incubating whole blood from CTEPH patients in serum tubes for 3 hours at room temperature and by subsequent snap-freezing of pieces of the formed clot in liquid nitrogen.
RNA preparation and Real-time PCR

Total RNA of mouse thrombi and isolated endothelial cells and monocytes was extracted using the RNeasy Mini Kit (Qiagen). For RNA extraction of white and red CTEPH thrombi, parent unthrombosed pulmonary artery, venous and arterial occlusions, coronary thrombi, in vitro whole blood clots from CTEPH patients and carotid plaques, the RNeasy Fibrous Tissue Mini Kit (Qiagen) was used. cDNA was synthesized from 2 µg of total RNA by reverse transcription (TaqMan Reverse Transcription Kit, Roche). Quantitative fluorogenic PCR was performed in an ABI PRISM 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Specific TaqMan primers and probes for murine vascular endothelial growth factor (Vegfa, Mm00437304_m1), vascular endothelial cadherin (VE-Cadherin, Cdh5, Mm00486938_m1), Kdr (Mm00440099_m1), common leukocyte antigen CD45 (Ptprc, Mm00448463_m1), CD68 (Mm00839636_g1) and connective tissue growth factor (Ctgf, Mm00515790_g1) were used (Applied Biosystems).

For analyzing human tissue samples, specific TaqMan primers and probes for angiopoietin-2 (ANGPT2, Hs00169867_m1), platelet endothelial cell adhesion molecule (PECAM1, Hs00169777_m1), vascular endothelial cadherin (CDH5, Hs00174344_m1), kinase-insert domain receptor (KDR, Hs00176767_m1), podoplanin (PDPN, Hs00362718_s1), endothelial tyrosine kinase Tie2 (TEK, Hs00176096_m1), vascular endothelial growth factor (VEGFA, Hs00173626_m1), bone morphogenetic protein receptor type II (BMPR2, Hs00176148_m1), transforming growth factor beta 1 (TGFβ1, Hs99999918_m1), connective tissue growth factor (CTGF, Hs00170014_m1), plasminogen activator inhibitor type 1 (SERPINE1, Hs01126606_m1), common leukocyte antigen (PTPRC, Hs04189704_m1), CD68 (Hs02836816_g1) and myeloperoxidase (MPO, Hs00924296_m1) were used. PCR was performed under standard conditions: i.e., 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 1 min. The mRNA expression levels of the genes were normalized to eukaryotic 18S rRNA levels (Hs00170014_m1, Applied Biosystems).

Histological Analysis

Three µm paraffin sections were prepared for (immuno-) histochemical analysis. A modified Trichrome stain was used as previously described. Both early fibrin and red blood cells were shown by lissamine fast yellow. Mature fibrin was stained by a combination of acid fuchsin, Biebrich scarlet and ponceau 2R (red), while collagen was visualized by a green color.

Immunohistochemical analyses were performed as previously described. An Olympus BX 50 microscope equipped with the imaging software Axio (Version 3.0-2002, Carl Zeiss Vision GmbH) was used.

Monoclonal antibodies for isoelectin B4 (Vector Laboratories, Burlingame, CA), F4/80 (eBioscience, San Diego, USA), von Willebrand factor (vWF) (M0616, DAKO) and platelet endothelial cell adhesion molecule (PECAM, CD31) (M0823, DAKO) were used.

Cell culture

HUVEC were isolated from umbilical cord vein by collagenase treatment as described previously and grown in endothelial cell basal medium (ECBM) containing 0.4 % endothelial cell growth supplement, 0.1 ng/ml recombinant human epidermal growth factor, 1 ng/ml recombinant human bFGF, 1 µg/ml hydrocortisone, 90 µg/ml heparin (all from PromoCell-supplement pack, Heidelberg, Germany) and 2 % fetal calf serum (FCS). HUVEC were used between passages 2 and 4.

Two-dimensional (2-D) gel electrophoresis

2-D gel electrophoresis was performed for red CTEPH thrombus, white CTEPH thrombus and unthrombosed CTEPH pulmonary artery. Frozen tissue samples (size 1 cm³) were individually processed by grinding with a mortar and pestle cooled with liquid nitrogen. Protein extraction was performed by ultrasonication in 6 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 4 % CHAPS (Roth, Germany), 1 % DL-Dithiothreitol (DTT) (Fluka,
Germany) and 2 % Pharmalyte 3-10 (GE Healthcare). Thereafter, the samples were precipitated with acetone and redissolved in TRIS-buffer (10 mM TRIS, 5 mM CaCl₂, 5 mM MgCl₂; pH 8.0). Protein quantification was performed with 2-D Quant kit (GE Healthcare). On each strip 400 µg protein were applied and isoelectric focusing was performed with 32.05 kVh. 2-D gel electrophoresis was performed as described elsewhere ⁷. Immobilized pH gradient (IPG) strips with non-linear pH 3-10 and 12.5 % SDS-gels were used. Gels were stained with fluorescent flamingo and scanned with a GS-800 densitometer (BioRAD, Germany).

Assay of mitogenic activity - BrdU assay
The effect of CTEPH thrombi lysates on endothelial cell proliferation was determined by the amount of the incorporated pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) using the cell proliferation BrdU-ELISA kit from Roche (Mannheim, Germany) according to the manufacturer’s instructions. Briefly, HUVEC were seeded in 96-well microtiter plates (5,000 cells/well) and starved over night in ECBM with 0.1% bovine serum albumin. After 24 h, HUVEC were incubated with different concentrations of lysates from red CTEPH thrombi, white CTEPH thrombi, unthrombosed CTEPH pulmonary arteries and healthy pulmonary arteries respectively (10 and 40 µg/ml) in absence or presence of 50 ng/ml VEGF diluted in ECBM with 0.2 % FCS for 24 h. After 18 h, BrdU labeling solution was added for the remaining 6 h. Incubation with 50 ng/ml VEGF served as positive control. Finally, labeling medium was removed, cells were fixed with FixDenat for 2 h at room temperature and subsequently blocked with 3 % BSA in PBS over night at 4°C. Cells were rinsed and incubated with anti-BrdU-POD for 2 h at room temperature. Cells were washed three times and incubated with the substrate solution for 30 min protected from light. Absorbance was measured at 450 nm (reference wavelength 650 nm).

Three-dimensional (3-D) in vitro angiogenesis assay - spheroid assay
HUVEC (3,000 cells/well) were seeded out in 96 round well plates in culture medium with 20 % methylcellulose and aggregates (spheroids) were formed over night. A fibrinogen stock solution (Calbiochem, Merck Millipore, Germany) was diluted to 1.8 mg/ml in Dulbecco's PBS, pH 7.4 (Invitrogen GmbH, Karlsruhe, Germany) containing 200 U/ml aprotinin (Bayer, Munich, Germany) as protease inhibitor. Washed spheroids were transferred to the fibrinogen solution and 0.65 U/ml thrombin was added to induce polymerization. After polymerization (20 min at 37°C), fibrin gels were equilibrated in endothelial cell medium with 1 % FCS for 1 hour. Then medium was replaced by ECBM with 1 % FCS and lysates from red CTEPH thrombi, white CTEPH thrombi, unthrombosed CTEPH pulmonary arteries and healthy pulmonary arteries respectively (10 and 40 µg/ml) for 48 h. VEGF (25 ng/ml) was added as a positive control. Another set of wells was stimulated with tissue lysates and VEGF together to see if those tissue lysates had any inhibitory effect on endothelial cell sprouting. Cells were fixed with 3 % (wt/vol) parafomaldehyde in phosphate-buffered saline for further analysis. The angiogenic response was quantified by determination of average total length of sprouts per spheroid. For each condition 10 spheroids were analyzed ⁸.

Statistical analysis
For murine data analysis, the significance of intergroup differences was determined by parametric statistics because all data were normally distributed and tested for normal variance. For single timepoint analysis as in Figure 1, we performed unpaired Students t-test. For analyzing intergroup differences at multiple timepoints, as in Figure 2 and 3, we used ANOVA with post hoc Tukey test. Results are expressed as means ± standard deviations (SD). For human data analysis, statistical differences were determined by Mann Whitney U-test. Statistical analyses were performed using IBM SPSS version 20, Chicago, USA. P<0.05 was considered statistically significant."
References for Methods


Supplemental Material

Defective angiogenesis delays thrombus resolution: a potential pathogenetic mechanism underlying chronic thromboembolic pulmonary hypertension

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Supplemental Table I. Microvessel and macrophage counts in thrombi of KdrΔend and controls. Data expressed as isolectin B4 or F4/80 positive cells of total cells per thrombus cross-sectional area (means ± SD, graphically represented in Figures 3E and 3M respectively). * indicates p<0.05.

<table>
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<tr>
<th>Day</th>
<th>Isolectin B4+ cells/total cells/thrombus area (%)</th>
<th>F4/80+ cells/total cells/thrombus area (%)</th>
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<tr>
<td></td>
<td>KdrΔend control</td>
<td>p</td>
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<tr>
<td>3</td>
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<td>3.1 ± 1.3 3.4 ± 0.9</td>
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<td>7</td>
<td>2.8 ± 0.3 6.2 ± 3.4</td>
<td>* 0.7 ± 0.2 1.5 ± 1.0</td>
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Supplemental Figure I. **Representative flamingo fluorescent stained 2-D SDS PAGE gels from CTEPH specimens.** Representative flamingo fluorescent stained 2-D SDS PAGE gels from (A) a red CTEPH thrombus, (B) a white CTEPH thrombus and (C) unthrombosed pulmonary artery from a single CTEPH patient are shown.