Role for Staphylococci in Misguided Thrombus Resolution of Chronic Thromboembolic Pulmonary Hypertension

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Objective—Acute pulmonary emboli usually resolve within 6 months. However, in 0.1% to 5% of cases thrombus transforms into fibrous masses. If vascular obstruction is severe, the resulting condition is chronic thromboembolic pulmonary hypertension (CTEPH). Patients who carry ventriculo-atrial (VA-) shunts for the treatment of hydrocephalus and report a history of shunt infection are at an increased risk for CTEPH. Because CTEPH lacks traditional plasmatic risk factors for venous thromboembolism, we hypothesized that delayed thrombus resolution rather than abnormal coagulation is important, and that bacterial infection would be important for this misguidance.

Methods and Results—Human CTEPH thromboemboli were harvested during pulmonary endarterectomy. The effects of Staphylococcal infection on thrombus organization were examined in a murine model of stagnant-flow venous thrombosis. Staphylococcal DNA, but not RNA, was detected in 6 of 7 thrombi from VA shunt carriers. In the mouse model, staphylococcal infection delayed thrombus resolution in parallel with upregulation of transforming growth factor (TGF) beta and connective tissue growth factor.

Conclusions—in the present work, we propose a mechanism of disease demonstrating that infection with Staphylococci enhances fibrotic vascular remodeling after thrombosis, resulting in misguided thrombus resolution. Thrombus infection appears to be a trigger in the evolution of CTEPH. (Arterioscler Thromb Vasc Biol. 2008;28:000-000.)

Key Words: ●●●

The natural history of acute pulmonary thromboemboli is to undergo almost complete resolution within 6 months.1 However, in 0.1% to 3.8% of survived acute events, thromboemboli undergo an organization process leading to permanent fibrotic obstruction of the pulmonary vascular bed. Chronic thromboembolic pulmonary hypertension (CTEPH) is characterized by predominantly major-vessel obstructions resulting in increased pulmonary vascular resistance. The molecular mechanisms underlying thrombus persistence are unknown.5

CTEPH is largely understood as of thromboembolic origin. However, patients with CTEPH lack classic plasmatic thromboembolic risk factors,6 and systemic7 or local8 imbalances of fibrinolytic proteins in the pulmonary arterial wall. In addition, it is impossible to induce the disease in animal models by repeated embolizations,9 suggesting alternative nonthromboembolic hypotheses.10 Increased plasminogen activator inhibitor (PAI)-111 and von Willebrand factor (vWF) (unpublished) expressions on endothelial cells of the thrombus surface support the concept of a localized abnormal prothrombotic gene expression pattern furthering in situ thrombosis.

We have recently observed that patients who carry ventriculo-atrial, (VA-) shunts for the treatment of hydrocephalus, or pacemaker leads and have a history of shunt/lead infection are at an increased risk for CTEPH12 and carry an unfavorable prognosis.13 Shunt infection is common in patients with VA-shunts, and Staphylococcus (S) aureus or epidermidis is responsible for up to one-half of these infections,14 leading to thrombosis and device failure.15 An association between tissue fibrosis and infection has been described in a variety of tissues, such as liver,16 lung,17 and intestine,18 and bacterial pathogens are drivers of unrestrained wound healing.19 Bacterial infection induces upregulation of TGF beta (TGFβ),20 a profibrotic cytokine, and connective tissue growth factor (CTGF),18 a matricellular protein that regulates collagen synthesis and maintains fibrosis.21,22 We hypothesized that bacterial infection triggers the transition from fresh thromboembolic material to fibrotic tissue in CTEPH.

Methods

Tissues

We collected thromboemboli from 19 consecutive CTEPH patients undergoing classical PEA23 between July 2005 and December 2006.
Table. Characteristics of VA-Shunt Carriers

<table>
<thead>
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<th>Parameter</th>
<th>1</th>
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<td>415</td>
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<td>848</td>
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<td>62</td>
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<td>3.0</td>
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<td>3.7</td>
<td>2.11</td>
<td>4.71</td>
<td>4.0</td>
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<tr>
<td>Staph. Species†</td>
<td>S epidermidis</td>
<td>S aureus</td>
<td>S aureus</td>
<td>ND</td>
<td>S aureus</td>
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<td>S aureus</td>
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<td>CRP, mg/dl**</td>
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<td>0.82</td>
<td>2.59</td>
<td>0.8</td>
<td>4.65</td>
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<td>Fibrinogen, mg/dl**</td>
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<td>Leukocytes, 10⁶/μl</td>
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<td>6.6</td>
<td>7.3</td>
<td>6.1</td>
<td>10.1</td>
<td>8.7</td>
<td>6.5</td>
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</table>

*Pacemaker carrier; †Ebola or atrial thrombus culture results at the time of VA-shunt/pacemaker lead infection; ‡Staphylococcus species identified in PEA specimens by rDNA amplification; **values at the time of PEA. NA indicates not available; ND, not detected.

at our institution, and examined all (n=7) surgical specimens from VA-shunt-infected pacemaker carriers in the CTEPH tissue database since 1994. Because of the limited number of acute pulmonary thromboemboli at our institution, only 5 acute pulmonary thromboemboli were available for analysis (Figure 2B, lanes 1’ to 5’). Therefore, we also collected fresh varicophlebitis thrombi from patients undergoing varicose vein surgeries (n=23). These samples were used as reference standards. Tissue aliquots were immediately fixed in 7.5% buffered formaldehyde and frozen in liquid nitrogen (LN₂). Patients and controls gave written informed consent under a study that was approved by the Ethics Committee of the Medical University of Vienna.

DNA Extraction and Staphylococcus-Specific Polymerase Chain Reaction

DNA was purified from chronic (n=26) and acute (n=28) thrombi using the QIAamp DNA Mini kit (Qiagen). Staphylococcus-specific polymerase chain reaction (PCR) was performed in the GeneAmp PCR System 2400. Bacterial 16S ribosomal DNA was amplified using the genus-specific forward primer 5'-CAGCTCTTGTTGCT-GAGATGT-3' and reverse primer 5'-CCAATCATTTGTCC-CAAATTCCTG-3'. The thermal profile consisted of an initial hot start at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 40 s and annealing/elongation at 60°C for 1 min. The resulting amplicon of about 420 bp contains hypervariable regions allowing not only detection of conventional, easy-to-grow microorganisms, but also of fastidious and intracellular pathogens.

RNA Preparation and Real Time PCR

After RNA isolation and cDNA synthesis, quantitative fluorogenic PCR was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Specific TaqMan primers and probes for human CTGF (TaqMan Gene Expression Assay, Applied Biosystems, Assay ID Hs00170014_m1), human TGF-β (Applied Biosystems, Assay ID Hs99999918_m1), and eukaryotic 18S rRNA endogenous control were used. PCR was performed under standard conditions: ie, 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 endogenous 18S-RNA levels. In parallel, the same RNA samples were used as a template for RT-PCR amplifications using Staphylococcus-specific 16S rDNA primers.

Preparation of S aureus Suspension

S aureus ATCC 12600 (American Type Culture Collection, USA) was cultivated on Columbia blood agar plates (Becton Dickinson) for 24 h at 35°C before injection. The bacterial suspension was dissolved in sodium chloride. Using spectrophotometry and the McFarland index, an optical density at 620 nm corresponding to 1.5 to 5×10⁹ cells per ml was achieved for the final suspension.

Murine Model of IVC Thrombosis

Adult female Balb/c mice (n=64; age: 8 wk, weight: 18 to 20g, Himberg, Vienna) were anesthetized with 100 mg/kg ketamine (Ketavet) and 5 mg/kg xylacine (Rompun). The IVC was exposed below the renal veins through a midline laparotomy incision. The intestines were retracted, and retroperitoneal blunt dissection of the infrarenal vena cava was performed to mobilize a 5-mm segment distal to the left renal vein. In contrast to the original description, endothelial denudation with a neurosurgical vascular clip was not performed because of a high rate of rupture of the IVC wall with this method. In our hands, injury to the endothelial layer that occurred as a consequence of the manipulation was a sufficient prothrombotic stimulus. A 5-0 Prolene suture was placed alongside the vena cava. A stenosis was produced in the vein by tying a 4-0 silk suture around a 5-0 Prolene suture was placed alongside the vena cava. A stenosis was produced in the vein by tying a 4-0 silk suture around the vena cava.
Collagen content is expressed as collagen in percent of total protein. Both dyes are eluted, and the absorbances obtained at 540 and 605 nm can be used to estimate the amount of collagen and total protein. As briefly described, the methodology relies on the binding of SiriusRed (Sigma) to collagen and FastGreen (Merek) to noncollagenous components. Both dyes are eluted, and the absorbances obtained at 540 and 605 nm can be used to estimate the amount of collagen and total protein. Collagen content is expressed as collagen in percent of total protein content.

Quantification of Collagen

Collagen quantification was performed as previously described. In brief, the methodology relies on the binding of SiriusRed (Sigma) to collagen, and FastGreen (Merek) to noncollagenous components. Both dyes are eluted, and the absorbances obtained at 540 and 605 nm can be used to estimate the amount of collagen and total protein. Collagen content is expressed as collagen in percent of total protein content.

(Immuno-) Histochemistry

For histochemical analyses, a modified Trichrome stain was used. Immunohistochemical analyses were performed as described. Purified rat anti-neutrophil monoclonal antibody (CL8993AP, Cedarlane Laboratories), monoclonal antihuman α-smooth muscle actin (α-SMA) antibody (M851, DAKO), monoclonal antibody against peptidoglycan (2E9, from Dr Laman), antihuman vimentin antibody (RM-9120-SO, NeoMarkers), and antimouse F4/80 glycoprotein antibody (M851, DAKO) were used. Two independent observers blinded to the experimental groups performed cross-sectional planimetry. Specific TaqMan primers and probes for mouse TGF-β (Applied Biosystems, Assay ID Mm00441724_m1) and CTGF (Applied Biosystems, Assay ID Mm00515790_g1) were used. The Austrian Ministry of Science approved the animal studies.

Statistics

The significance of intergroup differences was determined by ANOVA with repeated measures. All results are expressed as mean±SD. Nominal variables were compared with the Chi square test. The Spearman test was used for correlation of continuous variables. P<0.05 was considered statistically significant.

Results

Patient Characteristics

Mean age of CTEPH patients was 50±17 years, donors of control thrombi were 55±12 years old (P=0.15). There were 9 males among the CTEPH patients, and 12 males among donors of fresh clots. Seven CTEPH patients carried either a VA-shunt or pacemaker, and 5 of those had a history of single or recurrent shunt/lead infection with positive blood/atrial thrombus cultures at the time of a clinically apparent infection (Table). Device implantation occurred 103±87 months before CTEPH diagnosis. Of the controls with acute pulmonary embolism, 1 patient had papillary bladder tumor, 1 suffered from perforated stomach ulcer, 1 female patient was an obese smoker, and 2 patients had recently undergone major surgery. Patients had no overt signs of infection at the time of CTEPH diagnosis. Mean C-reactive protein was 1.1±0.8 mg/dL, blood leukocyte count was 9.3±4.7G/L, and fibrinogen was 382±103 mg/dL, compared with a C-reactive protein of 3.6±4.6 mg/dL, (P=0.09), a blood leukocyte count of 11.6±6.5G/L, (P=0.08), and fibrinogen of 443±157 mg/dL (P=0.07) in controls.

Characterization of CTEPH Thrombi

Both macroscopically (Figure 1A and 1B) and by histological analysis, acute pulmonary emboli differ from CTEPH thrombi. Reddish soft casts of the pulmonary arterial tree in acute pulmonary embolism contrast the fibrotic whitish material of CTEPH. Trichrome-stained paraffin sections (Figure
IC and ID) illustrate fresh fibrin strands with scattered inflammatory cells as their main components, whereas CTEPH specimens are mainly composed of collagen and α-SMA-positive cells.

The relative quantity of CTGF mRNA as a molecular marker of fibrosis was elevated in CTEPH thromboemboli compared with acute pulmonary thrombi (0.23 ± 0.32 versus 1.69 ± 0.98 on a logarithmic scale, P = 0.013, Figure 1E). The relative quantity of TGF-β mRNA was low in CTEPH specimens (0.84 ± 0.61), compared with acute thrombi (2.06 ± 0.41, P = 0.006).

Human CTEPH Specimens Contain Staphylococcus-Specific DNA
Whereas control samples (n = 28) were negative, a Staphylococcus-specific 420-bp product was present in 7 of 26 CTEPH thrombi (or 6 of 7 VA-shunt/pacemaker carriers, P = 0.019, Figure 2). All but 1 patient in whom Staphylococcus-specific DNA was detected were VA-shunt or pacemaker carriers with a history of device infection (Table). Sequencing revealed the presence of S aureus in 5 cases, and S epidermidis in 1 case (Table). A comparison between bacterial DNA positive and negative human CTEPH samples revealed a statistically significant difference in collagen content (10.52 ± 5.11 versus 5.23 ± 3.68% collagen, P = 0.036; Figure 3A) and in the relative CTGF mRNA levels (1.61 ± 0.59 versus 0.83 ± 0.56, P = 0.027; Figure 3B). Furthermore, a strong correlation was found between thrombus CTGF mRNA levels and thrombus collagen content (R² = 0.853, P < 0.001; Figure 3C).

Delayed Resolution of Murine Thrombi After Infection With S aureus
To study thrombus resolution, we used a mouse model of IVC thrombosis. After infection, Staphylococcus-specific DNA was detectable in all thrombus specimens at all time points by culture and specific RT-PCR from infected mice, but in none of the control mice (data not shown). Thrombi from infected mice were significantly larger than thrombi from uninfected animals, with larger cross-sectional thrombus areas (Figure 4A through 4H, and Figure 5D, P < 0.001). A marked increase in α-SMA immunoreactive, vimentin-positive cells was noted on days 14 and 28 after infection (Figure 4I through 4P, and Figure 5E, P < 0.001), as well as an increased neutrophil cell count per mm² cross-sectional thrombus area (Figure 5F, P < 0.001). By contrast, macrophage counts were lower in infected thrombi than in control samples (P = 0.001, data not shown), most prominently by day 14 after vena cava ligation. The relative CTGF mRNA level was elevated on day 28 after infection (Figure 5G, P < 0.001). By comparison, relative
TGF-β mRNA levels were elevated by day 7 after bacterial infection (Figure 5H, P<0.001).

Discussion

The generation of thrombin from its precursor prothrombin is the central event of blood coagulation, which is essential to hemostasis and the culprit in thrombosis. This is a highly regulated, dynamic, and rapid process. By contrast, the timely removal of thrombus results from the concerted action of plasmatic fibrinolysis and a complex vascular remodeling process, which is time-consuming and involves circulating cells and cells of the vessel wall. Compared with the scientific interest in mechanisms of thrombus formation, few studies have addressed the vascular biology of thrombus resolution. We hypothesized that CTEPH is a disorder of misguided thrombus resolution.

CTEPH evolves over years, and decades may lie between the initiating thromboembolic event, the onset of symptoms and diagnosis4 (Table). Thus, CTEPH specimens represent the end-stage of a vascular remodeling process involving the intimal and medial layers of an affected pulmonary artery. In fact, CTEPH thrombi markedly differ from acute pulmonary emboli, and also from chronic pulmonary arterial thrombi seen for example in about 20% of Eisenmenger patients, as a consequence of chronic slow flow attributable to depressed right ventricular function. New vessel formation and fibrosis are histological hallmarks of CTEPH thrombi. Given the uncertainties regarding a simple mechanistic thromboembolic origin of CTEPH, a specific in situ vascular remodeling process is proposed.

All but one Staphylococcus DNA-positive CTEPH case in this study were VA-shunt/pacemaker carriers, and all but one had had positive bacterial cultures previously, either from right atrial thrombi or from whole blood (Table). By contrast, none of 28 fresh venous thrombi were culture-positive for *S aureus* or *epidermidis* or contained staphylococcal DNA. Neither patients nor controls had clinical signs of active infection at the time of sampling, nor was there evidence for active bacterial metabolism based on bacterial cultures of CTEPH specimens, peptidoglycan immunostaining, and *S aureus* RNA analyses (data not shown). Mildly increased C-reactive protein levels in patients #1, 3, 5, and 6 (Table) without concomitant leukocytosis are more likely attributable to the inflammatory state associated with chronic heart failure34 than a sign for an active bacterial infection. These data suggest that bacterial DNA within chronic thrombi represents remnants of previous infection.

A tight correlation between the amount of bacterial DNA and the degree of fibrosis or CTGF mRNA seems unlikely, because pathogen virulence as well as host susceptibility factors determine the extent of infectious disease. However, the comparison between bacterial DNA positive and negative human CTEPH samples revealed a significant difference in collagen content and relative CTGF mRNA levels (Figure 3), strengthening the concept that bacterial infection is a major determinant of thrombus fibrosis and remodeling.

Human thrombi were characterized by features of a fibrotic process6 (Figure 1) with an increased collagen content compared with fresh thrombi. CTGF mRNA was found significantly elevated by day 28 (Figure 5G) in the model, which is considered the most relevant time point for this...
comparison, and a strong CTGF signal was detected in human CTEPH samples (Figure 1E). By contrast, TGF-β mRNA was markedly lower in CTEPH specimens than in acute thrombi (Figure 1F). These findings are in accord with TGF-β expression patterns in a variety of wound healing and fibrosis models.\textsuperscript{37} On injury, TGF-β is rapidly induced and may induce CTGF expression.\textsuperscript{38} Hypoxia-inducible factor 1 that is activated on bacterial infection\textsuperscript{39} induces CTGF expression, possibly via a hypoxia-responsive element in the CTGF promoter.\textsuperscript{40} Peptidoglycan from \textit{S. aureus} causes rapid release of tumor necrosis factor (TNF-α) and other inflammatory cytokines resulting in TGF-β induction.\textsuperscript{41} Similarly, \textit{S. aureus} protein A, a highly expressed surface component, can mimic TNF-α to activate its receptor and initiate subsequent proinflammatory\textsuperscript{42} and profibrotic signaling.\textsuperscript{43}

Later in the course of healing, TGFβ expression is confined to the leading edge of the scar tissue.\textsuperscript{25} The differences in the TGF-β expression profile between human CTEPH thromboemboli and infected murine thrombi illustrate one of the limitations of the model. An observation period of 28 days is contrasted by a time interval of 103±87 months between device implantation and the diagnosis of CTEPH. In addition, vascular remodeling in pulmonary vessels may be subjected to different rules than in the caval vein. In particular, it has to be taken into account that bacterial infection in the mouse model involved the entire wall of the IVC, resulting in a thrombophlebitis.

A decreased neutrophil count in the early thrombotic process is associated with increased collagen and impaired thrombus resolution, which was not observed when occurring 24 h after thrombus formation.\textsuperscript{44} In our model, bacterial infection was performed 24 h after thrombus formation, confirming that the role of neutrophils in thrombus resolution is time-dependent.

Another major finding was that macrophages that appear to be essential for thrombus recanalization and resolution\textsuperscript{23,45} were significantly reduced in number in infected murine thrombi compared with controls. In vitro, monocytes/macrophages have been shown to undergo apoptosis within 2 to 4 h after phagocytosis of \textit{S. aureus}.\textsuperscript{46}

Based on the analysis of patients with a history of intravenous catheter infection, we conclude that CTEPH is a disorder of misguided thrombus resolution with disproportionate fibrosis driven by \textit{S. aureus} infection.

**Sources of Funding**

This research project received financial support from the European Commission under the 6th Framework Programme (Contract No: LSHM-CT-2005-018725, PULMOTENSION). In addition, this research was in part supported by FWF S9406-B11 (to I.M.L.), and the Österreichischer Herzfonds (to D.B.).

**Disclosures**

None.

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Arterioscler Thromb Vasc Biol. published online January 31, 2008;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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