Platelet endothelial cell adhesion molecule 1 (PECAM-1) deficiency misguides venous thrombus resolution

Joerg Kellermair¹, Bassam Redwan², Sherin Alias³, Joerg Jabkowski⁴, Adelheid Panzenboeck³, Lukas Kellermair³, Max P. Winter³, Ansgar Weltermann¹ and Irene M. Lang³

¹Department of Internal Medicine I, Elisabethinen Hospital, Linz, Austria; ²Department of Thoracic Surgery, General Hospital Muenster, Medical University of Muenster, Germany; ³Department of Internal Medicine II, Vienna General Hospital, Medical University of Vienna, Austria; ⁴Department of Dermatology, Elisabethinen Hospital, Linz, Austria.

Correspondence should be directed to: Irene M Lang; Department of Cardiology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria; telephone number 0043 1 40400 4614; fax number 0043 1 40400 4216; email irene.lang@meduniwien.ac.at
Keypoints

1) PECAM-1 deficiency misguides venous thrombus resolution

2) PECAM-1 cell surface shedding occurs at the site of venous thrombosis
Abstract

Platelet endothelial cell adhesion molecule 1 (PECAM-1) is involved in leukocyte migration and angiogenesis, which are key components of venous thrombus resolution. This study investigated the effect of PECAM-1 deficiency on thrombus resolution in FVB/n mice and the extent to which levels of soluble PECAM-1 (sPECAM-1) correlate with delayed thrombus resolution in humans after acute symptomatic deep vein thrombosis (DVT).

In a mouse stagnant flow venous thrombosis model Pecam-1−/− thrombi were larger, persisted for longer periods of time, and displayed attenuated macrophage invasion and decreased vessel formation in the presence of increased fibrosis. In humans, higher levels of truncated plasma sPECAM-1 possibly cleaved from cell surfaces, were found in patients with delayed thrombus resolution (assessed via duplex-based thrombus scoring) relative to those whose thrombi resolved (median (25th/75th percentile): 92.5 (87.7/103.4) ng/ml versus 71.5 (51.1/81.0) ng/ml; p<0.001). Furthermore, unresolved human deep vein thrombus specimens stained positively with antibodies specific for the extracellular but not the cytoplasmic domain of PECAM-1, consistent with accumulation of cleaved PECAM-1.

Our data suggest a regulatory role of PECAM-1 in venous thrombus resolution and suggest a predictive value of sPECAM-1 for post-thrombotic syndrome (PTS) after acute DVT.
Introduction

Venous thromboembolism (VTE, i.e., deep-vein thrombosis [DVT] or pulmonary embolism) is a common disorder with an annual incidence of approximately 1 or 2 cases per 1000 persons in the general population\(^1\). Short-term treatment is effective, but long-term complications clinically manifest as post-thrombotic syndrome (PTS) or chronic thromboembolic pulmonary hypertension (CTEPH). While CTEPH is rare\(^2\), one of 2-3 patients with DVT will develop PTS within 2 years\(^3\), which leads to significant socio-economic consequences due to high morbidity and treatment costs. Clinical manifestations of PTS are caused by increased hydrostatic pressure due to venous valve dysfunction and persistent thrombus, and range from chronic pain, heaviness, itching, edema and cramping to ulcers in the affected leg. Treatment is limited to palliation with mechanical leg compression and symptomatic ulcer care.

Cellular mechanisms of impaired venous thrombus resolution remain unclear. A transient pro-inflammatory status is regarded to be beneficial for thrombus clearance, but might contribute to the damage of vessel wall structures. Physiologic resolution comprises reorganization of the extracellular matrix at the site of thrombosis via fibrinolysis and angiogenesis. Innate immune cells coordinate these mechanisms: Neutrophil granulocytes are the first to invade a newly formed thrombus and promote fibrinolysis and collagenolysis\(^4\). Consequently, neutropenia is associated with larger venous thrombi and increased fibrosis in various animal models\(^5\). Subsequently, neutrophil granulocytes are replaced by monocytes. Pro-inflammatory signaling molecules such as monocyte chemotactic protein 1 (MCP-1)\(^6\) and interleukin 8 (II-8)\(^7\) stimulate their recruitment and accelerate thrombus resolution \textit{in vivo}. Leukocyte migration to the site of inflammation and thrombosis is not fully understood, but is thought to be mediated by cell adhesion molecules, one of them being platelet endothelial cell adhesion molecule 1 (PECAM-1)\(^8\text{-}10\).

PECAM-1 is a single chain glycopeptid receptor of 130 kDa relative molecular weight and is expressed on platelets, endothelial cells, macrophages, neutrophils, lymphocytes and bone marrow cells\(^11\). The molecule’s cytoplasmatic domain participates in intracellular signaling.

cascades via the immunoreceptor tyrosine-based inhibitory motif (ITIM)\textsuperscript{12}. PECAM-1 is involved in leukocyte transmigration, regulation of leukocyte responses to inflammatory stimuli\textsuperscript{13}, and vascular development\textsuperscript{14-17}, which are major determinants of venous thrombus resolution\textsuperscript{18}. A soluble form of PECAM-1 (sPECAM-1) is generated either by PECAM-1 proteolytic cleavage at the cell surface or by alternative splicing upon cell activation\textsuperscript{19,20}.

Hence, sPECAM-1 exists at least in two isoforms: A truncated form that lacks the cytoplasmic tail and results from cell surface shedding, and a transmembrane-less form that comprises both the extracellular and cytoplasmic domains and originates from splicing out the transmembrane region.

The aim of our study was to elucidate the role of PECAM-1 in venous thrombus resolution. We hypothesized that PECAM-1 deficiency impacts leukocyte thrombus invasion and angiogenesis, and consequently misguides thrombus resolution.
Materials and Methods

Murine Model of Stagnant Flow Venous Thrombosis

Wild type and Pecam-1^{-/-} mice in the FVB/n background were housed at the animal facility of the Medical University of Vienna (protocol approved by the Austrian Ministry of Science). Pecam-1^{-/-} mice were generated as previously described by Schenkel et al.\textsuperscript{21}, and were obtained from William A Muller (Department of Pathology; Medical College of Cornell University New York, USA). PECAM-1 deficient mice do not exhibit a general defect in leukocyte trafficking. The requirement of PECAM-1 in this process is rather stimulus-dependent\textsuperscript{10} and strain-specific. In contrast to the C57BL/6 Pecam-1^{-/-} strain, the inbred FVB/n Pecam-1^{-/-} strain is not able to compensate for the loss of PECAM-1 regarding leukocyte function\textsuperscript{22}, and was therefore chosen for the experiments.

In the murine model, thrombi were induced in the infrarenal vena cava of narcotized mice (n=110; 8 to 12 week-old animals weighing 21 to 32 g were given 100mg/kg ketamine and 5mg/kg xylacine intraperitoneally) via midline laparotomy incision. A 5-0 suture (prolene) was placed alongside the vena cava and a stenosis was produced in the vein by tying a 4-0 silk suture around the vena cava to include the prolene. The prolene was then pulled to allow blood to continue to pass up the vein. Abdominal wall was sutured and infrarenal venous thrombi of both groups were subsequently excised and prepared for analysis at fixed time intervals (days 3, 7, 14 and 28) after ligation. Tissues were fixed in 7,5% buffered formaldehyde. 3 μm paraffin sections were prepared for (immuno)histochemical analysis. For thrombus area quantification an Olympus BX 50 microscope equipped with the imaging software Axio (Version 3.0, Carl Zeiss Vision GmbH, Germany) was used. Thrombus lengths, cross-sectional areas and volumes (lengths x cross-sectional areas) were determined on days 3, 7, 14 and 28 after vena cava ligation. Mean relative volume changes were calculated as the differences of mean thrombus volumes between two subsequent timepoints.
**Modified Trichrome Stain**

For histological analysis, a modified trichrome stain was employed as previously described\(^{23}\). Both early fibrin and red blood cells are shown by lissamine fast yellow. Mature fibrin is stained by a combination of acid fuchsin, biebrich scarlet and ponceau 2R (red), while collagen is visualized by a green color.

**Immunohistochemistry**

Immunohistochemical analysis was performed as previously described\(^{24}\). For the labeled-streptavidin-biotin technique we used a Histostain SP kit (95-6543 AEC Mouse Kit; Zymed Laboratories Inc., San Francisco, USA). Cell characterization was performed using the following primary antibodies: Anti murine F4/80 glycoprotein antibody (14-4801, Ebioscience), anti murine neutrophil monoclonal antibody (CL8993AP, Cedarlane Laboratories), monoclonal anti murine α-smooth muscle actin (α-SMA) antibody (M851, DAKO), and monoclonal anti murine isolectin B4 antibody (B-1205, Vector Laboratories).

Immunohistochemical staining with domain-specific antibodies for the intracellular portion (iPECAM-1; mAb 235.1) and the extracellular domain (ePECAM-1; anti CD31 M0823, DAKO) of human PECAM-1 was performed using Histostain-SP Broad Spectrum Kit (959943 AEC, Invitrogen) according to the manufacturer’s instructions. Cleaved PECAM-1 is visualized by N-terminal PECAM-1 (M0823) immunoreactivity in the absence of C-terminal PECAM-1 (mAb 235.1) immunoreactivity.

**Measurement of Collagen Concentration**

Collagen concentration in paraffin-embedded sections was quantified as previously described\(^{25}\). The method is based on the selective binding of Sirius Red F3BA and Fast Green FCF to collagen and non-collagenous proteins. The dyes have maximum absorbances at different wavelengths. One milliliter of 0,1 M NAOH in absolute methanol is added to elute the color from the sections. Eluted dye is read in a spectrophotometer (Nano...
Drop ND 1000) at 540 and 605nm. Collagen content is expressed as collagen in percent of total protein content.

**Real Time PCR**

Total RNA was extracted from murine/human tissue using RNeasy Mini Kit and RNeasy Fibrous Tissue Kit (Qiagen). cDNA was synthesized from 2μg of total RNA by reverse transcription (TaqMan Reverse Transcription Reagents, 8080234, Invitrogen). Quantitative fluorogenic real time PCR was performed in an ABI PRISM 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Specific TaqMan primers and probes for murine common leukocyte marker *CD68* (ID: Mm00839636_g1) and human *PECAM-1* (ID: Hs00169777_ml) were used (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA, USA). mRNA expression level was normalized to endogenous eukaryotic 18S ribosomal RNA levels. Results are expressed as ratios of specific gene expression.

**Study Population and Procedures**

Consecutive patients (n=48; Caucasians) with a diagnosis of acute, symptomatic popliteal or proximal DVT were enrolled after providing written informed consent in accordance with the Declaration of Helsinki under a protocol reviewed by the ethics committee of the General Elisabethinen Hospital, Linz, Austria. Exclusion criteria were an expected life span less than 1 year and coexisting symptomatic pulmonary embolism (assessed either via computer tomography pulmonary angiography or standard 6-view perfusion lung scan).

Thrombus score (reflecting the burden of leg vein thrombus) was calculated using manual complete compression ultrasonography (CCUS)\(^\text{26}\). The score for each affected venous segment (except for the deep femoral) was adjusted for thrombus diameter (assessed under full compression and compared with the diameter of the corresponding artery). The segment score was increased 1.5-fold if its compressed diameter was ≥1.5 times the arterial diameter, and reduced 0.5-fold if this was ≤ 0.5 times the arterial diameter. Thrombus score was calculated as the sum of each segment: external iliac vein (8), common femoral vein (4),
deep femoral vein (2), proximal (4) and distal (3) superficial femoral vein, popliteal vein (2), posterior tibial veins (2) and peroneal veins (2). A score ≥ 4 at baseline was an inclusion criterion.

A follow-up duplex scan was scheduled 28 (±3) days after diagnosis in order to evaluate residual vein thrombus and to calculate change of thrombus score (Δ = thrombus score baseline-thrombus score on day 28). A Δ thrombus score < 4 was defined as delayed thrombus resolution. All patients received compression stockings, therapeutic dose of low molecular weight heparin (LMWH) and/or subsequent oral anticoagulation therapy (OAT). Adequate OAT therapy was defined as an INR (international normalized ratio) value of 2-3.

Blood samples for soluble PECAM-1 enzyme linked immunosorbent assay (ELISA) were collected within 24 hours after diagnosis and at follow-up appointment and stored at -80°C until final analysis. After 12 (±0.5) months included patients were evaluated for PTS using the Villalta scale: Points from 0 (absent) to 3 (severe) for five patient-rated symptoms (pain, cramps, paraesthesia, pruritus, heaviness) and six clinician-rated symptoms (pretibial edema, redness, skin induration, hyperpigmentation, venous ectasia, pain during calf compression) were summed into a total score (range 0-33). PTS was defined by a Villalta score ≥ 5. Investigators performing duplex scan and PTS evaluation did not have access to the results of blood analyses at any time.

**Soluble PECAM-1/Soluble ICAM-1 Sandwich ELISA**

Total soluble PECAM-1 measurements were performed utilizing indirect enzyme-linked immunosorbent assay (Platinum ELISA Bender MedSystems BMS 229) according to manufacturer’s instructions. The monoclonal anti-human mouse antibody directed against the cytoplasmic tail of PECAM-1 (235.1 provided by Peter J Newman, PhD, Blood Research Center of Wisconsin, Milwaukee, WI, USA) was used to detect the soluble sub-form comprising the cytoplasmic domain: The IgG antibody was diluted in HEPES to 25μg/ml and adsorbed onto microtiter plates overnight (4°C). Wells were rinsed (PBS 0.05% Tween 20) and blocked with 1% BSA (1 hour, room temperature), followed by a 1 hour incubation of the
plasma sample. HRP-conjugated anti-human soluble PECAM-1 monoclonal detection antibody directed against the extracellular domain was added and incubated for 3 hours (room temperature). Microwells were rinsed and color was developed by addition of substrate solution (tetramethyl-benzidine). Absorbances were read on spectrophotometer using 450nm as primary wave length after 10 minutes.

For soluble intercellular adhesion molecule 1 (sICAM-1) determination we used the sICAM-1 Platinum ELISA (Ebioscience BMS 201) according to manufacturer’s instructions.

**Human Tissue Samples**

Human tissue samples of nonresolving DVT thrombi (n=4) and venous vessel wall (n=4), that were harvested in the course of variceal surgeries, were fixed in 7.5% formaldehyde for immunohistochemistry. Pulmonary endarterectomy specimens from patients with CTEPH (n=6), and unthrombosed pulmonary arterial vessel wall (pulmonary artery, n=4) were harvested during respective surgeries and were immediately snap frozen in liquid nitrogen for RT-PCR.
Statistics

The statistical package SPSS Version 18.0.2 was used. Murine data was expressed as means and standard deviations (SD), and significance of intergroup differences was examined using student’s unpaired t-test. Levels of sPECAM-1, sICAM-1 and D-Dimer were described by medians and inter-quartile-ranges (25th percentiles/75th percentiles) with an intergroup (Δ thrombus score < 4 versus ≥ 4, PTS yes versus no) difference determined by Mann-Whitney U Test. Differences regarding PTS status in patients with normal and delayed thrombus resolution were determined using Fisher’s Exact Test. Significance of intergroup gene expression differences was determined by ANOVA. A p-value <0.05 was considered statistically significant. Univariate logistic regression was used to determine the predictive value of baseline sPECAM-1. Odds-Ratios (OR) and Risk-Ratios (RR) are provided with 95% confidence intervals and p-values. In order to identify potential confounders, we simultaneously added baseline patient characteristics to the univariate model to calculate adjusted OR. Correlation between sPECAM-1 levels and Δ thrombus score was determined using Bravais-Pearson’s correlation coefficient. For the correlation between sPECAM-1 levels and D-Dimer levels the Spearman’s Rho was used.
Results

*Pecam-1−/−* venous thrombi are larger, more persistent, and characterized by less inflammatory cells and fewer vessels

We were able to harvest thrombi in 96% of *Pecam-1−/−* mice and in 84% of controls resulting in 110 thrombi (n=8 to 20 per each group and individual time point).

Quantitative thrombus analysis revealed that *Pecam-1−/−* mice exhibited significantly larger thrombi on days 0-28 (Figure 1A/C/D). Mean thrombus volume peaked by day 3 and regressed to its minimum value by day 14. In contrast to controls, *Pecam-1−/−* mice showed overall impaired thrombus resolution with a period of thrombus length (Figure 1C) and volume increase (Figure 1A) between days 14-28. Mean relative thrombus volume changes are illustrated in Figure 1B. By day 28 the mean cross sectional area of *Pecam-1−/−* thrombi was nearly four-fold that of controls (1.5 ± 0.2 mm² vs. 0.4 ± 0.2 mm²; p<0.001) (Figure 1D).

Staining characteristics indicated that *Pecam-1−/−* thrombi (Figure 2) contained significantly larger areas of collagen compared to controls on day 28 (17.6 ± 1% vs. 10.2 ± 1%; p<0.001) (Figure 1H). Furthermore, *Pecam-1−/−* macrophages were less frequently found in thrombi (Figure 1F; Figure 3I-P); (day 7: 9.5 ± 4.7 cells/mm² vs. 1.5 ± 0.7 cells/mm²; p=0.019 / day 28: 5.8 ± 2.9 cells/mm² vs. 0.6 ± 0.8 cells/mm²; p=0.013). Accordingly, expression of the leukocyte marker *CD68* was reduced at the site of thrombosis in *Pecam-1−/−* mice (Figure 1G). By contrast, numbers of smooth muscle cells and neutrophils (data not shown) were comparable to those in controls at all timepoints.

Finally, *Pecam-1−/−* thrombi were characterized by diminished thrombus vessels (Figure 1E; Figure 3A-H). Newly formed microvessels were detectable in the periphery of control thrombi by day 3 (34.3 ± 16.9 isolectin B4-positive cells/mm² in control thrombi vs. 2.7 ± 1.8 isolectin B4-positive cells/mm² in *Pecam-1−/−* thrombi; p=0.014), while *Pecam-1−/−* thrombi showed less vascularization until day 14 (day 7: 58.0 ± 18.6 cells/mm² vs. 8.5 ± 7.4 cells/mm²; p=0.01/ day 14: 97.8 ± 53.0 cells/mm² vs. 32.3 ± 10.1 cells/mm²; p=0.87).
Serum of patients with delayed thrombus resolution contains elevated sPECAM-1 levels at baseline

Characteristics and results of patients with acute, symptomatic DVT who completed 1-year of follow up are shown in Table 1. Right-sided DVT was diagnosed in 46.9%, left-sided in 46.9% and bilateral DVT in 6.2% of all patients. Malignancy was observed in 27.1%, and 39.6% of patients had had a previous episode of VTE. Duration of anticoagulation therapy ranged between 3 months and indefinite depending on individual risk of recurrence. 45.8% of all patients displayed delayed thrombus resolution one month after diagnosis. Cumulative incidence of PTS in 46 patients after one year was 32.6%.

In 16.7% of patients with ∆ thrombus score ≥ 4 PTS developed within one year. These patients had median (25th/75th percentile) sPECAM-1 baseline plasma levels of 71.5 (51.1/81.0) ng/ml and median D-Dimer baseline plasma levels of 4.5 (3.1/11.0) μg/ml (Table 1). Patients with ∆ thrombus score < 4, however, developed PTS in 50% of cases [RR of 3.00 (95% CI 1.108-8.124; p=0.002)], and showed median sPECAM-1 baseline plasma levels of 92.5 (87.7/103.4) ng/ml (p<0.001), and median D-Dimer baseline plasma levels of 3.3 (2.1/4.4) μg/ml (p=0.069) (Table 1; Figure 4). On follow-up median sPECAM-1 levels were 90.0 (73.0/102.6) ng/ml vs. 71.9 (57.2/87.0) ng/ml (p=0.049) in patients with ∆ thrombus score < 4 versus ≥ 4 (Table 1). Pearson’s coefficient for correlation between ∆ thrombus score and sPECAM-1 baseline levels was -0.467 (p=0.001); Spearman’s Rho for correlation between sPECAM-1 baseline levels and baseline D-Dimer levels was -0.326 (p=0.027).

Baseline sPECAM-1 levels predicted thrombus persistence [OR of 1.134 (95% CI 1.046-1.229; p=0.002)] both in univariate logistic regression analysis and multivariate logistic regression analysis corrected for age, gender and malignancy [Adjusted OR of 1.139 (95% CI 1.051-1.235; p=0.001)]. In addition, baseline sPECAM-1 plasma levels were predictive of PTS [Adjusted OR of 1.350 (95% CI 1.049-1.739; p=0.020)]. Baseline soluble ICAM-1 plasma levels did not differ significantly between patients with subsequent PTS and those
who did not develop PTS (565.8 (480.1/771.3) ng/ml vs. 499.1 (466.2/589.3) ng/ml; p=0.210), however levels tended to be higher in the PTS group, which has been described\textsuperscript{27}.

Patients with PTS showed median Villalta-Score of 6.0 (5.0/7.0) and median baseline sPECAM-1 levels of 88.0 (85.2/110.2) ng/ml compared to median Villalta-Score of 2.0 (1.0/4.0) and median baseline sPECAM-1 levels of 79.2 (51.5/88.2) ng/ml (p=0.007) in patients without PTS (Figure 4).

Total thrombus burden expressed by the most proximal thrombus edge (Table 1) was not significantly different between the groups.

Use of antibody 235.1 directed against the cytoplasmic domain as coating antibody in the ELISA assays failed to detect sPECAM-1 in patients with $\Delta$ thrombus score $< 4$ and in patients with $\Delta$ thrombus score $\geq 4$ suggesting that sPECAM-1 levels in our patients represented exclusively the truncated form.

\textit{Immunohistochemistry with domain-specific antibodies suggests PECAM-1 shedding at the site of venous thrombosis}

The truncated form of sPECAM-1 results from proteolytic cell surface cleavage. In order to identify the source of elevated truncated sPECAM-1 plasma levels in patients with delayed thrombus resolution, we analyzed human nonresolving DVT tissue specimens in comparison to human unthrombosed saphenous vein vessel wall specimens. We used domain-specific PECAM-1 antibodies directed against ePECAM-1 (N-terminal) and iPECAM-1 (C-terminal) to determine presence of both domains indicating expression of full-length PECAM-1. In unthrombosed venous vessel sections immunoreactivity for the extracellular and intracellular domain of PECAM-1 was found suggesting the presence of full-length PECAM-1 (Figure 5: arrows in panel C2 and C3), while nonresolving deep vein thrombi stained positively with antibodies specific for ePECAM-1 (Figure 5A2/B2) but not iPECAM-1 (Figure 5A3/B3), suggesting cleaved forms of PECAM-1. A discordance of extracellular and intracellular
PECAM-1 domain expression (starlet in Figure 5A3/B3) was previously interpreted as accumulation of truncated forms of PECAM-1\textsuperscript{28}.

**Gene expression analysis demonstrates diminished PECAM-1 expression in chronic venous thrombosis**

Analysis of *PECAM-1* expression in vascular tissues confirmed significant down-regulation of *PECAM-1* in nonresolving thrombi, compared with non-thrombosed pulmonary artery (Figure 6) (p=0.009). Endarterectomy specimens from patients with CTEPH were employed as extreme examples for persistent/recurrent thrombosis.
Discussion

Current data suggest that physiological venous thrombus resolution after VTE depends on leukocyte migration and angiogenesis, and resembles wound repair. The recruitment of innate immune cells is important in the remodeling process because misguided activation triggers both initiation and propagation of venous thrombosis. An impaired resolution process after thrombosis is also associated with PTS and its sequela. Understanding mechanisms regulating thrombus resolution is necessary for the development of effective treatment strategies.

We employed Pecam-1−/− mice to investigate the role of this glycoprotein in venous thrombus resolution in a stagnant flow thrombosis model. Our data demonstrate that PECAM-1 deficiency led to significantly larger thrombi at all time-points. Mechanisms underlying larger thrombus sizes between days 0-3 in Pecam-1−/− mice were not addressed in the present study. Recent findings have emphasized the importance of platelets in venous thrombosis. Because platelet PECAM-1 has been shown to inhibit arterial thrombus formation, and because PECAM-1 deficient platelets displayed enhanced aggregation and granule secretion in response to various stimuli, PECAM-1 deficiency of platelets likely contributed to significantly larger thrombi immediately after ligation.

Similar initial thrombus sizes in controls and Pecam-1−/− mice would have been easier to interpret with regard to resolution rates. However, we calculated volume changes over time. We found that similar size decreases occurred between days 3 to 14 (Figure 1 A/B/C/D), while thrombus volumes increased in Pecam-1−/− mice between days 14 and 28 (Figure 1B). Neutrophil numbers did not differ between controls and Pecam-1−/− thrombi on days 3 and 7 (data not shown), suggesting that similar neutrophil recruitment in the beginning may have contributed to similar resolution rates. However, subsequent thrombus characteristics significantly changed in PECAM-1 deficient thrombi with significantly less macrophages (Figure 1F, Figure 3I-P), less thrombus vessels (Figure 1E, Figure 3A-H) and increased fibrosis (Figure 1H). Endothelial cell PECAM-1 deficiency reduces angiogenesis by inhibition of endothelial cell filopodia. Neovascularization and macrophage recruitment are important
for thrombus resolution in vivo\textsuperscript{34}. In our study PECAM-1 deficiency led to a dampened inflammatory response and less vascularization at the site of thrombosis in FVB/n strain mice, which may account for delayed resolution between days 14-28, resulting in thrombus persistence. Histologically, organizing mouse thrombi bear close similarities with nonresolving human thrombi, for example human complicated DVT, and CTEPH.

Based on the murine data of delayed thrombus resolution between days 14-28, we aimed to investigate the role of PECAM-1 in a prospective observational clinical study of patients with acute symptomatic DVT. We monitored thrombus resolution via duplex-based thrombus scoring and development of PTS within 12 months. We found significantly elevated baseline sPECAM-1 plasma levels in patients with subsequently delayed thrombus resolution. The difference was also detectable on day 28 (±3), suggesting continued cleavage of PECAM-1, and underscoring the importance of serial blood sampling because baseline commonly represents a condition in which unspecific inflammatory processes are transiently up-regulated.

At first glance the association between elevated baseline sPECAM-1 levels and delayed thrombus resolution seems to contradict the data in the murine model. However, elevated human plasma sPECAM-1 derives exclusively from its cleaved form, which was confirmed by ELISA assays employing a monoclonal antibody selectively directed to the PECAM-1 cytoplasmic domain. PECAM-1 must have been cleaved at the surface of cells involved in the resolution process. Previous studies\textsuperscript{28,35} have demonstrated PECAM-1 proteolytic cleavage at the cell surface and potential competitive inhibition of membrane-bound PECAM-1\textsuperscript{36} during inflammation, thus mimicking a lack of surface expression. This would be in agreement with the observation in mice lacking PECAM-1.

To confirm this hypothesis, we analyzed nonresolving human DVT thrombi in comparison to unthrombosed human venous wall by immunohistochemistry. Domain-specific analysis for the extracellular and intracellular portion of PECAM-1 revealed little co-distribution at the luminal thrombus side, suggesting accumulation of cleaved extracellular PECAM-1, which is most likely the source of elevated sPECAM-1 plasma levels. We suggest that human
PECAM-1 activity is suppressed either by its soluble form or by surface shedding. This condition would favour thrombus persistence, similar to the observation in the animal study. Our experiments do not identify the cellular source of sPECAM-1, but we propose that it derives from cells located at the site of thrombosis.

In addition, we evaluated PECAM-1 gene expression in human nonresolving DVT thrombi and chronic thrombi endarterectomized from patients with CTEPH. These thrombi contain more extracellular matrix and less cells compared with unthrombosed pulmonary artery vessel wall. Consequently, housekeeping genes are at a lower level of expression. However, PECAM-1 mRNA levels were normalized to housekeeping genes and still show significant downregulation in nonresolving thrombi, particularly in CTEPH thrombi (Figure 6). It is plausible that decreased numbers of PECAM-1 expressing cells impact expression levels, however, PECAM-1 downregulation on a single cell level cannot be excluded. Based on these data, gene down-regulation on a mRNA level is likely to enhance the loss of PECAM-1 activity on a protein level, thus, exacerbating PECAM-1 deficiency.

Persistent thrombus with consecutive vessel occlusion is known to be associated with PTS. Clinical trials have focused on soluble cell adhesion molecules (i.e. ICAM-1, VCAM-1) in DVT and their potential role as marker molecules for the development of PTS. In our study univariate regression analysis revealed that baseline sPECAM-1 levels are able to predict delayed thrombus resolution, and also the development of PTS potentially more reliably than sICAM-1. C-reactive protein (CRP), an acute-phase protein, did not show a significant difference between the groups (data not shown), indicating that sPECAM-1 results are not simply due to a general inflammatory response.

Patients with Δ thrombus score < 4 have a threefold higher risk to develop PTS compared to patients with Δ thrombus score ≥ 4. If our findings can be confirmed in larger sample size studies, plasma sPECAM-1 level measurement at the time of DVT diagnosis might predict which patients need more aggressive treatment for PTS prevention and which patients possibly benefit from longer periods of anticoagulant treatment or, at least, CCUS...
reevaluation before anticoagulant therapy withdrawal. Based on the novel informations in this manuscript, plasma sPECAM-1 level as a biomarker and potential therapeutic implications should be addressed in systematic clinical trials.

This is the first study to provide evidence for the involvement of PECAM-1 in venous thrombus resolution. In a mouse model mimicking human DVT, PECAM-1 deficiency led to significantly larger thrombi and misguided thrombus resolution. In patients with DVT and subsequently delayed thrombus resolution, plasma sPECAM-1 levels are significantly higher, possibly due to enhanced surface cleavage at the site of thrombosis, and predict development of PTS. One limitation of our study is the use of an animal model with a primary deficiency of PECAM-1 comparing with a human model of “secondary” PECAM-1 deficiency. Our data are further limited by the relatively small sample size, and by speculation regarding precise mechanisms of sPECAM-1 biological function in patients suffering from nonresolving venous thrombosis. Further studies will have to clarify cellular sources of sPECAM-1, and verify its predictive value.
Acknowledgements

The study was supported by a fellowship grant of the Austrian Society of Cardiology. We would like to thank Dr. Sebastian Schellong, University of Dresden, Germany, for his assistance in thrombus scoring, and Dr. Peter J Newman, Blood Research Institute of Wisconsin, Milwaukee, USA, for providing monoclonal antibody 235.1.
Authorship

Contribution: J.K., B.R., S.A., L.K., M.P.W., and A.P. performed experiments; J.K., I.M.L. and A.W. designed the research; J.K., L.K., A.P. and I.M.L. analyzed results; J.K. made the tables and figures; J.J. performed CCUS; J.K. and I.M.L. wrote the paper

Conflict-of-interest disclosure: The authors declare no competing financial interests
References


### Tables and Table Legends

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Δ thrombus score ≥ 4 (n=26)</th>
<th>Δ thrombus score &lt; 4 (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>46.2%</td>
<td>45.5%</td>
</tr>
<tr>
<td>Age (years ± SD)</td>
<td>68 (±9)</td>
<td>68 (±11)</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>15.4%</td>
<td>13.6%</td>
</tr>
<tr>
<td>Body Mass Index (± SD)</td>
<td>28.5 (±2)</td>
<td>31.7 (±2)</td>
</tr>
<tr>
<td>Immobilization/surgery (%)</td>
<td>15.4%</td>
<td>18.2%</td>
</tr>
<tr>
<td>Malignancy (%)</td>
<td>23.1%</td>
<td>31.8%</td>
</tr>
<tr>
<td>Thrombophilia (%)</td>
<td>0%</td>
<td>4.5%</td>
</tr>
<tr>
<td>Previous VTE (%)</td>
<td>42.3%</td>
<td>36.4%</td>
</tr>
<tr>
<td>PTS 12 months after diagnosis (%)</td>
<td>16.7%</td>
<td>50%*</td>
</tr>
<tr>
<td>Median Villalta-Score (score; percentile 25/75)</td>
<td>2.5 (2.0/4.0)</td>
<td>4.5 (2.0/7.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatments</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of anticoagulation (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 3 to 6 months</td>
<td>0%</td>
<td>9.1%</td>
</tr>
<tr>
<td>- 6 to 12 months</td>
<td>15.4%</td>
<td>22.7%</td>
</tr>
<tr>
<td>- &gt;12 months</td>
<td>84.6%</td>
<td>68.2%</td>
</tr>
<tr>
<td>Vitamin K antagonists(%)*</td>
<td>69.2%</td>
<td>72.7%</td>
</tr>
<tr>
<td>Therapeutic dosage of low molecular weight heparin (%)*</td>
<td>26.9%</td>
<td>27.3%</td>
</tr>
<tr>
<td>Dabigatran (%)*</td>
<td>3.9%</td>
<td>0%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D-Dimer and sPECAM-1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median D-Dimer baseline (µg/ml; percentile 25/75)</td>
<td>4.5 (3.1/11.0)</td>
<td>3.3 (2.1/4.4)</td>
</tr>
<tr>
<td>Median D-Dimer day 28 (±3) (µg/ml; percentile 25/75)</td>
<td>1.2 (0.7/1.5)</td>
<td>0.7 (0.5/1.5)</td>
</tr>
<tr>
<td>Median sPECAM-1 baseline (ng/ml; percentile 25/75)</td>
<td>71.54 (51.05/81.04)</td>
<td>92.50 (87.73/103.36)**</td>
</tr>
<tr>
<td>Median sPECAM-1 day 28 (±3) (ng/ml; percentile 25/75)</td>
<td>71.96 (57.22/87.03)</td>
<td>90.07 (73.02/102.62)**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thrombus burden</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean thrombus score baseline (± SD)</td>
<td>16.7 (±9.1)</td>
<td>10.5 (±2.0)</td>
</tr>
<tr>
<td>Mean thrombus score day 28 (±3) (± SD)</td>
<td>10.0 (±9.1)</td>
<td>10.3 (±2.5)</td>
</tr>
<tr>
<td>Mean Δ thrombus score (± SD)</td>
<td>6.7 (±2.5)</td>
<td>0.3 (±2.4)**</td>
</tr>
<tr>
<td>Proximal segment baseline (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- popliteal vein</td>
<td>11.5%</td>
<td>9.1%</td>
</tr>
<tr>
<td>- distal femoral vein</td>
<td>19.2%</td>
<td>18.2%</td>
</tr>
<tr>
<td>- femoral vein (mid-segment)</td>
<td>30.8%</td>
<td>36.4%</td>
</tr>
<tr>
<td>- proximal femoral vein</td>
<td>30.8%</td>
<td>36.4%</td>
</tr>
<tr>
<td>- external iliac vein</td>
<td>7.6%</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Pharmacological substance was evaluated 28 (±3) days after diagnosis

**p-value <0.05

***p-value <0.01
Table 1. Clinical data of study patients. Patient characteristics, treatments, laboratory findings, and thrombus burden of patients with normal (Δ thrombus score ≥4) and delayed thrombus resolution (Δ thrombus score <4) at baseline, after 28 (±3) days and 12 (±0.5) months after DVT diagnosis are shown.
Figures and Figure Legends

Figure 1. Temporal changes of thrombus characteristics in Pecam-1-/- and wild-type mice. Thrombus volumes (A), relative thrombus volume changes (B), thrombus lengths (C), cross-sectional areas (D), endothelial cell counts (E) and macrophage counts (F), relative CD68 mRNA expression levels (G) and collagen deposition (H) on days 3, 7, 14 and 28 after vena cava ligation are shown (*p<0.05, all means with ± standard deviations). Black symbols represent Pecam-1-/- mice, white symbols represent controls.
Figure 2. Trichrome stains of wild-type and Pecam-1–/– thrombi. Representative thrombus cross-sections of wild-type (A-D) and Pecam-1–/– (E-H) mice on days 3 (A/E), 7 (B/F), 14 (C/G) and 28 (D/H) after vena cava ligation are shown. Initially, thrombi are fibrin-rich (red color) and fragile, and subsequently become solid due to increased collagen synthesis (green color). Scale bar represents 100μm.
Figure 3. Immunohistochemical analyses of wild-type and Pecam-1⁻/⁻ thrombi illustrating misguided vascular remodeling of thrombus resolution in the absence of PECAM-1. Isolectin-B4 immunoreactivities representing endothelial cells in wild-type (A-D) and corresponding Pecam-1⁻/⁻ thrombi (E-H) are shown on days 3 (A/E), 7 (B/F), 14 (C/G) and 28 (D/H) after vena cava ligation on the left side of the panel. The right side of the panel shows F4/80 immunoreactivities representing macrophages (examples of immunopositive cells are pointed out with small arrows) in wild-type (I-L) and corresponding Pecam-1⁻/⁻ thrombi (M-P) on days 3 (I/M), 7 (J/N), 14 (K/O) and 28 (L/P) after vena cava ligation. Scale bar represents 100μm.
Figure 4. Baseline sPECAM-1 plasma levels (ng/ml) in study patients. Box plots illustrating baseline sPECAM-1 levels (ng/ml) in patients with subsequent PTS versus patients without PTS (shown on the left side of the figure; p-value =0.007). The two box plots on the right side of the figure show sPECAM-1 levels (ng/ml) in patients with subsequently delayed thrombus resolution (Δ thrombus score < 4), compared with patients undergoing normal thrombus resolution (Δ thrombus score ≥ 4, p-value <0.001).
Figure 5. Differential domain-specific antibody immunoreactivities suggesting PECAM-1 cell surface shedding at the site of thrombosis. The left side of the panel shows trichrome stains of two cases of nonresolving human deep vein thrombus (specimen A and B, A1/B1) and vessel wall from a human saphenous vein (C1). These tissue samples were immunohistochemically stained using domain-specific antibodies directed against the extracellular domain (N-terminal) of PECAM-1 (ePECAM-1: A2/B2/C2) and intracellular portion (C-terminal) of PECAM-1 (iPECAM-1: A3/B3/C3). Cleaved PECAM-1 is characterized by the presence of immunoreactivity derived from the antibody against ePECAM-1 in the absence of immunoreactivity derived from the antibody against iPECAM-1. The area of discordant PECAM-1 expression (A2 vs. A3; B2 vs. B3) is highlighted with a starlet (A3/B3). By contrast, the endothelial cell layer of saphenous vein wall stains positively with both antibodies, indicating full length PECAM-1 expression (arrows in panels C2 and C3). Scale bar represents 100μm.
Figure 6. Low relative expression of *PECAM-1* mRNA within nonresolving human thrombi. The figure shows real-time PCR analysis of CTEPH thrombi (n=6) and nonresolving DVT thrombi (n=3) compared with pulmonary artery (n=4). Chronic nonresolving thrombi are characterized by significant down-regulation of *PECAM-1* mRNA (normalized to 18S RNA).
Platelet endothelial cell adhesion molecule 1 (PECAM-1) deficiency misguides venous thrombus resolution

Joerg Kellermair, Bassam Redwan, Sherin Alias, Joerg Jabkowski, Adelheid Panzenboeck, Lukas Kellermair, Max P. Winter, Ansgar Weltermann and Irene M. Lang