Elevation of Endothelial Microparticles, Platelets, and Leukocyte Activation in Patients With Venous Thromboembolism

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OBJECTIVES The purpose of this research was to determine the levels of platelet, leukocyte, and endothelial activation and markers of cellular interactions in patients with venous thromboembolism (VTE).

BACKGROUND The details of interactions between endothelium, platelets, and leukocytes in VTE are not well understood.

METHODS We studied 25 patients with VTE and compared 25 healthy controls. We used flow cytometry to measure: 1) endothelial microparticles (EMP) identified by CD31+/CD42b− (EMP31) or E-selectin (EMP62E); 2) platelet microparticles (CD31+/CD42b+); 3) surface expression of P-selectin in platelets and CD11b in leukocytes; 4) EMP-monocyte conjugates (percentage of monocytes positive for E-selectin); and 5) platelet-leukocyte conjugates (PLC) expressed as percentage of leukocytes positive for CD41.

RESULTS Patients with VTE had marked elevations of EMP31 (2,193 vs. 383 counts/μl; p = 0.003), EMP62E (368 vs. 223 counts/μl; p = 0.001), and EMP-monocyte conjugates (3.3% vs. 2.5%; p = 0.002), as well as increased activation of platelets (35.2 vs. 5.0 fluorescence intensity units for P-selectin; p < 0.0001) and leukocytes (13.9 vs. 7.7 U for CD11b; p = 0.004). Also elevated in VTE were PLC (61.7% vs. 39.6%; p = 0.01). Expression of CD11b in leukocytes strongly correlated with PLC (r = 0.74; p < 0.0001).

CONCLUSIONS Marked activation of endothelium, platelets, and leukocytes occurs in VTE, and VTE, or the accompanying inflammatory process, involves the release of EMP and formation of EMP-monocyte conjugates and PLC. These findings support prior studies suggesting that release of EMP and their binding to monocytes are key events in thrombogenesis. Our findings also support the concept that the formation of PLC regulates leukocyte activation and participates in linking thrombosis with inflammation. (J Am Coll Cardiol 2005;45:1467–71) © 2005 by the American College of Cardiology Foundation

The pathophysiology of venous thromboembolism (VTE) involves endothelial damage, blood stasis, and hypercoagulability (1). Significant advances have been achieved in the understanding of humoral factors of hypercoagulability. However, the details of interactions between endothelium, platelets, and leukocytes in VTE have been less well studied.

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Recently, flow-cytometric analysis of microparticles from platelets and from the endothelium has emerged as a powerful method for assessing the status of endothelial cells (EC), platelets, leukocytes, and their interactions (2–4). Endothelial microparticles (EMP) are small (<1.5 μm) vesicular fragments of the EC membrane released during EC activation or apoptosis (2,3). Similarly, platelet microparticles (PMP) are released by platelets upon activation (4). Both endothelial (5,6) and platelet (7) microparticles can interact with leukocytes. By employing strategic combinations of fluorescent-labeled antibodies, it is possible to detect EMP-leukocyte and platelet-leukocyte conjugates (2,4). In addition, leukocyte expression of activation marker CD11b and platelet expression of activation marker CD62P can be readily measured by flow cytometry.

In this study, we aimed to determine whether these markers of activation of EC, platelets, and leukocytes and their interactions are increased in patients with VTE.

METHODS

Study subjects. A convenience sample of 25 patients with deep vein thrombosis (DVT) and/or pulmonary embolism (PE) was studied and compared to 25 healthy controls studied in the same period of time. Patients with a clinical suspicion of VTE were prospectively enrolled at Jackson Memorial Hospital/University of Miami. The research protocol was approved by our institutional review board, and informed consent was obtained from all patients.

Important demographic, clinical, and laboratory characteristics were prospectively recorded upon enrollment. Patients were followed, and results of diagnostic tests were recorded. We selected patients with DVT confirmed by...
prepare platelet-poor plasma.

Blood samples consisted of two citrate Vacutainers (light-blue top; Becton-Dickinson, Franklin Lakes, New Jersey) from a peripheral vein upon enrollment. Samples were processed for assay within 4 h. One was saved for whole-blood tests, and the other was centrifuged 10 min at 160 × g to prepare platelet-rich plasma; then the platelet-rich plasma was centrifuged for 8 min at 1,000 × g to prepare platelet-poor plasma.

FREE PMP AND EMP. The method of EMP assay was previously described (8,9); PMP and EMP were measured simultaneously in platelet-poor plasma by the pair of fluorescent monoclonal antibodies (mAb), PE-labeled anti-CD31, and FITC-labeled anti-CD42b, both from BD/Pharmingen (Franklin Lakes, New Jersey). Events were counted by triggering on the red fluorescence signal of PE, above background noise, on the y-axis of the dot-plot, while the green signal of CD42b was on the x-axis (four-decade log scales, both x and y). Because CD31 occurs on both PMP and EMP but CD42b occurs only on platelets, PMP were defined by events CD31+/CD42+ and EMP by events CD31+/CD42−. Values are reported as counts/µl; CD31 occurs also on some leukocyte subsets, but we observed (using PE/Cy5-labeled CD45) that leukocyte microparticles accounted for a negligible fraction of CD31+/CD42− microparticles, in both normal and thrombogenic patients. To further validate this procedure, known amounts of pure PMP and EMP prepared in vitro were mixed in various proportions, and then measured by this method; results were essentially the same as measuring the pure components individually.

ACTIVATION STATUS OF PLATELETS AND LEUKOCYTES. Total expression of P-selectin (CD62P) was measured by FITC-anti-CD62P mAb (Pharmingen) in dilute whole blood as the sum of three bit-map populations: free platelets, microaggregated platelets, and platelet-leukocyte complexes (10). The population of each was multiplied by the mean fluorescent intensity (arbitrary units) of that population, then summed, then divided by the total number of events in the three bit-maps, yielding the mean fluorescent intensity of P-selectin per event. The rationality for this is that activated platelets avidly bind to each other and to some leukocytes; therefore, measuring free platelets alone does not reflect total platelet activation status. Leukocyte activation was measured by their expression of CD11b.

MEASUREMENT OF LEUKOCYTES CONJUGATED WITH PMP OR EMP. The method for measuring platelet-leukocyte conjugates has been described elsewhere (7) and is based on the co-expression of fluorescent pan-leukocyte marker CD45 (PE label) with platelet marker CD41 (FITC label); neutrophils were selected (gated) based on forward- and side-scatter; EMP-monoceyte conjugate assay was based on expression of mAb markers of ECs, CD62E, appearing on leukocytes (coincident with CD45); monocytes, neutrophils, and lymphocytes were selected based on forward- and side-scatter (5).

Statistical analysis. Given the non-normal distribution of the markers measured, values are expressed as median and interquartile range (IQR) throughout the article and were compared between the two groups using the Mann-Whitney U test. The linear relationship between continuous variables was analyzed with linear regression, and the corresponding Pearson correlation coefficient (r) and r squared (r²) values were calculated. Logarithmic transformation of continuous variables (CD11b and platelet-leukocyte conjugates) was undertaken to improve normality before entering regression models. All reported probability...
values are two-tailed. Statistical significance was defined as a p value <0.05. Analyses were performed with the statistical package NCSS for Windows (Kaysville, Utah).

RESULTS

Baseline characteristics of our population of patients with VTE are shown in Table 1. The mean age was 54 years; PE was present in 44% of patients, whereas DVT without a suspicion or diagnosis of PE was present in the remaining 56%. About one-half of the patients had recent surgery as a precipitating factor for VTE, and one-third had an underlying malignancy.

EMP and EMP-monoocyte conjugate levels (Figs. 1A to 1C). Patients with VTE had markedly increased levels of EMP31 (2,193 counts/µL; IQR = 1,000 to 6,222) compared to normal controls (383 counts/µL; IQR = 243 to 2,403; p = 0.003). Similarly, patients with VTE had higher levels of EMP62E (368 counts/µL; IQR = 253 to 888) than normal controls (223 counts/µL; IQR = 84 to 315; p = 0.001); EMP-monoocyte conjugates were also significantly higher in patients with VTE (3.3 fluorescence intensity units [FLIU]; IQR = 2.96 to 3.91) than in controls (2.5 FLIU; IQR = 2.13 to 2.83; p = 0.002).

PMP, platelet P-selectin, leukocyte CD11b, and platelet-leukocyte conjugates (Figs. 2A to 2C). Platelet expression of CD62P was markedly increased in patients with VTE (35.2 FLIU; IQR = 23.5 to 58.2) compared to normal controls (5.0 FLIU; IQR = 1.5 to 21.6; p < 0.0001). Unexpectedly, levels of PMP were not significantly elevated in patients with VTE (8,212 vs. 5,395 counts/µL; p = 0.52); CD11b expression in leukocytes was also higher in patients with VTE (13.9 FLIU; IQR = 9.6 to 21.2) than in controls (7.7 FLIU; IQR = 4.9 to 10.7; p = 0.004). Increased levels of platelet-leukocyte conjugates were also seen in the VTE group (61.7%; IQR = 41.4 to 73.2) compared to normal controls (39.6%; IQR = 21 to 58; p = 0.01). As shown in Figure 3, there was a strongly and highly significant correlation between CD11b expression in leukocytes and platelet-leukocyte conjugates (r = 0.74; r² = 0.55; p < 0.0001).

Finally, to rule out the possibility that the differences observed were driven by the postoperative state present in some of our patients, we repeated all the comparisons excluding patients who had undergone surgery in the preceding 30 days (n = 12). All differences were confirmed in these analyses. Probability values for these comparisons were as follows: EMP31 (p = 0.01), EMP62E (p = 0.004), EMP-monoocyte conjugates (p = 0.004), P-selectin expression in platelets (p = 0.0004), platelet-leukocyte conjugates (p = 0.01), and CD11b expression in leukocytes (p = 0.005). No significant differences were found when these markers were compared between patients with VTE who had recent surgery versus those who did not.

DISCUSSION

This study demonstrates that marked activation of the endothelium, platelets, and leukocytes occurs in VTE, and that venous thrombosis, or the accompanying inflammatory process, involves release of circulating EMP and formation of EMP-leukocyte and platelet-leukocyte conjugates.

The presence of elevated EMP in this study reflects increased endothelial activation in VTE, consistent with prior studies (11,12). Under physiological conditions, the

### Table 1. Baseline Characteristics of Patients With VTE

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>VTE (n = 25)</th>
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<tr>
<td>Age</td>
<td>53.9 ± 19</td>
</tr>
<tr>
<td>Male gender</td>
<td>12 (48%)</td>
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<tr>
<td>Prior episodes of VTE</td>
<td>4 (16%)</td>
</tr>
<tr>
<td>PE</td>
<td>11 (44%)</td>
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<tr>
<td>Isolated DVT</td>
<td>14 (56%)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>9 (36%)</td>
</tr>
<tr>
<td>Recent surgery (within 30 days)</td>
<td>12 (48%)</td>
</tr>
<tr>
<td>White cell count (×10³/mm³, ± SD)</td>
<td>9.72 ± 4.17</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6 (24%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>7 (28%)</td>
</tr>
<tr>
<td>Platelet count (×10³/mm³, ± SD)</td>
<td>297 ± 144</td>
</tr>
<tr>
<td>Hematocrit (% ± SD)</td>
<td>31.7 ± 7.2</td>
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<tr>
<td>Antiplatelet drug use</td>
<td>5 (20%)</td>
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DVT = deep vein thrombosis; PE = pulmonary embolism; VTE = venous thromboembolism.
vascular endothelium plays a potent antithrombotic role, but upon endothelial activation is shifted toward a prothrombotic state (13); EC activation is associated with EMP release (2). Additionally, activated EC express tissue factor (TF), the main trigger of thrombin generation. Endothelial microparticles express many receptors of the parent EC and can support thrombin generation by way of the TF/factor VIIa pathway (14). Coagulation is supported by the presence of phosphatidylserine (15), which is translocated from the inner to the outer leaflet of the cell membrane during EMP formation (6). Hence, EMP provides a source of TF as well as a catalytic surface for assembly of the prothrombinase complex (16). Taken collectively, these considerations suggest that EMP are not only a marker of endothelial activation in VTE, but play an active role in the thrombotic process. They may also link thrombosis to inflammation insofar as EMP, like PMP, have been shown to function as vectors for many inflammatory mediators (17). Further research is needed to define whether the circulating concentrations of EMP in VTE are sufficient to facilitate thrombus formation and mediate inflammation.

The increased levels of platelet activation marker P-selectin (CD62P) (Fig. 2) are consistent with previous reports (18–20). Previous studies measured soluble P-selectin, as opposed to P-selectin expression on platelets as we did. However, the majority of so-called soluble P-selectin is in reality largely bound to microparticles (2). Although venous thrombosis has been traditionally associated with red blood cells and fibrin-rich “red clot,” platelets are key in the pathophysiology of VTE (21). Our study demonstrates increased formation of platelet-leukocyte conjugates in VTE. Furthermore, we found a strong correlation between CD11b expression in leukocytes and platelet-leukocyte conjugates in these patients (Fig. 3). This finding is consistent with in vitro studies showing that platelet adhesion to leukocytes upregulates Mac-1 (CD11b/Cd18), which itself supports interactions with platelets (7,22), and support the concept that the formation of platelet-leukocyte conjugates regulate leukocyte activation and participate in linking thrombosis with inflammation in vivo.

Activation of human platelets by diverse stimuli results in shedding of microparticles (4,23). In vitro studies have suggested that microparticles shed by aggregating platelets may, in turn, cause platelet activation and EC activation through the transcellular delivery of arachidonic acid (23) or other mediators (17), and that PMP provide a catalytic phospholipid surface for assembly of the prothrombinase complex (24,25) as discussed above. In addition, we have shown that PMP bind and activate neutrophils in vitro (7). The finding of normal circulating levels of PMP in patients with VTE in our study is intriguing. This finding is not necessarily inconsistent with the above cited studies, because platelet activation in VTE may result in PMP formation followed by their rapid binding to leukocytes.

Finally, our study demonstrates markedly increased leukocyte activation (Fig. 2B) and binding of EMP to monocytes in patients with VTE (Fig. 1C). Platelets, leukocytes, and ECs co-localize and interact in the milieu of a forming thrombus (26). The release of EMP by EC has the potential to amplify coagulation events through the induction of TF (6). The activation of leukocytes and their adhesive interaction with EC are key events in vein wall inflammation and thrombus organization.
Conclusions. Our study has demonstrated that activation of the endothelium, platelets, and leukocytes occurs in patients with VTE, and that venous thrombosis (or the accompanying inflammatory process) involves release of EMP and formation of EMP-leukocyte and platelet-leukocyte conjugates. To the best of our knowledge, this is the first study to demonstrate elevated circulating EMP, platelet-leukocyte conjugates, and EMP-leukocyte conjugates in VTE. Microparticles released from EC and their interactions with leukocytes are likely to play a central role on clot formation, but further research is needed to clarify this role for the different species studied here (e.g., EMP$_{33}$, EMP$_{62E}$) of EMP and their interactions with leukocytes in VTE and whether the formation of EMP represents a therapeutic target to prevent and limit thrombus formation.

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REFERENCES