Factor XI as target for antithrombotic therapy
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Chapter 7

Circulating nucleosomes and neutrophil activation as risk factors for deep vein thrombosis

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Abstract

Objective: The formation of neutrophil extracellular traps (NETs) and the exposure of nucleosomes on these NETs contribute to coagulation activation and the propagation of deep vein thrombosis (DVT) in animal models. However, no data are available on the role of NETs or nucleosomes in patients with thrombosis.

Methods and results: We conducted a case-control study, in which levels of circulating nucleosomes and neutrophil elastase–α1-antitrypsin (EA) complexes were assessed in plasma from 150 patients with objectified symptomatic DVT (cases) and compared with 195 patients with a clinical suspicion of DVT, but in whom DVT was excluded (controls). We explored the association between both nucleosomes and EA complexes, and the presence of DVT by calculating the odds ratio (OR) with corresponding 95% confidence intervals (CI). Elevated levels of both circulating nucleosomes and EA complexes were associated with a 3-fold risk of DVT, and the associations remained similar after adjustment for potential confounders (malignancy, smoking, recent immobilization, recent hospitalization). The risk increased with higher nucleosome and EA complex levels, suggesting a dose-dependent relationship between circulating nucleosomes, activated neutrophils and DVT.

Conclusions: Our study suggests an association between circulating nucleosomes, activated neutrophils and presence of DVT in humans, which might have implications for treatment and prevention.
Introduction

Venous thromboembolism (VTE), the collective term used for deep vein thrombosis (DVT) and pulmonary embolism (PE), is the most common vascular disease after myocardial infarction and ischemic stroke. The incidence of symptomatic and objectively confirmed VTE is 2 to 3 per 1000 people per year, and increases with age. Historically, VTE is caused by three main aspects, i.e. changes in the blood flow, changes of the vessel wall and changes in the composition of the blood (Virchow’s triad). Recently, it has been reported that activation of neutrophils, consequently leading to the formation of neutrophil extracellular traps (NETs), and the exposure of nucleosomes on these NETs, contribute to coagulation activation in vivo and development of DVT in animal models. In an elegant mouse model, NET formation by thrombus-resident neutrophils was shown to be indispensable for thrombus propagation through factor (F)XII activation. Neutrophil depletion as well as disintegration of NETs inhibited thrombus propagation in this model. Hence, based on these animal studies, NET formation and nucleosome exposure on NETs form a crucial step in the pathogenesis of DVT, especially in thrombus propagation.

Nucleosomes exposed on NETs are crucial in the prothrombotic potential of NETs. Nucleosomes consist of a core octamer of two copies each of the histones H2A, H2B, H3 and H4, around which a stretch of helical DNA of 146 base pairs is wrapped. Beside the exposure on NETs, nucleosomes can be actively released from dead cells as a result of the activity of endonucleases and factor-VII activating protease (FSAP). Circulating nucleosomes detected in sepsis and meningococcal sepsis have been reported to correlate with severity of inflammation and mortality, as well as with markers for coagulation and neutrophil activation. Recently, circulating nucleosomes have been reported to be a suitable marker for NET formation in plasma in baboons and humans. However, no studies are available yet on the correlation between nucleosomes and NETs in patients with clinical established forms of thrombosis, such as DVT.

Therefore, the aim of this study was to assess levels of circulating nucleosomes and systemic neutrophil activation, as evidenced by presence of human neutrophil elastase–α1-antitrypsin (EA) complexes, in plasma of patients with established symptomatic DVT and in controls without DVT.
Methods

Identification of patients and controls
Plasma samples were obtained from consecutive patients of at least 18 years old who were referred for suspicion of acute symptomatic DVT of the leg to the Academic Medical Center in Amsterdam, The Netherlands, between September 1999 and May 2006. DVT was diagnosed when a proximal leg vein was not compressible on ultrasound or by the presence of an intraluminal-filling defect on venography. Proximal DVT was defined as a thrombus in the popliteal vein, superficial femoral vein, or common femoral vein. If compression ultrasound showed no venous thrombosis and the D-dimer plasma level was ≥0.5 mg/L, compression ultrasound was repeated after 7 days. DVT was ruled out in case of a Wells score ≤1 in combination with a low D-dimer plasma level (<0.5 mg/L). DVT was also ruled out in case of a normal venography or negative compression ultrasound in combination with a low D-dimer plasma level (<0.5 mg/L), and after a repeated negative ultrasound.

For this analysis, we randomly selected 150 plasma samples of patients with DVT (cases) and 195 plasma samples of patients with a clinical suspicion of DVT but in whom DVT was objectively excluded (controls). Controls did not have a history of previous VTE and were individually matched to the cases for sex, age (±5-year intervals) at the time of blood sample collection, and time of DVT evaluation. Patients with DVT were selected irrespective of presence of thrombophilia or provoking co-morbidities. Blood samples were taken after having obtained written informed consent. The presence of clinical risk factors for VTE and other patient characteristics were assessed using a structured questionnaire in cases and controls, prior to establishing or ruling out the diagnosis of DVT. Collection of plasma was approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam, The Netherlands.

Blood collection
Venous blood was drawn in citrate-containing vials (3.2%), and plasma was prepared by centrifuging twice at room temperature for 15 minutes at 1500xg. Plasma was aliquoted and stored at -80°C until assayed.

Nucleosome Enzyme-Linked Immunosorbent Assay (ELISA)
Nucleosome levels were assessed with an ELISA as recently described. Briefly, monoclonal antibody CLB-ANA/60 (Sanquin, Amsterdam, The Netherlands), which recognizes histone 3 was used as a catching antibody. Biotinylated F(ab)2 fragments of monoclonal antibody CLB-ANA/58 (Sanquin, Amsterdam, The Netherlands), which recognizes an epitope exposed on complexes of histone 2A, histone 2B and dsDNA,
Circulating nucleosomes and neutrophil activation as risk factors for deep vein thrombosis

in combination with poly-horseradish peroxidase-labeled streptavidin (Sanquin, Amsterdam, The Netherlands) was used for detection. As a standard we used culture supernatant of Jurkat cells (1*10⁶ cells/mL), cultured for an additional week to obtain 100% apoptotic cells. One unit is the amount of nucleosomes released by approximately 100 Jurkat cells. The lower detection limit of the assay was 2.5 U/ml. The reference range for circulating nucleosomes in our laboratory is <10.3 U/ml. The inter- and intra-assay coefficient of variation is 8.5% and 4.3%, respectively.

Neutrophil activation
Elastase-α₁-antitrypsin (EA) complexes were measured by an ELISA that has been adapted from a previously described radioimmunoassay. Briefly, plates were coated with a polyclonal rabbit anti-human neutrophil elastase antibody (1.5 μg/mL; Sanquin, Amsterdam, The Netherlands). Standard and samples were diluted in high-performance ELISA buffer (HPE) (Sanquin, Amsterdam, The Netherlands) + 40 μg/mL bovine IgG. Bound complexes were detected by incubation with biotinylated monoclonal anti-α₁-antitrypsin antibody (1 μg/mL) in combination with poly-horseradish peroxidase-labeled streptavidin. Results were expressed in ng/mL by reference to a standard curve of normal human citrated plasma in which EA complexes were generated by incubating with porcine elastase (final concentration 2 μg/mL, Sigma Zwijndrecht, The Netherlands) for 15 minutes at room temperature. The lower detection limit of the assay was 2 ng/mL. The reference range for EA complexes in our laboratory is 8.5 – 55.7 ng/ml. The inter- and intra-assay coefficient of variation is 9.5% and 5.7%, respectively.

Statistical analysis
Descriptive statistics were used for data of all included patients. Levels of circulating nucleosomes and EA complexes were described by medians and interquartile ranges (IQR). Mann-Whitney U-tests were used to assess differences in levels of nucleosomes and EA complexes between cases and controls. The Spearman rank test was used to determine the correlation between circulating nucleosomes and EA complexes.

The associations between both circulating nucleosomes and EA complexes, and presence of DVT were explored by means of logistic regression analysis and were expressed as odds ratios (OR) with corresponding 95% confidence intervals (CI). For these analyses, patients were divided into two groups based on the 80th percentile for controls for circulating nucleosome and EA complex levels, separately, with ≤80th percentile being the reference category. The analyses were repeated with cut-points based on quintiles of levels of nucleosomes and EA complexes of controls, separately. For both analyses, the lowest quintile (≤20th percentile) was used as the reference. The potential confounding influence of four clinical established risk factors for DVT (i.e. malignancy, smoking, recent
immobilization (defined as having been bedridden >3 days and/or having had surgery within 4 weeks prior to confirming or refuting the diagnosis of DVT), and hospitalization <6 months prior to confirming or refuting the diagnosis of DVT) on the associations between nucleosomes and EA complexes, and the presence of DVT, were evaluated using multivariable logistic regression models. Statistical analyses were performed with SPSS (version 16.0) software (SPSS, Chicago, Illinois, USA). Statistical significance in all analyses was set at \( P<0.05 \).

Results

Patient characteristics
We included 150 patients with objectively diagnosed DVT (cases) and 195 controls in this study. In one case and twelve controls, circulating nucleosomes and activated neutrophils were not determined due to insufficient plasma availability, leaving 149 cases and 183 controls for analysis. Table 1 shows the patient characteristics of cases and controls. Patients with DVT were more often exposed to established clinical risk factors for DVT than their matched controls. Furthermore, among females, oral contraceptives were more frequently used by cases compared to controls (Table 1).

<table>
<thead>
<tr>
<th>Cases (DVT) n=149</th>
<th>Controls (no DVT) n=183</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age upon DVT diagnosis, years (SD)</td>
<td>58 (16)</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>69 (46)</td>
</tr>
<tr>
<td>Body Mass Index, median kg/m² (IQR)</td>
<td>26 (24-29)</td>
</tr>
<tr>
<td>Malignancy at time of DVT diagnosis (on treatment / recently treated), n (%)</td>
<td>24 (16)</td>
</tr>
<tr>
<td>Smoking at time of DVT diagnosis, n (%)</td>
<td>39 (26)</td>
</tr>
<tr>
<td>Paralysis, paresis or recent plaster immobilization lower extremities, n (%)</td>
<td>15 (10)</td>
</tr>
<tr>
<td>Recent immobilization (bedridden &gt;3 days and/or major surgery &lt;4 weeks), n (%)</td>
<td>26 (17)*</td>
</tr>
<tr>
<td>History of recent trauma (&lt;60 days), n (%)</td>
<td>19 (13)</td>
</tr>
<tr>
<td>Hospitalization &lt;6 months, n (%)</td>
<td>35 (24)*</td>
</tr>
<tr>
<td>Oral contraceptive use at time of DVT diagnosis, n females (% of females)</td>
<td>23 (29)*</td>
</tr>
</tbody>
</table>

* indicates that there was a significant difference between cases and controls for the particular parameter (\( P<0.05 \))
Circulating nucleosomes and activated neutrophils

Patients with DVT had significantly higher levels of circulating nucleosomes and activated neutrophils as measured by EA complexes, compared to controls (Figures 1A and 1B). Median levels of nucleosomes in cases were 17 U/mL (IQR 9-35) versus 9 U/mL (IQR 5-17) in controls. Median levels of EA complexes were 53 ng/mL (IQR 43-71) in cases versus 45 ng/mL (IQR 33-55) in controls. Levels of circulating nucleosomes and EA complexes in cases and controls showed a significant correlation with each other (Spearman correlation coefficient, 0.436; P<0.001, and 0.359; P<0.001, respectively).

Patients with levels of nucleosomes >80th percentile of controls had a statistically significant increased risk of DVT, as compared to patients with levels ≤80th percentile (OR 3.0; 95% CI 1.5 to 3.9). In addition, patients with levels of EA complexes >80th percentile of controls, had an increased risk of DVT, as compared to patients with levels ≤80th percentile (OR 2.4; 95% CI 1.5 to 3.9). After adjustment for potential confounders (i.e. malignancy, smoking, immobilization due to bed rest >3 days and/or surgery within the last 4 weeks prior to confirming or refuting the diagnosis of DVT, and hospitalization <6 months prior to confirming or refuting the diagnosis of DVT), ORs did not change significantly in both models (adjusted OR 3.0; 95% CI 1.7 to 5.0, and adjusted OR 2.3; 95% CI 1.4 to 3.9, respectively). As malignancy is associated with an increased risk of DVT as well as with nucleosomes expression,18 we analyzed cases and controls, either with or without malignancy, separately for nucleosome levels (Figure 1C). These results confirm that high nucleosome levels are an independent risk factor for DVT irrespective of presence of malignancy.

Subsequently, we divided the patients into quintiles according to levels of circulating nucleosomes and EA complexes of controls, separately. Results of the analysis of the association between these quintiles and the presence of DVT are shown in Table 2. For both circulating nucleosomes and EA complexes, the OR clearly increased with a higher cut-off level, indicating a dose-dependent relationship between these factors and the risk of DVT. Adjustment for potential confounders (i.e. malignancy, smoking, recent immobilization, and recent hospitalization prior to confirming or refuting the diagnosis of DVT) did not alter the findings substantially.
Circulating nucleosomes and activated neutrophils

Patients with DVT had significantly higher levels of circulating nucleosomes and activated neutrophils as measured by EA complexes, compared to controls (Figures 1A and 1B). Median levels of nucleosomes in cases were 17 U/mL (IQR 9-35) versus 9 U/mL (IQR 5-17) in controls. Median levels of EA complexes were 53 ng/mL (IQR 43-71) in cases versus 45 ng/mL (IQR 33-55) in controls. Levels of circulating nucleosomes and EA complexes in cases and controls showed a significant correlation with each other (Spearman correlation coefficient, 0.436; P<0.001, and 0.359; P<0.001, respectively).

Figure 1: levels of nucleosomes and human neutrophil elastase-α1-antitrypsin (EA) complexes, in cases and controls

Median levels of circulating nucleosomes (A) neutrophil elastase-α1-antitrypsin (EA) complexes (B) in cases and controls. Median levels of circulating nucleosomes stratified into groups with and without malignancy, as well as into cases and controls *P=0.049; **P=0.007; ***P=0.003; ****P<0.001 (C). Bars represent interquartile ranges (IQR).
Table 2. Association between levels of circulating nucleosomes and activated neutrophils (as measured by presence of elastase-α1-antitrypsin (EA) complexes) and presence of DVT with different cut-off levels in comparison with lowest quintile†

<table>
<thead>
<tr>
<th></th>
<th>Cases (n=149)</th>
<th>Controls (n=183)</th>
<th>Adjusted odds ratio* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Circulating nucleosomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quintile 1; n (upper cut-off 4.0 U/ml)</td>
<td>13</td>
<td>41</td>
<td>1 (ref)</td>
</tr>
<tr>
<td>Quintile 2; n (upper cut-off 7.0 U/ml)</td>
<td>20</td>
<td>36</td>
<td>1.9 (0.8-4.6)</td>
</tr>
<tr>
<td>Quintile 3; n (upper cut-off 11.0 U/ml)</td>
<td>24</td>
<td>41</td>
<td>2.3 (1.0-5.3)</td>
</tr>
<tr>
<td>Quintile 4; n (upper cut-off 20.0 U/ml)</td>
<td>26</td>
<td>23</td>
<td>3.7 (1.6-8.7)</td>
</tr>
<tr>
<td>Quintile 5; n</td>
<td>66</td>
<td>42</td>
<td>6.4 (2.8-14.6)</td>
</tr>
<tr>
<td><strong>Activated neutrophils</strong> (EA-complexes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quintile 1; n (upper cut-off 32.0 ng/ml)</td>
<td>9</td>
<td>40</td>
<td>1 (ref)</td>
</tr>
<tr>
<td>Quintile 2; n (upper cut-off 40.0 ng/ml)</td>
<td>20</td>
<td>34</td>
<td>3.7 (1.3-10.2)</td>
</tr>
<tr>
<td>Quintile 3; n (upper cut-off 49.0 ng/ml)</td>
<td>33</td>
<td>38</td>
<td>5.7 (2.2-15.1)</td>
</tr>
<tr>
<td>Quintile 4; n (upper cut-off 61.2 ng/ml)</td>
<td>32</td>
<td>35</td>
<td>6.0 (2.3-16.0)</td>
</tr>
<tr>
<td>Quintile 5; n</td>
<td>55</td>
<td>36</td>
<td>9.4 (3.6-24.3)</td>
</tr>
</tbody>
</table>

†≤20th percentile; reference category
*Odds ratios (ORs) are adjusted for four established clinical risk factors for DVT (malignancy, smoking, recent immobilization, recent hospitalization)

Discussion

In this study, we identified an association between both circulating nucleosomes and activated neutrophils, as evidenced by EA complexes, and the presence of DVT. Levels of nucleosomes and neutrophil activation above the 80th percentile of controls were associated with a 3-fold risk of DVT, and the associations remained similar after correction for potential confounders such as presence of malignancy, smoking, recent immobilization and recent hospitalization. The thrombotic risk increased with higher nucleosome and EA complex levels, suggesting a dose-dependent relationship between circulating nucleosomes, activated neutrophils and DVT.

Previous animal studies have shown the critical role of neutrophil activation, NET formation and nucleosome exposure in coagulation activation⁴ and thrombus propagation,⁵ but this is the first study in patients demonstrating a significant association with DVT. We carefully matched DVT cases with controls without DVT on sex, age and date of DVT evaluation. None of the female patients were pregnant. However, controls often had co-morbidities and other health conditions, e.g. malignancy or recent surgery, which contributed to a high clinical suspicion of DVT upon presentation, as is represented
in Table 1. This partially explains the increased levels of nucleosomes and EA complexes that were also found in our controls, contrary to other studies that assessed levels in a control group.5, 17, 19, 20 Although significantly increased levels of circulating nucleosomes and EA complexes were demonstrated in patients with DVT, their origin and role is still unclear. Levels of circulating nucleosomes in DVT patients may reflect the degradation of NETs in the thrombus, but could also derive from other cell sources within or outside the thrombus.5, 13 NET formation was recently reported to induce endothelial cell death.21, 22 Hence, nucleosome release from activated or damaged endothelial cells could also contribute to nucleosome levels measured in the circulation of these patients.

As we showed a significant correlation in DVT patients between levels of circulating nucleosomes and neutrophil activation, as evidenced by EA complexes, our data provides indirect evidence that measured nucleosomes may originate from NET degradation, as both nucleosomes and elastase form part of NETs.3 However, we measured circulating nucleosomes in the plasma and not NET formation locally, at the site of the thrombus. This is in accordance to recent literature where nucleosomes have been measured as marker for NET formation in humans and baboons6, 15.

It is still unknown how nucleosomes and/or neutrophil activation contribute to thrombus propagation.23 Massberg et al. suggested that nucleosomes exposed on NETs form a template for neutrophil proteases to inactivate tissue factor pathway inhibitor (TFPI), thereby propagating coagulation.4 A similar inactivation mechanism could be postulated for antithrombin or protein C,24 while coagulation activation via NET-induced FXII activation represents another pathway.5, 23 The question remains whether circulating nucleosomes and activated neutrophils are causative for thrombus formation, or rather a consequence of its development.

The demonstrated association suggests that circulating nucleosomes and EA complexes might be a suitable plasma marker for NET formation and might be involved in pathological thrombus formation. Therefore, inhibition of circulating nucleosomes could be a potential target for treatment and prevention of DVT, as was suggested in animal models with DNase1.5 In conclusion, increased levels of circulating nucleosomes and neutrophil activation, as evidenced by presence of EA complexes, were associated with a 3-fold risk of DVT. Future studies are necessary to clarify their origin and role in thrombus formation, as well as their potential as new therapeutic targets.
References


