Effects of Cigarette Smoke Exposure on Clot Dynamics and Fibrin Structure
An Ex Vivo Investigation

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Objectives—The purpose of this study was to examine the effect of cigarette smoke exposure (CSE) on clot dynamics and fibrin architecture and to isolate the relative contribution of platelets and fibrinogen to clot dynamics.

Methods and Results—From young healthy males smokers (n = 34) and nonsmokers (n = 34) a baseline blood was drawn, and smokers had another blood draw after smoking 2 regular cigarettes. Using thromboelastography (TEG) the degree of platelet-fibrin interaction was measured. In additional experiments, abciximab (20 μg/mL) was added to the smokers samples (n = 27) to reduce the effects of platelet function from the TEG parameters. The maximum clot strength (G) obtained with abciximab measured mainly the contribution of fibrinogen to clot strength (GF). By subtracting GF from G, the contribution of platelets to clot strength (GP) was presumed. A significant difference was found for all TEG parameters between nonsmokers versus postsmoking and pre- versus postsmoking samples. Postsmoking both GF and GP were significantly higher as compared to presmoking. On electron microscopy and turbidity analysis, postsmoking fibrin clots were significantly different compared to presmoking and nonsmoking samples.

Conclusions—Acute CSE changes clot dynamics and alters fibrin architecture. Both functional changes in fibrinogen and platelets appear to contribute to heightened thrombogenicity after acute CSE. (Arterioscler Thromb Vasc Biol. 2009; 29:00-00.)

Key Words: smoking • thrombosis • platelet • fibrinogen

Cigarette smoke exposure (CSE) increases the risk for acute myocardial infarction and sudden cardiac death, and thrombosis is responsible for most of these acute events. It has been suggested that cigarette smoke exposure induces an imbalance between various hemostatic molecules in the circulating blood or at the vessel-wall interface producing a hypercoagulable state. However, the exact mechanism(s) for initiation and propagation of thrombosis in terms of platelet-fibrin interaction in cigarette smokers remains to be defined. Furthermore, although there has been extensive research regarding platelet function and CSE, to our knowledge no study has examined the functional contribution of fibrin to clot dynamics after CSE and how these parameters relate to fibrin architecture.

In this study, using thromboelastography (TEG), GP IIb/IIIa inhibition, and electron microscopy, the effect(s) of CSE on the dynamics of clot formation and fibrin architecture were examined in vitro. Furthermore, an attempt was made to differentiate the relative functional contribution of platelets and fibrinogen on clot dynamics in this setting.

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Clot samples of 14 nonsmokers and 17 smokers were evaluated and images digitally recorded at 10K×20K magnification with a Hitachi S-3500N scanning electron microscope (Hitachi High Technologies America Inc) at an accelerating voltage of 10kV. To assess fiber thickness, a series of random coordinates (20 x/yr coordinates) were computer generated by Image J version 1.40g software (National Institute of Health) and applied to the SEM image of each clot sample. An independent observer measured the fiber diameter at or nearest to each of the 20 coordinates and the average was taken. Only those fibers with clearly defined margins were measured. Similarly, 6 sets of randomly generated coordinates were used to assess the average number of fibers per 1-μm² SEM image area on each of the clot samples. All measurements were performed with Image J software.

**Determination of Levels of Cotinine, Procoagulant, and Inflammatory Markers**
Plasma cotinine levels, hemostatic variables including t-PA (Imubind, American Diagnostica Inc), PAI-1 (Imubind, American Diagnostica Inc), Factor XIII antigen levels (Assaypro), and C reactive protein (Imuclose CRP [hs] ELISA Kit, American Diagnostica Inc) levels were determined using commercially available ELISA kits.

**Turbidimetric Clotting Assay**
Turbidimetric clotting assay was performed following a method published previously after minor modification. Twenty-five μL of platelet poor plasma, diluted with 50 μL of 0.05 mol/L Tris-HCl, 0.12 mol/L NaCl, pH 7.5, and 50 μL of activation mix (final concentrations: 0.4 U/mL thrombin [Aniara], and 7.5 mmol/L CaCl₂ in assay buffer) was added to each column of the 96-well plate using a multi-channel pipette at 10 second intervals. Plates were read at 405 nm at 10-minute intervals until maximum absorbency was reached in a Bio-Tek EL×800 microplate reader. Turbidity for samples reached a plateau at 40 minutes of analysis. Maximum change in absorbency (max Δ Abs) expressed as absorbency at 40 minutes minutes minus the baseline. Two replicate measurements were performed for each sample.

**Statistical Analyses**
Results are presented as the mean±SEM. Unpaired and paired Student’s t tests were used to compare the group differences where appropriate P<0.05 was considered statistically significant.

**Results**
Clinical Characteristics of the Study Population
Baseline clinical characteristics and levels of prothrombotic and inflammatory markers from both smokers and nonsmokers are shown in the Table 1. There was no significant difference between the 2 groups with the exception of WBC count and plasma cotinine levels, which were significantly higher in smokers.

Clot Dynamics Data by TEG
As represented in Table 2, all TEG parameters were similar between pre- and nonsmoking samples. However, postsmoking samples differed significantly in all the measured parameters of clot generation by TEG as compared to nonsmoking samples.
Table 1. Baseline Clinical Characteristics and Levels of Prothrombotic and Inflammatory Markers of Nonsmokers and Smokers

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoker (n=34)</th>
<th>Smoker (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>29±1</td>
<td>27±1</td>
</tr>
<tr>
<td>Cigarette/day</td>
<td>0</td>
<td>14±1*</td>
</tr>
<tr>
<td>Pack/ys</td>
<td>0</td>
<td>9.8±1.7*</td>
</tr>
<tr>
<td>Cotinine, ng/dL</td>
<td>4.6±2.1</td>
<td>47±3.1*</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>15.9±0.1</td>
<td>15.7±0.2</td>
</tr>
<tr>
<td>White blood cell, K/uL</td>
<td>6.4±0.2</td>
<td>7.6±0.4*</td>
</tr>
<tr>
<td>Platelet, K/uL</td>
<td>228±6</td>
<td>242±8</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>237±8</td>
<td>259±11</td>
</tr>
<tr>
<td>Total Cholesterol, mg/dL</td>
<td>176</td>
<td>177</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>107</td>
<td>120</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>44</td>
<td>45</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>111</td>
<td>115</td>
</tr>
<tr>
<td>hCRP, ug/mL</td>
<td>0.73±0.16</td>
<td>0.72±0.12</td>
</tr>
<tr>
<td>t-PA, ng/mL</td>
<td>13.2±1.5</td>
<td>10.2±1.5</td>
</tr>
<tr>
<td>PAI-1, ng/mL</td>
<td>85.5±5</td>
<td>72±5</td>
</tr>
<tr>
<td>Factor XII, ug/mL</td>
<td>19±3</td>
<td>21±3</td>
</tr>
</tbody>
</table>

Results are presented as mean±SEM. Unpaired Student t test. *P<0.02 for nonsmoker vs smoker.

(P<0.05) as well as presmoking (P<0.05) samples (Table 2 and Figure 2), leading to a higher clot strength in the postsmoking samples.

Clot strength (GF) obtained after addition of abciximab, a function of the contribution of fibrinogen to GF, was significantly higher in the postsmoking sample (n=27) as compared to the presmoking sample (n=27, P<0.05; Table 2 and Figure 2). The contribution of platelet function on clot strength (GP), estimated by subtracting GF from G, was also significantly higher in the postsmoking sample as compared to the presmoking sample (P<0.05; Table 2 and Figure 2), suggesting both functional changes in platelets and fibrinogen contributed to the increased clot strength in the postsmoking samples.

Architecture of the Fibrin Clots
As represented in Table 3, postsmoking fibrin clots had significantly thinner fibrin fibers and more fibrin fibers per

Table 2. TEG parameters of Nonsmokers and Smokers

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoking (n=34)</th>
<th>Presmoking (n=34)</th>
<th>Postsmoking (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R, min</td>
<td>5.7±0.3</td>
<td>5.5±0.3</td>
<td>4.8±0.3*</td>
</tr>
<tr>
<td>K, min</td>
<td>2.9±0.1</td>
<td>2.8±0.1</td>
<td>2.4±0.1*</td>
</tr>
<tr>
<td>Alpha Angle, degree</td>
<td>54±1</td>
<td>55±1</td>
<td>59±1*</td>
</tr>
<tr>
<td>G, dyne/cm² (n=27)</td>
<td>6.5±0.2</td>
<td>6.8±0.2</td>
<td>7.3±0.3*</td>
</tr>
<tr>
<td>GF, dyne/cm² (n=27)</td>
<td>n/a</td>
<td>0.95±0.07</td>
<td>1.01±0.07*</td>
</tr>
<tr>
<td>GP, dyne/cm² (n=27)</td>
<td>n/a</td>
<td>5.95±0.2</td>
<td>6.30±0.3*</td>
</tr>
</tbody>
</table>

Results are presented as mean±SEM. Unpaired Student t test. *P<0.05 vs nonsmoker and paired Student t test; **P<0.05 vs presmoking.

CSE increases the risk for arterial thrombosis and in the coronary arteries for an acute coronary syndrome (ACS). Thrombosis results largely from platelet aggregation and fibrin formation on the surface of a ruptured or eroded atheromatous plaque and, in the case of ACS, within an epicardial coronary artery. Activated platelets adhere to the exposed vascular subendothelium, release storage granules, and aggregate to form a platelet-rich thrombus. This is further supported by activation of the clotting cascade and the generation of a fibrin red-cell mesh leading to complete coronary occlusion. Activation of platelets is accompanied by the increased expression of GPIIb/IIa receptors which bind fibrinogen or von Willebrand Factor and facilitate further platelet aggregation.

Discussion
Multiple studies have shown that CSE affects platelet function. On the other hand, data on CSE and fibrinogen are limited. A majority of data only report increased circulating plasma fibrinogen levels in association with CSE. Although epidemiological studies have shown that an elevated level of fibrinogen is an independent cardiovascular risk factor for adverse events, the mechanisms of CSE and platelet-fibrin interaction are unknown. Specifically, the relation between CSE and the dynamics of clot formation, and whether there is an accompanying fibrin architecture change have not previously been elucidated. To investigate these questions, an automated assay (TEG) was used which evaluated the viscoelastic properties of a clot by measuring the degree of platelet-fibrin interaction at low shear and calculating initial fibrin formation time, kinetics of clot formation, rapidity of fibrin build-up, as well as maximum clot...
strength. To help identify the specific contribution of fibrin molecules to clot strength, GPIIb/IIIa receptor blockade (abciximab) was used.

The results of the study suggest for the first time that acute CSE is associated with functional changes in both platelets as well as fibrin. This affects the kinetics of clot formation, the rapidity of fibrin build-up, and clot strength. With the addition of abciximab, functional changes in both platelets and fibrin were found contributing to the increased thrombogenicity after acute CSE.

Fibrinogen influences thrombogenesis, affecting the rheology of blood flow, blood viscosity, and platelet aggregation. The structural support of a thrombus is provided by a matrix of cross-linked fibrin. The present study found that postsmoking fibrin clots had thinner fibers and more fibrin fibers per $1 \mu m^2$ field as compared to non-smoking and presmoking samples. We also found that postsmoking samples had significantly higher clot turbidity. In general, decrease in fiber thickness is associated with a decrease in final clot turbidity. However, it was reported that turbidity of clot is not only related to fiber diameter but also was related to the number of fibrin fiber branching points, fibrin fiber density, as well as uniformity of fiber distribution. Indeed, in our study, although there was a decrease in fiber thickness, the fibrin fiber density was significantly higher and their distribution was more uniform, in postsmoking samples as compared to the presmoking and nonsmokers samples.

Table 3. Fibrin Thickness and Average No. of Fibrin Strands per 1 $\mu m^2$ Area, and Clot Turbidimetric Assay of Nonsmokers and Smokers

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoker (n=14)</th>
<th>Presmoking (n=17)</th>
<th>Postsmoking (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrin thickness</td>
<td>92±6 nm</td>
<td>77±5 nm</td>
<td>66±5 nm*</td>
</tr>
<tr>
<td>Average no. of fiber</td>
<td>13.67±1.51</td>
<td>15.38±1.37</td>
<td>18.66±1.37*</td>
</tr>
<tr>
<td>Clot turbidity (max Δ Abs)</td>
<td>0.068±0.002</td>
<td>0.082±0.009</td>
<td>0.086±0.008*</td>
</tr>
</tbody>
</table>

*P<0.05 vs nonsmoker and paired Student t test; *P<0.05 vs presmoking.

A relationship between fibrin clot structure and fibrinolysis has been previously reported in several studies. The majority of data suggests a link between dense fine fibrin networks and hypofibrinolysis. Furthermore, several investigators have reported similar changes in fibrin architecture in relation to diabetes as well as end-stage renal disease and suggested that these altered clot features may be associated with increased cardiovascular mortality.

Additionally, it has been suggested that high sensitive hs-CRP, fibrinogen, and PAI-1 levels may affect the fibrin structure and function as well. In the present study, hs-CRP, fibrinogen, PAI-1, t-PA, and Factor XIII levels were not different among smokers and nonsmokers. Indeed, there were no significant differences in all the measured parameters except for WBC count, which was higher in smokers compared to nonsmokers.

All differences in the TEG parameters were found only after acute exposure of 2 cigarettes. The mechanism(s) for these changes in clot dynamics remain unknown, but it can be speculated that increased oxidative stress may have contributed at least in part to these findings. Multiple investigators including our group have shown that acute CSE increases cellular oxidative stress. Furthermore, in a recent article 8-epi-prostaglandin F2α, a marker of oxidative stress, was reported to be increased in patients

Figure 3. Electron microscopic image of clots from a nonsmoker (a) and smoker (b and c, pre- and postsmoking samples from the same subject) at 20K magnification.
with ACS, and in vitro fibrin clots from these individuals had dense fibrin networks.\textsuperscript{7}

It is unclear as to why in our study population there were no significant differences in multiple measured parameters between nonsmoking and presmoking samples. Perhaps this was related to the young age of our population of healthy smokers (mean age=27 years) and a relatively low pack years of CSE.

Our study had several limitations: (1) The study population was young males, with low pack years of cigarette exposure, who had no increases in baseline markers of inflammation and procoagulant factors. Therefore, our results are only applicable to this specific group. It is possible that with greater pack years of cigarette exposure, the baseline inflammatory and procoagulant markers would have been elevated, leading to significant differences between nonsmoking and presmoking samples. (2) In the present study, abciximab was used in a method as reported by Kettner et al.,\textsuperscript{21} to identify the contribution of fibrin to overall clot dynamics. Platelets treated with abciximab may still support thrombin generation. Therefore, in this model, the influences of platelet mediated thrombin generation may not have been totally excluded. Thus, our TEG data with abciximab have to be interpreted within in the context of this possible limitation. Nevertheless, our EM as well as turbidity data support that there was a significant change in fibrin architecture after acute smoke exposure which likely contributed to overall clot strength as well. (3) The experiments were performed in vitro; therefore, this may not accurately represent the in vivo situation.

In conclusion, acute CSE is associated with shortening of the time for fibrin formation and augmented clot strength. These changes are associated with an alteration in fibrin architecture. Both functional changes in fibrin molecules and heightened platelet activity appear to contribute to the higher clot strength observed after acute CSE. These findings suggest a new mechanism regarding the dynamics of clot formation and heightened thrombogenicity after acute CSE.

Disclosures

None.

References

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