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

Rajat S. Barua, Fridolin Sy, Sundararajan Srikanth, Grace Huang, Usman Javed, Cyrus Buhari, Dennis Margosan, John A. Ambrose

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.

Subjects and Study Design
All subjects were requested to abstain from smoking, food, and drink except for water for the 6 to 8 hours (overnight) before arrival at the study center. On arrival in the morning, subjects rested for 20 minutes in a quiet temperature-controlled study room. The medical and family history, blood pressure, and heart rate were recorded.

Nonsmokers had single blood draw and smokers had 2 blood draws. The first blood draw was on arrival after overnight abstinence from smoking. The second blood draw was done immediately after smoking 2 cigarettes (Marlboro Reds).

Whole blood was used for thromboelastography. Platelet poor plasma was used to determine cotinine levels and other hemostatic variables. For platelet poor plasma, whole blood was collected in a 3.8% trisodium citrate tube and centrifuged at 4600 g for 20 minutes at 4°C, then stored at −70°C until use. For the biochemical analysis such as complete blood count (CBC), coagulation profile, lipid profile, chemistry, the blood was sent to the hospital clinical laboratory.

Thromboelastography
Five milliliters of whole blood were collected in 3.8% trisodium citrate tube and analyzed using TEG (Hemoscope Corporation) following the manufacturer’s instruction as described below.
Clot formation is initiated and sustained by the interaction of platelets and fibrin. The strength of a clot is graphically represented over time as a characteristic cigar shape figure. There are 4 parameters of the TEG clot lysis. 

1. **R:** Period of time from initiation of the test to initial fibrin formation. 
2. **k:** Measure of time from beginning of clot formation until the amplitude of thromboelastogram reaches 20 mm and represents the dynamics of clot formation; alpha angle, the acceleration (kinetics) of fibrin build up and cross-linking; G, strength of a clot, which is dependent on function of platelets and its interaction with fibrin.

TEG allows measurement of clot-shear elasticity in response to the formation of thrombin and platelet activation. It is a viscoelastic test that characterizes the formation and strength of the blood clot over time. It determines the time to clot initiation and formation, its acceleration phase, strengthening, retraction, and finally the onset of clot lysis.

The strength of a clot is graphically represented over time as a characteristic cigar shape figure. There are 4 parameters of the TEG tracing: R, k, alpha angle, and G, which measures the different stages of clot development (Figure 1).

One ml of blood was transferred to a vial containing kaolin and mixed by inversion; 360 μL of the activated blood was immediately added to a cup supplied by the manufacturer and assayed in the TEG analyzer according to the manufacturer’s instructions to obtain the thrombin-induced clot.

To eliminate platelet function from the thromboelastogram, the above experiment was repeated for each individual sample with the addition of abciximab (20 μg/mL; GPIIb/IIIa receptor blocker) to the sample. The G for each sample obtained this way became a parameter of clot generation by TEG as compared to nonsmoking smokers. However, postsmoking smokers showed significantly higher in smokers.

### 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 XII 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. 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 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.

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**Figure 1. TEG parameters.** R indicates period of time from initiation of the test to initial fibrin formation; k, measure of time from beginning of clot formation until the amplitude of thromboelastogram reaches 20 mm and represents the dynamics of clot formation; alpha angle, the acceleration (kinetics) of fibrin build up and cross-linking; G, strength of a clot, which is dependent on function of platelets and its interaction with fibrin.
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</td>
<td>72±5</td>
</tr>
<tr>
<td>Factor XIII, ug/mL</td>
<td>19±3</td>
<td>21±3</td>
</tr>
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</table>

Results are presented as mean±SEM. Unpaired Student t test. *P<0.05 vs 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 1-μm² field compared to presmoking (P<0.05) and nonsmoking (P<0.05) samples. Postsmoking samples had more uniform fibrin fiber distribution as compared to presmoking and nonsmoking samples. Figure 3 shows the scanning electron microscopic images of fibrin architecture from a nonsmoker and pre- and postsmoking samples from the same smoking subject at 20K magnification.

Turbidimetric Clotting Assay

As seen in Table 3, postsmoking fibrin clots had significantly greater change in clot turbidity on spectrophotometry compared to presmoking and (P<0.05) nonsmoking (P<0.05) samples.

Discussion

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 GP IIb / IIIa receptors which bind fibrinogen or von Willebrand Factor and facilitate further platelet aggregation.10,11

Multiple studies have shown that CSE affects platelet function.12–14 Platelets isolated from smokers exhibit increased aggregation as compared to nonsmokers.13 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.15 Although epidemiological studies have shown that an elevated level of fibrinogen is an independent cardiovascular risk factor for adverse events,15 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 formation.
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.16–18 The present study found that postsmoking fibrin clots had thinner fibers and more fibrin fibers per 1-μm² 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.19,20 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.

A relationship between fibrin clot structure and fibrinolysis has been previously reported in several studies.7,15–17 The majority of data suggests a link between dense fine fibrin networks and hypofibrinolysis.15 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.15,16 Additionally, it has been suggested that high sensitive hs-CRP, fibrinogen, and PAI-1 levels may affect the fibrin structure and function as well.7,15,16 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.1,2,13,14 Furthermore, in a recent article 8-epi-prostaglandin F2α, a marker of oxidative stress, was reported to be increased in patients

<table>
<thead>
<tr>
<th>Table 3. Fibrin Thickness and Average No. of Fibrin Strands per 1 μm² Area, and Clot Turbidimetric Assay of Nonsmokers and Smokers</th>
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<tbody>
<tr>
<td>Nonsmoker (n=14)</td>
</tr>
<tr>
<td>Fibrin thickness</td>
</tr>
<tr>
<td>Average no. of fiber strands per field</td>
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<tr>
<td>Clot turbidity (max Δ Abs)</td>
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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.

Figure 3. Electron microscopic image of clots from a non-smoker (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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