Role of Risk Factors in the Modulation of Tissue Factor Activity and Blood Thrombogenicity

Antonia Sambola, MD; Julio Osende, MD; James Hathcock, PhD; Michael Degen, BSc; Yale Nemerson, MD; Valentin Fuster, MD, PhD; Jill Crandall, MD; Juan Jose Badimon, PhD

Background—Several studies suggest a role for an increased circulating pool of tissue factor (TF) in atherothrombotic diseases. Furthermore, certain cardiovascular risk factors, such as diabetes, hyperlipemia, and smoking, are associated with a higher incidence of thrombotic complications. We hypothesized that the observed increased blood thrombogenicity (BT) observed in patients with type 2 diabetes mellitus may be mediated via an increased circulating tissue factor activity. We have extended our study to smokers and hyperlipidemic subjects.

Methods and Results—Poorly controlled patients with type 2 diabetes mellitus (n=36), smokers (n=10), and untreated hyperlipidemic subjects (n=10) were studied. Circulating TF was immunocaptured from plasma, relipidated, and quantified by factor Xa (FXa) generation in the presence of factor VIIa. BT was assessed as thrombus formation on the Badimon perfusion chamber. Patients with improvement in glycemic control showed a reduction in circulating TF (362±135 versus 243±74 pmol/L per min FXa, P=0.0001). A similar effect was observed in BT (15 445±1130 versus 12 072±596 μm/mm², P=0.01). Two hours after smoking 2 cigarettes, TF was increased (217±72 versus 283±106 pmol/L per min FXa, P=0.003). Hyperlipidemic subjects showed higher TF (237±63 versus 195±44 pmol/L per min FXa, P=0.035) than healthy volunteers.

Conclusions—These findings suggest that high levels of circulating TF may be the mechanism of action responsible for the increased thrombotic complications associated with the presence of these cardiovascular risk factors. These observations strongly emphasize the usefulness of the management of the patients based on their global risk assessment.

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Key Words: cardiovascular diseases ■ risk factors ■ thrombosis ■ coagulation

Tissue factor (TF) is considered to be a major regulator of normal hemostasis and thrombosis.1,2 TF is highly expressed in atherosclerotic plaques, and its content has been related to plaque thrombogenicity.3-9 Recent evidence suggests the existence of a blood-borne pool of TF that may play a critical role in the propagation of thrombosis.10,11 Moreover, it has been reported that polymorphonuclear leukocytes might be involved in the transport of circulating TF to platelets by a CD15-dependent mechanism.12 High plasma levels of TF antigen have been reported in patients with acute coronary syndromes (ACS) versus patients with stable angina or non-coronary artery disease.13 Furthermore, circulating TF-positive microparticles with procoagulant activity have been described in patients with ACS.14,15 It is well established that atherosclerotic lesion disruption and subsequent acute thrombus formation play a key role in ~70% of patients dying from an ACS.16-18 Risk factors such as diabetes mellitus type 2 (DMT2), hypercholesterolemia, and smoking have been known to exacerbate the progression of atherosclerotic disease and trigger atherothrombotic complications.17,18 The presence of these risk factors seems to play a critical role in patients who die from cardiovascular causes while having only plaque erosion, and this suggests that an increased blood thrombogenicity (BT) combined with endothelial erosion is capable of triggering an ACS event in the absence of more severe plaque disruption.19,20 These observations seem to highlight not only the pathological role of the lipid-rich vulnerable plaque but also the role of increased BT (high-risk “vulnerable” blood) in the pathogenesis of ACS.

Our data indicate that the described hyperthrombotic or procoagulant state responsible for the increased rate of atherothrombotic complications among diabetic, hyperlipidemic, and smoker populations could be mediated via increased levels of circulating TF.

Methods

Study Population

The present study involved 3 different populations: T2DM patients (n=36) from a previously published study,21 subjects (n=10) who...
TABLE 1. Clinical Characteristics at Baseline in the Diabetes Study

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/male</td>
<td>7/8</td>
<td>15/21</td>
</tr>
<tr>
<td>Age, y</td>
<td>54±14</td>
<td>57±8</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>NA</td>
<td>11±9</td>
</tr>
<tr>
<td>Smoking</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>88±13</td>
<td>164±50</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>189±27</td>
<td>194±35</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>123±26</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
<td>44±11</td>
<td>46±11</td>
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<tr>
<td>Statins</td>
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<td>8</td>
</tr>
<tr>
<td>Aspirin</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Blood thrombogenicity</td>
<td>11 354±570</td>
<td>14 389±762*</td>
</tr>
<tr>
<td>Tissue factor activity</td>
<td>216±35</td>
<td>371±161*</td>
</tr>
</tbody>
</table>

*P<0.01; **P<0.001.

smoked ≥10 cigarettes per day with a smoking history of ≥10 years, and untreated hyperlipidemic subjects (n=10) with total cholesterol ≥200 mg/dL and LDL cholesterol ≥100 mg/dL. Furthermore, a group of healthy volunteers (n=15) without the risk factors was also included to serve as a control group.

Study Design

Characteristic Baseline of Study Population

Poorly controlled patients with DMT2 and hemoglobin A1c (HbA1c >7.5%) were selected for the study. While maintaining their present hypoglycemic therapies, they were randomized in a double-blind design into a conservative (diet modification plus placebo) or intensive (diet modification plus troglitazone) hypoglycemic regimen for 3 months, as previously described.21 Troglitazone was given at a dose of 600 mg/d to the intensive group. BT and circulating TF activity were measured at baseline and 3 months after treatment. The repeated measurements allowed every patient to serve as his or her own control. Clinically significant improvement in glycemic control was defined as a reduction in HbA1c ≥0.5 percentage points. TF was determined after overnight fasting both at baseline and 3 months after treatment. The baseline characteristics of both groups are presented in Table 1; no significant differences between these groups were found in diabetes duration, concomitant medication, or presence of cardiovascular risk factors.

TABLE 2. Demographic Characteristics of the Smokers and Hyperlipidemic Groups and Respective Controls

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Smoker Group</th>
<th>Hyperlipidemic Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/male</td>
<td>5/5</td>
<td>6/4</td>
<td>3/7</td>
</tr>
<tr>
<td>Age, y</td>
<td>32±10</td>
<td>31±10</td>
<td>49±11</td>
</tr>
<tr>
<td>Smoking</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>176±18</td>
<td>180±8</td>
<td>179±20</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>110±8</td>
<td>105±10</td>
<td>100±11</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48±16</td>
<td>52±11</td>
<td>54±20</td>
</tr>
<tr>
<td>Tissue factor activity</td>
<td>168±40</td>
<td>224±69*</td>
<td>191±40</td>
</tr>
</tbody>
</table>

*P<0.05.

The baseline characteristics of the hyperlipidemic and smoker groups and their respective control groups are presented in Table 2. The group of untreated hyperlipidemic subjects was comprised of 7 males and 3 females. A normolipemic group of healthy volunteers (9 males and 6 females) served as a reference control group. The group of smokers (age 31±10 years) presented an average of 17±9 cigarettes per day and smoking time of 13±4 hours. Plasma levels of circulating TF were assessed after 12 hours without smoking and 2 hours after smoking 2 cigarettes.

Assessment of Blood Thrombogenicity

The effect of glycemic control on BT was assessed at baseline and after 3 months of treatment as changes in the thrombus area using the Badimon perfusion chamber. The perfusion chamber and BT studies have been previously described.8,21–23

Statistical Analysis

Data are expressed as mean±SEM. Differences between the treatment groups were assessed using nonpaired Student’s t test or Mann-Whitney U test when distribution was not normal. Follow-up Pearson correlations between Δ scores were conducted. Finally, two-tailed significance level was set at <0.05. Statistical analyses were performed with SPS 10.01.

Results

Circulating TF Activity

Levels of circulating TF activity were determined using a modification of a previously described technique.10 The modified technique requires only 480 μL of plasma. Adjusted titration of phospholipid concentration for optimal TF activity was required because of the low plasma volume being used. The titration curve from 2.5 to 250 μmol/L of total phospholipids (30:70 phosphatidylycholine:phosphatidylserine) is presented in Figure 1. The concentration of 75 μmol/L of phospholipid gave optimal activity for typical plasma samples. A batch of pooled plasma obtained from 21 healthy volunteers and a separate batch of 6 μmol/L recombinant TF (Genentech) served as standardized controls of TF activity for all experiments. The interassay and intra-assay coefficients of variation were 10% and <5%, respectively.

Figure 1. To determine the concentration of phospholipid that yielded the optimum TF activity, various concentrations of phosphatidylycholine:phosphatidylserine (30:70%) were added to the elution buffer and the resulting TF activity was measured.
Citrated plasma samples (480 μL) were mixed with 20 μL of Triton X-100 and centrifuged for 10 minutes (2000g at reverse transcriptase). The solubilized TF protein was captured by a polyclonal antibody immobilized to Affigel (Bio-Rad; 0.25 mg polyclonal antibody TF per mL of Affigel). The rabbit polyclonal antibody anti-sTF was raised against the solubilized TF mutant (residues 1 to 218), encompassing most of the extracellular domain of wild-type human TF.24 TF was eluted from the gel by adding guanidine-HCl (5 mol/L), BSA (0.2%), n-octyl-β-D-glucopyranoside (100 mmol/L), and phospholipid mixture (75 μmol/L), all final concentrations. The eluate was dialyzed for at least 4 hours against HEPES-buffered saline (10 mmol/L HEPES, pH 7.4; 140 mmol/L NaCl). TF was assessed by adding factor X (150 nmol/L), factor VIIa (1 nmol/L), and calcium (5 mmol/L) and then periodically removing samples of the reacting mixture and storing them in EDTA. The concentration of factor Xa (FXa) in each removed solution was determined by adding a chromogenic substrate (Spectrozyme-Xa; American Diagnostics) and comparing the rate of change in absorbance (optical density 405 nm) to a standard curve obtained with known concentrations of FXa. Hence, circulating data are reported as the increase in the concentration of FXa per unit time (pmol/L per min). In control experiments, the omission of factor VIIa from the reaction mixture completely abolished FXa generation, and in separate experiments, preincubation of the dialyzed samples with an inhibitory anti-TF antibody resulted in a 91% inhibition in TF. In addition, standard curves performed with twice the normal volume of plasma and with serial dilutions of plasma showed a dose-dependent response in the measured levels of TF activity (r=0.94).

TF Activity and Blood Thrombogenicity in DM:
Effect of Glycemic Improvement

DM was associated with significantly higher baseline levels of TF (371±161 versus 216±35 pmol/L per min FXa, P<0.0001) and BT (14 389±762 versus 11 354±7098 μm2/mm2, P<0.001) than the control group. To additionally investigate the effect of glycemic control, the patients with DM were separated into 2 groups based on their improvement in glycemric control (Figure 2). Improved glycemic control was defined as ≥0.5% reduction in HbA1c. The improvement in glycemic control was associated with a significant reduction in levels of circulating TF activity (362±161 versus 286±163 pmol/L per min FXa, P=0.0001). On the other hand, patients without glycemic improvement showed no change in TF activity (387±191 versus 354±221). Interestingly, these effects were independent of the treatment.

We have previously reported a similar pattern of BT in patients depending on whether they did or did not have improved glycemic levels.21 A significant reduction in thrombus formation was observed in the group with improved glycemic control (15 140±4963 versus 11 904±2927 μm2/mm2, P=0.002), whereas patients without improvement did not have any change in BT (14 236±2310 versus 15 362±3718 μm2/mm2; NS). Again, those changes were dependent on the glycemic improvement rather than the treatment received. Furthermore, glycemic control was a predictive variable for TF in T2DM as a group. Reduction in HbA1c after 3 months of treatment was positively correlated with reduction of TF (r=0.40, P=0.016). A positive correlation was also found between changes in BT and TF after 3 months of treatment (r=0.46, P=0.007).

Circulating TF Activity in Smoker Patients

Active smoking, despite 24 hours of being smoke-free, was associated with significantly higher levels of circulating TF activity than not smoking (224±69 versus 168±40 pmol/L per min FXa; P<0.05). Baseline levels of circulating TF in current smokers were significantly increased 2 hours after smoking 2 cigarettes (217±72 pmol/L versus 283±106 pmol/L per min FXa, P=0.003). There were no sex-related differences at baseline or after 2 hours (209±36 versus 221±78 pmol/L per min FXa at baseline and 255±90 versus 315±110 pmol/L per min FXa 2 hours after smoking). TF baseline levels showed a strong correlation with TF levels detected 2 hours after smoking (r=0.88). Despite the reduced
number of subjects involved in the group, a strong correlation ($r=0.76, P=0.010$) was observed between the average number of smoked cigarettes per subject and baseline (12 hours without smoking) levels of circulating TF activity.

**Circulating TF Activity in Hyperlipidemic Patients**

Circulating TF activity in hyperlipidemic subjects was significantly higher than in healthy donors ($237\pm63$ versus $194\pm40$ pmol/L per min FXa, $P=0.035$). Interestingly, despite the reduced number of subjects involved in this group, a significant correlation was observed between plasma levels of LDL cholesterol and circulating TF ($r=0.44, P<0.05$). In addition, an interesting trend, even though not statistically significant, was seen between TF and HDL cholesterol ($r^2=-0.32$).

**Discussion**

This study correlates increased levels of circulating TF activity associated with DM, hyperlipidemia, and smoking. These cardiovascular risk factors have been epidemiologically linked to a high incidence of atherothrombotic complications. Similar to the well-established concept of vulnerable high-risk atherosclerotic lesion, these observations bring to our attention the concept of a high-risk or “vulnerable” blood (procoagulant state?) associated with certain cardiovascular risk factors. High levels of circulating TF activity could be responsible for the observed increase in BT associated with these pathologic conditions. Furthermore, our observations provide evidence that the adequate management of hyperglycemia in T2DM significantly reduces the increased BT in these patients.

Similar correlations between circulating TF activity and hyperlipidemia and smoking were obtained. These observations highlight the impact of certain cardiovascular risk factors on platelet and blood reactivity. The concept of high-risk blood is supported by pathological evidence of coronary thrombosis without plaque disruption. Patients showing only endothelial erosion were associated with DM, cigarette smoking, or an atherogenic lipid profile (high total cholesterol or low HDL cholesterol). Again, the concept of hyperreactive blood linked to high levels of circulating TF would help explain the incidence of acute thrombotic events in the absence of plaque disruption.

An increased content of thrombotic material in atherosclerotic lesions obtained from diabetic patients by using atherectomy has been reported. The description of increased TF and BT among T2DM could help explain the increased incidence of thrombosis among these patients. In vitro studies have demonstrated the induction of TF production by advanced glycation end products in human cells. Therefore, this stimulatory effect of glycated proteins on TF synthesis could be one of the potential mechanisms of action responsible for an enhanced platelet reactivity and thrombosis in T2DM.

The relationship between glycemic control and hypercoagulability in T2DM is a controversial issue. We and others have suggested that glycemic control may reduce the risk of thrombotic complications. We are now suggesting that a reduction in plasma levels of circulating TF activity could be the mechanism responsible for the reported normalization of the increased BT associated with glycemic control.

Smoking is considered a major risk factor for increased atherothrombotic complications. We have found a strong association between the number of cigarettes smoked per day and circulating TF activity, indicative of a possible dose-response relationship. In agreement with our results, it has recently been reported that mice exposed to cigarette smoke had increased TF expression in aort atherosclerotic lesions. By combining these observations with our own observations correlating the TF content of human atherosclerotic lesions and its thrombogenicity when exposed to flowing blood, we speculate that smoking may enhance circulating TF activity and BT.

Others authors have documented enhanced TF expression mediated by monocytes in the presence of platelets in patients with hypercholesterolemia, but they did not determine TF in the plasma. Hyperlipidemia is associated with the generation of oxidized LDL, macrophage, and monocyte activation and cell apoptosis. Such phenomena may generate TF released in the circulation as procoagulant particles. We have reported an increased BT in hyperlipidemic patients and normalization of the increased thrombogenicity after the normalization of plasma cholesterol levels by statin administration. More recently, it has been reported that lipid lowering by statin therapy has reduced TF expression and macrophage recruitment in atherosclerotic rabbits and reduced cholesterol-induced thrombogenicity.

In conclusion, our data indicate the following: (1) Increased BT in poorly controlled T2DM seems to be related to plasma levels of a circulating pool of activatable TF activity; (2) improvements in glycemic control are associated with a reduction in plasma levels of circulating TF activity and BT; and (3) risk factors such as hyperlipidemia and smoking have a significant modulatory effect on plasma levels of circulating TF and BT. Therefore, the importance of an effective and global management of all of the existing risk factors on the prevention and treatment of cardiovascular diseases is clearly emphasized by these observations, showing a strong correlation between some cardiovascular risk factors and both increased TF activity and BT. Furthermore, the described circulating pool of TF activity could be the rationale for the development of specific inhibitors of the TF pathway for the prevention and treatment of thrombotic complications.

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**References**

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