

## Atherothrombosis: Role of tissue factor Link between diabetes, obesity and inflammation

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*Received 7 July 2006*

Atherothrombotic vascular disease is a complex disorder in which inflammation and coagulation play a pivotal role. Rupture of high-risk, vulnerable plaques with the subsequent tissue factor (TF) exposure is responsible for coronary thrombosis, the main cause of unstable angina, acute myocardial infarction, and sudden cardiac death. Tissue factor (TF), the key initiator of coagulation is an important modulator of inflammation. TF is widely expressed in atherosclerotic plaques and found in macrophages, smooth muscle cells, extracellular matrix and acellular lipid-rich core. TF expression can be induced by various stimulants such as C-reactive protein, oxLDL, hyperglycemia and adipocytokines. The blood-borne TF encrypted on the circulating microparticles derived from vascular cells is a marker of vascular injury and a source of procoagulant activity. Another form of TF, called alternatively spliced has been recently identified in human and murine. It is soluble, circulates in plasma and initiates coagulation and thrombus propagation. Evidence indicates that elevated levels of blood-borne or circulating TF has been associated with metabolic syndrome, type 2 diabetes and cardiovascular risk factors and is a candidate biomarker for future cardiovascular events. Therapeutic strategies have been developed to specifically interfere with TF activity in the treatment of cardiovascular disease.

**Keywords:** Atherothrombosis, Inflammation, Tissue factor

Acute atherothrombotic complications are the consequence of atherosclerotic lesion disruption with superimposed thrombus formation. Occurrences of metabolic and vascular abnormalities associated with metabolic syndrome (MetS), obesity and diabetes are the major risk factors for coronary heart disease<sup>1</sup>. The incidence of obesity has become epidemic and is associated with increased cardiovascular mortality and morbidity, mainly through insulin resistance<sup>2</sup>. Coagulation and fibrinolytic pathways are altered in the presence of insulin resistance and type 2 diabetes<sup>3</sup>. Tissue factor (TF), the most potent trigger of the coagulation cascade is increased in patients with acute coronary syndromes<sup>4</sup> and diabetes mellitus<sup>5</sup>. Improvement in glycemic control (reduction in HbA1c >0.5%) showed significant reduction in blood thrombogenicity<sup>6</sup>.

Inflammation, vasoconstriction and rheological factors are involved in the process of plaque destabilization and rupture<sup>7,8</sup>. The presence of inflammatory mediators correlates with an increased

risk of acute coronary events and a predictor of the existence of vulnerable plaques<sup>9</sup>. In addition activation of innate immunity offers a potential unifying pathophysiology for insulin resistance and dyslipidemia in MetS<sup>10,11</sup>. LDL, modified by oxidation, glycation and aggregation is a major cause of injury to the endothelium. It contributes to atherothrombosis by inducing endothelial cell apoptosis, thus plaque erosion, by impairing the anticoagulant balance in endothelium<sup>12,13</sup>. Recently Hutter *et al.*<sup>14</sup> from our group have reported the colocalization of TF and caspase-3 in monocytes and apoptosis of macrophages by oxLDL. Apoptotic macrophages lead to shedding of membrane microparticles with potent procoagulant activity, mediated by TF<sup>15</sup>. Prospective studies show strong correlation between proinflammatory biomarkers, such as C-reactive protein (CRP) interleukin 6 (IL-6) and TNF- $\alpha$  and perturbations in glucose homeostasis and obesity. CRP contributes to acute atherothrombotic events by stimulating plasminogen activator-1 (PAI-1) in endothelial cells and TF in mononuclear cells and smooth muscle cells<sup>16</sup>.

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Adipocytokines (adiponectin, CRP, TNF- $\alpha$ , IL-6 and PAI-1, leptin and resistin) secreted by adipocytes constitute a critical link between obesity, inflammation and cardiovascular diseases<sup>17</sup>.

Tissue factor is a 47-kDa transmembrane cell-surface glycoprotein and the primary initiator of the coagulation cascade. Full-length TF protein consists of a 219-amino-acid (AA) extracellular region, a 23-residue transmembrane domain, and a 21-residue intracellular region. TF, the receptor for factor VII (FVII) initiates the activation of both FX and FIX. Activation of FXa along with FVa as cofactor leads to cleavage of prothrombin to thrombin, finally resulting in the generation of fibrin, platelet activation, and thrombus formation<sup>18,19</sup>. TF is expressed in macrophages, intimal smooth muscle cells (SMC) and endothelial cells of the atherosclerotic plaque, and is particularly abundant within the lipid core<sup>20</sup>. In addition, elevated levels of blood-borne TF with procoagulant activity have been found in the peripheral circulating blood of patients with acute coronary syndromes and metabolic syndrome<sup>21</sup>. Further, both cellular and extracellular TF could play an important role in atherothrombosis (Fig. 1). Platelets from diabetic patients exhibit increased activation, aggregation, and degranulation with higher

thromboxane synthesis<sup>22</sup>. Activated platelets have been shown to enhance the procoagulant activity of monocytes, and this may be linked to the generation of monocyte-derived MPs with decrypted TF activity<sup>19,22</sup>. The purpose of this review is to evaluate the molecular mechanism involved in the vascular cell-mediated TF expression leading to prothrombotic state and acute coronary events.

### Effect of hyperglycemia, inflammation and obesity on vascular cells

#### Implication in tissue factor expression and activity

Tissue factor is normally absent from cells that come in contact with blood. TF expression by vascular cells induces intravascular thrombosis. In vessel wall, tissue factor is almost exclusively confined to the adventitia. In physiological condition the vascular system forms a protective anticoagulant envelope to inactivate the coagulation cascade. Pathological expression of TF occurs in activated monocytes, macrophage-derived foam cells, smooth muscle cells in atherosclerotic lesion and endothelial cells. Some of these pathological conditions, including hyperglycemia and obesity, induce inflammatory and apoptotic processes at the cellular level of vascular tissue. This potentially accelerate the

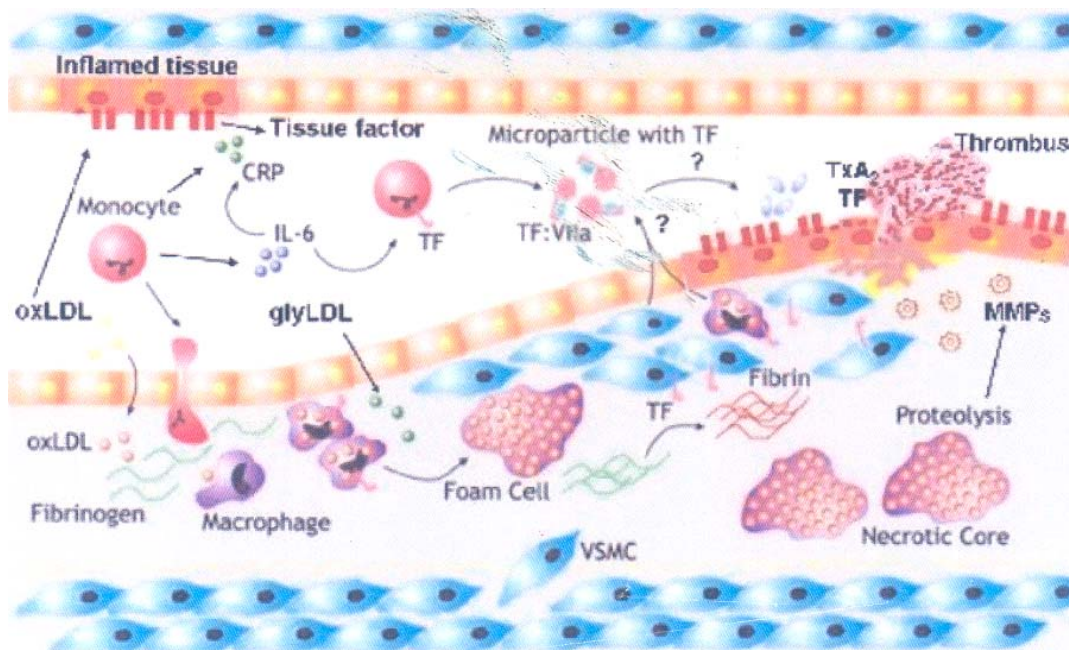


Fig. 1—Molecular and pathophysiological process involved in the production of circulating or blood-borne tissue factor, lesion initiation and thrombus formation.

atherosclerotic process by release of TF from apoptotic macrophages and smooth muscle cells that results in the occurrence of TF in the necrotic core of the plaque leading to atherothrombosis. TF is the key initiator in the *in vivo* generation of thrombin that is critical in atherothrombotic disease condition at least in two main ways, as an ultimate coagulation and platelet-activating enzyme and as an important cell-signaling effector molecule in inflammatory and angiogenic pathways<sup>23</sup>.

TF in cells exist in three different pools, surface TF, encrypted TF and intracellular TF. Evidence indicates the release of soluble TF isoform and full length TF from endothelial cells in culture in response to inflammatory cytokines<sup>24</sup>. Similarly, the accumulation of active TF in the medium of cultured human SMCs has been reported<sup>25</sup>. In addition increase in TF expression in THP-1 monocytes and human peripheral blood derived monocytes exposed to CRP and Ox-LDL have been observed. These results indicate that vascular cells may be the major source for the circulating as well as the vessel wall TF.

#### Endothelial cells

Endothelial cells play a wide variety of critical roles in the control of vascular function. It participates in all aspects of the vascular homeostasis including physiological or pathological processes like thrombosis, inflammation, or vascular wall remodelling. Endothelial cells secrete a variety of molecules important for the regulation of blood coagulation and platelet functions. Vessel damage or exposure to certain cytokines or proinflammatory stimuli or other kind of mediators impair the endothelial function and shifts the balance towards a procoagulant/prothrombotic phenotype. These do not normally express the primary trigger of the coagulation system, tissue factor. It is known that several cytokines (TNF- $\alpha$ , IL-1 $\beta$ , CD40 ligand)<sup>26,27</sup>, biogenic amines (serotonin, histamine)<sup>28,29</sup> and mediators such as thrombin, oxidized LDL, vascular endothelial growth factor<sup>30,31</sup>, C-reactive protein<sup>32</sup>, and nicotine<sup>33</sup> induce TF expression in endothelial cells. Most of these mediators use the same intracellular signaling to regulate TF expression (MAP kinases p38, p44/42, *c-jun* amino terminal kinase). The final step is the activation of transcription factors such as AP-1, nuclear factor (NF)- $\kappa$ B and ERG-1<sup>27,34</sup> that, by the stimulation of TF

promoter upregulate the TF mRNA. The expression of TF in endothelial cells is not always correlated with its activity because of the tissue factor pathway inhibitor (TFPI) secretion, the distribution of TF into cellular compartments<sup>29,30,35</sup> and the production of inactive form (encrypted TF) regulate TF activity. Intracellular TF represent a pool released on cell damage, while TF located at the cell surface is biologically active.

Hyperglycemia and hyperinsulinemia induce oxidative stress and inflammatory state that result in endothelial dysfunction. Endothelial cells exposed to high glucose *in vitro* increase the production of extracellular matrix components, such as collagen and fibronectin, and of procoagulant proteins, such as vWF and tissue factor, and show decreased proliferation, migration and fibrinolytic potential, and increased apoptosis<sup>36,37</sup>. Further, high glucose influences endothelial cell function indirectly by the synthesis of advanced glycosylation end products (AGEs), growth factor and vasoactive agents<sup>38</sup>. It has been shown that the components of the metabolic syndrome can affect endothelial function<sup>17</sup>, probably by the release of adipocytokines that contributes to create a subinflammatory status, which could explain the endothelial dysfunction and the disturbances in the haemostatic and fibrinolytic systems. However, further studies are needed to elucidate how inflammation, hyperglycemia and obesity interact together to induce endothelial dysfunction.

#### Vascular smooth muscle cells

In vessel wall, tissue factor is almost exclusively confined to the adventitia. This confinement led to the concept that normally distributed TF represents a haemostatic envelope ready to activate coagulation whenever vascular integrity is disrupted. It has been shown that in physiological condition vascular smooth muscle cells express TF *in vitro* and *in vivo* at low levels<sup>29</sup>. Several mediators such as TNF- $\alpha$ , CD40 ligand, histamine, thrombin, LPS, PDGF-BB, aggregated LDL, Ox LDL, nicotine, CRP<sup>27,29,39,40</sup> and activated platelets<sup>41</sup> induce TF in SMCs. The mechanisms of this induction involve MAP kinases, PI3 kinase-pathway and ERK as in ECs, but the expression level appears to be lower than in ECs and in monocytes. Recent data indicate that the interaction between TF and its specific ligand, factor VII activated (FVIIa), may trigger different intracellular signals, culminating in a variety of cell responses, thus

suggesting a role for TF as a "true" cell membrane receptor. In addition, it has been shown that binding of FVIIa to TF stimulates SMC proliferation via activation of the p44/42 MAP kinase (ERK 1/2) and JNK pathways<sup>42</sup>. Hyperglycemia may also affect the smooth muscle cells function. Actually it has been shown that high glucose levels may accelerate the smooth muscle accumulation in lesions of atherosclerosis and impair the tone of vasculature<sup>43</sup>. The effects of mediators secreted by adipose tissue on smooth muscle cells function are not completely clear, and some data obtained in human are controversial. However, further studies are necessary to investigate these effects and the role of hyperglycemia.

#### **Platelets**

Although it has been suggested that activated platelets possess active TF<sup>44</sup>, the notion of TF as an integral platelet component is contested by more recent data. Actually the source of platelet TF is not absolutely clear. Despite the absence of a nucleus, various gene transcripts have been found in platelets, and there is a debate as to whether or not platelets are capable of protein synthesis by utilizing mRNA derived from megakaryocytes<sup>45</sup>. Rather, platelets may be very important in decrypting monocyte TF activity in a process entailing transfer of TF to activated platelets<sup>46</sup>. It has been reported that leukocytes co-incubated with platelets generate more procoagulant activity than either cell type alone. These data suggest that leukocytes were involved in the transfer of TF particles to platelets and that this transfer was mediated by CD15<sup>47</sup>. There is emerging evidence of encrypted TF in normal blood associated with platelets and monocytes<sup>22</sup>.

It has been known that platelets hyperreactivity is associated with hyperglycemia and hypoadiponectinemia<sup>48</sup>. The osmotic effect of hyperglycemia can directly increase platelet reactivity<sup>49</sup>. Another mechanism potentially contributing to increased platelet reactivity in hyperglycemia is vascular dysfunction. Decreased vascular endothelial production of prostacyclin and nitric oxide in patients with insulin resistance promotes increased activation of platelets<sup>50</sup>. Platelets from patients with diabetes have a greater propensity to adhere after vascular injury because of greater platelet surface expression of glycoprotein IIb-IIIa and increased concentration and activity of vWF in blood<sup>51</sup>.

#### **Monocytes and macrophages**

In monocytes very little to no basal level of TF are found. However, it has been shown that different mediators such as CRP, CD40 ligand, OxLDL, angiotensin II<sup>52-54</sup>, can induce TF in monocytes. Recent data indicated that CRP is unable to induce directly TF expression in monocytes and MDMs. The presence of CRP-induced TF expression in PBMCs suggests that CRP can induce TF, probably through cross-talk between cells<sup>55</sup>. Monocyte-derived macrophages that are enriched with lipids or intracellular free cholesterol (foam cells) produce greater amounts of TF compared to non-lipid-enriched macrophages<sup>56</sup>. Lymphocytes may interact with monocytes and macrophages to induce TF expression by specific cytokines like interleukin (IL)-2, tumor necrosis factor- $\beta$  and interferon- $\gamma$  and by T cell binding to the CD40 receptor present on the membranes of monocytes and macrophages<sup>57</sup>. Increased monocyte TF expression is seen in patients with an acute coronary syndrome and less pronounced, in patients with chronic stable angina<sup>58</sup>. It has been shown that prolonged exposure to hyperglycemia induce nonenzymatic glycosylation of proteins and lipids. Glycosylated proteins interact with a specific receptor present on monocyte-derived macrophages. This interaction results in the induction of oxidative stress and pro-inflammatory responses<sup>59</sup>. Further efforts with experimental and clinical studies are needed to better understand the role of adipokines on monocytes and macrophage.

#### **Adipocytes**

Obesity is associated with increased cardiovascular mortality and morbidity. Most recent evidences suggest that the adipocytes may be implicated in atherogenesis and atherothrombosis<sup>60</sup>. Adipocytes are an important source of various adipokines with favourable and unfavourable cardiovascular effects<sup>17,61</sup>. They have very important functions in cholesterol homeostasis and are an important determinant of a low level chronic inflammatory state as reflected by levels of IL-6, TNF- $\alpha$  and CRP<sup>62</sup>. Animal models showed that adipose tissue express considerably higher levels of TF mRNA that probably could contribute to a hypercoagulable state<sup>63</sup>.

#### **Blood borne tissue factor**

The paradigm that arterial thrombosis occurs after vascular damage when vessel-wall TF is exposed has been challenged by the occurrence of blood-borne or circulating TF (cTF) encrypted on the microparticles

(MPs)<sup>64</sup>. TF encryption is the post-translational suppression of TF procoagulant activity (PCA) on the cell surface. MPs are submicron vesicles that are released from cells undergoing activation or apoptosis<sup>22,65,66</sup>. A thrombus forming potential has been demonstrated for nonfunctional or encrypted TF present in MPs in blood from healthy subjects<sup>67</sup>. TF encryption may be the primary mechanism preventing procoagulant activity in the circulating blood.

An elevated level of TF associated MPs have been demonstrated in numerous disease conditions such as type 2 diabetes and acute coronary syndromes<sup>4,68</sup>. In patients with type 2 diabetes, the soluble TF concentration above 300 pg mL<sup>-1</sup> has been considered as 15-fold higher risk for the presence of microvascular disease when compared with those with concentrations below 100 pg mL<sup>-1</sup><sup>24,69</sup>. Many cell types can synthesize TF antigen including vascular smooth muscle cells, endothelial, monocytes/macrophages and also found in shed apoptotic microparticles in extracts of atherosclerotic plaques<sup>4,25</sup>. The source may be different in different disease conditions. Furthermore, TF-rich microvesicles from monocytes were found to fuse with the activated platelets, and transfer contents to the platelets<sup>70</sup>. Hence, MPs derived from activated platelets are thrombogenic and express adhesion receptors including P-selectin (P-sel). P-sel, translocated from granules to the cell surfaces of activated platelets and endothelial cells, was recently found to play multiple roles in hemostasis. Signaling by P-sel through its receptor on leukocytes, P-selectin glycoprotein ligand 1 (PSGL-1) induces the generation of TF-positive, highly procoagulant MPs<sup>71</sup>. These studies indicate that the blood borne TF originate from encrypted TF, which upon interaction with activated platelets becomes decrypted.

These MPs are procoagulant because of exposure of negatively charged phosphatidylserine (PS) on their surface<sup>71</sup>. In the plasma membrane of quiescent cells phosphatidylserine (PS) is sequestered on the inner leaflet of the bilayer<sup>72</sup>. When cells are activated (platelet activation), or enter apoptosis by different stimulus, lipid asymmetry can be perturbed and this exposes phosphatidylserine (PS) at the cells' outer surface and serves as a potent procoagulant surface<sup>73</sup>. The coincidence of TF decryption and PS exposure does not prove these events are coupled<sup>70</sup>. However, it has been known that PS accelerated coagulation reactions on membrane surfaces<sup>73</sup>. Furthermore, PS

also appears on the surfaces of cells undergoing apoptosis<sup>74</sup> and TF PCA is decrypted when cells become apoptotic. Hutter *et al.*<sup>14</sup> reported the colocalization of TF and caspase-3 in macrophages present in atherosclerotic plaque<sup>14</sup> and we found increased TF expression and induction of apoptosis in monocytes/macrophages exposed to oxLDL in culture. These studies suggest that the regulation of PS distribution in cell membranes may be critical in controlling coagulation and in determining the survival of pathologic cells in the circulation.

#### **Alternatively spliced TF**

Alternatively spliced variant of TF (as TF) has recently been identified in humans and mice<sup>75,76</sup>. This soluble form of TF circulates in blood and exhibits procoagulant activity. It contains most of the extracellular domain of TF but lacks a transmembrane domain and terminates with a unique 3'peptide sequence. Up to 30% of the TF antigen found in circulating blood was derived from alternative splicing of the primary RNA transcript<sup>75</sup>. These forms are not bound to MPs and represent a distinct form of circulating TF. The presence of alternatively spliced TF (asTF) mRNA was detected in human tissues - kidney, brain, heart, islet cells, placenta and in the lungs<sup>69</sup>. Pro-inflammatory cytokines induce procoagulant activity by releasing asTF into cell supernatant<sup>24</sup>. In addition, a recent report confirms the presence of TF variant in arterial thrombi<sup>77</sup>, establishing a potential role in coagulation-related (patho) physiology. Further studies are needed to find out the relative contribution of soluble TF, microparticle-bound and vessel wall-associated TF in the initiation and propagation of thrombus formation.

#### **Therapeutic implications and future directions**

During the past years in addition to cellular TF, an important role of circulating TF is emerging. However, the relative contribution of vessel wall-associated versus blood-borne TF to thrombus formation and/or propagation is debated. Future studies should aim at elucidating the physiological and pathophysiological role of circulating TF and its implication as therapeutic target. Given the primary role of TF in intravascular thrombosis, several therapeutic strategies have been developed to specifically interfere with its action. Novel antithrombotics are emerging to target specific steps in coagulation cascade and in pathways of platelet adhesion, activation and aggregation. Several

inhibitors have been tested out in various settings, such as animal models of arterial injury and in *in vitro* models of thrombus formation and propagation. Because of the key position of TF as an initiator of the coagulation, inflammation, cell growth and proliferation, specific interventions targeted to interfere with the activity of the complex TF:FVIIa have been identified and developed. The potential advantages in inhibiting the TF:FVIIa complex is that this inhibits the initial step of the coagulation pathway, resulting in the inhibition of new thrombin formation. In addition these inhibitors may prevent the occurrence of other TF-mediated effects, including inflammation and cell proliferation, and finally, they may act only where they are needed, that is, at the site of vascular injury, thus leaving intact the physiological hemostasis.

Some of the most relevant or promising of these inhibitors include: *recombinant tissue factor pathway inhibitor (TFPI)*, the only physiological inhibitor of the TF:FVIIa complex. Another approach is the inhibition of propagation of coagulation by drugs that block factors IXa or Xa or by agents that inactivate their respective cofactors, factor VIIIa or factor Va. In addition, direct thrombin inhibitors interfere in thrombin mediated inflammatory and angiogenic pathways and offer several advantages over heparins/heparinoid drugs. Further, modulation of platelet function has been a strategy for the control of cardiovascular disease. Platelet adhesion to a damaged blood vessel is the initial trigger for arterial hemostasis and thrombosis. Several drugs have been tested and used in clinical practice but other trials are needed in order to ascertain the safety and efficacy of the most promising antithrombotics in various clinical contexts.

### Acknowledgement

The authors are indebted to Dr Valentin Fuster, Director of the Cardiovascular Institute, Mount Sinai Medical Center, for his invaluable guidance in this work.

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