



## Cytokines and hemostasis

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### ABSTRACT

**Background and Objectives.** Cytokines are low molecular weight polypeptides that act as pleiotropic mediators of inflammation and may contribute significantly to regulation of hemostatic balance in both physiologic and pathologic conditions. The purpose of this review is to underline the most significant progresses recently achieved in this rapidly growing area.

**Design and Methods.** The authors have been involved both at home and abroad in experimental and clinical research in this field for years and have contributed original papers in peer-reviewed journals. In addition, the material examined in the present review includes articles published in journals covered by the Science Citation Index and Medline.

**Results.** Tissue factor, a transmembrane glycoprotein that serves as a surface receptor for coagulation factor VIIa, plays a key role in the initiation of coagulation processes. Very little, if any, tissue factor activity is detectable in normal conditions on the cell surface of monocytes and endothelial cells. However, upon proper stimulation by a number of agents such activity may be expressed in these cells, which can then contribute significantly to clotting activation. Pro-inflammatory cytokines. Interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF) are effective inducers of tissue factor upregulation and may trigger endothelial cells to change their antithrombotic properties into a procoagulant, clot-promoting state. Indeed, much experimental and clinical evidence has been accumulated to suggest that cytokines play a key role in the pathophysiology of hemostatic abnormalities in different disease states. These include, *inter alia*, the coagulopathy observed during septicemia, the veno-occlusive disease of the liver after bone marrow transplantation, the prothrombotic state associated with atherosclerotic vessels, the occurrence of deep venous thrombosis after major abdominal surgery and the thrombotic tendency of patients with cancer. Several new antithrombotic strategies based on these new concepts have been attempted in experimental models of thrombosis and also in man. Examples of new possible antithrombotic agents are the tissue factor pathway inhibitor, Fab fragments of monoclonal antibodies directed against factor VII or factor VIIa,

mutant forms of biologically inactive tissue factor and inhibition of cytokines involved in the regulation of tissue factor expression. Many of these studies have produced positive or interesting results, although more must be learned before the appropriate drug and the adequate dose are defined in the different clinical situations.

**Conclusions.** Pro-inflammatory cytokines (IL-1, IL-6 and TNF) play a key role in tissue factor expression on monocytes and on endothelial cells and contribute significantly to regulation of hemostatic balance in physiologic and pathologic conditions. This effect is of great interest from both speculative and practical viewpoints.

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Key words: cytokines, hemostasis, tissue factor, endothelial cells, monocytes

Cytokines are low molecular weight polypeptides that act as pleiotropic mediators of inflammation and immunity. Leukocytes and vascular cells are both sources of cytokines and targets for them. It is known that hemostatic balance depends on complex interactions among endothelial cells, blood cells and the coagulation-fibrinolytic system and cytokines play an important role in such interactions. Indeed, in the last decade much experimental and clinical evidence has been accumulated to suggest that cytokines may contribute significantly to regulation of hemostatic balance in both physiologic and pathologic conditions.

The purpose of this review is to underline the most significant progresses which have been achieved in this rapidly growing area. After a short description of the mechanisms responsible for regulation of tissue factor expression, emphasis will be given to what has been learnt recently about the role of cytokines in the pathophysiology of hemostatic abnormalities in different clinical situations. Finally, new possible antithrombotic strategies based on these new concepts will be described and discussed.

### Regulation of tissue factor expression

#### **Tissue factor: structure and function**

Tissue factor is a 46 kD transmembrane glycoprotein that serves as a cell surface receptor and essential cofactor for coagulation factor VII and its active form VIIa.<sup>1-4</sup> On binding of factor VIIa to tissue factor, the complex acquires catalytic activity and converts factors IX and X to their active derivatives IXa

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and Xa, respectively, thus leading to thrombin generation and fibrin formation.<sup>5</sup> Most interestingly, tissue factor does not change conformation upon binding to VIIa.<sup>6,7</sup> This suggests that tissue factor forms a rigid scaffold for immobilizing the otherwise flexible VIIa<sup>8</sup> and positions the active center at the correct distance from the cell membrane. Therefore, association of VIIa with cell surface tissue factor results in catalytic enhancement of the VIIa catalytic domain. Further enhancement of the reaction rate is achieved by preferential recognition of phospholipid bound factors IX and X by the tissue factor-VIIa complex.<sup>1</sup>

Thus, little reason exists today to doubt that the binding of factor VII to tissue factor and the subsequent reactions so triggered play a *prima ballerina* role in the initiation of coagulation process in both physiologic and pathologic conditions.<sup>9</sup>

Although the initiation of coagulation is considered to be the main physiologic function of tissue factor, recent evidence suggests that tissue factor might participate in other biological activities, including the induction of intracellular Ca<sup>2+</sup> signals,<sup>10-12</sup> the regulation of the metastatic behavior of melanoma cells<sup>13</sup> and angiogenesis.<sup>5</sup> The results of these studies imply that tissue factor is a true receptor with signaling abilities, which is an attractive concept in the light of the structural similarity of tissue factor to members of the cytokine receptor superfamily.<sup>3</sup> Interestingly, targeted disruption of the murine tissue factor gene is associated with impaired vascular development and lethal embryonic bleeding.<sup>14</sup>

Anatomical localization of tissue factor is of importance to understand its crucial role in hemostasis and thrombosis.<sup>15</sup> Tissue factor is constitutively present on the cell membrane of fibroblasts, of pericytes in and around blood vessels, of glomerular epithelial cells and of tumor cells.<sup>15-17</sup> Such localization accounts for clotting activation in sites of endothelial damage with blood extravasation and for hemostatic abnormalities in patients with cancer. Contrariwise, very little, if any, tissue factor activity is detectable in normal conditions on the cell surface of monocytes and endothelial cells. However, upon proper stimulation by a number of agents such activity is expressed in these cells, which can then contribute significantly to clotting activation.

#### **Tissue factor expression on monocytes**

The different stimuli able to induce tissue factor expression on monocytes are reported in Table 1. It is known that monocytes become activated for inflammatory response upon exposure to bacteria, viruses, endotoxins or immune complexes and such activation is accompanied by expression of tissue factor activity on cell membrane.<sup>18-20</sup> Pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF) and IL-6 are also very well known inducers of tissue factor expression in monocytes<sup>19,21,22</sup> and the same effect has been observed after exposure of these cells to IL-8 or interferon (IFN)- $\gamma$ .<sup>23,24</sup> The ability of infectious agents, immune complexes and pro-inflammatory cytokines to activate monocytes for the inflammatory response and at the same time to induce tissue factor expression on their membrane is of great interest, as it could represent the biological basis to explain the strong rela-

**Table 1. Agents able to induce tissue factor expression on monocytes.**

Infectious agents (viruses, bacteria)
Endotoxins
Immune complexes
Reactive C protein
C5a (anaphylotoxin)
Different cytokines (IL-1, TNF, IFN- $\gamma$ , IL-6, IL-8)
Interaction of monocytes, mediated by adhesion receptors, with other cells (platelets, polymorphonucleated leukocytes, endothelial cells, tumor cells) or with adhesive proteins (fibrinogen)

tionships existing between inflammation and the coagulation system.<sup>21,25,26</sup> Upregulation of tissue factor expression in monocytes upon adhesion to other cells or to adhesive proteins is also likely to be relevant in this context.<sup>20,27,28</sup>

#### **Tissue factor expression on endothelial cells**

Endothelial cells were long considered little more than a *layer of nucleated cellophane*, endowed with negative properties, the most important of which being that of a non-thrombogenic substrate for blood. This view has changed radically: it is now evident that hemostasis, inflammatory reactions and immunity involve close interactions between immunocompetent cells and vascular endothelium. Upregulation of tissue factor expression upon exposure of endothelial cells to appropriate stimuli is of great importance in understanding their role in hemostatic balance. A list of agents able to induce *in vitro* the expression of tissue factor activity on endothelial cells is reported in Table 2. It is of interest to note that several inducers able to trigger tissue factor expression on monocytes (see above) show the same activity on endothelial cells; these include infectious agents, endotoxins, immune complexes and pro-inflammatory cytokines IL-1 and TNF.<sup>29-31</sup> Such cytokines are known to influence endothelial cell function profoundly, as they not only induce procoagulant activity, but also inhibit the thrombomodulin/protein C anticoagulation pathway and affect fibrinolysis by upregulating both urokinase type plasminogen activator and type I inhibitor of such activation. Furthermore, IL-1 and TNF stimulate endothelial cells to produce and release important vasoactive agents such as PGI<sub>2</sub>, nitric oxide, PAF and endothelin. Such a complex series of molecular events which take place in endothelial cells upon exposure to pro-inflammatory cytokines is considered to represent the induction of a *prothrombotic pro-inflammatory program* in such cells.<sup>29</sup>

Other agents act as specific inducers of tissue factor expression on endothelial cells, as they are not effective with monocytes. Advanced glycosylation end products (AGEs), which are formed in patients with poorly controlled diabetes mellitus, show this property.<sup>32</sup> AGE formation results from the spontaneous covalent reaction of circulating glucose with free amino groups with subsequent rearrangement and production of fluorescent moieties irreversibly bound to proteins. AGEs promote the formation of oxidized

**Table 2. Agents able to induce tissue factor expression on endothelial cells.**

Infectious agents (viruses, bacteria)
Endotoxins
Immune complexes
Pro-inflammatory cytokines (IL-1, TNF)
Advanced glycosylation end products (AGEs)
Reactive oxygen species
Thrombin
Binding of CD40 to its ligand (CD40L)

LDL, induce cytokine and growth factor release from macrophages, and stimulate the expression of tissue factor and of adhesion molecules on endothelial cells. They are, therefore, considered to play an important role in progression of atherosclerosis in diabetes.

Reactive oxygen species are other important specific inducers of tissue factor expression on endothelial cells.<sup>29</sup> Their effect is mediated by stimulation of NF- $\kappa$ B, a transcription factor which acts as a regulator of genes involved in inflammation.<sup>29,33</sup>

Interestingly, thrombin has also been reported to be able to induce tissue factor expression on endothelial cells,<sup>14</sup> thus leading to further clotting activation.

Furthermore, it has been recently demonstrated that ligation of CD40, an important signal transduction molecule present on endothelial cells, elicits tissue factor dependent procoagulant activity and this pathway has been suggested to be involved in the development of prothrombotic states during diseases associated with endothelial cell activation.<sup>34, 35</sup>

Therefore, a wide spectrum of agents may trigger *in vitro* tissue factor expression on endothelial cells: pro-inflammatory, dysmetabolic, oxidative stimuli may induce endothelial cells to change their anti-thrombotic properties into a procoagulant, clot-promoting state. Tissue factor mRNA and protein levels decline in endothelial cells despite continued exposure to agonists, and this could represent a mechanism to contain the extent of clotting activation. This behavior could also explain why it has been difficult to demonstrate expression of tissue factor by endothelial cells *in vivo* even in response to potent provocations when expression was expected.<sup>14</sup> However, tissue factor is found *in vivo* associated with endothelial cells after vascular injury, within atherosclerotic plaques and in tumor-derived vessels.<sup>14</sup>

### Cytokines and hemostatic abnormalities in different disease states

Septicemia is a dramatic, very convincing demonstration of the close relationship existing between inflammation and the hemostatic system. The spectrum of hemostatic changes during systemic infection may vary from subclinical clotting activation to disseminated intravascular coagulation associated with consumption coagulopathy, microvascular thrombosis and subsequent multiorgan failure.<sup>36,37</sup> Recent *in vivo* studies in humans and non-human primates have documented the pivotal role of cytokines

in hemostatic abnormalities of patients with sepsis.<sup>21,38</sup> Cytokines involved in the altered hemostatic properties in infection and inflammation are TNF, IL-1, IL-6 and IL-10. Indeed, both clinical and experimental studies have documented enhanced production of these cytokines during systemic infection.<sup>39-42</sup> Activation of coagulation during septicemia proceeds through the extrinsic tissue factor-mediated pathway. Activation of the contact system does not contribute to coagulation abnormalities, but is involved in the development of shock.<sup>21</sup> Available experimental and clinical evidence suggests that TNF is mainly involved in activation of fibrinolysis,<sup>40</sup> IL-6 in activation of coagulation<sup>41,43</sup> and IL-1 in activation of both.<sup>44,45</sup> In contrast, IL-10 may serve a protective function during septicemia, at least in part related to its capacity to inhibit the production of pro-inflammatory cytokines.<sup>21, 46</sup>

Pro-inflammatory cytokines could also be involved in the pathophysiology of thrombotic complications in a number of other disease states: these include the antiphospholipid syndrome,<sup>47-49</sup> veno-occlusive disease of the liver after bone marrow transplantation,<sup>50,51</sup> the vasculitides observed in autoimmune connective tissue diseases,<sup>19</sup> the prothrombotic states associated with atherosclerotic vessels,<sup>30, 52-54</sup> the occurrence of deep venous thrombosis after major abdominal surgery,<sup>19,55</sup> and acute rejection after solid organ transplantation.<sup>19,56-59</sup>

Finally, cytokines are involved in hemostatic abnormalities and in the thrombotic tendency of patients with cancer. The systemic activation of coagulation that occurs in malignancy is well known and has been described under the name of Trousseau's syndrome.<sup>60-62</sup> The mechanisms of this systemic coagulation activation have been extensively investigated and were shown to involve the tumor cells and the host response. First of all cancer cells are able to promote blood clotting by several tumor-associated procoagulant agents, including tissue factor<sup>63,64</sup> and cancer procoagulant, a cysteine protease which directly activates factor X.<sup>64-66</sup> Furthermore, tumor-associated macrophages stimulate the production of growth and angiogenic factors and express tissue factor-like procoagulant activity.<sup>67,68</sup> Indeed, cytokine production is greatly increased in such patients, as a result of activation of host cells such as monocytes and endothelial cells and of cytokine release by tumor cells themselves.<sup>68-72</sup> In turn, cytokines such as IFN- $\alpha$ , IFN- $\gamma$  and TNF have been shown to be able to increase tumor cell procoagulant activity,<sup>73,74</sup> thereby enhancing clotting activation in cancer patients. Finally, the close interaction of tumor cells and endothelial cells may induce surface expression of tissue factor in the latter<sup>75</sup> and this effect could play an important role in the two-way interaction of tumors with the hemostatic system.

### New possible antithrombotic strategies

Several therapeutic approaches to inhibiting tissue factor/VIIa complex or to limiting cytokine production or activity have been attempted in experimental models of thrombosis and also in man.

Examples of possible new antithrombotic agents

are the tissue factor pathway inhibitor, the physiologic Kunitz domain inhibitor of the tissue factor/VIIa complex, and Fab fragments of a monoclonal antibody directed against factor VII or factor VIIa. Several animal studies support the therapeutic usefulness of these molecules in thrombosis and Gram-negative sepsis.<sup>76-78</sup> Another potential agent is TFAA, a mutant form of human soluble tissue factor (Lys165Ala, Lys166Ala) which binds to VIIa and forms an inactive complex. Antithrombotic activity was shown by TFAA in a rabbit arterial thrombosis model without affecting general hemostasis.<sup>5</sup> Together, these data indicate that pharmacologic intervention at the initial stage of the coagulation cascade might be efficacious as well as safe.

Another attractive approach is inhibition of cytokines involved in the regulation of tissue factor expression. Several possibilities have been explored in this context: antibodies directed against pro-inflammatory cytokines, cytokine inhibitors, drugs capable of inhibiting cytokine production.

Treatment with anti-TNF antibodies was found to reduce mortality rates and coagulation abnormalities after injection of endotoxin or live bacteria into monkeys,<sup>21,39</sup> although this effect has not been confirmed in patients with sepsis.<sup>79</sup> Furthermore, administration of an anti-IL-6 antibody prevented coagulation activation after administration of a low dose of endotoxin to chimpanzees, whereas it did not affect activation of the fibrinolytic system.<sup>41-43</sup> In the same line, recombinant IL-1ra, the naturally occurring inhibitor of IL-1, has been administered in baboons with fatal bacteremia, and in patients with sepsis.<sup>44,45</sup> In both cases activation of coagulation and fibrinolysis was clearly reduced by the treatment.

Inhibition of cytokine production is also a potentially interesting approach. Glucocorticoids, theophylline and pentoxifylline are powerful suppressors of cytokine release<sup>19,80</sup> and this property could be of clinical benefit in selected situations of clotting activation. Furthermore, FR167653, a low molecular weight inhibitor of IL-1 and TNF production, markedly improved thrombocytopenia and plasma coagulation parameters after injection of endotoxin in rats.<sup>81</sup>

New avenues are being opened by fundamental discoveries regarding the relationship between cytokines and thrombosis, forming a rationale for new therapeutic approaches. However, more must be learnt before the appropriate drug and the adequate dose are defined in the different clinical situations.

#### **Contributions and Acknowledgments**

*Authors of the present paper were similarly involved in the design, bibliographic research, writing and discussion of the manuscript.*

#### **Disclosures**

*Conflict of interest: none.*

*Redundant publications: no substantial overlapping with previous papers.*

#### **Manuscript processing**

*Manuscript received April 27, 2000; accepted August 3, 2000.*

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