Blood-borne tissue factor

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A B S T R A C T

Existing paradigms of coagulation have been challenged in recent years by new experimental evidence for the existence of intravascular (blood-borne) tissue factor (TF). A revised model of coagulation to explain the mechanisms of initiation and propagation of coagulation has been proposed, in which physiological hemostasis -- and probably certain types of pathological thrombosis -- are critically dependent on circulating intravascular TF. Accumulating evidence suggests that this form of TF may be predominantly associated with cell-derived microparticles (MPs), small membrane units shed from activated and/or apoptotic cells. However, other forms of circulating TF, including soluble form(s), and cell-bound forms may also contribute to the intravascular pool of TF. While these observations are leading to a renaissance in the interest in blood-borne TF in the pathogenesis of thrombosis associated with cancer, TF may play a critical role in several other malignant cell functions, including angiogenesis and metastasis. The purpose of this communication however is to review the theoretical and practical considerations to be considered in designing assay(s) that might be used to measure circulating TF procoagulant activity in cancer and other disease states.

Newer models of hemostasis incorporating blood-borne TF

TF is the cellular receptor for coagulation FVII(a), and formation of the TF/VIIa complex is the physiological signal for initiation of coagulation. In the past 5 years, there has been an evolving paradigm shift in the accepted role of TF in hemostasis. Cellular TF expression may not be limited to cells located in the extravascular space, but rather may also be present in the intravascular compartment, albeit at very low concentrations. While binding of factor VII(a) to extravascular TF may be the key event in the *initiation* of physiological clot formation, it is now postulated that intravascular TF is subsequently required for normal *propagation* of coagulation.[1] There may in fact be several species of intravascular TF, including soluble and/or alternatively spliced forms.[2] However, because of the absolute requirement of TF for membrane anionic phospholipids for full PCA, it seems likely that only cell- or MP-bound forms of intravascular TF are functionally relevant in hemostasis. Similarly, pathological cellular TF expression by malignant or innate immune host cells is likely be of functional importance in thrombotic states.

Monocyte-derived MPs expressing phosphatidylserine and TF not only attach, but also fuse with the membrane of activated platelets in solution, resulting in transfer of both proteins and lipid. This process may be blocked by either annexin-V or by an antibody to PSGL-1.[3] Similarly, it has been demonstrated that MP-borne TF may be transferred to activated platelets adherent to collagen[4] or injured endothelium[5] in a P-selectin-dependent manner. Elegant *in vivo* studies have also shown that circulating MPs derived from hematopoietic -- but not vessel wall cells localize to the surface of adherent platelets in growing microvascular thrombi in mice.[6] However, the role of blood-borne TF in hemostasis remains controversial, and some *in vitro* systems have been unable to corroborate the existence of the minimal amounts of TF that would be theoretically required to partake in hemostasis.[7] Furthermore, not all animal models support a role for intravascular TF in coagulation. Transplantation of wild-type bone marrow into mice expressing about 1%

human TF did not accelerate large vessel venous or arterial thrombosis.[8] Therefore the role of intravascular TF in hemostasis and/or thrombosis may be highly model-dependent; important variables may include the specific vasculature size, composition, and shear rate, and the procoagulant stimulus responsible for initiation of coagulation.

MP-borne TF may be important in cancer-associated thrombosis. Dvorak *et al.*, demonstrated that cultured cancer cell lines release MP-associated TF.[9] Yu and Rak recently re-affirmed the observation that TF is secreted from cultured tumor cells in the MP-borne functional form, rather than as the alternatively spliced soluble form.[10]

Tissue factor encryption and de-encryption

Cellular TF procoagulant activity (PCA) is regulated by several mechanisms. First, cells that are normally in contact with blood (such as endothelial cells or monocytes) probably express significant quantities of TF only when activated or apoptotic. Second, the multivalent Kunitz-type inhibitor tissue factor pathway inhibitor (TFPI) rapidly inhibits TF/VIIa complexes in a FXa-dependent manner. Third, the phenomenon of TF *encryption*, whereby membrane-bound TF expresses only a fraction of its full procoagulant potential in the resting cell compared to the disrupted cell, limits the ability of the TF/VIIa complex to activate substrate FX or FIX. Encrypted TF is available on the surface of unperturbed cells to bind FVII/FVIIa despite its inability to promote coagulation. Activation or apoptosis of many cell types mediated by an increase in cytosolic [Ca^{2+}] is associated with a series of plasma membrane changes, including exposure of membrane anionic phospholipids that promote coagulation by supporting assembly of procoagulant vitamin K-dependent enzyme complexes. Loss of membrane phospholipid asymmetry may be linked to cellular cytoskeletal disruption and vesiculation of MPs. While phosphatidylserine (PS) exposure in the external membrane leaflet accounts for much of the TF PCA de-encryption phenomenon, there is also a PS-independent component. Transition of membrane TF from a dimer to the monomeric form results in exposure of a macromolecular-substrate binding site for factors IX and X that coincides with enhanced TF function; this process might therefore explain the phospholipid-independent component of de-encryption.[11] De-encryption of TF may result in up to a 100-fold increase in procoagulant activity compared to the encrypted (but antigenically detectable) form. To date, little is known about the encryption status of cell- or MP-bound TF *in vivo*, but the implication is that the presence of (any form of) intravascular TF antigen should not be assumed to equate with functionally active TF.

Assays for blood-borne tissue factor

Pre-analytical Issues

In theory, assays for TF may be affected by a number of pre-analytical variables, including: 1] method of venipuncture (potential contamination by TF-bearing dermal cells); 2] anticoagulant used (calcium-chelating anticoagulants may promote dissociation of TFPI from TF-VIIa); 3] centrifugation (which may produce cell-free or MP-free plasma, depending on the centrifugation force used); 4] the use of fresh vs. frozen samples (the latter may de-encrypt membrane-bound TF PCA).

Assay characteristics

The reproducibility, sensitivity and specificity of assays for blood-borne TF should be routinely considered and defined. Specificity should be established by demonstrating inhibition of PCA by the addition of a well-characterized blocking antibody to TF, and/or by the absolute dependence on FVII(a). Furthermore, the reference material (standard) and reference method for the assay of TF PCA have not been defined.

Location of blood-borne tissue factor

TF may circulate in blood either as a component of blood cells and MPs or as a soluble plasma protein. TF antigen has been most commonly measured in standard platelet-poor plasma using ELISA, with a typically quoted reference range of 100-200 pg/mL (2-3 pM). Unfortunately, very little is known about the specific forms of TF antigen detected by the most commonly used commercial assays. When specifically evaluated, several studies have demonstrated that one third to one half of TF antigen in *cell-free* plasma may be sedimented by ultracentrifugation, implying that it is probably MP-associated and may be functionally intact. It has also been suggested that TF antigen may reflect endothelial injury rather than intravascular TF expression.

MPs are usually isolated from cell-free plasma by ultracentrifugation (using 'g' forces of 17,500 to >100,000 x g). Using flow cytometry, most groups -- including our own -- have defined circulating MPs in plasma according to at least 2 criteria; 1] size below a pre-defined threshold – most commonly < 1.0 μm; and 2] specific binding of monoclonal antibodies to cell-specific antigens. In addition to these 2 criteria, many investigators mandate the presence of two additional criteria to define MPs, namely; 3] PS exposure (detected by annexin V binding); and 4] that ultracentrifugation will remove particles from platelet-free plasma. Although this more restrictive definition increases the specificity of detection, it is possible that a potentially important MP subset, namely those that do not bind annexin V, is being overlooked. It may be

the case that MPs bearing *encrypted* TF are primary responsible for shuttling of the molecule between cells, whereas those bearing *de-encrypted* TF PCA do directly participate in the initiation/propagation of coagulation. Thus, a consensus definition of what actually constitutes a MP is urgently needed. In the meantime, many groups have demonstrated the presence of MPs in plasma derived from various cell types (e.g. monocytes, endothelial cells, or platelets) that are positive for TF antigen by flow cytometry. While of interest, these studies do not address the question of whether the TF is functionally intact and/or encrypted.

We recently developed a novel functional assay to measure circulating intravascular TF PCA. An immobilized antibody (1B10) was used to capture circulating MPs in platelet-free plasma derived from a variety of cell sources, including monocytes, granulocytes, endothelial cells, smooth muscle cells, and fibroblasts. Notably however, because they do not express the antigen recognized by 1B10, MPs derived from platelets or erythrocytes were not captured. TF PCA was measured on the immobilized MPs using a chromogenic assay of FXa generation. MP-associated TF PCA was detectable in the majority of healthy subjects, and increased rapidly but transiently *in vivo* in response to endotoxin administration.[12]

Monocytes have long been recognized to be the most relevant probable source of intravascular TF. Recently, in keeping with *in vitro* models of MP-borne TF transfer from monocytes to platelets, we demonstrated that an increase in monocyte-associated TF PCA was followed by a more modest and delayed increase in platelet-associated TF PCA *in vivo* in the human endotoxemia model.[13]

Conclusions

At present, there is no standardization of methodology for the measurement of blood-borne TF. The minute quantities of circulating TF PCA *in vivo* present a challenge for the development of accurate and reproducible assays. In the absence of a *gold standard*, it would seem appropriate whenever possible to compare several methodologies, including at least one antigen and one activity assay. Lastly, it is imperative that we understand more about the *in vivo* counterpart of the *in vitro* TF encryption phenomenon that may vastly influence the display of TF procoagulant activity.

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