From Virchow’s triad to metastasis: circulating hemostatic factors as predictors of risk for metastasis in solid tumors

E. Beleva, J.Grudeva-Popova

Department of Medical Oncology, Medical University, Plovdiv, Bulgaria

Summary

Tumor growth is characterized by disturbances of hemostatic mechanisms towards subclinical activation of coagulation. In cancer patients there is an increased risk up to 4-6 fold for developing idiopathic thrombosis due to the tumor-associated prothrombotic status. The molecular basis for the interrelationship between malignant phenotype and hemostatic disbalance includes several specific properties of tumor cells such as production of proinflammatory and proangiogenic cytokines, exhibition of procoagulant / fibrinolytic activities by tumor cells themselves and direct cell-to-cell interactions between tumor cells and cellular components of the hemostatic system.

Aberrant tissue factor (TF) expression is a hallmark of human cancers and has been linked to their metastatic potential and neoangiogenesis. TF is a multifunctional molecule which controls the pro-/antiangiogenic balance in tumor tissue both by its procoagulant properties and via coagulation-independent mechanism inducing production of angiogenic cytokines by tumor cells.

Components of the fibrinolytic system urokinase plasminogen activator (uPA) and urokinase plasminogen activator receptor (uPAR) facilitate targeted proteolysis of the basement matrix in order neovascularization to occur. Overexpression of these factors in tumor tissue has been identified as prognosticator for metastatic spread and overall survival in many human cancers, including breast, gastrointestinal, lung, urological and gynecological malignancies. Measurement of circulating TF and the soluble isoform of uPAR in plasma may be useful for evaluating the risk of cancer metastasis, monitoring for tumor recurrence and prediction of response to cancer therapy.

Key words: angiopoietin, cancer, microparticles, uPAR, tissue factor
Introduction
Tumor growth is associated with the development of hypercoagulable state. Thromboembolic disease (TED) is an early sign of malignancy and cancer patients are at increased risk of developing thrombotic complications in the course of their disease. According to population-based case-control studies the 2-year cumulative incidence of venous thromboembolism (VTE) among cancer patients is between 0.8 and 8% [1,2]. It has also been noted that the presence of VTE is a sign of higher disease aggressiveness, whereby in 40% of cancer patients with concurrent deep vein thrombosis (DVT) at presentation there is already evidence of distant disease [3]. Virchow's triad remains an useful concept for understanding thrombogenesis as a process resulting from alterations in three broad categories: endothelial injury, stasis and activation of clotting. Moving away from this classic perception towards understanding the molecular basis for thrombophilia in cancer, alterations of the three fundamental factors could be viewed as a distant reflection of tumor-inherent processes. Endothelial homeostasis can be disrupted by tumor-induced endothelial overexpression of tissue factor (TF) and proadhesive molecules, downregulation of the anticoagulant molecules thrombomodulin (TM), thrombospondin (TSP), tissue factor pathway inhibitor (TFPI) and altered angiopoietin-2/-1 proportion.

Tumor masses can cause stasis by infiltration of the vessel wall, endoluminal growth, and vascular compression. Tumor cells can directly trigger clotting through secretion of procoagulants such as TF cancer procoagulant (Figure 1). Thus, a two-way relationship between the hemostatic system and malignancy has been established. First, the malignant disease itself promotes the development of hypercoagulable state through secretion of procoagulant substances, disruption of endothelial homeostasis and alteration of blood flow [4]. Second, the hemostatic system with its components and their interactions facilitates cancer progression-related processes such as tumor growth, invasion and neoangiogenesis (Figure 2) [5]. So, one could consider the hemostatic system in the concept of malignancy as emanation of cancer progression in which molecular components of hemostasis play the key roles.

Tissue factor
TF is a multifunctional transmembrane glycoprotein from the class II cytokine receptor family [6]. Under normal conditions it is expressed on the surface of perivascular cells (pericytes, adventitial fibroblasts).

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Figure 1. Virchow's triad in cancer.
and epithelial cells (parenchymal organs, body surfaces), but not in resting endothelium [7]. Inducible TF expression in endothelial cells occurs upon stimulation with proinflammatory cytokines (IL-1, TNF-α, LPS), while constitutive TF expression is the hallmark of malignant cells [8]. Circulating TF has been identified at very low levels in the blood of healthy individuals in the form of TF+ microparticles (MP) and on the surface of a subset CD14+ monocytes [9]. Serving as the key initiator of coagulation, TF exerts its physiological role by binding to its natural ligand f.VII and converting it to f.VIIa. The complex TF:VIIa proteolytically cleaves f.X to f.Xa, initiating the generation of thrombin and formation of the hemostatic clot.

The study was approved by the National Ethics Committee and by the Regulatory Authority from Romania and all patients gave written informed consent to participate in the study.

Besides its major role in hemostasis TF has been identified as an important signaling receptor in cancer biology. Ample preclinical evidence has accumulated over the past decade, implicating TF as an important effector in the processes of tumor initiation, growth, angiogenesis and metastasis. Moreover, this has led to the development of approaches exploring TF as a potential target for anticancer therapy [10,11]. TF is overexpressed in many types of human tumors, including breast cancer, pancreatic cancer, gastric cancer, prostate cancer, colorectal cancer, non-small-cell lung cancer, melanoma, leukemia, lymphoma, esophageal cancer, hepatocellular carcinoma, brain glioblastoma, but not in their normal tissue counterparts [6]. TF overexpression in cancer cells has been correlated with tumor progression and unfavorable prognostic indicators such as increased angiogenesis, advanced disease stage and resistant phenotype [12-14]. Therefore TF overexpression in situ could be considered a biomarker for solid tumors.

Enhanced TF expression in cancer has been reported to be an oncogenic driven event. In colorectal cancer activation of the K-ras oncogene and loss-of-function mutation of p53 result in constitutive activation of MAPK and PI3K pathways leading to increased TF expression [15]. In turn inhibition of the PI3K and MAPK by restoration of the PTEN tumor suppressor gene in glioma cells downregulates TF expression dependent on EGFR amplification [16]. In medulloblastoma cell lines TF expression has been shown to result from mutation in the c-Met oncogene and subsequent activation of Src kinases [17]. It has been observed that a certain subset of tumor cells, known as cancer stem cells, which constitutively express activated oncogenes and are capable of undergoing multilineage differentiation, are characterized by TF abundant phenotype [18]. Moreover, enhanced TF expression is observed during the processes of epithelial-to-mesenchymal-transition, whereby epithelial cells acquire a mesenchymal, more aggressive and motile phenotype [19]. This indicates that TF is possibly involved in maintaining cancer cell self-perpetuation.

**Figure 2.** Hemostatic system modulates the malignant potential of tumor cells.
There is a structure-function dependency in TF mode of action. TF plays a role in cancer progression both by initiating tumor growth and by promoting efficient tumor cell dissemination. Tumor-promoting activities of TF occur via non-hemostatic mechanism and can be attributed to the cytoplasmic domain signaling dependent mostly on the activation of the protease activated receptor 2 (PAR2). Prometastatic properties of TF can rather be coupled with its extracellular domain and the subsequent generation of thrombin, which, as a potent growth factor, exhibits further pleiotropic cellular effects.

TF-mediated signaling is critical for both physiological and pathological angiogenesis. TF deficiency in murine knock-out experiments caused early embryonic lethality due to impaired vasculature development [20]. Zhang et al. demonstrated that tumors overexpressing TF become highly vascularized once implanted into mice and the observed growth induction could not be inhibited despite maximal anticoagulation [21]. It has been revealed that involvement of TF cytoplasmic domain in several transduction cascades accounts for the production by tumor cells of angiogenic cytokines and contributes to increased angiogenesis in a paracrine fashion [22]. Formation of the complex TF/VIIa leads to increase of intracellular Ca2+ and phosphorylation of serine residues on TF cytoplasmic tail. This triggers signaling via the G-protein coupled membrane receptor PAR2 that activates MAPK and PI3K transduction cascades, resulting in increased gene expression of the angiogenic cytokines VEGF, VEGF-C, CXCL1, II-8, and Cyr61 [23-25]. In addition to PAR2 signaling, the cytoplasmic domain of TF can be phosphorylated independently of f.VII ligand binding by protein kinase C (PKC) resulting in the transcriptional activation of VEGF, VEGFR, TGF and suppressed expression of anti-angiogenic molecules such as thrombospondin [26,27]. The relationship between TF and VEGF has been extensively studied and is manifested by reciprocal co-stimulation of their expression profiles. VEGF induces TF expression by orchestrating the binding of nuclear factors NFAT and AP-1 to the promoter region of TF gene [28]. Regulation of VEGF is in turn dependent on the TF cytoplasmic domain signaling, which is demonstrated by the finding that tumor cells transfected with truncated TF cDNA lacking the cytoplasmic domain fail to produce VEGF, but preserve the TF procoagulant function [27]. Experimental ex vivo and in vivo studies have further supported the TF-VEGF interrelationship by finding increased co-expression on tumor sections and their association with increased angiogenesis and malignant potential in human tumors [13-15,29,30].

Intriguing is the question whether TF has a role in angiopoietin/Tie2 receptor system regulation, since angiopoietins are considered regulators of VEGF function, and dysregulation of angiopoietin -1/-2 (Ang-1/-2) balance is critical for the angiogenic switch in tumors. A study by Kim et al. found an inhibitory effect of Ang-1 on TF induction by VEGF through activation of the PI3K pathway, but no direct effect on TF expression itself [31]. Another recent study found decreased vascular remodeling and expression of Ang-1 after anti-TF treatment in a mouse model of small intestine vascular remodeling system [32]. The precise mechanisms of the interaction between Ang/Tie2 receptor system and TF and of their interdependent contribution to tumor angiogenesis remain to be elucidated.

TF enhances primary tumor growth via TF/VIIa/PAR2 signaling. This is evidenced by studies on cancer cell lines, where overexpression of TF by cancer cells conferred growth advantage compared to cell lines expressing low levels of TF [11,27]. Delay in primary and metastatic tumor growth was observed after specific TF/VIIa inhibition with the anticoagulant rNAPc2 but not after inhibition of f.Xa [10]. Studies on selective targeting of different domains of the complex TF:VIIa revealed that PAR2 signaling and integrin ligation is sufficient for TF tumor promoting properties [33].

Prometastatic properties of TF can be attributed to its extracellular domain, which is required for its major role in triggering coagulation. The extracellular mutant domain (TFmut) has markedly diminished function for activation of f.X, while full-length or cytoplasmic tail-deleted TF retains its procoagulant activity [26,34]. The expression of TF in tumors can induce downstream coagulation activation via the TF/VIIa/Xa pathway leading to fibrin and tumor stroma formation. Tumor cells become encapsulated in fibrin and platelet rich throm-
bi, being protected from the host immune defense and arrest in the microcirculation. Local thrombin formation facilitates arrest of tumor cells to the vessel wall by upregulation of adhesion molecules and strengthening cell-to-cell junctions [35]. Fibrin matrix in the tumor stroma builds itself a multifunctional scaffold rich on growth factors such as PDGF, TGF, FGF, that is not only protective, but also promotes matrix-cell interactions necessary for neovascularization [36]. Thrombin, constitutively generated by the activated coagulation cascade in the tumor surrounding, mobilizes the adhesion molecules αIIbβ3-integrin, P-selectin, CD40 ligand and enhances tumor cell interactions to platelets, endothelial cells and matrix [37-39]. Thrombin has also an important function in angiogenesis by inducing the activation of endothelial secreted collagenase type IV, which degrades basement matrix proteins and collagen during neoangiogenesis [40]. In vivo experimental models of metastasis demonstrated dramatic increase of lung metastasis with thrombin-treated tumor cells compared with untreated cells [41].

In summary, TF-mediated effects either in a coagulation independent mechanism via direct cytoplasmic domain signaling and TF/VIIa/PAR2 or dependent on the coagulation products induce diverse sets of cellular responses inherent to tumor cells including tumor growth, neoangiogenesis, cell migration and metastasis. Taking into account the specific biologic role of TF in the malignant tissue, detecting circulating TF in cancer patients might be informative of active disease and ongoing processes of matrix reorganization, cell destruction and neovascularization.

The circulating pool of TF consists of TF antigen, microparticles (MP) bearing TF activity and complexes of TF/VIIa and TF/VIIa/Xa. Quantitative measurement of TF in body fluids is possible with the available clotting and chromogenic assays, TF-ELISAs and methods for detection of TF-bearing microparticles [42,43]. Even though various assays have been developed, they are challenged by the lack of an international standard for TF.

Clotting assays measure TF procoagulant activity as determined by the time of recalcified fresh blood to clot in the presence or absence of anti-TF antibody. Chromogenic assays differentiate between generation of VIIa, Xa and thrombin by using different substrates. A significant issue with these assays is the large amount of intra-assay variability [44].

TF antigen in plasma can be measured by several commercially available and in-house developed ELISA assays using anti-TF mono- or polyclonal antibodies. The major problem with these assays is that they do not specify the state of TF [44]. Of concern is also the wide range of reported TF values in healthy individuals. For instance, a mean level of 27 pg/ml in healthy individuals was reported in one study, while other investigators found a range of 61 to 187 pg/ml [44,45]. Several studies reported elevated levels of TF in various disease states including unstable angina, type 2 diabetes mellitus, vasculitis, cancer, chronic renal and hepatic failure, suggesting that increased amounts of TF release into the circulation upon massive immune and tissue remodeling processes [42].

Microparticles (MPs) are defined as submicron membrane-encapsulated vesicles released from activated apoptotic cells. They are highly procoagulant and serve as a source of functionally active TF in blood. Elevated levels of TF MPs have been found in ischemic disease, diabetes, disseminated intravascular coagulation and cancer, and it is considered that their role goes way beyond functioning as a source for procoagulant activity, but rather serving as messenger of biologic information between different cells [46,47]. The most widely used method of detecting MPs is flow-cytometry, though it harbors several limitations. In order to accurately define MPs among other events with characteristic flow cytometry profile there is a need for a global marker for MPs. Since it is considered that MPs are generated upon loss of membrane phospholipid asymmetry, phosphatidylserine (PS) expression on the released vesicles is most commonly used as a marker. In freeze-thawed stored samples PS expression can be enhanced and MPs number significantly altered. Moreover, certain cells can release MPs without PS expression. Standardizing flow cytometers and including all MPs of appropriate size (MP range between 0.1–1 μm) presents a challenge for accurate quantitative assessment [44,48].

Because of the presence of various assays for detecting circulating TF and the lack of international standard,
it seems reasonable to perform comparison assays using different approaches of TF measurement and comparing TF antigen with TF-MP levels. Another issue is that most of the studies performed address the involvement of the various TF forms in thrombosis in different disease states. There are fewer studies relating TF levels and activity to prognosis of cancer patients, particularly those who had undergone surgery and receive adjuvant chemotherapy. It is of particular interest to determine the dynamics of TF in the adjuvant setting in order to reveal whether there is a link between TF levels and the risk of metastatic disease.

**Fibrinolytic system and cancer**

The physiologic role of fibrinolysis is dissolution of the fibrin clot and collagen degradation exerted by the action of plasmin. Generation of plasmin, the main enzyme in fibrinolysis, occurs upon activation of plasminogen by the tissue plasminogen activator (tPA) and uPA. tPA functions in the intravascular space, orchestrating fibrinolysis of the thrombotic clot and maintaining vascular patency. In contrast, uPAs primary role is in processes such as degradation of the extracellular matrix and cell migration during wound healing, tissue repair and physiologic angiogenesis [49]. Binding of uPA to its receptor uPAR on the cellular surface is of high affinity and potentiates uPAs activity. The complex uPA/uPAR on the cellular surface ascertains targeted degradation of the basement matrix. Moreover, uPAR is motile within the cellular membrane, which allows its allocation at the cellular front of desired direction for proteolysis [50]. Under normal conditions the process of active proteolysis is tightly controlled by the proteolytic systems.

In cancer biology activation of uPA/uPAR system is a prerequisite for efficient focal proteolysis, adhesion and migration and enables penetrating tumor cells to invade and metastasize [51]. Tissue overexpression of uPA/uPAR has been detected in many human tumors like breast, prostate, gastrointestinal and lung cancers [52]. Oncogenes responsible for enhancement of uPA/uPAR expression in malignant tissue include ras, jun, myc, fos, rel and ets. uPAR expression can also be stimulated by the expression of TF and the epidermal growth factor receptor (EGFR) [53].

There is ample body of evidence correlating overexpression of proteolytic factors and the clinical prognosis of many malignancies. In breast cancer uPA and uPAR from primary tumor tissue have been established as independent prognostic factors for disease-free survival and overall survival [53]. In colorectal cancer expression of both uPA and uPAR is an important predictor of overall survival and metastasis, while in lung cancer the combination of elevated uPAR and plasminogen activator inhibitor-1 (PAI-1) is predictive of short survival. Thus, components of the fibrinolytic system have been recognized as invasiveness factors in cancer biology and reflect the metastatic potential of tumors.

Difficulty in utilizing these markers in the routine clinical setting arises from the methodology used to determine uPA/uPAR antigen levels. Commercially available ELISAs determine the antigen levels in extracts of primary tumor tissue [54]. Even though available tests are robust for clinical use and of guaranteed quality, their wide application might be prevented by the necessity of preparing extracts of primary tumor tissue.

Recently, a soluble fraction of the uPAR has been identified. Soluble uPAR (suPAR) originates from cleavage and release of the membrane-bound uPAR by different proteases including uPA, plasmin, chymotrypsin, different matrix metalloproteinases and elastases [50]. Its presence in plasma, urine, blood, serum and cerebrospinal fluid in various concentrations makes it an attractive and flexible biomarker for measurement of disease activity in certain disease settings.

Increased serum suPAR levels have been documented in pathological conditions including infections (malaria, pneumococcal and streptococcal pneumonia bacteremia, sepsis, HIV-infection, active tuberculosis), cardiovascular disease, diabetes and solid tumors (breast, colorectal, prostate, ovarian, non-small cell lung cancer) [50]. Furthermore, high blood concentrations of suPAR are independent predictors of increased mortality both in healthy individuals and patients [55]. A general population-based prospective study that followed up 2532 Danish patients during 1992-2006 determined an association of elevated suPAR levels with increased risk of pulmonary and other cancers, but not with gastrointestinal malignancies [56]. Measurement of preoperative
levels of suPAR in a retrospective study of 591 patients with colorectal cancer demonstrated an increasing risk of mortality with increasing plasma suPAR levels [57]. Elevated preoperative suPAR levels in patients with gynecologic malignant tumors compared to patients with benign tumors or healthy blood donors confer substantial risk of progression and relapse [58]. In breast cancer higher suPAR levels in serum and tumor cytosol are independently correlated with worse prognosis in terms of relapse-free and overall survival [59]. In patients with newly diagnosed acute myeloid leukemia, consecutive measurements of suPAR pre-chemotherapy, during remission and at relapse determine significant correlation of suPAR plasma levels and the amount of circulating blast cells and thus might be informative of disease activity [60].

However, in the adjuvant setting there is fewer data present on the informative utility of suPAR. Whether suPAR levels fluctuate during the course of adjuvant chemotherapy, reflecting disease activity and how these variations can be exploited in the successful management of certain cancer types remains a matter of investigation. Consecutive measurement of suPAR during systemic chemotherapy might be useful in order to reveal biologically active disease and imminent metastatic spread.

**Conclusions**

Thrombogenesis in cancer patients as a phenomenon even though studied for more than a hundred years continues to serve as a source for intriguing research concepts. Since it has been recognized that cancer–hemostatic system interrelationship goes way beyond the mere clinical presentation as VTE in cancer patients, research agendas have evolved into revealing the molecular mechanism behind these interactions and identifying novel determinants of cancer expansion. Experimental studies and few clinical studies have demonstrated the role of components of the hemostatic system such as TF and uPA/uPAR in tumor biology and invasion. Implication of these hemostatic factors in the processes of tumor growth, angiogenesis and metastasis has identified them as potential targets for anticancer therapies. Measurement of circulating levels of TF and suPAR may prove useful for evaluating the risk of cancer metastasis, monitoring for tumor recurrence and predicting response to cytotoxic therapy. Therefore, establishing TF and suPAR as plasma biomarkers and standardizing methods for their measurement requires further investigation. Last, but not least, looking into the era of personalized medicine and witnessing the progress of targeted therapies, the development of individual biomarkers to reflect the efficacy of targeting molecules further necessitates the establishment of reliable and easily accessible plasma surrogates.

**References**

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