Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2009 December ; 29(12): 1989–1996. doi:10.1161/ATVBAHA.108.177402.

Tissue Factor in Coagulation: Which? Where? When?:

Butenas Role of Tissue Factor in Blood Coagulation

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Abstract

Tissue factor (TF) is an integral membrane protein, normally separated from the blood by the vascular endothelium, which plays a key role in the initiation of blood coagulation. With a perforating vascular injury, TF becomes exposed to blood and binds plasma factor VIIa. The resulting complex initiates a series of enzymatic reactions leading to clot formation and vascular sealing. In some pathologic states, circulating blood cells express TF as a result of exposure to an inflammatory stimulus leading to intravascular clotting, vessel occlusion and thrombotic pathology. Numerous controversies have arisen related to the influence of structural features of TF, its presentation and its function. There are contradictory reports about the synthesis and presentation of TF on blood cells and the presence (or absence) of functionally active TF circulating in normal blood either on microparticles or as a soluble protein. In this review we discuss TF structure-function relationships and the role of TF during various phases of the blood coagulation process. We also highlight controversies concerning the expression/presence of TF on various cells and in blood in normal and pathologic states.

Keywords

tissue factor; monocytes; posttranslational modifications; platelets; thrombin generation

Tissue factor (TF) is an integral membrane protein that is the essential cofactor component of the TF-factor VIIa complex enzyme. TF is expressed in the vascular adventitia, in astroglial cells, in organ capsules and is found in the central nervous system, lungs, and placenta at relatively high concentrations.[1–3] Many cells produce detectable amounts of TF when they are stimulated *in vitro* by various agents.[4,5] Monocytes and macrophages are known to express TF after stimulation, primarily by inflammatory cytokines.[6–8] In addition to its expression by normal tissues and cells, it is also known to be present on tumor cells, where its expression appears related to the metastatic potential of those cells.[9–11] Furthermore, it has been identified in atherosclerotic plaques, which has suggested a role for TF in the progression of cardiovascular disease.[12,13] Under normal circumstances, however, cells in contact with blood do not express physiologically active TF.[14] When mechanical or chemical damage of the vascular wall occurs, subendothelial TF is expressed/exposed to blood and binds plasma factor VIIa, which circulates as an operationally inactive enzyme at a concentration of approximately 0.1 nM (1% of plasma factor VII)[15] and escapes the inhibitors of coagulation proteases because of its poor enzymatic qualities. The TF-factor VIIa complex initiates blood coagulation by activating the zymogens factor IX and factor X to their respective serine proteases, factor IXa and factor Xa.

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Disclosures
None.

Structure-Function Relationships (Which?)

TF is a 263/261 amino acid transmembrane protein containing three domains (Figure 1): 1) an extracellular domain representing the NH_2-terminal part of the molecule (residues 1–219) and composed of two fibronectin type III domains; 2) a transmembrane domain, which anchors TF to the membrane (residues 220–242); and 3) a cytoplasmic COOH-terminal domain (residues 243–263).[16] The extracellular domain of TF is involved in complex formation with factor VIIa increasing, in a membrane dependent fashion, the activity of the protease toward its natural substrates factor IX, factor X, and factor VII by several orders of magnitude.[17,18] Thus, two of the three domains of TF (extracellular and transmembrane) play distinct roles in the blood coagulation process. The major role of the cytoplasmic domain is related to signal transduction.[19] As a consequence, it has been generally accepted that TF lacking the cytoplasmic domain is functionally identical to the full-length protein in the initiation of thrombin generation. On the other hand, recombinant TF lacking both the cytoplasmic and transmembrane domains cannot bind to the membrane, and therefore, while forming a complex with factor VIIa, does not activate factor VII and has decreased catalytic efficiency towards factor IX and factor X.[17,18]

Over 20 years ago, sufficient natural TF was isolated to identify, clone and express the recombinant protein (rTF) in human kidney 293 cells and in *E. coli*.[16,20,21] Subsequently, various forms of rTF ranging from the full-length protein to the extracellular domain of TF with different levels of posttranslational modifications have been expressed in a variety of vectors including yeast and insect cells (Figure 1). Mutational studies[22] have been performed, and an x-ray structure[23] has been derived using these rTFs. Although these rTFs have been used extensively as surrogates for the natural protein, the limited availability of purified natural TF has not allowed certification of results obtained with rTF.

The contributions of various regions of the primary structure of TF on its activity are relatively well established. However, data related to the influence of posttranslational modifications on the TF function are scarce, if available at all. The amino acid sequence data related to the structure of rTF indicate that the extracellular domain of protein has potential glycosylation sites at Asn^{11}, Asn^{124} and Asn^{137}.[16,24] There are also two disulfide bonds (Cys^{49}-Cys^{57} and Cys^{186}-Cys^{209}) located in this domain.[25] The carboxyterminal cytoplasmic domain of TF contains a single Cys^{245} residue and three Ser residues. The Cys^{245} residue is linked to a palmitate or stearate fatty acyl-chain,[25] while one of the Ser residues can be phosphorylated by a protein kinase C-dependent mechanism.[26] Although the sites of glycosylation of the extracellular domain are established and a partial identification of carbohydrates attached to those sites has been accomplished,[24] a complete analysis of the carbohydrate side chain structure is lacking. In addition, no systematic analyses have been reported which examine the influence of glycosylation on TF affinity for factor VIIa, or on the affinity of the TF-factor VIIa complex for its natural substrates factor IX and factor X, or on its effects on TF-factor VIIa catalytic efficiency. The apparent lack of interest related to TF glycosylation may have been caused by two early publications addressing the subject. In the only reported activity comparison for glycosylated and non-glycosylated rTFs by Paborsky and coworkers,[21] it was suggested that TF glycosylation is not required for procoagulant activity. However, since no data were provided in the report, it is not established whether glycosylation influences TF activity. Waxman *et al*. reported that the activity of $rTF_{1–263}$ is identical with that of natural TF from brain,[27] however this also was not supported by data included in the publication. Similarly, studies suggesting that glycosylation could be essential for TF activity do not provide experimental evidence to support this hypothesis.[28,29] Thus the question whether the glycosylation of TF has an effect on its function remains open due to the absence of relevant data.

A controversial issue associated with the activity of TF is related to a hypothetical "encryption-decryption" process associated with TF activity presentation. It has been suggested that the majority of TF molecules located on the cell surface have low activity (are "encrypted") and that "decryption" is essential for the expression of TF function.[30] Several contradictory mechanisms have been hypothesized in attempts to explain the "encryption-decryption" and presentation of TF activity.

One established method for inducing TF activity on the cell surface consists of the treatment of quiescent TF-bearing cells with calcium ionophore.[31–34] Ionophore treatment increases TF activity by 2 to 10-fold. While some authors assign this increased TF activity to increased expression of TF protein,[35] others suggest this arises from changes in the cell membrane environment, particularly in an increased expression of acidic phospholipids,[31,34,36] sometimes related to cell death.[32,37] Several studies hypothesize a role for cholesterol in cell lipid rafts contributing to the "encryption-decryption" of TF activity,[38–40] although there is little agreement between the proposed mechanisms for this process. An increase in TF activity has been reported when lipopolysaccharide (LPS)-stimulated monocytes are treated with platelets.[41–43] However the observed increase in activity was quite limited (2 to 3-fold) and could be (in part) assigned to an increase in TF antigen expression by monocytes.[43]

It has been suggested[44] that an "encryption" of TF preexisting and residing on the cell membrane is related to the reduction status of the Cys^{186}-Cys^{209} bond, which leads to impaired TF activity. The presumed re-formation of this bond using an oxidizing agent ($HgCl_2$) appears to restore TF activity. Unfortunately, structural data have not been provided in support of the re-formation of the disulfide bridge from a hypothesized reduced state. In addition the proposed mechanism is not supported by relevant studies that conclude that in general $HgCl_2$ will oxidize only a single thiol group.[45,46] Moreover, an increase in TF activity on cell surfaces similar to that caused by $HgCl_2$ can be achieved by treating TF-bearing cells with other metal compounds, such as $AgNO_3$ and phenylmercuric acetate,[47] with the authors concluding that this increase is related to the elevated exposure of phosphatidylserine.[48,49] Similar controversy surrounds publications related to the putative role for protein disulfide isomerase (PDI) in TF activity. Ahamed *et al*. [49] postulate that PDI disrupts the Cys^{186}-Cys^{209} bond and, as a consequence, suppresses TF procoagulant activity. Reinhardt and co-workers, however, suggest in their study[50] that PDI promotes TF activity, whereas Pendurthi *et al*.[51] reports that PDI plays no role in TF activity, and that the observed increase in TF activity is related to the contamination of PDI with phospholipids.[52]

A soluble form of TF circulating in blood (alternatively spliced TF) was identified several years ago.[53] It has been suggested that this form of TF is procoagulant[54] and stimulates clot growth.[53] However subsequent studies showed that this form of TF has no procoagulant activity[55,56] but could promote tumor growth and angiogenesis.[56] The potential origin of this discrepancy could be assigned to the physiologically-irrelevant conditions used for the detection of alternatively spliced TF activity and the lack of validated commercial assays for the detection of TF activity at its physiologic concentrations.[57–59] The role of soluble TF remains problematic.

The Controversy Regarding Blood-Borne TF (Where?)

During the last several years, numerous conflicting studies related to the presence, concentration and functional activity of TF circulating in blood as a soluble protein and on/in various blood cells and platelets have been published. Several groups of investigators reported the presence of TF antigen circulating in blood at the concentrations as high as 5–10 nM[60] and those of active protein reaching (sub)nanomolar concentrations.[61] It has been reported that this blood-borne TF is located on blood cells, platelets and microparticles or that it circulates as a

soluble protein. Frequently these reports have been developed using non-validated commercial assays. In contrast, data published by several other groups indicate that if there is TF-related activity either in blood or plasma from healthy humans, the concentration of active TF does not exceed 20 fM[62–64] (Figure 2). Additionally, based upon the experience accumulated in our laboratory as well as on reports from other laboratories, blood or plasma activated with (sub) picomolar concentrations of functional TF clots within several minutes.[65–69]

Another subject of controversy related to blood-borne TF is the location of this protein. It is generally agreed that TF can be expressed/exposed by monocytes upon cytokine stimulation. It has been also in general been accepted that the source of circulating TF in pathologic conditions could be cell-derived microparticles.[70–74] More controversial is a reported presence of TF in/on platelets.[75–78] In some of those publications it has been suggested that TF is transferred to platelets from the cells,[75,78] whereas others suggest that TF is synthesized by platelets.[76,77,79] In contrast to these publications, it has been reported that neither TF activity nor antigen were detected on resting and calcium ionophore stimulated platelets.[64] In that study, no TF antigen-related signal was observed in resting or ionophore treated platelets using flow cytometry (Figures 3a and 3b) although 91% of platelets were activated upon treatment with the calcium ionophore (Figure 3c). Similarly, there is little agreement related to the presence of TF on granulocytes. Maugeri *et al.* suggested in their publication that granulocytes produce TF upon stimulation[80] while other authors have reported the expression of TF in neutrophils[81] and eosinophils.[82] However, data from Osterud's laboratory show no evidence of TF expression in any granulocytic cells.[43,83,84]

The major causes for the discrepancies related to the presence and concentration of TF are, most likely, the lack of validated and reliable assays for TF antigen and activity.[57–59] The majority of studies reporting high concentrations of TF in plasma and the presence of TF in platelets and blood cells use commercial assays. We developed and validated in-house assays for the quantitation of TF antigen[58] and activity.[85] Using our assays, we have reported that the TF antigen concentrations in plasmas from patients with acute coronary syndrome are at low picomolar levels, with an average functional concentration less than 0.4 pM.[85] In contrast, in a study by Bis *et al*., which used a commercial TF assay, nanomolar concentrations of TF in plasma from patients with a similar diagnosis were reported.[86] Until there is agreement in the scientific community concerning the validity of the assays used by various laboratories incongruent reports will continue to accumulate in the literature.

TF Requirement Throughout the Process of Blood Coagulation (When?)

Although there is consensus on the requirement for TF for the initiation of the coagulation process and on the proteolytic coagulation complexes that emerge in response to TF,[87] there is less agreement on the overall mechanism by which TF functions. In one construct of normal hemostasis, TF is found outside of blood vessels,[1,2] requiring the disruption of blood vessel integrity to exert its effects, and within circulating blood cells, requiring specific signaling events to promote its intravascular expression.[84,88] When an adequate TF challenge is presented, a full coagulant response follows; if the TF challenge is insufficient, the procoagulant response is arrested, primarily by the synergistic activities of the TF pathway inhibitor (TFPI), antithrombin and the protein C pathway.[89,90] A competing hypothesis of TF biology has been advanced in which the initiating TF stimulus requires constant supplementation to the ongoing reaction with newly available TF, providing a mechanistic rationale for blood-borne TF in normal hemostasis.

Eliminating one of these hypotheses requires resolving two basic areas of dispute: the constitutive presence of TF in blood; and the identity of the procoagulant catalysts required to propagate clot growth. As has been noted, the controversy concerning the presence and activity

of TF species in blood and on blood cells continues, and ultimately has become an important debate about the rigor of the quantitative methods used. The mechanistic argument for a requirement for ongoing supplementation of coagulation reactions with TF depends on three interdependent contentions: 1) that the maintenance of the coagulation process requires a continual contribution from additional TF cofactor activity (extrinsic factor Xase complex). 2) that the developing platelet/fibrin plug isolates the procoagulant complexes initially formed at the site of vascular injury from further supply of fresh reactants, thus eliminating participation of the triggering TF supply as the reaction proceeds;[91] and 3) that TF is present in blood at levels below the threshold to support a coagulant response[92] or in some cryptic state, but accumulates to an effective level on the vascular face of a forming thrombus.[53,93] In this regard Panes *et al*.[77] recently reported that activation of platelets leads to rapid *de novo* synthesis of TF and its expression. In this model, thrombus growth is viewed as self-limiting in the absence of an ongoing supply of TF to the outer face of the thrombus.

Other data consistent with this overall view of how a coagulant response is propagated include immunochemical dependent demonstrations of TF embedded in human[94] and mouse thrombi,[95,96] suggesting that some type of circulating TF species contributes to *in vivo* thrombus formation. The *in vitro* observation that supplementation of blood with a concentration of lipidated TF that is subthreshold in a static blood context but that results in increased fibrin formation when blood is flowed over immobilized TF[97] also supports a role for circulating TF in the growth of thrombi.

On the other hand, substantial evidence supports the view that in normal hemostasis TF functions primarily in the initial phase of the clotting process and that other catalysts are involved in the propagation and maintenance of fibrin platelet clots. Our laboratory has explored the time dependence of the requirement for TF during the progress of a blood coagulation reaction using mathematical, synthetic coagulation proteome and whole blood models.[98] When TF activity was eliminated either using inhibitory antibodies for factor VII and TF or mathematically at various times during the initiation phase, the results in all three models indicated that the progress of the reaction rapidly loses an absolute dependence on the presence of a functioning TF-factor VIIa complex and becomes fully independent of TF by the onset of the propagation phase of thrombin generation. In addition these studies indicated that the catalysts generated by transient expression of TF cofactor activity were sufficient to maintain a TF-independent procoagulant response as long as reactants were available and that this catalyst pool could reinitiate coagulation without input from the TF-factor VIIa complex.

Figure 4 shows an example of this type of experiment using the synthetic coagulation proteome where inhibitory antibodies to TF and factor VIIa were added at the onset of the reaction or 10, 60 or 240 s post initiation. No thrombin generation is seen when antibodies are present at the beginning of the reaction. Conversely, addition of inhibitory antibodies at the onset of the propagation phase had no effect. However, when added 60 s after the start of the reaction, there is a slight prolongation of the initiation phase and almost no effect on other parameters of thrombin generation. Thus in several *in vitro* models of TF- initiated coagulation, the procoagulant response becomes independent of TF cofactor activity prior to the onset of clot formation, reflecting the emergence of the more efficient intrinsic factor Xase complex,[98] and suggesting that transient expression of TF is sufficient to successfully achieve the first phase of hemorrhage control, formation of an impermeable platelet fibrin barrier.

Figure 5 shows a synthetic coagulation proteome experiment testing the stability of the procoagulant catalysts generated by an episode of TF-initiated thrombin generation. A TF-initiated reaction in which thrombin production had ceased and no detectable thrombin remained (due to inhibition by antithrombin) was subdivided after 20 minutes, with individual aliquots then resupplied at various later times with mixtures containing prothrombin,

antithrombin, phospholipid with or without factor VIII. In the absence of FVIII (closed symbols) thrombin generation by resupplied reactions was observed to decline slowly as the time period before resupply increased, reflecting a slow decline in the prothrombinase concentration. However inclusion of factor VIII (open symbols) into the resupply mixture yielded time courses of thrombin generation that appeared unaltered even after an additional 100 min of incubation prior to resupply.

These studies indicate that prothrombinase and factor IXa formed during an episode of TF-initiated coagulation persist and also that they can function to restart thrombin generation. Complementary studies using our whole blood model have verified the importance of the prothrombinase complex in reinitiating coagulation.[99] Numerous other studies have implicated fibrin bound thrombin as a relatively stable, localized, procoagulant product of TF-initiated coagulation, capable of activating procofactors, cleaving fibrinogen and activating platelets, and thus functioning to propagate thrombus growth.[100–107]

Thus, work from our laboratory and others[100,101,103,104,106,107] has led us to propose a model of hemorrhage control (Figure 6),[98,99] which contrasts with models requiring constant infusions of TF. In this model, two procoagulant compartments emerge as a consequence of the impermeable barrier formed by platelets and fibrin: an extravascular one, isolated from the blood, with quiescent (reactant starved) procoagulant catalysts that can respond immediately if the barrier fails; and a vascular side where the accumulated ensemble of procoagulant catalysts, exposed to flowing blood, continue the process of clot growth. On this side, however, these catalysts are exposed to the active anticoagulant properties of the vasculature that eventually neutralize them, rendering the vascular face of the clot inert. Thus, in this model, hemorrhage control in a healthy vasculature involves not only the formation of an effective barrier and appropriate control of clot growth on the vascular side but also involves the presence of a persisting, TF-independent procoagulant potential on the extravascular side including clot bound thrombin[100,106,107] and the prothrombinase complex.[98,99]

Acknowledgments

Sources of Funding

This work was supported by P01 HL46703 grant from the National Institutes of Health.

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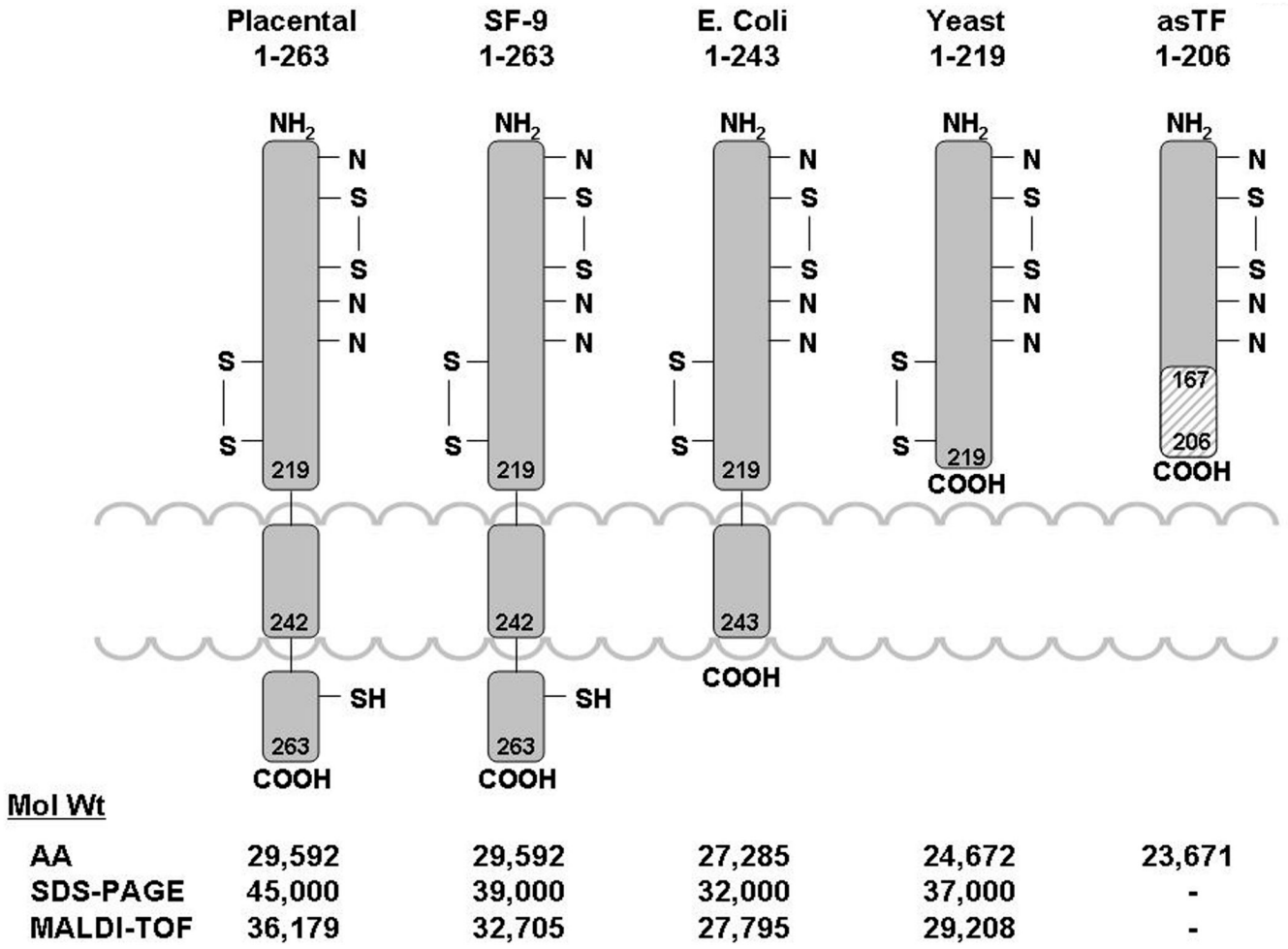

Figure 1.

The structure of various TF species. Indicated molecular weights were determined from the amino acid composition (AA), gel electrophoresis (SDS) and mass-spectroscopy (MALDI-TOF). (This figure was originally published in *Surgery*[108]).

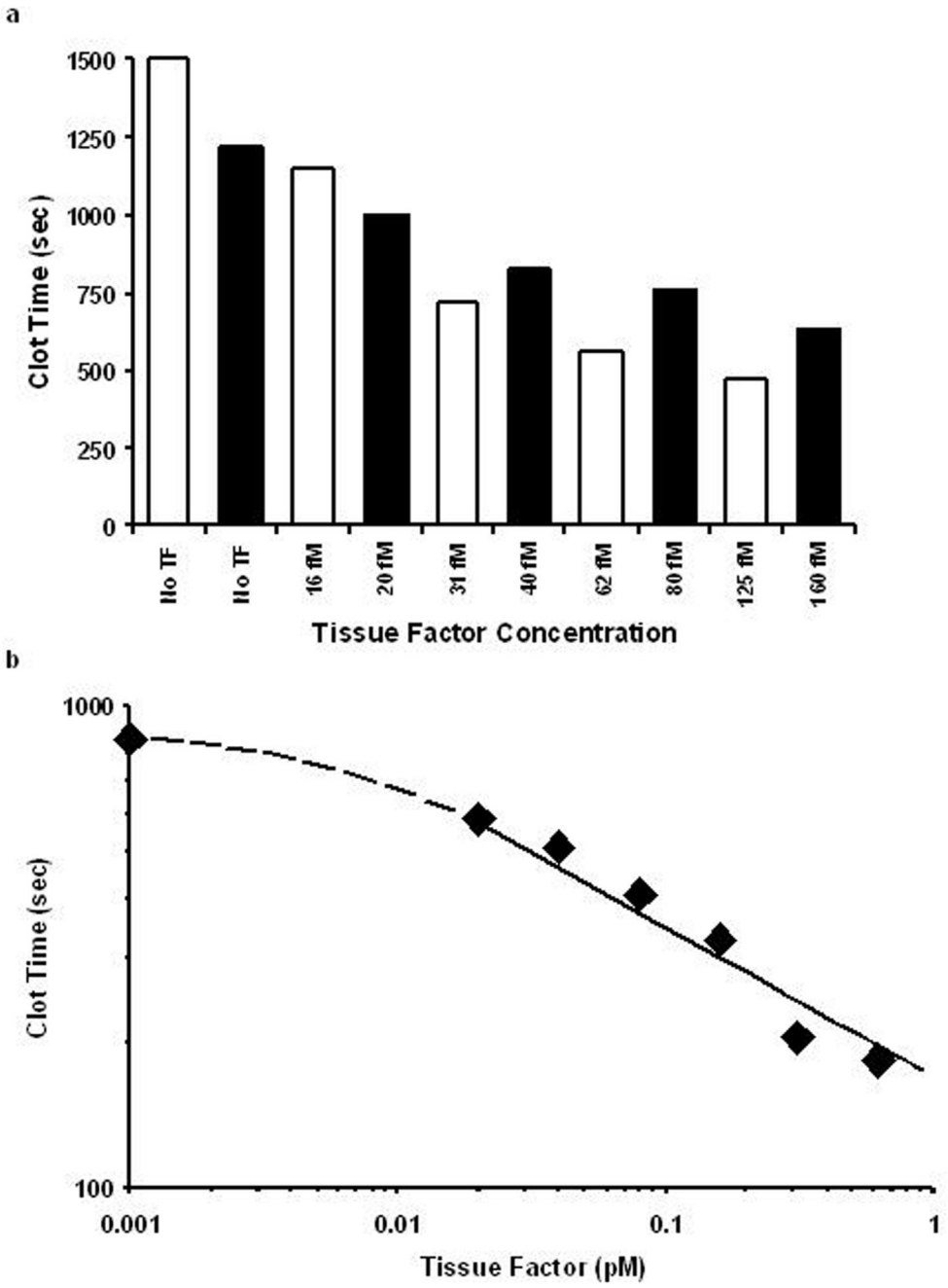

Figure 2.
TF titrations in contact pathway inhibited whole blood (**A**) and plasma (**B**) from healthy individuals. Black and white bars represent two healthy donors. (This figure was originally published in *Blood*[64]).

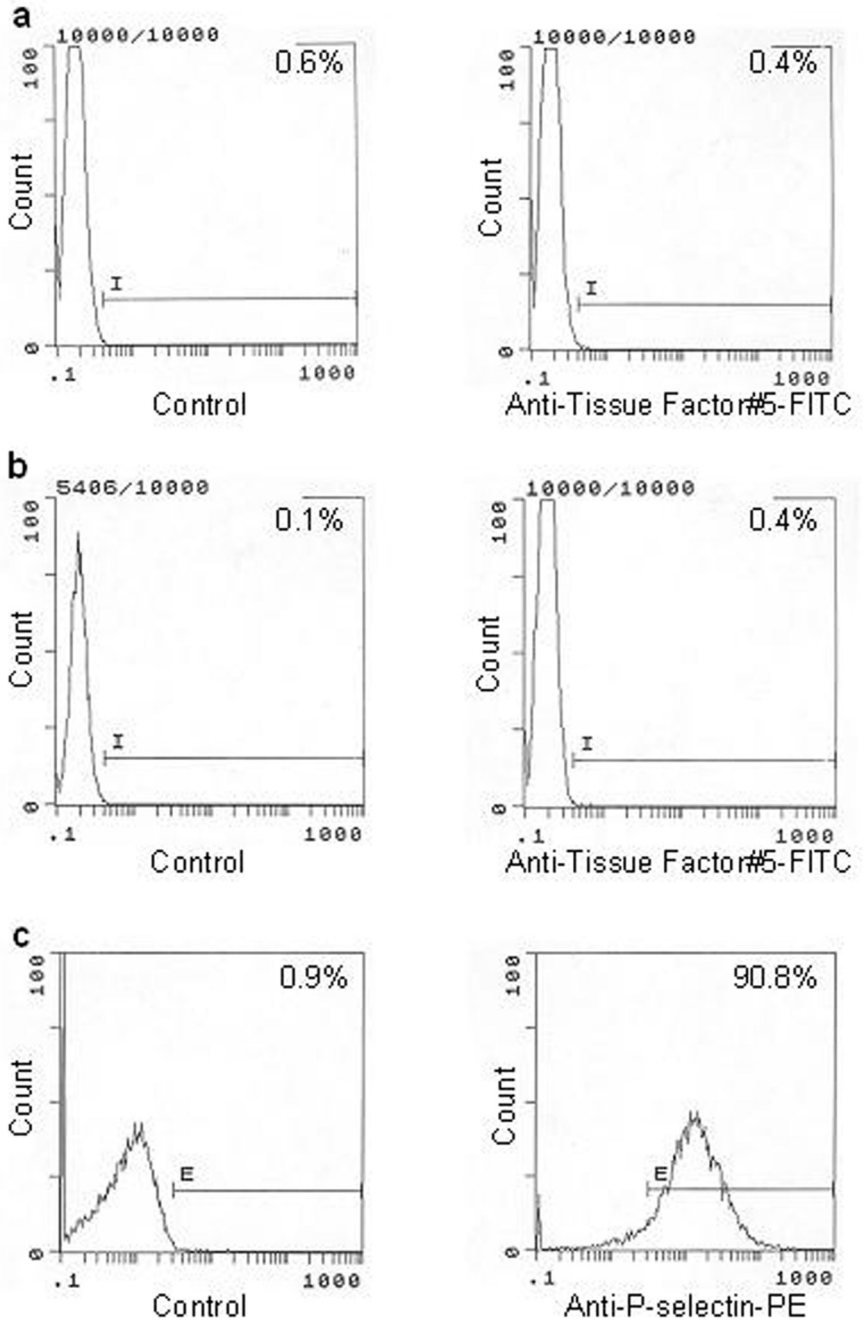

Figure 3.

Flow cytometric analyses of resting and calcium ionophore A23187-treated platelets. Resting (**A**) or A23187-treated platelets (**B**) were immunostained with anti-TF-5 monoclonal antibody. A23187-treated platelets were also treated with an anti-P-selectin antibody (**C**). An irrelevant, isotype-matched mouse IgG was used in the control experiments. (This figure was originally published in *Blood*[64]).

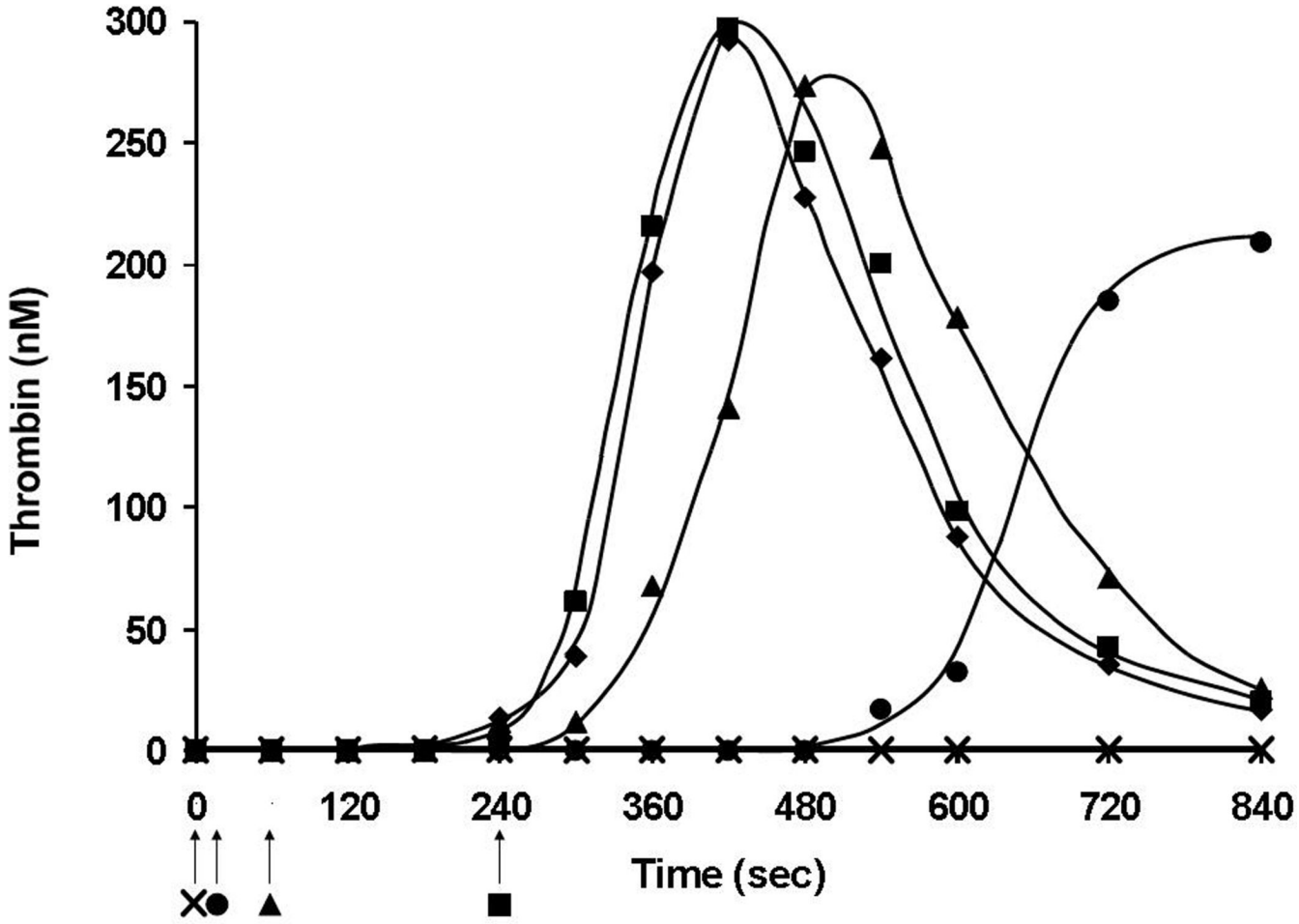

Figure 4.
Termination of TF and factor VIIa activity in TF (5 pM) initiated thrombin generation in Synthetic Coagulation Proteome. Anti-TF and anti-factor VIIa inhibitory antibodies were added at 0 s (✖), 10 s (●), 60 s (▲) and 240 s (■) after the initiation of the reaction or not added at all (◆). Arrows indicate antibody addition time-points. (This figure was originally published in *J Biol Chem*[98]).

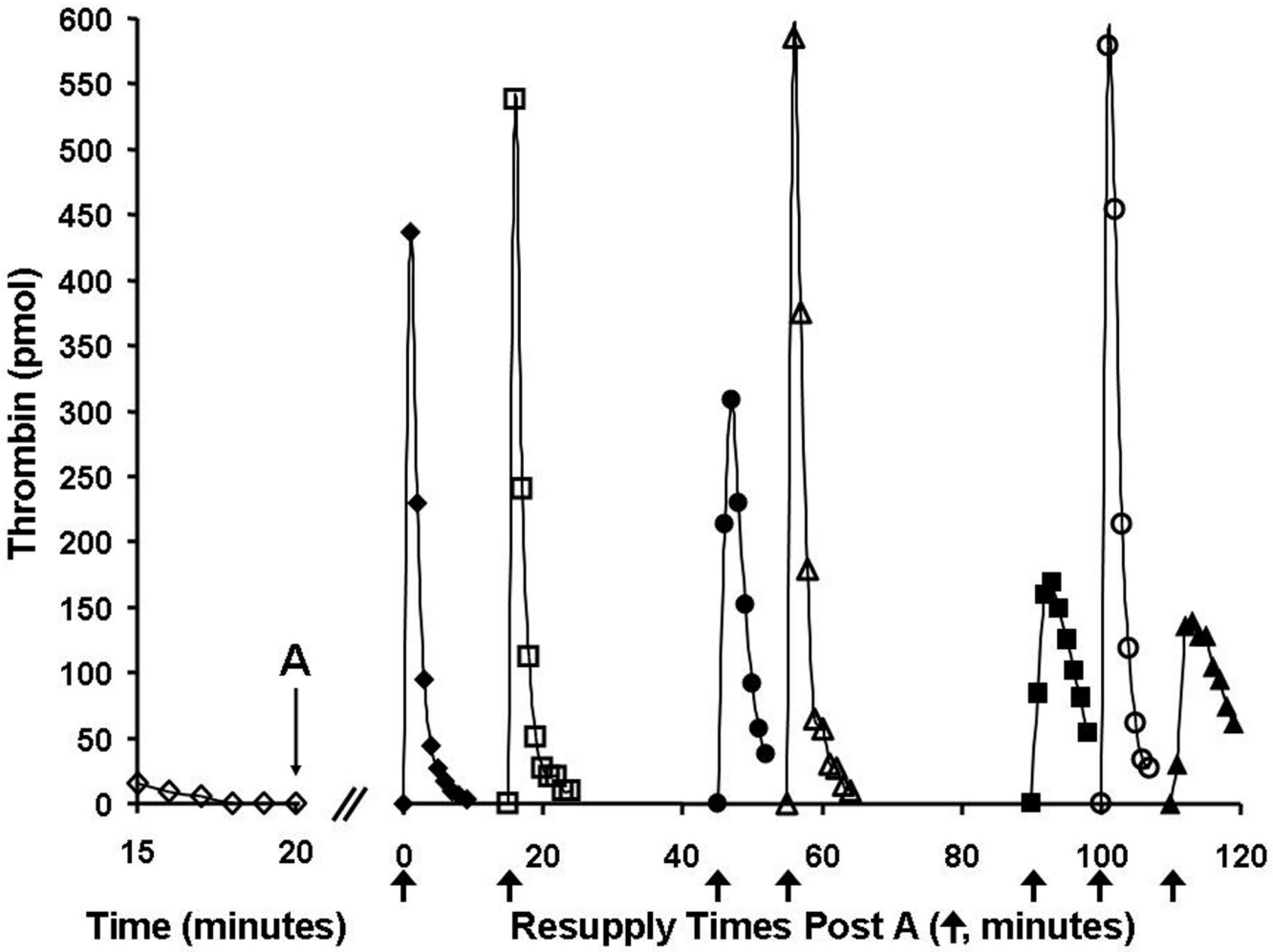

Figure 5.

Resupply of the synthetic coagulation proteome—the effect of factor VIII on the stability of the response. A 5 pM TF-initiated reaction mixture was subdivided after 20 min (A), and the eight separate aliquots subsequently resupplied at different times with an equal volume of a mixture containing 1.4 μM prothrombin/3.4 μM antithrombin/2 μM phospholipids either without factor VIII (closed symbols) or with 0.7 nM factor VIII (open symbols). The resulting time courses of thrombin generation are presented. Resupply with the mixture without factor VIII was conducted immediately (20 min → t=0, (◆) and 45 (●), 90 (■) and 110 (▲) min later. Resupply with the mixture supplemented with factor VIII was conducted at 15 (□), 55 (△) and 100 (○) min after the subdivision of the TF-initiated reaction. Thrombin levels for the final 5 min of the TF-initiated episode are also shown (◊). Thrombin levels are expressed as total picomoles of active thrombin to normalize for the volume change. An arrow indicates the resupply time for each aliquot. Reproduced with permission from Orfeo et al.[99]

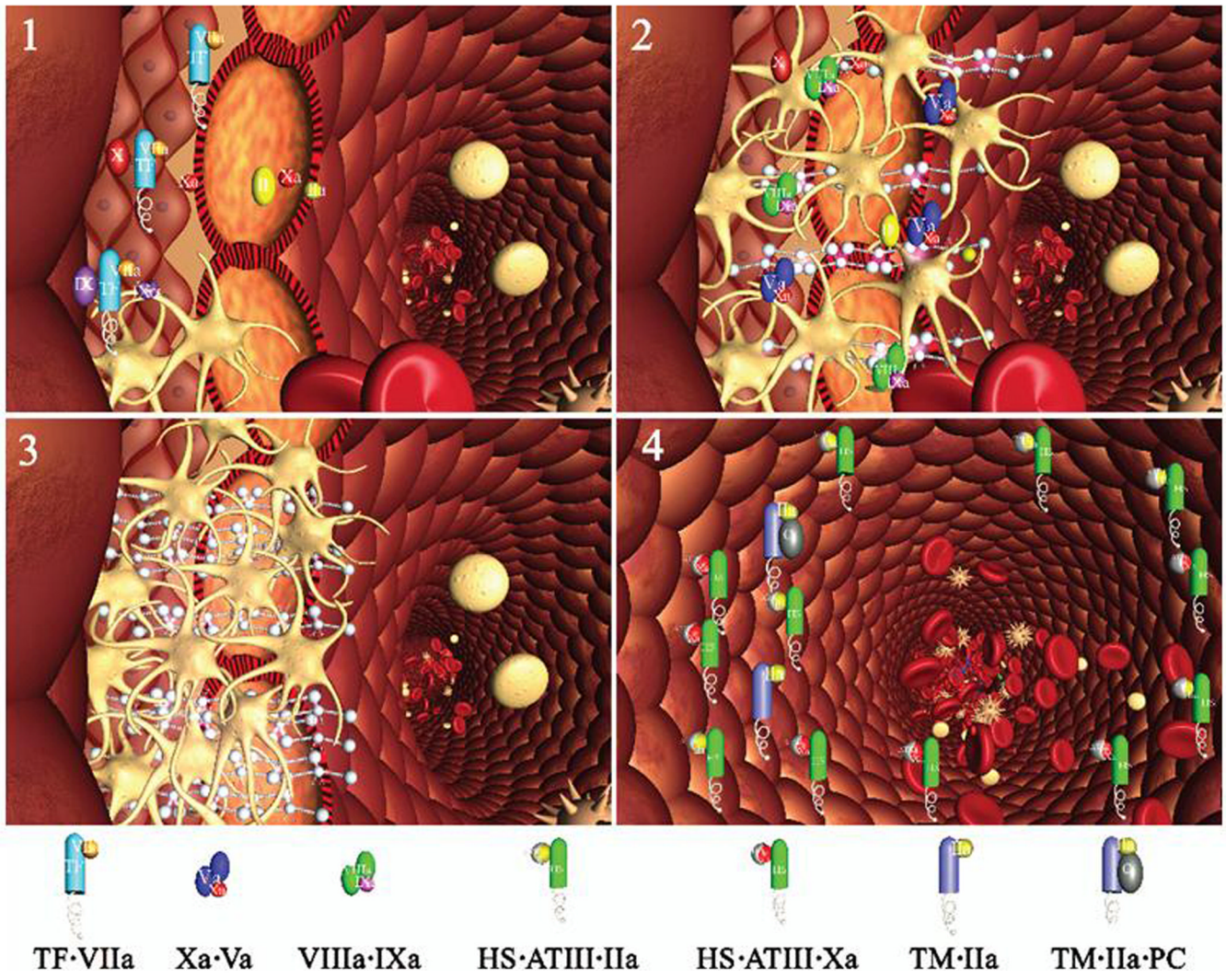

Figure 6.

Schema of a two compartment model of the regulation of TF-initiated blood coagulation. A cross section of a blood vessel showing the luminal space, endothelial cell layer and extravascular region is presented at the site of a perforation. The blood coagulation process in response is depicted in four stages. Tissue factor-factor VIIa complex, TF•VIIa; prothrombinase complex, Xa•Va; intrinsic factor Xase, VIIIa•IXa; ATIII-endothelial cell heparan sulfate proteoglycan complex bound to thrombin or factor Xa, HS•ATIII•(IIa or Xa); protein C bound to thrombomodulin-thrombin, TM•IIa•PC.

Stage 1. Perforation results in delivery of blood, and with it circulating factor VIIa and platelets, to an extravascular space rich in membrane bound TF. Platelets adhere to collagen and von Willebrand factor associated with the extravascular tissue, and TF binds factor VIIa , initiating the process of factor IX and factor X activation. Factor Xa activates small amounts of prothrombin to thrombin that activates more platelets and converts factor V and factor VIII to factor Va and factor VIIIa.

Stage 2. The reaction is propagated by platelet-bound intrinsic factor Xase and prothrombinase with the former being the principle factor Xa generator. Initial clotting occurs and fibrin begins to fill in the void in cooperation with activated platelets.

Stage 3. A barrier composed of activated platelets ladened with procoagualant complexes and enmeshed in fibrin scaffolding is formed. The reaction in the now filled perforation is terminated by reagent consumption attenuating further thrombin generation but functional procoagulant enzyme complexes persist because they are protected from the dynamic inhibitory processes found on the intravascular face.
Stage 4. View downstream of the perforation. Enzymes escaping from the plugged perforation are captured by antithrombin-heparan complexes and the protein C system is activated by residual thrombin binding to endothelial cell thrombomodulin, initiating the dynamic anticoagulant system. These intravascular processes work against occlusion of the vessel despite the continuous resupply of reactants across the intravascular face of the thrombus. (This figure was originally published in *J Biol Chem*[98]).