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Tissue factor and the extrinsic pathway of coagulation during infection and vascular inflammation

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Tissue factor is a potent initiator of blood coagulation. In tissue sections, it has been immunologically demonstrated in cells normally not in contact with circulating blood, and elevated activity has been repeatedly demonstrated in peripheral blood monocytes of patients considered to be at risk for thrombosis. Studies with endothelial cells and monocytes in culture have documented the induction of tissue factor synthesis by biochemical mediators of the inflammatory process. Lytic processes, such as those caused by complement activation or viral infections, increase the tissue factor activity several fold over the basal level of the affected cells. Diminished anti-thrombotic properties of endothelium, and induced tissue factor expression in endothelium and monocytes/macrophages, combined with the increased specific procoagulant activity resulting from cell membrane damage, may endow inflammatory foci with dramatically elevated procoagulant activity. Levels of tissue factor activity at which procoagulant mechanisms escape regulation by natural anticoagulant mechanisms and produce thrombosis remain to be determined.

Introduction

Haemostasis requires a balance between procoagulants, anticoagulants, and fibrinolysis. Coagulation is most effectively initiated via the extrinsic, or tissue factor, pathway[1]. Indeed, the recognition that factor XII (intrinsic pathway) deficiency is not accompanied by a bleeding disorder, that factor IX can be activated by the tissue factor pathway, and that factor XI can be activated by thrombin have led to consideration of the extrinsic pathway as the principal route for physiological initiation of blood clotting[2–6]. A current, but probably temporary, version of the coagulation cascade is illustrated in Fig. 1.

Initiation of coagulation via the extrinsic pathway involves tissue factor (TF), three serine porteases and their zymogens, and the tissue factor pathway inhibitor (TFPI)[5–9]. Tissue factor is a membrane protein which serves as a cofactor for the activation of factor VII (fVII) to factor VIIa and as a cofactor for fVIIa in the activation of factors IX and X to their active protease forms, fIXa and fXa[2,9,10]. Tissue factor is differentially expressed in a number of cell types, being constitutively expressed in some, inducible in others, or not known to be expressed[10–13]. Tissue factor pathway inhibitor can combine with fXa, inactivating the enzyme, and the TFPI-fXa complex is a potent inhibitor of the TF-fVIIa complex[7]. Three pools of TFPI are known. Some of the TFPI circulates in association with plasma lipoproteins, and some is sequestered in platelets, and released following platelet activation. The third pool is released into the circulation in response to heparin infusion, and its source is not yet known[7,14,15]. A variety of cells produce TFPI in vitro, including endothelial cells[16]. The interactions of these proteins in the coagulation system are illustrated in Fig. 1, in which TF is shown to be derived from activated cells, and positive feedback reactions involving thrombin and factor XIa are included.

Cells which constitutively produce tissue factor are not normally in contact with blood, but can be found in perivascular tissues and stroma[12,13,17,18]. Only monocytes and endothelial cells are known to be capable of tissue factor expression of the cells normally in contact with blood[11,19–21]. Extensive in vitro studies have shown that inflammatory agents and mediators of the inflammatory response induce tissue factor expression, as well as other responses, in both monocytes and endothelial cells[19,22–24]. Figure 2 summarizes a number of inflammatory stimuli and cellular responses that have been investigated. Simplified to the least detail, the endothelial cells lose anticoagulant properties, gain procoagulant properties, and express adhesive molecules recognized by platelets and leukocytes[23–25]. Both endothelial cells and adherent monocytes may contribute procoagulant properties by production and expression of tissue factor. The associations among cardiovascular disease, thrombosis, and inflammation, and the procoagulant response of vascular and circulating cells to inflammation-related stimuli have prompted this discussion.

Tissue factor expression by monocytes in vitro

Monocytes freshly isolated from peripheral blood of normal donors, or maintained in teflon in the absence of stimuli, contain essentially no detectable tissue factor activity[11,19]. These same cells, allowed to adhere to glass or plastic, express tissue factor activity over the several hours required for mRNA and protein synthesis[26]. Much greater procoagulant activity is

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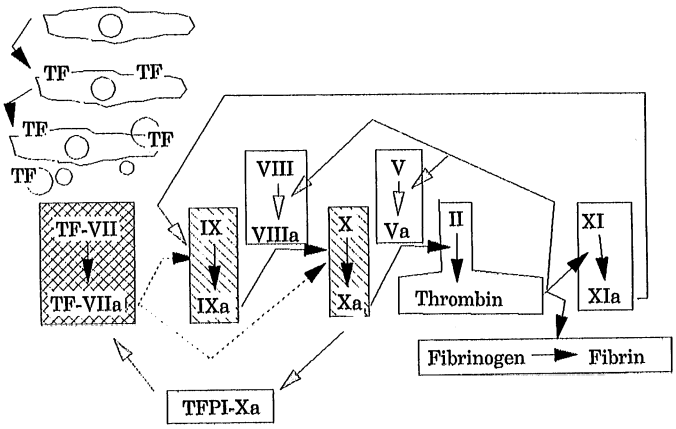

Figure 1 Current model of the coagulation cascade. The 'extrinsic pathway', involving tissue factor (TF), VII, IX, X, and tissue factor pathway inhibitor (TFPI), controls the initiation of coagulation, while only XI of the 'intrinsic pathway' is shown participating in a positive feedback loop. The role of other intrinsic factors (XII, high molecular weight kininogen, and prekallikrein, which can also activate XI) in the coagulation cascade remains to be redefined. TFPI forms a proteolytically inactive complex with Xa, and this complex combines with and completely inhibits TF-VIIa (hatched box). Some factor IX activation continues via the XIa feedback loop, and some X activation continues via IXa-VIIIa. This secondary activation of factors IX and X may persist until the thrombomodulin-protein C system inactivates factors V and VIII.

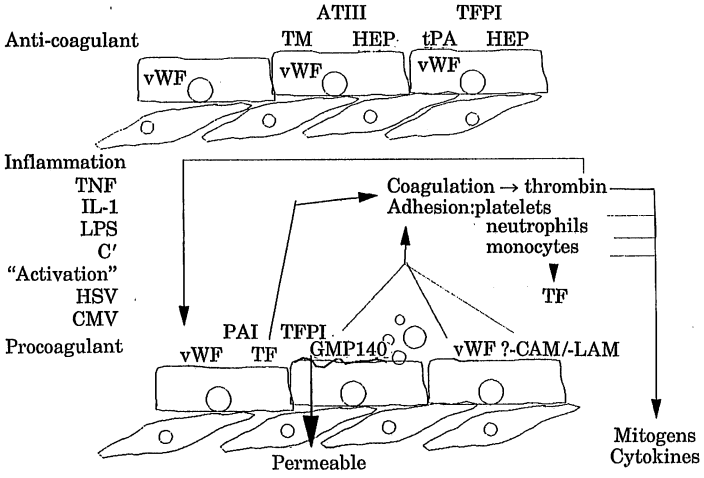

Figure 2 Summary schematic depicting inflammatory mediators and their effects on vascular cells with respect to haemostasis. The symbols depict thrombomodulin (TM), antithrombin III (ATIII), heparin-like proteoglycans (HEP), tissue plasminogen activator (tPA), tissue factor pathway inhibitor (TFPI), von Willebrand Factor (vWF), plasminogen activator inhibitor (PAI), granule membrane protein 140 (GMP140), cell adhesion and leukocyte adhesion molecules (CAM/LAM), tumour necrosis factor (TNF), interleukin-1 (IL-1), lipopolysaccharide (LPS), complement (C'), herpes simplex virus (HSV), and cytomegalovirus (CMV).

expressed, with or without adherence, if the monocytes are incubated with a variety of inducing agents, including bacterial lipopolysaccharide (LPS), products of complement activation, immune complexes, an as yet uncharacterized molecule called monocyte procoagulant-inducing factor (MPIF), and tumour necrosis factor[11,27–31]. The tissue factor response may be augmented by platelets or serum and by specific cytokines, including interferon-gamma and GMCSF[32–35]. Under some conditions, lymphocytes may participate in the induction of monocytes to produce tissue factor. MPIF may be T cell-derived, and monocyte-T cell interactions have been shown to enhance tissue factor production in some experiments[19,22,30,36]. Monocytes

are less responsive to LPS when stimulated after overnight culture, but co-culture with lymphocytes can restore tissue factor induction by LPS[19]. This observation partially explains disagreements in studies which reported different requirements for lymphocytes in tissue factor expression by monocytes.

Tissue factor synthesized by stimulated monocytes accounts for the procoagulant activity of these cells[11]. It is mostly, if not completely, present on the cell surface, and is recognized by specific antibodies as well as by fVIIa[37,38]. As with other cells which express tissue factor, the tissue factor activity is dramatically increased following cell disruption[38,39]. This increased activity is presumed to be due to redistribution of phospholipids in the membrane, the composition of which can profoundly influence the specific activity of tissue factor[40].

Endothelial cell tissue factor in vitro

As with monocytes, most of our knowledge regarding endothelial cell tissue factor expression has come from in vitro studies. 'Healthy' endothelial cells are considered to be anti-thrombotic, expressing thrombomodulin, tissue plasminogen activator, TFPI, proteoglycans which can bind antithrombin III and presumably TFPI, and arachidonate metabolites that inhibit platelet aggregation[7,23,24]. Endothelial cell responses to inflammatory stimuli include loss of many of these anti-thrombotic properties and acquisition of prothrombotic features, illustrated in Fig. 2[23,24,41,42]. The expression of von Willebrand factor and GMP140 can occur relatively quickly, resulting from redistribution of these proteins from storage pools (e.g. Weibel-Palade bodies)[43]. Other responses, such as the loss of thrombomodulin, are slower, requiring down-regulation of existing protein[44,45]. Expression of some new functions, such as tissue factor procoagulant activity, requires transcription and protein synthesis, and proceeds over the course of several hours[44]. Production of TFPI and release into the culture supernatant does not appear to change very much[46]. Endothelial cells produce tissue factor in response to many of the same stimuli that induce tissue factor in monocytes, including LPS, tumour necrosis factor, and thrombin. Additionally, interleukin-1 induces tissue factor expression in cultured endothelial cells[21,47–50].

Epidemiological links between atherosclerosis and herpes viruses (reviewed in[51]) prompted several studies of herpes simplex virus (HSV) and cytomegalovirus (CMV) effects on the properties of cultured endothelial cells. Both HSV- and CMV-infected endothelial cells express elevated procoagulant activity, which can include tissue factor[52–54]. Other procoagulant activities, such as factor X-binding and acceleration of its activation by the viral gC protein, are also expressed on the surface of HSV infected cells[54]. Loss of heparan sulphate proteoglycan from the surface of infected cells, down-regulation of thrombomodulin, and increased adhesiveness toward leukocytes also contribute to the prothrombotic changes associated with viral infection[52,55,56].

Cell–cell interactions

A number of the stimuli discussed above have parallel effects on both endothelial cells and monocytes. There are interesting differences between monocyte and endothelial responses elucidated in vitro. Monocytes can produce interleukin-1, which induces tissue factor expression in endothelial cells, but not in monocytes themselves[24,49]. Co-culture experiments found that the presence of monocytes increased the endothelial cell response to LPS dramatically, and the augmentation was due largely to the production of interleukin-1 beta by the stimulated monocytes[57]. Considering that stimulated endothelial cells express adhesive molecules which increase the adherence of monocytes, and that those same monocytes are capable of producing interleukin-1, tumour necrosis factor and tissue factor, the possiblity of localized high concentrations of cytokines and induced procoagulant activity becomes evident. Further, activated coagulation enzymes generate thrombin, which can also induce tissue factor production[50,58]. Perturbed endothelium has been reported to be more responsive to tumour necrosis factor[59], and lymphocytes, which accumulate in inflammatory lesions, can alter tissue factor production by stimulated monocytes[19].

While endothelial TFPI production is only marginally affected by inflammatory stimuli[46], leukocyte elastase, released by cells adherent to the activated endothelial cells, not only inactivates TFPI but can reactivate TFPI-inhibited tissue factor-fVIIa complexes[60]. Platelets which adhere to and aggregate at these procoagulant and adhesive sites both secrete TFPI, which would result in short-term inhibition of existing tissue factor, and contribute agents which augment long-term production of additional tissue factor[7,61]. These interactions and responses, documented in vitro, clearly implicate cellular expression of procoagulant properties, particularly tissue factor, in and near inflammatory lesions.

Lytic events: complement and viruses

As mentioned above, the tissue factor activity is considerably higher in 'damaged' cells than in their intact counterparts. This is an old observation from results with protease-damaged or freeze–thaw treated cells[62–63]. Tissue factor activity levels approaching those expressed after disruption have been measured under several circumstances relevant to infection and inflammation. Elevation of cytoplasmic calcium by treatment with exogenous calcium and calcium ionophore dramatically and reversibly increases tissue factor activity in the absence of additional tissue factor synthesis[64].

Our laboratories have found similar, though probably irreversible, increases in tissue factor activity following complement fixation on cultured fibroblasts[65]. Similar

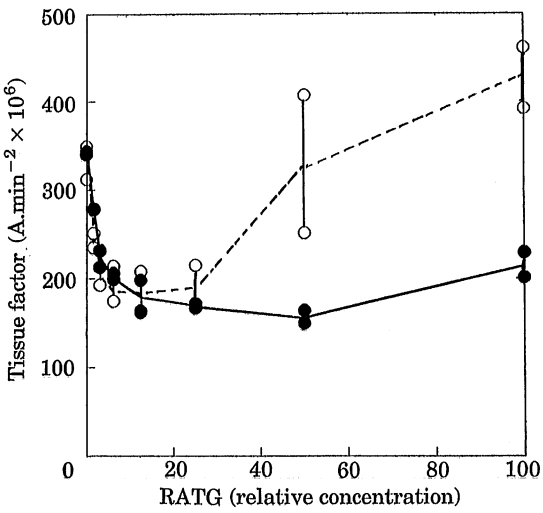

Figure 3 The effect of human complement on TF activity expressed in human fibroblasts (GM5758, NIGMS) was addressed in experiments similar to previous studies which used newborn rabbit serum as a complement source[65]. Freshly drawn human blood was clotted by addition of human thromboplastin (clotted in <5 min), and the clot was allowed to retract at 4 °C for 30 min. The serum was adsorbed with barium sulfate (100 mg . ml^{-1}) to remove vitamin K-dependent clotting factors. This serum was used as a source of complement. The rabbit anti-thymocyte globulin (RATG) was also barium sulfate-adsorbed. The cells, grown in 96-well plates, were incubated with serial dilutions of RATG (1/10 to 1/640, where 1/10 is defined as a relative concentration of 100) at 37 °C for 30 min (100 µl/well). Serum was added to experimental wells (100 µl/well). After 70 min, the wells were gently emptied and rinsed, and TF activity was determined[63,79] using human factor VIIa and factor X (○). Control wells included cells with RATG and no serum (●), or complete reactions with TF-inhibitory monoclonal antibody, HTF1 (not shown)[80].

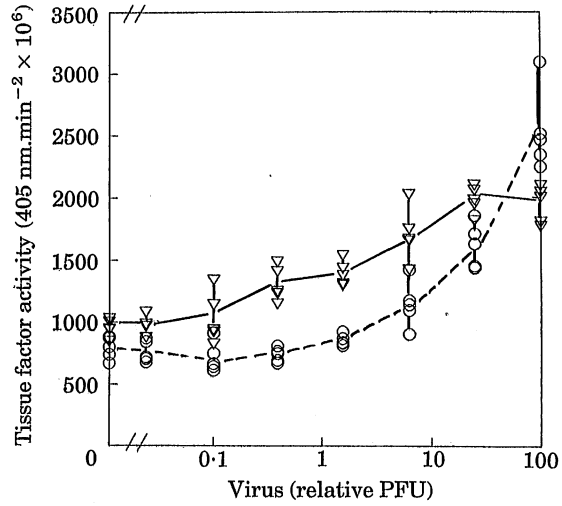

Figure 4 Tissue factor in virus-infected fibroblasts. Human fibroblasts (GM5759A, NIGMS) were grown to near confluency in 96-well plates. In the first experiment, half of the wells (○) were inoculated with serial dilutions of polio virus (PV1-Sabin) and half (▽) with vaccinia (stock dilution 1 to 5 × 10^5 plaque forming units). After approximately 16 h, representative wells were photographed by phase contrast microscopy. At 17 h post-inoculation, the cells were gently rinsed and assayed for tissue factor activity. One well at each virus dilution contained inhibitory anti-tissue factor monoclonal antibody HTF1 to assess non-tissue factor procoagulant activity.

increases in prothrombinase activity have been documented with complement-damaged platelets and cells[66,67]. Our studies of complement damage and tissue factor activity revealed that tissue factor activity preceded, but roughly paralleled cytolysis, implicating membrane damage as the causative event[65]. Antibody-mediated complement fixation on cultured fibroblasts, using human complement, confirmed results previously obtained using rabbit complement. As expected, human complement fixation on human cells gave less dramatic changes in activity (Fig. 3). Although the use of human factors VIIa and X gave higher baseline TF activity, and the added antiserum reduced the measured tissue factor activity, the increased tissue factor activity was dependent on antiserum concentration and complement. HTF1 anti-tissue factor monoclonal antibody inhibited more than 85% of the activity in control wells, indicating that the increased activity was due to tissue factor.

The reports of tissue factor induction and expression in virus-infected endothelial cells, discussed above, led us to investigate the effects of lytic viral infections on the activity of tissue factor in constitutively procoagulant cells. Cultured fibroblasts were infected with polio virus or vaccinia. While both viruses halt synthesis of host cell proteins soon after infection, vaccinia contains a DNA-based genome and has a membrane coat whereas polio has an RNA-based genome and a protein capsid. Infection with either virus produced dose-dependent increases in tissue factor activity, as shown in Fig. 4. Microscopic examination of the cells revealed that the increase in tissue factor activity was qualitatively related to the overt cell damage. Wells inoculated with low virus titres (or none) contained nearly confluent sheets of adherent cells, whereas wells inoculated with higher virus titres contained mostly rounded cells, cell clumps, and debris. Activity present in wells containing HTF1 monoclonal antibody was less than 5% of that in the corresponding experimental wells, confirming that the increased procoagulant activity was due to tissue factor. The time-course experiment, shown in Fig. 5, illustrates the time-dependent increase in tissue factor activity caused by vacinnia, and shows that the activity in this experiment was still increasing at 24 h post-infection. The control wells, lacking factor VIIa or containing HTF1, show that the activity measured was due predominantly, if not exclusively, to tissue factor.

By studying cells which constitutively express tissue factor, we have been able to study changes in tissue factor activity due to cell damage, independent of the expression of new protein induced by complement or viral infection. These observations indicate that the tissue factor activity in regions with extreme cell damage and cytolysis may be even higher than that due to expression of newly acquired tissue factor alone. Regions of cytolysis, due to complement fixation or

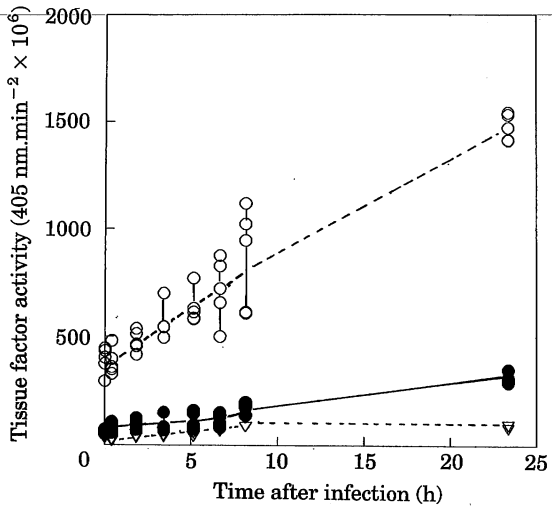

Figure 5 Tissue factor activity increases with time after vaccinia infection of fibroblasts. Fibroblasts (GM5758) were inoculated with vaccinia at timed intervals over 24 h. For each post-infection time period, tissue factor activity was determined in five wells (○), five wells were assayed with added HTF1 (●), and two wells were assayed without factor VIIa (▽).

direct viral effects, may be particularly rich in tissue factor activity in addition to increased prothrombinase activity.

Tissue factor in tissues

Establishing the physiological relevance of the in vitro findings requires correlative findings in patients, patient-derived materials, and animal studies. It has been reassuring that immunohistological studies of tissue factor distribution in human tissues revealed that this potent procoagulant is geographically separated from blood in normal tissues[12,13,17,18]. Tissue factor is notably absent from vascular endothelial cells, circulating blood cells, and placental syncytiotrophoblast. These findings are consistent with belief that tissue factor exposed to blood will initiate coagulation and that normal vessels provide a non-thrombogenic conduit for this vital fluid. Tissue factor distribution in vascular adventitia and organ capsules has been described as a hemostatic 'envelope', providing procoagulant activity in the event that the conduit is compromised[12]. It is interesting that little or no tissue factor antigen has been documented in the intima or media of vessels, yet functional studies have provided evidence of subendothelial tissue factor activity[68,69]. This suggests that methods used to detect tissue factor antigen may not be sufficiently sensitive to reveal very low levels of the protein which are, nevertheless, functionally demonstrable and possibly relevant to hemostasis.

In view of the well established tissue factor production by stimulated endothelial cells in vitro, several investigators have attempted to detect tissue factor in vessels associated with pathological lesions. Immunohistology and in situ hybridization detected tissue factor protein and mRNA in atherosclerotic regions of carotid artery[17]. Within the diseased tissue, tissue factor was present in intimal cells, macrophages, monocytes, and extracellular matrix, but not in endothelial cells. Results of that initial study have recently been confirmed in studies of atherosclerotic sections of human aorta[70]. Studies of human placenta, on the other hand, have provided evidence of tissue factor in endothelial cells of vessels within inflammatory foci referred to as villitis of unestablished aetiology[71,72]. Labarrere and Faulk have used monoclonal antibody HTF1, against human tissue factor, and endothelial cell-specific antibodies in double label-immunofluorescence studies of placentae. Foetal stem vessels in inflammatory foci contained tissue factor 'around endothelium' in one study and coincident with endothelium in the second study. In consideration of the different sensitivities of immunochemical versus functional methods for detecting tissue factor, and the vagaries of immunofluorescence in frozen sections, the presence of endothelial cell tissue factor in tissue specimens remains controversial.

The expression of tissue factor in peripheral blood monocytes isolated from patients with infections is not controversial, perhaps in part due to the ease with which activity, rather than antigen, can be determined with these cells. Monocytes from patients with a variety of infections have been found to have dramatically elevated tissue factor activity[73–75]. What remains unclear from these studies is whether overt changes in haemostasis are associated with the increased monocyte tissue factor, and what level of monocyte tissue factor activity can, or will predictably, result in thrombosis or disseminated coagulation.

Studies with animals indicate that tissue factor does indeed have a significant physiological role. Baboons and rabbits infused with lethal amounts of LPS survived the LPS-induced toxic shock if they were first infused with inhibitory antibodies against tissue factor[76,77]. These results implicate tissue factor induction as an active principal in toxic shock. Separate studies of tissue factor pathway inhibitor found that rabbits immunologically depleted of this specific coagulation inhibitor developed disseminated intravascular coagulation at levels of LPS which produced no such effect in otherwise normal rabbits[78]. These studies strongly support a role for tissue factor induction as part of the inflammatory response in vivo. While both endothelial cells and monocytes produce tissue factor in response to LPS in vitro, the mechanism(s) underlying these in vivo findings remain to be elucidated.

Conclusions

In vitro studies have clearly documented the potential for monocyte and endothelial cell procoagulant responses, both with respect to loss of anti-thrombotic properties and the acquisition of prothrombotic molecules, particularly tissue factor. It remains to be determined incontrovertably whether endothelial cells ever express tissue factor in vivo. On the other hand, tissue

factor is clearly elevated in the circulating monocytes of donors experiencing inflammatory reactions, but the physiological effect, significance, and meaningful levels of the monocyte tissue factor remain to be defined. Tissue factor antigen is widely distributed in human tissues, but its functional activity or potential within those tissues are presumed, not proven. The roles of tissue factor in homeostasis and perturbations of homeostasis caused by infection and inflammation remain to be determined.

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