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Visions & Reflections

The search for a unified theory of coagulation and inflammation

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Coagulation is an essential phenomenon in the living macro-organism. For centuries, theories have been developed by scientists to describe clotting and fibrinolysis and to predict the physiology and pathophysiology of this system. Almost all of these theories, including the so-called cascade model developed by Davie and Ratnoff [1] and MacFarlane [2] in 1964, exhibited the properties of what philosopher Sir Karl Raimund Popper called a good scientific theory: they made definite predictions which could be tested by observation and, possibly, rejected.

Unfortunately for the theories, they were rejected along with many others. To have a theory rejected is hard for scientists who have worked with or, even worse, developed the theory to accept. However, rejection of theories almost always implies significant progress in thinking and results in a new theory which extends the old theory in accomodating a wider range of observations in one model. The result, therefore is both scientific progress and another theory waiting to be falsified. Refuting basic beliefs paves the way for advancement.

The cascade theory described blood clotting as two series (intrinsic and extrinsic) of proteases cleaving and activating the following factor converging at the activation of factor X (FX) to FXa with the following activation of prothrombin (FII) into thrombin (FIIa) and subsequent activation of fibrinogen to fibrin by FIIa. While being useful tool to describe pathologic results in the in vitro coagulation tests prothrombin time (PT) and activated partial thromboplastin time (aPTT), the theory was not consistent with the clinical observations that deficiencies of some proteins in the cascade remain almost nonsymptomatic, whereas lack of others causes severe hemophilia. Additionally, the observation that some proteases not only activate the following factor in the cascade but also factors upstream and in the other pathway disturbed our classical understanding of coagulation as a row of dominoes. The initiator of the extrinsic pathway, the tissue factor/factor VII (TF/FVII) complex, can not only activate FX but also one of the enzymes of the intrinsic pathway, factor IX (FIX). These features, in combination with the observation that factor XI (FXI) can be directly activated by thrombin on the surface of activated platelets in vivo explain why factor XII (FXII), as well as high-molecular-weight kininogen (HMK) and pre-kallikrein (PK) might not be required for successful hemostasis. Inspired by this contradiction, almost 40 years after the cascade model, a cell-based model of coagulation was developed by Hoffman and Monroe in 2001 [3].

In this model, the process of hemostasis is described in three phases: initiation, amplification and propagation. The mainstay of hemostasis initiation in this model is the exposure of a TF-bearing cell to the blood flow. Fibroblasts in the subendothelial matrix and, in sepsis and endotoxin exposure, endothelial cells and blood mononuclear cells rapidly express TF which is an integral membrane protein and does not usually circulate in plasma. However, one possible source of TF in plasma are microparticles (MPs) originating from platelets or granulo-

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cytes. These particles with a size of $<1.0-1.5$ µm and negatively charged phosphatidylserines in the outer leaflet of the membrane bilayer express CD14 and TF and induce marked thrombin generation in vitro. Their physiological and pathophysiological properties remain to be elucidated, but there is some evidence that MPs play a role in cell-to-cell cross-talk. In patients with meningococcal sepsis and in experimental human endotoxinemia, MPs were found to be an important source of TF for the initiation of coagulation [4, 5]. Circulating FVII binds rapidly and tightly to TF. Once complexed with TF, FVII is activated by proteases. The resulting TF/FVIIa complex activates FX as well as FIX. If FXa dissociates from the cell surface, it is immediately inactivated by antithrombin (AT) and tissue factor pathway inhibitor (TFPI). On the cell surface, small amounts of thrombin are generated by FXa, alone or in complex with its cofactor, activated factor V (FVa). These small amounts of thrombin are required for the activation of platelets and factor VIII (FVIII) in the following amplification phase. The key role of FVII, TF and the TF/FVIIa complex in the initiation of coagulation is highlighted by the fact that molecules like active-site-inhibited FVIIa (rFVIIai) as well as TFPI or antibodies against TF or FVII blunt the coagulant response to endotoxin exposure and bacteremia, both in vitro and in vivo [6, 7]. Recently, an inhibitor of TF/FVIIa, recombinant nematode anticoagulant protein (rNAPc2), significantly lowered mortality of Ebola virus infection in monkeys, thereby highlighting the importance of this initiation of coagulation in viral hemorrhagic fever [8].

In the amplification step, exposure of matrix proteins to the plasma leads to the binding of circulating platelets to the site of injury. Platelets also bind to leukocytes once they stop rolling and adhere to the endothelium. The small amounts of thrombin which are generated in the initiation step on TF-bearing cells, which may be fibroblasts in the case of vessel wall injury or endothelial cells or leukocytes in the clinical setting of sepsis, activate the adhering platelets. Once activated, platelets release FV in a partially activated form from their alpha granules to be fully activated by thrombin or FXa. Thrombin molecules, bound to GPIb/IX receptors, among others, will cleave FVIII from von Willebrand factor (vWF) and activate it to FVIIIa. The coagulation process has now moved to the surface of activated platelets which have the procoagulant proteins FVa and FVIIIa bound to their surface ready to facilitate massive thrombin generation. Recent flow cytometry investigations showed that platelets, once they are activated by thrombin and collagen simultaneously, reveal two different subpopulations, of which one, named COAT platelets, express high levels of procoagulant proteins like FV, fibrinogen, fibronectin and vWF on their surface [9]. While the final physiologic significance of COAT platelets needs to investigated, this subpopulation of platelets might play a critical role in initiating platelet-platelet aggregations and thus supporting amplification and the propagation phase in the cell-based model of hemostasis.

To facilitate large-scale thrombin generation, the 'tenase' and 'prothrombinase' complexes are assembled on the surface of activated platelets in the propagation phase. The tenase complex of FVIIIa and FIXa is formed when FIXa moves from the TF-bearing cell where it is activated to the binding receptor expressed on activated platelets. Since FIXa is not rapidly attacked by AT and other plasma protease inhibitors, it can diffuse from TF-bearing cells toward activated platelets without being inactivated. Plasma FXI binds to a specific receptor on the platelet surface and is activated by thrombin. FXIa will activate more FIX to FIXa directly on the platelet surface. Once the FVIIIa/FIXa complex is formed, it activates FX with the resulting FXa complexing directly with FVa to build the prothrombinase complex. This complex will now produce a burst of thrombin necessary to form a hemostatic fibrin clot. Thrombin converts the soluble fibrinogen into fibrin, and activates the fibrin-stabilizing FXIII, plus the most important inhibitor of the coagulation system, protein C (PC), as well as the thrombin-activatable fibrinolytic inhibitor (TAFI). Receptors on the cell surface of the vessel wall and on activated platelets are important in the cell-based model of coagulation, since these receptors restrict thrombin generation to those cells which can assemble the procoagulant coagulation factors on their surface. While TF is the major binding site on the injured vessel wall, the platelet surface provides the receptors for the propagation phase. Thrombin binds to the GPIb/IX receptor, as does vWF, however, the binding sites for the other factors of the tenase and prothrombinase complex have not been identified, although there is strong evidence for their existence.

One of the significant advances of the cell-based model of coagulation is to localize the process of protease activation on the cell surface. On this platform, coagulation factors have been found to be involved in a number of processes other than thrombin generation. As early as the initiation phase, the binding of FVIIa to TF activates intracellular signaling. The structure of TF has close homology to the superfamily of cytokine receptors, especially to the interferon α, β and γ receptors [10]. However, still unclear is whether the short intracellular tail is involved in the signal transduction events or, for which there is more evidence, the TF/FVIIa complex cleaves protease-activated receptor 2 (PAR2) with subsequent signal transduction via phosphoinositide 3 kinase (PI3K). The downstream pathways from PI3K are involved in angiogenesis, cell migration and inflammatory responses. These proinflammatory effects of TF/FVIIa binding were already described in macrophages in 1999 [11], whereas the promotion of cell migration as well as the inhibition

of apoptosis (inhibiting caspase 3 activity) by FVIIa binding to TF was described most recently in cancer cells which over-express TF 1000 fold [11, 12]. In contrast, the binding of rFVIIai to TF impairs experimental metastasis and did not protect cancer cells from cell death as did rFVIIa.

The enormous clinical importance of activation of the inflammatory response through activation of the coagulation system is highlighted by a recent study of Miller and colleagues on the effects of blocking coagulation activation by rFVIIai in acute lung injury (ALI) after intratracheal lipopolysaccharide (LPS) instillation [11, 13]. In this rat model, both ALI and proinflammatory cytokine release, together with NF-κB pathway activation were inhibited by blocking initiation of coagulation with rFVIIai at the time of the LPS insult. Furthermore, rFVIIai given 6 h after the LPS insult also protected the lung from inflammation and ALI. To exclude the possibility that rFVIIai was only protective by blocking the generation of the important downstream product of the coagulation system, thrombin, the experiment was repeated and rFVIIai was replaced by a thrombin inhibitor (Hirulog) given intravenously. Although intra-alveolar thrombin activity was significantly decreased by Hirulog compared to control animals, ALI was not prevented by blocking thrombin in contrast to blocking the TF/FVIIa complex.

Although the initiator, the TF/FVIIa complex is not the only protein linking coagulation and inflammation by intracelluar signaling. FX, once activated to FXa, plays a pivotal role in hemostasis but also activates endothelial cells via the effector cell protease receptor (EPR1), intracellular signal transduction and increased cytokine expression [14]. EPR1, however, does not contain sites sensitive to proteolysis so that the speculation is that the attenuation of cytokine expression by FXa is induced by FXa binding to EPR1 with subsequent cleavage of PAR 1 and PAR2 [15, 16].

In contrast, the FXa inhibitor AT presents anticoagulant as well as anti-inflammatory effects. AT binds to glycosaminoglycans on the surface of endothelial cells and inhibits the adhesion of rolling leukocytes to the endothelial surface. Whereas the anticoagulant effect of AT by inactivating FXa is well known, there is little insight into the anti-inflammatory processes induced by AT. In contrast to the other important inhibitor in the coagulation system, PC, AT inhibits only the release of interleukin (IL)-10, IL-6 and IL-8, whereas the levels of tumor necrosis factor-α (TNF-α) were reported to increase under treatment with recombinant AT in a baboon model of *Escherichia coli* sepsis [17]. One speculation is that anti-inflammatory effects are mediated by prostacyclin which is released from endothelial cells once AT binds to the glycosaminoglycans on their surface. The strong affinity of AT to these heparin-like molecules highlights the importance of the AT/heparin interaction. In the presence of heparin, AT action is restricted to the cofactor function for the anticoagulant effects of heparin [18]. Of interest is that while AT failed to reduce mortality due to sepsis in a large clinical randomized trial, subgroup analysis revealed a clear survival advantage in favor of AT compared to placebo in those patients who did not receive heparin comedication [19].

Once certain amounts of thrombin have been generated by activation of coagulation, a fraction of it binds to thrombomodulin (TM), another transmembrane protein. The thrombin/TM complex is able to activate PC to activated protein C (APC) when PC binds to the endothelial protein C receptor (EPCR). The thrombin/TM complex, parallel to its PC/EPCR interaction, is able to induce antiapoptotic intracellular signaling via the PAR1 receptors [20].

In addition, APC exceeds its anticoagulant (inhibiting the prothrombinase and tenase complex) and profibrinolytic [inhibiting plasminogen activator inhibitor (PAI 1)] properties by initiating intracellular signaling modulation of inflammation. Recent studies have shown that APC initiates signaling through inhibition of the NF-κB pathway, thereby not only decreasing the liberation of proinflammatory cytokines like TNF-α, IL-1β, IL-6 and IL-10 in monocytes as well as in animal models of endotoxinemia and spinal cord injury, but also reducing the expression of CD14 and toll-like receptor 4 (TLR4), the receptors for endotoxin on the surface of monocytes [21–23]. Once liberated, the proinflammatory cytokines, as well as thrombin itself, contribute to the expression of adhesion molecules like ICAM1 and 2 as well as the E-selectin family which stick leukocytes on the surface of the endothelium. Furthermore, APC-induced intracellular signaling inhibits the encryption of TF on the surface of monocytes, thereby closing an obviously important loop back to the initiation step in the cell-based model of coagulation [24].

The hypothesis that the action of APC as a link between coagulation and inflammation might ultimately prove to be more important than its function as an anticoagulant was strengthened by the recent observation that patients with a resistance of FV to APC (FV Leiden mutation) survive severe sepsis better than those who do not have this mutation [25]. Work with mice in the same paper confirmed that generation of enough thrombin to activate the endogenous PC pool and subsequent APC action focused solely on its anti-inflammatory and profibrinolytic properties is associated with increased survival in sepsis. With respect to this clinical observation, there is growing evidence that the model of APC as an anticoagulant protein within the model of coagulation really refers, at least in septic patients, to a side effect. The overall importance of APC in inhibiting the devastating effects of systemic activation of coagulation and infection was presented in a randomized clinical trail showing a significant reduction

in mortality of patients with severe sepsis when treated with APC [26, 27].

Fibrin, the product of thrombin action on fibrinogen, can induce a proinflammatory cytokine response in macrophages [28]. Together with other proteins of the extracellular matrix like fibronectin, type 1 collagen and laminin, fibrin induces intracellular signaling via the activation of protein kinase C (PKC) and activation of mitogen-activated protein kinases (MAPKs) [29]. Interestingly, this signaling seems not to involve the NF-κB pathway.

The other important amendment to the cell-based model of coagulation is the addition of another dimension, a time axis. Full thrombin generation is necessary for the formation of a tight, stable hemostatic fibrin plug. The thrombin generated in a certain amount of time seems to be an important factor influencing the architecture of the fibrin gel formed by conversion of fibrinogen into fibrin by thrombin [30]. Fast conversion initiated by a powerful thrombin impulse results in a tight meshwork made of thin fibrin fibers, whereas a weak impulse with a slower generation of thrombin results in a clot made of thicker and coarse fibers, even if the total amount of thrombin as well as that of generated fibrin are identical. Whereas the first, tight clot is resistant to premature fibrinolysis and able to sustain hemostasis, the latter is easily degraded by plasmin [31].

Clot architecture has been investigated by electron microscopy which gave valuable insight into the three-dimensional structure of the fibrin gel. The clinical observation that compounds like recombinant activated factor VII (rFVIIa) are able to act as a universal hemostatic agent by enhancing the speed of the FII to FIIa conversion highlights the importance of the time axis in the model.

Finally, coagulation and inflammation are actors on the stage set by the unique genetic predisposition of each individual. Numerous mutations have been described to be either protective or deleterious in inflammation and coagulation. In patients with sepsis, the most important genetic variations influencing outcome are the PAI 1 polymorphism leading to a higher concentration of PAI 1 and thereby an inhibited fibrinolysis, and the TNF-α polymorphism which results in a high expression of TNF-α by activated monocytes. Other mutations known to aggravate the outcome of severe sepsis are mutations in the IL6, IL10 and IL1RN genes and in the TLR4, TLR2 and CD14 genes [32, 33]. The importance of the FV Leiden mutation in sepsis has already been mentioned, but mutations in FXIII, the fibrinogen gene as well as the prothrombin mutation have so far mainly been discussed in the context of inherited thrombophilia. In a recent study, the interaction of the FXIII Val34Leu mutation with mutations in the fibrinogen gene was assessed by a combination of electron microscopy studies of fibrin meshwork architecture and penetration experiments through a fibrin gel [34]. These experiments showed that the FXIII 34Leu mutation can either protect from thrombosis (at high fibrinogen concentrations, e.g. induced by an accompanying fibrinogen Aα Thr312Ala mutation, smoking or inflammation) or have harmful effects by forming a tight, fibrinolysis-resistant clot structure at low to normal fibrinogen concentrations. These results demonstrate that combinations of two or possibly more mutations, which alone are potentially debilitating, do not necessarily lead to a cumulative effect but may also counterbalance each other.

The cell-based model of coagulation was a significant leap forward in our understanding of the process of blood clotting. However, in contrast to its predecessor, the cascade model, the cell-based model of coagulation is unlikely to remain unchanged for the next 40 years. The model is already evolving from a static to a very dynamic understanding of generating fibrin and, most probably, coagulation and inflammation as two independent concepts will not survive the next 5 years. The close links between coagulation and inflammation are not only present at the molecular level but also at the bedside where patients almost always demonstrate a simultaneous activation of both systems (fig. 1). Almost all procoagulant factors in coagulation have complex proinflammatory properties, whereas the important inhibitors in the coagulation system show pronounced anti-inflammatory effects in vivo and in vitro.

Once the two models are unified in a combined theory of inflammation and coagulation (fig. 2), we shall be in a better position to understand the interaction at the interface of coagulation and inflammation. Progress of the model into a unified theory will finally allow predictions as to how interventions within the system will modify the network of interactions both internally and externally. This knowledge will take us the further step needed to generate hypotheses to be tested in clinical trials.

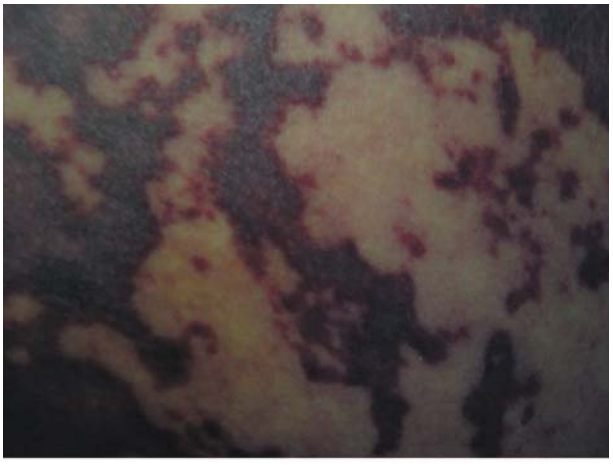

Figure 1. Skin lesions of a patient with maximal activation of coagulation (purpura fulminans) due to severe meningococcal sepsis. Note the inflammatory red border area surrounding the microcirculatory thrombosis with tissue necrosis, indicating the simultaneous activation of both systems, coagulation and inflammation.

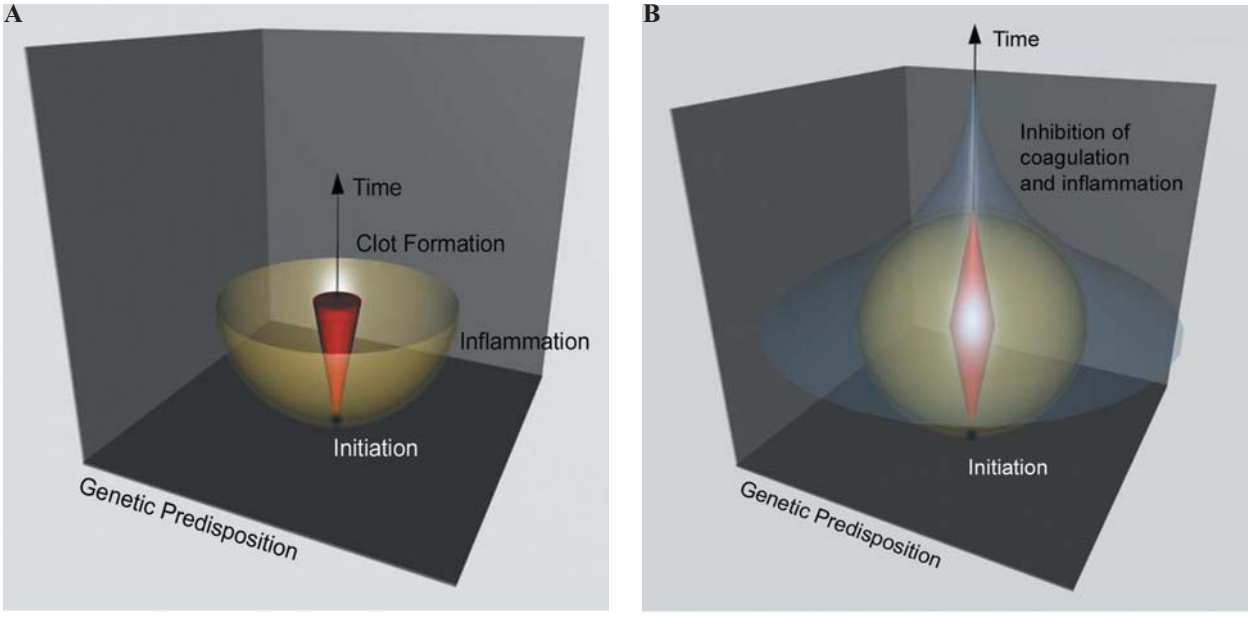

Figure 2. In this illustration, coagulation is expanding in a cone depending on the inital thrombin activation impulse on a time axis against the background of an individual genetic profile, here displayed as a box. The cone of the activation of coagulation is surrounded by the corona of the simultaneous activation of inflammation. When thrombin generation reaches its maximum, inhibitors (PC and AT) shut down both coagulation and inflammation simultaneously; however, clinically, the effect on inflammation seems to be even more important than the anticoagulant function.

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