MECHANISMS IN HEMOSTASIS AND THROMBOSIS- III

TISSUE FACTOR, PLATELETS AND POLYPHOSPHATE: NEW WAYS TO MODULATE BLOOD CLOTTING AND FIBRINOLYSIS

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Background. Blood clotting is triggered when the integral membrane protein, tissue factor, binds the serine protease, factor VIIa, to form the TF:VIIa complex on cell surfaces. Membrane-anchoring is required for tissue factor function, since the isolated ectodomain (sTF) is orders of magnitude less active. In addition, dense granules of human platelets were recently shown to contain (and secrete) inorganic polyphosphate. Polyphosphate is also abundant in many unicellular organisms, including human pathogens. Additional studies in our laboratories have therefore focused on how polyphosphate modulates blood clotting. Aims: Develop new methods for assembling and studying the TF:VIIa complex on membrane surfaces. Understand how platelet polyphosphate modulates blood clotting and fibrinolysis. *Methods.* We reasoned that adding a hexahistidine tag to the C-terminus of sTF would allow it to bind tightly to liposomes containing nickel ions that are bound to metal-chelating lipids. With regard to polyphosphate, numerous methods were employed to identify the point(s) in the blood clotting cascade at which it acts. Results: Reversible attachment of sTF to metal-chelating lipids in liposomes restored 100% biologic activity. Interestingly, metal-chelating lipids also potently activated the contact pathway of blood clotting. Polyphosphate was found to accelerate blood clotting and inhibit fibrinolysis by acting at two points in the clotting cascades: it activated the contact pathway of blood clotting, and it greatly accelerated the proteolytic conversion of factor V to Va. The latter resulted in complete abrogation of the anticoagulant effect of tissue factor pathway inhibitor (TFPI), and delayed fibrinolysis in a manner dependent on thrombin-activatable fibrinolysis inhibitor (TAFI). Conclusions: These studies identify novel ways to regulate and manipulate the blood clotting system, and may provide new insights into control of blood clotting and fibrinolysis in hemostasis, thrombosis and host-pathogen interactions.

THROMBIN GENERATION: AGONISTS AND ANTAGONISTS

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It is well appreciated that hemostasis is initiated at wound sites due to exposure of tissue factor and some components to which cells adhere. In recent studies, the concept has been forward that thrombus growth at such sites is enhanced by microparticles, probably derived primarily from leukocytes. These particles contain tissue factor and also adhesion molecules and thus can *pile on* to the growing thrombus. In addition to clot development at a wound site, the process can be stimulated by inflammatory mediators, some of which are derived from adipocytes and atherosclerotic lesions. Furthermore, such mediators elevate tissue factor expression on leukocytes and facilitate microparticle release, both of which would be expected to increase the rate and extent of thrombus growth. Fortunately, in healthy individuals, thrombus growth is limited by natural anticoagulants, many of which function primarly on the vasculature, particularly on the microvasculature. Expression of many of these anticoagulants is reduced by inflammation, ischemia or atherosclerosis, thus likely contributing to the development of a hypercoaguable state. To correct hypercoaguable situations, anticoagulants are employed. Coumadin and heparin are standard currently, but synthetic direct thrombin and factor Xa inhibitors are now in development. From *in vitro* considerations, one would predict thrombin inhibitors would have a narrow therapeutic window. In reality, excluding liver toxicity, they appeared to behave in a relatively predictable and reasonably safe fashion clinically. One can explain this apparent discrepancy through consideration of how the reversible thrombin inhibitors probably function *in vivo*. Thrombin formed in the large vessels would normally be inhibited by antithrombin or participate in clot formation. While thrombin is inhibited with the reversible thrombin inhibitor, neither clot formation nor thrombin inhibition by antithrombin can occur. As a result, such thrombin has an increased opportunity to migrate to the microcirculation, at which time it becomes bound primarily to natural anticoagulants like thrombomodulin or vascular heparin-like molecules. Upon dissociation of the inhibitor, the bound thrombin would elicit an anticoagulant response and be rapidly inactivated. Assuming this model explains the mechanisms through which thrombin inhibitors function *in vivo*, the model predicts that efficacy of the thrombin inhibitors would be reduced and their predictable behavior. In contrast, factor Xa inhibitors have a much better predicted therapeutic window and, with our current understanding, lack the complex regulatory machinery involved with thrombin. Thus, I would predict that factor Xa inhibitors will ultimately prove superior to thrombin inhibitors.

FACTOR V: A JANUS-FACED ACTOR OF BLOOD COAGULATION

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The generation of thrombin by the prothrombinase complex constitutes a crucial step in haemostasis, thrombin being important for amplification of blood coagulation, fibrin formation and platelet activation. The activated form of coagulation factor V (FVa) is an essential cofactor to the enzyme-activated factor X (FXa), FXa being virtually ineffective in the absence of its cofactor. Besides its procoagulant potential, intact FV has an anticoagulant cofactor capacity functioning in synergy with protein S and activated protein C (APC) in APC-catalyzed inactivation of FVIIIa. The expression of both pro- and anticoagulant cofactor functions of FV is dependent on proteolysis, procoagulant FVa being the result of thrombin- or FXa-mediated proteolysis, whereas the expression of anticoagulant FV activity depends on APC-mediated proteolysis of intact FV. Thus, FV has the potential to function in both pro- and anticoagulant pathways, its functional properties being modulated by proteolysis exerted by pro- and anticoagulant enzymes. The procoagulant activity of FVa is down regulated by APC together with its cofactor protein S. In fact, regulation of thrombin formation proceeds primarily through the up- and down regulation of FVa cofactor activity and failure to control FVa activity may result in either bleeding- or thrombotic complications. A prime example is APC resistance, which is the most common genetic risk factor for thrombosis. It is caused

by a single point mutation in the FV gene (FVLeiden) that not only renders FVa less susceptible to the proteolytic inactivation by APC but also impairs the anticoagulant properties of FV. The presentation will give a description of the dualistic character of FV, a discussion about structure-function relationships of FV, and the details of a recently created model of the prothrombinase complex.

THROMBIN SIGNALING IN HEMOSTASIS AND THROMBOSIS

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Thrombin is a critical effector of hemostasis and thrombosis. In addition to mediating fibrin formation, thrombin activates platelets and other cells via G protein-coupled protease-activated receptors (PARs). PARs are necessary for activation of human and mouse platelets by thrombin, and recent studies in knockout mouse models suggest that PARs and fibrin formation can together account for the importance of thrombin in hemostasis. Studies in a variety of mouse models of thrombosis also suggest that thrombin signaling via PARs is particularly important in settings in which propagation of a thrombus is required and less important for the formation of extramural and juxtamural thrombi. These results raise a number of interesting questions. What accounts for initial platelet activation in juxtamural thrombi in the absence of thrombin signaling? How is the thrombin that normally activates platelets at a distance from the vessel wall generated? How does thrombin activation of platelets interact with thrombin generation and fibrin formation? These and other questions will be discussed. In addition to activating platelets, PARs trigger responses to coagulation proteases that are potentially both proinflammatory and cytoprotective in endothelial cells and other cell types. Thus, it has been tempting to hypothesize that PARs may help orchestrate inflammatory and/or cytoprotective responses in the setting of disseminated intravascular coagulation in endotoxemia. Studies that attempt to define the roles of PARs in a mouse model of endotoxemia will be presented.

MECHANISMS IN HEMOSTASIS AND THROMBOSIS - IV

PLATELETS AND ATHEROTHROMBOSIS: NEW MOLECULAR INSIGHTS

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It has been known for many years that platelets play an important role in hemostasis and thrombosis. Recently, however, it has become evident that platelets also have relevant functions in inflammation. Thrombosis and inflammation are intrinsically linked processes sharing several key molecular mechanisms and in this contest platelets represent an important linkage between inflammation, thrombosis and atherogenesis. Although it was previously thought that platelets were relatively inactive cells, it is now known that this is not the case. Platelets secrete and express a large number of substances that are crucial mediators of both coagulation and inflammation and, as recently recognized, they synthesize proteins from constitutive mRNAs such as IL-1β, Bcl-3, PAI-1 and also Tissue Factor (TF). TF is the key initiator of the blood coagulation cascade; it binds factor VIIa resulting in activation of factor IX and factor X, ultimately leading to fibrin formation. Over the last decade it has become clear that in addition to its well established role in coagulation, TF participates in other cellular processes including inflammation and is involved in the pathogenesis of cardiovascular diseases such as acute coronary syndromes. It is worth mentioning that functionally active platelet-associated TF is significantly higher in unstable angina compared to stable angina patients. Recently, an alternative spliced form of TF (asTF) has been discovered, which is soluble, circulates in the blood and exhibits procoagulant activity. A consistent expression of asTF mRNA levels has been observed in linfomonocytes from patients with acute coronary syndromes compared to controls. These data provide evidence that different TF mRNA isoforms present in linfomonocytes and platelets of ACS patients can contribute to the hypercoagulability associated with the disease. In view of the inflammatory and thrombotic nature of cardiovascular complications, interruption of the coagulation-inflammation cycle is of great interest in cardioprotection.

ENDOTHELIAL PROGENITOR CELLS FOR VASCULAR REPAIR AND TUMOR ANGIOGENESIS

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Evidence supports the existence of a common hematopoietic and endothelial stem cell (hemangioblast) during embryonic development, and suggests that cells with hemangioblast activity perisist into adult life in humans. Asahara *et al.* first hypothesized that endothelial progenitor cells (EPCs) contribute to vessel production. EPCs can be assessed by their immunophenotype or capability to form endothelial colonies *in vitro* (CFU-End). Immunophenotype identification of EPCs relies on co-expression of specific cell-surface proteins, in particular endothelial markers, including CD34 and VEGFR-2 (KDR), and the human stem cell marker CD133. Lower numbers of EPCs were reported in patients with coronary artery disease compared to healthy controls and in men free of a history of clear atherosclerosis an