Myocardial infarction and the balance between fibrin deposition and removal

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When fibrin deposition and removal are properly balanced, the organism is protected from both a catastrophic loss of blood at the site of injury and the inappropriate loss of fluidity within the vascular system. When these activities are not properly balanced, however, severe bleeding or thromboses can occur. Myocardial infarction is a common and morbid consequence of the latter. The thrombin/thrombomodulin complex plays an essential role in regulating this balance because it generates both an anticoagulant substance, activated protein C, and an antifibrinolytic substance, activated TAFI (thrombin activatable fibrinolysis inhibitor, also known as plasma carboxypeptidase B or carboxypeptidase U). Thus, the coagulation and fibrinolytic cascades are explicitly linked by virtue of thrombin catalyzed activation of TAFI, either by the thrombin/thrombomodulin complex or, in the absence of thrombomodulin, by the massive amounts of thrombin generated through the factor XI-dependent pathway after clotting. Some potential targets for diagnosis, prognosis and therapy related to the balance between fibrin formation and removal include: development of a convenient global assay for plasma fibrinolytic potential; an assay for plasma or urine thrombomodulin that had been oxidized at methionine 388 and thereby has lost its capacity to stimulate activation of protein C but not TAFI; an assay for activated TAFI; discovery of a means for tapping the tremendous potential of the vasculature to acutely release tissue-type plasminogen activator; and an assessment of the potential role of polymorphisms in the TAFI gene which might influence TAFI levels or the properties TAFIa. In addition, a much fuller and quantitative understanding of the properties of the coagulation and fibrinolytic cascades is needed in order to optimize diagnosis, prognosis and therapy in disorders such as myocardial infarction that are related to the balance between fibrin formation and removal.

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The need for fibrin formation and removal

The mammalian organism is faced with the challenge of eliminating the fluidity of blood at the site of injury, while maintaining its fluidity elsewhere. In part, these nearly incompatible events are accomplished through the tightly regulated deposition and removal of fibrin. The deposition of fibrin occurs through the action of the clotting enzyme thrombin, which is generated by activation of prothrombin through the coagulation cascade. The thrombin-catalyzed removal of fibrinopeptides A and B from the Aα and Bβ chains of fibrinogen allows the modified fibrinogen (fibrin) molecules to spontaneously polymerize into an immense, three-dimensional polymer. This causes the blood to gel, thus forming, along with entrapped red cells and platelets, the familiar blood clot. The removal of fibrin is accomplished through the action of the enzyme plasmin, which is activated from its precursor, plasminogen, through the fibrinolytic cascade. Plasmin catalyzes proteolysis in the α, β, and γ chains of fibrin, yielding a family of soluble fibrin degradation products, thereby rendering the blood clot soluble and restoring blood flow[1-3].

The regulation of the balance between fibrin deposition and removal ultimately depends on the regulation of the balance between the activities of the coagulation and fibrinolytic cascades and the generation and inhibition (or removal) of thrombin and plasmin. When these are properly balanced, the organism is protected, in the event of injury, from both a catastrophic loss of blood and an equally catastrophic loss of flow within the vascular system. When these are out of balance, however, pathological circumstances prevail, characterized by tendencies to either bleed or thrombose. Myocardial infarction is a common and morbid manifestation of the latter.

The activities of the coagulation and fibrinolytic cascades are generally latent, but are profoundly up-regulated when necessary. Their potentials are remarkably impressive. The coagulation cascade, for example, has the potential to generate, when

fully activated, a transient explosion of thrombin at a level of approximately 1500 nM[4]. Since thrombin at a 10 nM concentration will cause blood to clot in about 15 s, completely unregulated activation of the coagulation cascade would cause blood to clot in less than 3 s, and the polymerization of fibrin would be the rate limiting step[5]. Thus, within the blood clotting cascade, the latent potential exists to kill its host within seconds. Fortunately, this does not ordinarily happen. The potential of the fibrinolytic cascade is equally impressive. This was demonstrated, for example, by Giles et al.[6], who infused chimpanzees over a 30 s period with varying doses of a mixture of procoagulant phospholipid and factor Xa. At the relatively higher doses (sufficient to cause plasma to clot in 5 s), the combined procoagulant caused the entire fibrinogen content of the recipient to be converted to fibrin in less than 1 min. In addition, the platelet count decreased from normal to immeasurably low values over the same interval. These events were not catastrophic for the animals, however, because an equally potent fibrinolytic response was mounted. Two min after the infusion, the endogenous tissue-type plasminogen activator (t-PA) level had increased approximately 500 fold and soluble fibrin degradation products stoichiometric with lost fibrinogen were circulating. In addition, within 15 min the platelet count had returned to normal. Within 1 or 2 days all hemostatic parameters returned to normal. In spite of this dramatic insult, healthy animals survived it without apparent ill effects. Two of the older animals, however, did not survive this insult. They died virtually instantly with symptoms consistent with those of an acute heart attack. These experiments demonstrated *in vivo* the remarkable potential of both the coagulation and fibrinolytic systems, and in rare cases the catastrophic outcome of excess and acute fibrin deposition.

Thrombin/thrombomodulin, protein C, thrombin activatable fibrinolysis inhibitor and the intrinsic pathway of coagulation in the balance between fibrin formation and removal

The coagulation and fibrinolytic cascades have numerous sites at which their activities are regulated. One which is particularly notable involves the thrombin/thrombomodulin complex[7]. When thrombin binds thrombomodulin on the endothelial cell surface, its properties are changed in a fundamental manner. It loses its procoagulant function and adopts instead an anticoagulant function. This is achieved through the activation of protein C to the anticoagulant enzyme, activated protein C. The latter effectively shuts down thrombin generation by inactivating factors Va and VIIIa of the coagulation cascade. The physiologic significance of this pathway was established by the ability to rescue a small child with a genetic protein C deficiency from certain thrombotic death by supplying exogenous protein C[8].

Recently, another role of the thrombin/thrombomodulin complex has been disclosed[9-11]. It catalyzes activation of a plasma zymogen known as thrombin activatable fibrinolysis inhibitor (TAFI). This zymogen is also known as plasma procarboxypeptidase B or procarboxypeptidase U[12,13]. When cleaved at arginine 92 it generates TAFIa, a carboxypeptidase B-like enzyme. This enzyme removes the carboxyterminal lysine and arginine residues from plasmin-modified fibrin during the process of fibrinolysis[14]. It consequently prevents the positive feedback that normally occurs during fibrin-stimulated plasminogen activation, and thereby attenuates fibrinolysis. It also can be activated sufficiently to suppress fibrinolysis by thrombin in the absence of thrombomodulin, when thrombin is at the very high levels that occur transiently in plasma after clotting[15]. Activation by this route occurs in normal plasma, but not in plasmas deficient in factors VIII, IX or XI of the intrinsic pathway. *In vitro* work has shown that when clotting in plasma is initiated by the extrinsic pathway, in the presence of t-PA, subsequent fibrinolysis occurs 3-4 times sooner in plasmas deficient in factors VIII, IX or XI compared to normal plasma[15,16]. The hypothesis has been proposed that bleeding in hemophilia is more a consequence of premature lysis of clots than of failure to produce a clot[16]. If this turns out to be the case, the notion that the final outcome (clotting or bleeding) is highly dependent on the balance between fibrin deposition and removal will be substantially supported.

Because both protein C and TAFI are activated by thrombin, and particularly the thrombin/thrombomodulin complex, an explicit molecular connection exists between the coagulation and fibrinolytic cascades. Activation of the coagulation cascade, therefore, suppresses the activity of the fibrinolytic cascade. This suppression perhaps helps to stabilize an appropriately formed clot. The activity of TAFIa does not appear to be regulated by an opposing inhibitor. Instead, the enzyme is intrinsically unstable and, at body temperature, has a half life of 5 to 10 min[17]. Thus, its down-regulation appears to be dependent on the intrinsic instability of the enzyme.

That the TAFI pathway functions in an antifibrinolytic capacity *in vivo* is supported by several lines of experimentation. In a canine model of arterial thrombolysis, a transient carboxypeptidase B-like activity (TAFIa?) appeared coincident with infusion of t-PA[18]. Its level correlated positively with the time to achieve reperfusion. In other animal models of either arterial or venous thrombosis, the apparent potency of t-PA was enhanced 2-3 fold when a TAFIa inhibitor was co-infused[19,20]. In addition, spontaneous lysis of indwelling clots was promoted by an antibody which inhibits factor XIa[21], which would be expected to suppress the intrinsic pathway-mediated activation of TAFI.

The structural elements of thrombomodulin required for activation of TAFI are similar, but not identical, to those required for the activation of protein C[22,23]. This also holds for the structural elements of thrombin required for the two reactions[24,25]. Particularly striking is the fact that methionine 388 of the small peptide which joins the fourth and fifth epidermal growth factor-like domains of thrombomodulin is required for protein C, but not TAFI, activation[23]. When this residue is oxidized, activity with protein C is selectively lost. This residue can be oxidized by activated neutrophils *in vitro*[26], which raises the possibility that *in vivo* an inflammatory *milieu* could cause inhibition or elimination of the anticoagulant function of thrombomodulin, but not its antifibrinolytic function, thereby substantially tipping the balance in favor of fibrin deposition. This might contribute to thrombosis or disseminated intravascular coagulation often accompanying sepsis or trauma.

The potential for "all or none phenomena" in the coagulation and fibrinolytic cascades

The coagulation and fibrinolytic cascades both exhibit remarkable complexity. They are characterized by multiple zymogen to enzyme conversions that take place within a bath of inhibitors. They also show positive feedback in numerous steps, and down-regulation by negative feedback loops. In addition, they can be triggered by transient stimuli[1].

In general, systems which can be transiently stimulated, that have positive feedback, and are subject to inhibition can display exotic phenomena known as thresholding, priming and amplification. If a "stimulus" is applied to such a system, a "response" will occur (i.e. thrombin or plasmin generation in case of coagulation or fibrinolysis). The response will be sustained as long as the stimulus is present. If the magnitude of the response is below some threshold value, the response will decay through the action of the inhibitors when the stimulus is removed. If, however, the response is above the threshold value, the kinetics of response occurrence through positive feedback will overcome the kinetics of inhibition, and the response will continue to evolve, even in the absence of the stimulus. In this latter case, the response will continue to grow until a new level is obtained at which the kinetics of inhibition and positive feedback are in balance. In the long term, such systems display "all or none phenomena". That is, the system exhibits one of two states following transient exposure to the stimulus. It either remains "off" or stays "on". In addition, if a stimulus, sufficient to bring the response near the threshold, is applied transiently and then recedes, the system will be "primed", such that if another very small stimulus, insufficient to trigger the unprimed system, is applied before the system decays to its pre-stimulus "off" state, the threshold will be exceeded and the system will trip to the "on" state by application of the small stimulus. Such properties are invoked to rationalize obvious all or none phenomena such as cell differentiation or growth[27,28]. Whether such phenomena exist within the coagulation or fibrinolytic cascades is, to date, not known with certainty, although experimental and theoretical modeling suggests that they are present within the coagulation cascade[29]. In addition, the heart attack itself surely presents as an acute, all or none, catastrophic event.

Potential targets for diagnosis, prognosis and therapy in the balance between fibrin deposition and removal

A global assay for the fibrinolytic system, analogous to assays for clotting, does not appear to be routinely employed. However, several groups have used the time course of turbidity after clotting to assess fibrinolysis[9,15,16]. In these assays, plasma is clotted in the well of a microtiter plate by recalcification or thrombin addition; t-PA is also included, and, as a consequence, the clot is subsequently lysed. The process is conveniently monitored over time by the turbidity at a convenient wavelength. The time to achieve lysis would be expected to depend minimally on the levels of fibrinogen, plasminogen and antiplasmin. The assay would provide, through a single measurement, an index of the fibrinolytic potential of a test plasma.

Traces of thrombomodulin circulate in plasma and are perhaps found in urine[30]. An assay which could distinguish thrombomodulin species having intact or oxidized methionine 388 might reveal the extent to which vascular thrombomodulin had been subject to the effects of vascular inflammation, either acutely or chronically. The latter might provide an indicator of the future risk of a cardiovascular event, because chronic inflammation is associated with such a risk[31].

An assay for activated TAFIa might be useful. As shown by Redlitz et al.[18] in a canine model of arterial thrombolysis, an inducible carboxypeptidase B-like activity appears acutely upon infusion of the lytic agent. In addition, the higher this level, the greater the time required for reperfusion to occur. Perhaps the level of TAFIa circulating during thrombolysis would provide an index of efficacy.

Experiments in the chimpanzee have indicated that under the stress of systemic and acute fibrin deposition, the organism is capable of acutely releasing into the circulation t-PA at levels equal to or exceeding those that are achieved in thrombolytic therapy in humans[6]. The mechanism for the release is not currently understood. If a pharmacological means of tapping this potential were available, however, a very attractive alternative to exogenous t-PA would be available for thrombolysis. In addition, if this potential could be tapped in a con-

trolled, chronic fashion, a means of facilitating the fluidity of the vascular system would be available. Further studies on the mechanism of release of endogenous t-PA are clearly warranted.

The level of circulating TAFI in plasma is directly correlated with the time required to lyse a clot *in vitro* in that plasma[32]. Whether elevated TAFI levels correlate with the risk of thrombosis is currently under study in several laboratories and results to date indicate that polymorphisms in the non-coding regions of the gene correlate quite strongly with the plasma level[33]. Perhaps in the future, TAFI genotyping might provide a measure of the risk of cardiovascular events.

The down-regulation of the activity of TAFIa appears to be mediated by spontaneous decay of the enzyme, rather than through the action of an opposing inhibitor[12,17]. This is a property unique to TAFIa, in that it is not shared by the homologous pancreatic carboxypeptidase B. Studies of TAFIa *in vitro* have shown that the stability properties are associated with amino acids spanning position 302-330 in the enzyme[34]. Mutant forms of the enzyme which are less stable than the wild type enzyme are also less potent as an antifibrinolytic agent. The potential exists that naturally occurring mutations in the coding region of the TAFI gene could substantially alter its antifibrinolytic activity by altering its stability. A polymorphism at residue 325 (Ile or Thr) that influences stability by a factor of two, such that one of the forms has a half life of 8 min and the other a half life of 16 min at 37°C has been found in our laboratory. Besides, the more stable form is more potent as an antifibrinolytic (unpublished observations). Whether these two forms express different phenotypes with respect to cardiovascular risk is not yet known.

We are clearly in need of a fuller understanding of the global properties of the coagulation and fibrinolytic systems. If thresholding and priming exist, we would require a paradigm shift in our approach to assessing risk and prescribing treatment. We may, for example, wish to determine how close an individual is to a "threshold" for heart attack by measurements of the circulating levels of coagulation or fibrinolytic components. The vicinity to a threshold typically depends on the levels of more than one substance. As an example, an abnormally low level of antithrombin might be innocuous in some circumstances, but combined with an elevated level of prothrombin or TAFI might place an individual perilously close to a threshold for a heart attack. Generally, the species to be assayed would probably be those which: 1) stimulate the system; 2) inhibit the system, and 3) contribute to positive feedback. With a fuller understanding of the global properties of the coagulation and fibrinolytic systems, we should have a much better idea of which parameters to measure to assess risk and which parameters to modify or regulate pharmaceutically to reduce risk and provide long-term therapy.

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