Laboratory markers of hypercoagulability

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Investigations carried out over the last 40 years have demonstrated that coronary artery thrombosis is the critical event underlying myocardial infarction and unstable angina. The existence of a prolonged hypercoagulable state preceding the thrombotic event has been postulated for some time and significant associations have been established between the plasma concentrations of a number of hemostatic variables and the frequency of myocardial infarction. High plasma fibrinogen, factor VII/VIIa, tissue-type plasminogen activator and plasminogen activator inhibitor levels have been associated with at least as great a risk of developing myocardial (re)infarction or sudden death as high cholesterol levels, especially in the young. In the last year more sensitive assays have been developed, and they should allow a precise biochemical definition of hypercoagulable states. The significance of these new assays and their role in defining a hypercoagulable state in different conditions are analyzed.

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Clinicians have long sought blood tests that can be used to monitor the level of activation of the coagulation cascade, confirm or exclude the presence of thrombosis, and predict thrombotic events. The various possible approaches are based on the measurement of the plasma levels of coagulation substrates, coagulation enzymes or activation peptides.

In large epidemiological cohort studies, measurements of coagulation substrates (such as factor VII, fibrinogen) have provided information concerning the role of hypercoagulability in arterial thrombosis, but have proved to be of limited clinical use in the monitoring of thrombotic and prothrombotic states because there is a large excess of these species in plasma and only small amounts are converted to their active form *in vivo*. Furthermore, fibrinogen is an acute reactant that closely correlates with other cardiovascular risk factors such as age, smoking, diabetes and estrogens[1].

The measurement of coagulation enzymes is limited by the fact that most enzymes are neutralized by naturally occurring inhibitors or bound to cell receptors and are not easily measurable in plasma.

One of the most promising approaches has been the measurement of plasma levels of the peptides released by zymogen molecules when they are activated to enzymes or enzyme-inhibitor complexes. In the field of coronary artery disease the most frequently tested markers have been fibrinopeptide A, prothrombin fragment 1+2, factor IX and X peptides, and the thrombin-antithrombin complex. The present review discusses these new hemostatic markers and the mechanistic information they carry. The developed assays and *in vivo* half-lives of some of the species are listed in table I.

Markers of coagulation activation

Fibrinopeptide A. Nossel et al.[1,2] developed a sensitive radioimmunoassay for measuring fibrinopeptide A, a 16 amino acid peptide that is cleaved from the alpha chain of fibrinogen by thrombin, and is therefore a sensitive biochemical marker of thrombin activity and fibrin formation. The finding that small amounts of fibrinopeptide A were measurable even in the plasma of healthy individuals suggested the physiological existence of ongoing low level coagulation activation and fibrin formation, thus indicating that the hemostatic balance is in dynamic equilibrium.

The concentration of fibrinopeptide A can be determined by means of commercially available radioimmunoassays or immunoenzymatic assays that use antibodies with a significant but not absolute specificity for fibrinopeptide A over fibrinogen. Before performing these assays, it is therefore necessary to use simple procedures to

Table I. Laboratory markers of coagulation activation.

Biochemical step	Marker	Half-life (min)
Factor VII → factor VIIa	Factor VIIa	150
Factor XII → factor XIIa	Factor XIIa	Unknown
Factor IX → factor IXa	Factor IX activation peptide	15
Factor X → factor Xa	Factor X activation peptide	30
Prothrombin → thrombin	Prothrombin fragment 1+2	90
Thrombin inhibition by antithrombin III	Thrombin-antithrombin complex	15
Protein C → activated protein C	Protein C activation peptide	5
	Activated protein C-protein C inhibitor	40
	Activated protein C	20
Fibrinogen → fibrin	Fibrinopeptide A	3-5

remove the fibrinogen from plasma samples without altering peptide levels. Unfortunately, the usefulness of fibrinopeptide A is limited by the fact that the test is highly susceptible to *in vitro* artifacts occurring during blood sampling and often yields spuriously high values that make it difficult to interpret the results. Furthermore, fibrinopeptide A has a short half-life (3-5 min), and can therefore only provide insights into the activation of the coagulation system during the brief period preceding blood sampling.

In an attempt to overcome these problems, some investigators have proposed measuring urinary fibrinopeptide A. Approximately 70% of the total production of fibrinopeptide A is proteolytically degraded by intravascular and extravascular peptidases, but only 0.2-0.5% is excreted in urine. Twenty-four-hour urinary fibrinopeptide A levels correlate with plasma levels in normal subjects and spot urinary fibrinopeptide A levels correlate with those concomitantly obtained in plasma[3,4]. However, a major limitation of this measurement is the wide intrasubject variability in fibrinopeptide A catabolism, which may offset its potential advantages.

Prothrombin activation fragment 1+2. More recently, assays have been developed to measure prothrombin activation fragment 1+2, which is released from prothrombin when zymogen is activated by the prothrombinase complex to yield thrombin[5,6] (Fig. 1). Unlike fibrinopeptide A, which reflects a more distal step (the thrombin activation of fibrinogen), this polypeptide provides information concerning the upstream activation of the coagulation cascade. Normal individuals have finite plasma levels of prothrombin fragment 1+2, and its half-life of about 90 min makes it a less transient index of coagulation activation than fibrinopeptide A. It also has the advantage that the assay results are less susceptible to *in vitro* artifacts due to faulty blood sampling, although precautions must still be taken during venipuncture and plasma preparation[7].

In addition to the original double-antibody liquid-phase radioimmunoassays, enzyme-linked immunoabsorbent assay (ELISA) kits are now commercially available[8]. However, the intermethod correlation of individual values is poor, as is their classification as normal or abnormal[9]. Laboratories can therefore only compare

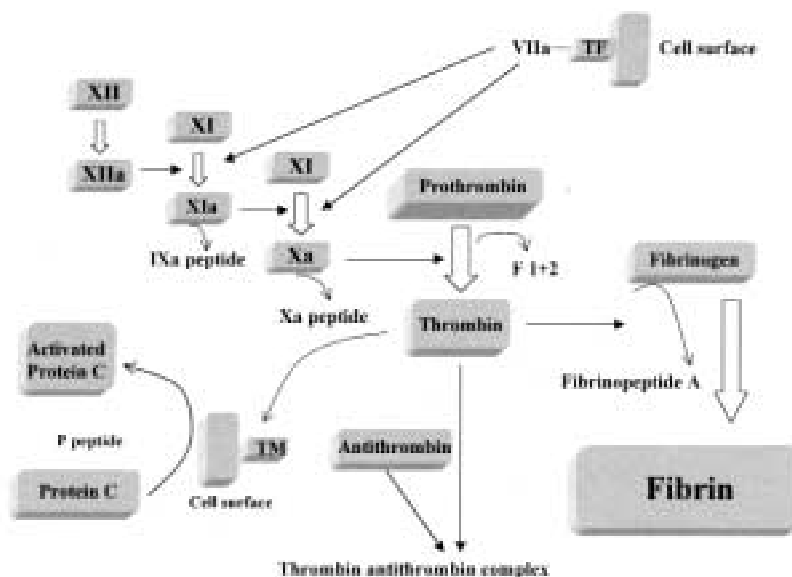

Figure 1. *The coagulation cascade activation. TF = tissue factor; TM = thrombomodulin.*

results obtained using the same method, and must ensure the presence of an age- and sex-matched control population in order to establish a local normal range of values.

IX and X activation peptides. Radioimmunoassays have been developed to measure the activation peptides of both factor X[10] (an indicator of increased enzymatic activity of the factor IXa-factor VIIIa-cell surface complex and the factor VIIa-tissue factor complex) and factor IX[11,12] (an indicator of the increased enzymatic activity of factor XIa and the factor VIIa-tissue factor complex). These assays can provide information on coagulation activation at an even earlier stage of the coagulation system than prothrombin fragment 1+2. However, although the use of these markers has clarified the role of the extrinsic pathway in activating blood coagulation activation *in vivo* under basal conditions (i.e. in the absence of vascular injury or thrombotic stimuli)[11-13], their clinical usefulness in defining a hypercoagulable state has not yet been validated.

Protein C activation peptide. Another activation marker is protein C activation peptide, the fragment released when protein C is transformed into activated protein C. Measurements of this marker have provided important mechanistic information concerning the physiological role of protein C and activated protein C. Protein C is a vitamin K-dependent zymogen that is activated by the thrombin bound to endothelial cell thrombomodulin and then inactivates the thrombin-activated cofactors of the coagulation cascade (factor Va and factor VIIIa); this is one of the main anticoagulant drivers of the hemostatic mechanism. Plasma protein C activation peptide levels can be taken as an indicator of thrombin/thrombomodulin function, and can be measured by means of radioimmunoassay[14]. In asymptomatic individuals with heterozygous protein C deficiency, plasma protein C activation peptide levels are reduced to about 50% of normal whereas plasma prothrombin fragment 1+2 levels are increased. This indicates increased thrombin generation[15]. In patients with homozygous protein C deficiency, plasma protein C activation peptide and prothrombin fragment 1+2 levels can be normalized by administering monoclonal antibody-purified protein C concentrate[16]. This demonstrates that an increase in the activity of the protein C anticoagulant pathway can inhibit prothrombin activation *in vivo*, and that the activation of protein C by the thrombin-thrombomodulin complex is a tonically active mechanism in the regulation of coagulation activation.

Thrombin-antithrombin III complex. A further assay of thrombin generation and thrombin neutralization is the measurement of thrombin-antithrombin III complexes. Since the thrombin-antithrombin complex is stable and has a half-life of approximately 15 min[17], immunological methods have been developed for its quantification in plasma. The original radioimmunoassay used a liquid-phase double-antibody method[18], whereas the commercially available method is a solid-phase ELISA[19]. The plasma levels of the complex are increased in a number of conditions associated with the activation of the coagulation cascade, such as venous thromboembolism[20], promyelocytic leukemia[21] and endotoxin infusion[22]. An intriguing difference between the two methods is that the range of normal values differs by a factor of 100: with the radioimmunoassay, the average normal concentration is 2 nmol/l; with the immunoenzymatic method, it is about 0.02 nmol/l. In stoichiometric terms, the enzymatic activation of 1 nmol of prothrombin will yield 1 nmol of thrombin and 1 nmol of prothrombin fragment 1+2. Assuming that all of the formed thrombin associates with antithrombin III, 1 nmol of thrombin will bind 1 nmol of antithrombin, thus leading to the formation of 1 nmol of complex. Since the half-life of the complex is one sixth that of prothrombin fragment 1+2, the plasma levels of the thrombin-antithrombin complex should be less than those of prothrombin fragment 1+2: about 0.2-0.3 nmol/l. However, as these theoretical values differ considerably from those actually obtained using either method, the biological significance of the measured values must be interpreted with caution until the reasons for these discrepancies, which may include the binding of thrombin to cell receptors and its subsequent internalization, are elucidated.

Definition of a biochemical hypercoagulable state

A hypercoagulable state can be defined biochemically if there is enhanced activation of the blood coagulation mechanism in the absence of thrombosis[23]. The laboratory detection of this condition, which is possible before overt thrombosis occurs, is potentially important for the identification of subjects who are at greatest risk of developing thrombotic events and who might benefit from prophylaxis.

Studies using simultaneous plasma measurements of prothrombin fragment 1+2 and fibrinopeptide A have facilitated the definition of one type of biochemical hypercoagulability. High prothrombin fragment 1+2 concentrations in the presence of increased fibrinopeptide A levels signify an increase in factor Xa production that is capable of generating sufficient free thrombin to initiate thrombus formation: i.e. a condition of overt thrombosis. However, high prothrombin fragment 1+2 concentrations in the presence of normal or only slightly increased fibrinopeptide A levels, while still signifying an enhanced production of factor Xa, do not result in the achievement of the threshold level necessary for the generation of sufficient free thrombin to initiate thrombus formation and, therefore, represent a condition which is biochemically defined as a hypercoagulable state. This condition of increased thrombin generation without fibrin formation may predispose affected individu-

als to the development of overt thrombotic events in response to relatively minor prothrombotic stimuli.

Theoretically, this transition from a prethrombotic state to a thrombotic event occurs if small increases in the generation of coagulation enzymes exceed the inhibitory threshold of an individual's endogenous anticoagulant mechanisms. Because the activity of the blood coagulation mechanism in these individuals is closer to the level at which the normal inhibitory processes are overwhelmed, thrombotic stimuli may induce the generation of slightly more thrombin via the factor VII-tissue factor pathway, which could then ignite the dormant intrinsic cascade by activating the factor IXa-factor VIIIa-cell surface complex, thus leading to the generation of increased amounts of free thrombin and the development of arterial or venous thrombosis.

The validity of markers of coagulation activation in clinically documenting the presence of a hypercoagulable state has been investigated in various models.

Acute coronary syndromes

The majority of patients with acute myocardial infarction and unstable angina have high levels of fibrinopeptide A in plasma[24-27], spot urine samples and 24-hour urine collections[3,4]. These findings are consistent with the results of angiographic, angioscopic and pathological studies clearly showing that intracoronary thrombosis plays a pivotal role in the pathogenesis of these coronary syndromes[28,29]. Abnormally high plasma prothrombin fragment 1+2 or fibrinopeptide A levels are found in nearly 50% of patients during the acute phase of the disease[27], their prevalence being higher in those with acute unstable angina or angiographic evidence of intracoronary thrombosis[3,4]. No difference in the levels of these peptides has been observed between patients with unstable angina and those with acute myocardial infarction[27]. This may indicate that plasma prothrombin fragment 1+2 and fibrinopeptide A levels are not dependent on the characteristics of the thrombus (which is subocclusive and platelet-rich in unstable angina, but occlusive and fibrin-rich in myocardial infarction)[30], but rather reflect a systemic condition of hypercoagulability. Whether this condition of hypercoagulability is associated with an unfavorable prognosis will be discussed in the following sections.

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