Markers of activated coagulation and their usefulness in the clinical laboratory

Armando Tripodi* and Pier Mannuccio Mannucci

Currently, information on hypercoagulability can be achieved directly—through measuring the enzymatic forms of coagulation zymogens generated during coagulation activation—or indirectly—through measuring the activation peptides generated when zymogens are activated or the enzyme–inhibitor complexes formed by inhibition of the enzymes by their plasmatic inhibitors. On the basis of published results, markers of activated coagulation are considered useful for investigating mechanisms that regulate hemostasis. They can also be used to better characterize patients at increased thrombotic risk. However, they should be considered indices of hypercoagulability, not of the risk of thrombosis, until prospective studies can demonstrate that alterations of these markers can predict the occurrence of thrombosis. For diagnosing acute thrombosis, their usefulness is questionable; they are less effective than markers of fibrinolysis activation such as the D-dimer. Finally, their use to monitor anticoagulant treatment is still premature and needs investigation in well-designed clinical studies.

INDEXING TERMS: hemostasis • thrombosis • anticoagulants • thrombomodulin • peptides • coagulation cascade • cardiopulmonary bypass • thrombophilia • autoimmune disorders • systemic lupus erythematosus

Coagulation is a highly integrated and regulated system that, in normal conditions, functions by maintaining a substantial procoagulant/anticoagulant balance. The procoagulant drive involves several zymogens and procofactors that, once activated by limited proteolysis, are able to generate thrombin, which in turn converts fibrinogen into fibrin (Fig. 1). The anticoagulant drive consists of powerful inhibitory systems such as antithrombin (AT), activated Protein C (APC), Protein S, and the tissue factor pathway inhibitor (Fig. 1).1 Heightened activation of coagulation zymogens may, by overwhelming the anticoagulant systems, cause a state of hypercoagulability. This can be defined biochemically as an imbalance in the coagulation cascade through pathological activation of the corresponding enzymes in the absence of clinical signs of thrombosis [1]. The laboratory detection of this condition, occurring before clinical thrombosis is apparent, is extremely important because of its potential to identify those subjects who would benefit most from prophylaxis. Over the last few years many articles have been written on hypercoagulability, and many tests have been made available for its detection. However, no precise guidelines have been given concerning the usefulness of these tests in the clinical laboratory.

Markers to Detect Hypercoagulability
Apart from markers of platelet and fibrinolysis activation, which will not be dealt with here, several approaches for laboratory detection of hypercoagulability are possible. One approach is to measure the concentrations of zymogens of the coagulation cascade, on the assumption that, when activated, their concentrations in plasma decrease. However, this approach is of little help because the concentrations of zymogens in plasma are high relative to the small amounts that are activated and consumed during the hypercoagulable state. This makes it difficult to quantify accurately very small decreases in zymogen concentrations.

Lately, other possibilities have been explored, including measurement of the fragments generated when zymogens are activated, measurement of the active enzymes, or measurement of the complexes that result from enzyme inactivation by naturally occurring plasma inhibitors. These activation markers are stable entities with finite lifetimes and circulate in normal plasma at low concentrations. If care is taken to avoid activation during or after blood collection, they can be used as indices of

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1 Nonstandard abbreviations: AT, antithrombin; PC, Protein C; Protein G; APC, activated Protein C; TF, tissue factor; F₁₋₂, factor (coagulation cascade); F—a, activated factor; FPA, fibrinopeptide A; PCP, PC activation peptide; TAT, thrombin–antithrombin; F₁₊₂, prothrombin fragment 1 + 2; FXIII and FX, factor IX and factor X activation peptides, respectively; PCC, prothrombin complex concentrate; and INR, International Normalized Ratio.
coagulation activation in clinical conditions in which hypercoaguability is suspected; they can also be used to study mechanisms that regulate hemostasis. For instance, activated factor XII (FXIIa) can be measured by an ELISA involving a monoclonal antibody that does not recognize the parent molecule, FXII. This assay has been proposed as a reliable index of the activation of coagulation through the contact system [2]. However, several lines of evidence suggest that the main trigger of coagulation is the tissue factor (TF)–factor VII (FVII) pathway (reviewed in ref. 3); thus, it would be desirable to have a simple and specific test to explore this function.

Under basal conditions, FVII circulates mainly as a zymogen, but trace amounts of activated FVII (FVIIa) are detectable in normal subjects [4, 5]. The relatively long life of FVIIa (half of the original concentration still being present in blood after ~2.5 h [6]) is consistent with the fact that no effective naturally occurring inhibitor for free FVIIa has been found [3]. A prospective study of middle-aged men assigned a predictive value for ischemic heart disease to increased concentrations of FVII in plasma [7]. However, FVII was measured with a functional clotting assay that was sensitive to both FVII and FVIIa. This raises the question of whether the predictive value should have been assigned to FVII or to FVIIa. Recently, two laboratories have independently developed functional clotting assays based on a truncated, soluble form of TF; this form lacks the cytoplasmic and transmembrane portions that enable TF to react with FVIIa, but not with intact FVII, in a prothrombin time-based clotting assay [4, 5]. Among the fragments generated from the activation of clotting factors, factor IX and factor X activation peptides (FXI and FXP) are now assayable by immunoassays. These fragments represent indices of upstream activation of the coagulation cascade [8, 9]. Prothrombin fragment 1 + 2 (F1+2), which results from the FXa-mediated activation of prothrombin [10-14], and fibrinopeptide A (FPA), which is cleaved from the alpha chain of fibrinogen by thrombin [15-17], are indices of thrombin generation and activity, respectively (Fig. 2). The times for their concentrations in plasma to halve are 90 and 3 min, respectively. Protein C (PC), a naturally occurring coagulation inhibitor, must be activated by thrombin bound to the endothelial receptor thrombomodulin to exert its anticoagulant action against FVIIIa and FVa. During activation, a small peptide is released from the amino-terminal end of the heavy chain of PC (Fig. 3). Some immunoassays are available for quantifying the PC activation peptide (PCP) or APC [18, 19]. For PC as for coagulation activation, normal subjects have low levels of activation that are responsible for the continuous production of APC and PCP. Therefore, these measurements can be regarded as indices of thrombin–thrombomodulin function. Finally, among the enzyme–inhibitor complexes, immunoassays have been developed for thrombin–antithrombin (TAT) [11, 20] and other complexes that result from the inhibition of APC by PC inhibitor and antiprotease [21, 22].

Assays for markers of coagulation activation have several potential uses. For example, they may (a) enhance our understanding of the mechanisms that regulate hemostasis; (b) further

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**Fig. 1.** Coagulation pathway and inhibitor systems. TPPI, tissue factor pathway inhibitor.

**Fig. 2.** Coagulation pathway and markers of activation.
characterize patients with clinical conditions known to predispose to thrombosis; (c) aid in the diagnosis of acute thrombotic events; and (d) monitor anticoagulant therapy.

**Activation Markers in Elucidation of Hemostatic Mechanisms**

Using an immunoassay to quantify FIXP, Bauer et al. [8] showed that FIX is activated in vivo under basal conditions by the TF-FVII mechanism. The concentration of FIXP is low in patients with severe FVII deficiency and FIX deficiency, but normal in patients with FXI deficiency [8]. This indicates that the generation of FIXa in vivo depends mainly on the extrinsic TF-FVII pathway, rather than on the contact phase. Two years later Bauer et al. measured activation peptides in a series of experiments designed to assess whether the FIXa–FVIIIa–cell surface complex contributes to the basal activation of the coagulation mechanism in vivo [23]. They infused recombinant FVIIa into patients with severe FVII deficiency and measured the generation of FIXP, FXP, and F1+2 at several time points after infusion. There was a substantial increase in the activation markers, which confirmed that the TF-FVII pathway is largely responsible for the activation of FIX and FX in the basal state. In another set of experiments, activation peptides were measured after infusion of purified FIX concentrate into hemophilia B patients. An increase in concentration of plasma FIXP was found without changes in FXP or F1+2. After infusion of highly purified FVIII concentrate into patients with hemophilia A, concentrations of FXP and F1+2 did not change. These data support the theory that, in basal conditions, FIXa generated by the TF-FVII pathway is unable to activate FX. Perhaps, during basal conditions the intrinsic system is quiescent and does not contribute to the basal activation of the coagulation system in vivo, probably because of the insufficient amount of FVIIIa bound to cell surfaces to interact with FIXa. Conversely, this system may function to amplify the generation of FXa at a time when a sufficient amount of thrombin, generated through the extrinsic TF-FVII pathway, converts FVIII to FVIIa and FXI to FXIa. However, direct evidence for the participation of TF in this chain of events is not available. Recently, ten Cate et al. [24], measuring FIXP, FXP, and F1+2 in plasma from normal chimpanzees infused with recombinant FVIIa, observed a significant increase of all activation peptides. The increase in peptides was suppressed by the concomitant infusion of a monoclonal anti-TF antibody. Metabolic studies with radiolabeled activation peptides have shown that the increased amounts of activation peptides were caused by accelerated in vivo conversion of the respective zymogens to active enzymes rather than by an altered clearance. These results suggest that recombinant FVIIa may trigger coagulation in vivo through an interaction with endogenous TF.

Recently, Boisclair et al. [2] studied the mechanism of thrombin generation during cardiopulmonary bypass by measuring perioperatively the concentration of activation peptides and FXIIa. They found that little FXIIa was generated during cardiopulmonary bypass, whereas F1+2 and FIXa increased substantially. More recently [25], some in this group found that a patient congenitally deficient in FXII was generated as much thrombin, measured by TAT complexes and F1+2, during cardiopulmonary bypass as nondeficient subjects did. They concluded that, during cardiopulmonary bypass, the main trigger for coagulation activation is provided by the TF-FVII mechanism rather than, as had been thought previously, by activation of the contact system through exposure of blood to foreign surfaces.

Conard et al. [26], by measuring activation markers, documented that the procoagulant imbalance associated with homozygous PC deficiency is normalized in vivo after infusion of purified PC concentrate. Two patients with homozygous PC deficiency had low basal concentrations of APC and PCP and high concentrations of F1+2. After infusion of PC concentrate, concentrations of APC, PCP, and F1+2 fell within normal ranges.

**Activation Markers to Characterize Patients at Increased Risk of Thrombosis**

Thromboembolic complications, such as myocardial infarction, venous thromboembolism, and disseminated intravascular coagulation, can occur when large and repeated doses of prothrombin complex concentrate (PCC) are infused into patients with hemophilia B. The thrombogenicity of PCC is still unexplained. However, as with FIX (the missing protein in hemophilia B), PCCs contain prothrombin, FX, and FVII, which may determine a state of hypercoagulability. Purified FIX concentrates have been developed in an attempt to avoid this complication. Mannucci et al. investigated ex vivo the thrombogenic potential of a highly purified FIX concentrate in comparison with the PCC by measuring activation peptides after infusion into hemophilia B patients [27, 28]. The concentrations of FXP, F1+2, and FPA increased after infusion of PCC, but not after infusion of purified FIX concentrate. These results indicate that infusion of PCC into hemophilia B patients can increase thrombin generation in vivo, and that this process can be abrogated by administration of more-purified FIX concentrate. Obviously, clinical studies are needed to determine whether purer FIX concentrates are also less thrombogenic than PCC.

Patients with inherited thromophilia related to defects of naturally occurring anticoagulants are at increased risk of devel-
opposing thrombosis [29]. Yet, only one-half of these patients experience thrombotic events during their life [29]. So far, attempts to identify those patients who are most susceptible to develop thrombosis have been frustrating. In theory, measurements of markers of coagulation activation should be useful. A cross-sectional study investigated the concentrations of markers of coagulation activation in a large number of patients affected by inherited thrombophilia [30]. About 25% of patients with deficiency of AT, PC, or Protein S had higher concentrations of either F1+2 or FPA, or both, than did normal controls. This and a subsequent study [31] confirmed and extended earlier findings that a procoagulant imbalance exists in these patients [28, 32]. However, the concentrations of activation markers found in patients with a previous thrombotic history were not significantly different from those found in patients without thrombotic history. These data suggest that measurement of activation markers is of limited value in detecting those patients with inherited thrombophilia who are most susceptible to develop thrombosis. To establish the absolute value of the activation markers in this situation would require a prospective study of a large number of patients.

Other patients at increased risk of thrombosis are those with autoimmune disorders [33]. In a cross-sectional study, we measured markers of coagulation activation in patients with systemic lupus erythematosus with and without lupus anticoagulant and in patients with lupus anticoagulant only [34]. About 25% of patients presented with F1+2 and FPA concentrations higher than the upper limit for normal controls. However, there were no differences between those who had a previous history of thrombosis and those who had been asymptomatic. Similar observations made independently by other authors [35] support the hypothesis that autoimmune disorders are associated with an ongoing thrombotic state. As for patients with inherited thrombophilia, prospective studies are needed to see whether alterations of activation markers predict the occurrence of thrombosis.

Acute myocardial infarction and unstable angina are clinical conditions associated with heightened activation of the hemostatic mechanism, as shown by the increased concentrations of FPA, D-dimer, and platelet factor 4 [36–38]. These findings might be interpreted as secondary to the presence of coronary thrombi, particularly because FPA concentrations have been shown to normalize after the acute event. Recently, Merlini et al. [39] confirmed that acute patients had increased concentrations of FPA accompanied by increased F1+2. Prospective investigations of patients who had an uneventful clinical course revealed that FPA, but not F1+2, concentrations returned after 6 months to the baseline value in the majority of patients. In some patients, therefore, a state of hypercoagulability—expressed by high concentrations of F1+2 but normal concentrations of FPA—apparently remains long after the acute event. Prospective studies are needed to see whether this hypercoagulable state has any predictive value for the occurrence or recurrence of thrombotic events and to determine the prophylactic value of anticoagulant treatment.

Aging is associated with signs of hypercoagulability, as shown by increases of markers of coagulation activation as a function of increasing age [40]. Mari et al. [41] studied 25 healthy centenarians in a cross-sectional investigation to see whether alterations of activation markers were also present in the very elderly who aged successfully. The concentrations of FVIIa, XFP, and FIXP in centenarians were strikingly greater than in two different control groups, ages 18–51 and 51–69 years. The other markers of activation, e.g., F1+2, FPA, and TAT, showed the same pattern. Mari et al. concluded that the very elderly do not escape the state of hypercoagulability associated with aging, but apparently this phenomenon is compatible with good health and longevity.

**Activation Markers to Diagnose Acute Thrombotic Events**

A few studies have been carried out to assess the usefulness of activation markers to diagnose acute thrombotic events [42–46]. The sensitivity of these markers is highly variable, depending on the time of blood sampling relative to the time course of the thrombotic event and to the treatment with antithrombotic drugs. In general, however, the sensitivity is lower than that of the D-dimer measured with ELISA. In one of these studies Boisclair et al. [42] measured TAT complexes in untreated patients and compared them with other markers of fibrinolysis activation. The majority of patients with disseminated intravascular coagulation had TAT complex concentrations higher than the upper limit of normal range. In this instance, sensitivity was comparable with that observed for the D-dimer test. In other conditions, e.g., deep venous thrombosis, myocardial infarction, acute leukemia, and liver disease, the sensitivity of the test was lower than that for D-dimer. Another study, carried out more recently by the DVTENOX study group [46], concluded that F1+2 and TAT have a lower sensitivity than D-dimer for the diagnosis of deep venous thrombosis, and that their time course values were not related to the clinical evolution.

**Activation Markers to Monitor Anticoagulant Treatment**

The usefulness of activation markers in monitoring anticoagulant therapy has been advocated mainly because Conway et al. first demonstrated [47] markedly suppressed concentrations of F1+2 in patients stabilized on oral anticoagulant therapy. Thus, investigators have surmised that the dosage of warfarin may be adjusted on the basis of F1+2 concentrations [48]. In principle, oral anticoagulant therapy could be monitored by measuring FVIIa concentrations, because this enzyme is increased in some conditions at increased thrombotic risk, e.g., pregnancy, and markedly reduced in patients taking oral anticoagulants [5]. However, although these approaches are plausible, no clinical studies have been carried out to assess them. In a cross-sectional study [49], we used a commercial kit to measure F1+2 in patients stabilized on oral anticoagulants and found that there was a close relationship between the concentrations of F1+2 and the degree of anticoagulation expressed as International Normalized Ratio (INR). In other reports, however, such a relationship has been questioned [45, 50]. On the one hand, these differences could be explained by the different times of blood samplings, i.e., whether the patients are on the stable phase of the therapy, still in the acute phase, or long past the thrombotic event. Alternatively, they might be related to the
different methods used. For example, some commercial immunoassays use monoclonal antibodies to capture F1+2 [13, 14], which conceivably might be affected by the variable gamma-carboxylation of the F1+2 released from the prothrombin molecules of warfarinized patients. Whatever the reason, this means that, even if clinical studies will demonstrate that F1+2 is superior to the INR, we shall have to tackle the standardization of this assay, a task that promises to be just as problematic as the standardization of prothrombin time. F1+2 determinations are clearly affected by the quality of venipuncture [51], by the different anticoagulants used to collect blood, and by the different standards (calibrators) used to construct calibration curves in the various methods reported [52–54]. Efforts to standardize F1+2 are in progress; we anticipate that this will be an area of growing interest for research workers and reagent manufacturers if the clinical usefulness of this marker is established. Finally, cross-sectional [44] and prospective [46] observations have shown that concentrations of F1+2 and TAT complexes are decreased in patients with acute thromboembolic events after heparin treatment. However, their clinical value is yet to be defined by appropriate clinical studies.

In summary, markers of coagulation activation have been used successfully to investigate mechanisms that regulate hemostasis. They can be used to better characterize biochemically the patients at increased risk of thrombosis. However, they should be considered indices of hypercoagulability, rather than the risk of thrombotic complications, at least until prospective studies have demonstrated that their alterations are predictive of the occurrence of thrombosis. One such prospective study is the Northwick Park Heart Study, which is currently assessing the predictive value of F1+2 for ischemic heart disease in middle-aged, apparently normal subjects [55]. Currently, the usefulness of markers of coagulation activation in diagnosing acute thrombotic events is questionable, being apparently less effective than other markers of fibrinolysis activation such as the D-dimer. Finally, the use of markers of coagulation activation to monitor anticoagulant treatment is still a premature indication and needs to be investigated by means of well-designed clinical studies.

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