Assays for thrombosis factors: relations to plasma lipids\textsuperscript{1,2}

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ABSTRACT Several coagulation and fibrinolytic factors appear to be associated with plasma lipids in both fasting, population-based studies and postprandial studies. The molecular mechanisms for these associations, however, are not understood. There are a variety of different assays available, which can be categorized into screening assays, type 1 assays (which estimate the circulating concentration of individual zymogen, substrate, or inhibitor), type 2 assays (which measure molecular species that are formed as the result of in vivo enzymatic activity), and type 3 assays (which directly measure plasma enzymes). Although all of these types of assays have been used in human studies exploring the relation of dietary fats to thrombosis, in most cases the interpretation of the results has been hindered by one or more of the following: the potential for in vitro artifacts with blood collection, the technical limitations of clot-based assays, the different reagent systems that are available for many of these assays, and the lack of agreed-on standards. Work is needed in these areas as well as in the development of an expanded repertoire of type 2 and 3 assays, provocative tests that will allow us to determine how sensitive one's system is to procoagulant stimuli and the gene-gene and gene-environment interactions that play a role. Am J Clin Nutr 1997;65(suppl):1669S–73S.

KEY WORDS Thrombosis, thrombosis factors, thrombosis assays, fibrinolysis, fibrinolysis factors, fibrinolysis assays, plasma lipids

INTRODUCTION

Coagulation and fibrinolytic factors have been studied in relation to lipids in three contexts: epidemiologic studies, which have revealed stronger-than-expected relations between factors and plasma lipid concentrations; dietary studies, which have revealed that different dietary intakes can have a long-term effect on factor concentrations; and postprandial studies, which have shown that there are also immediate effects of fat ingestion.

Research projects have used various coagulation and fibrinolytic factor assays to assess the effects of dietary lipids. The four major types of assays are screening assays, type 1 assays (which estimate the circulating concentration of individual zymogen, substrate, or inhibitor), type 2 assays (which measure molecular species that are formed as the result of in vivo enzymatic activity), and type 3 assays (which directly measure plasma enzymes) (Table 1) (1). All of these types of assays have been used in human studies exploring the relation of dietary fats to thrombosis.

There are four general considerations regarding blood samples for thrombosis factor assays. First, the quality of the venipuncture is important. A wide-gauge needle should be used asatraumatically as possible. Evacuated collection tubes can be used if the appropriate anticoagulant is present (e.g., a standard citrate tube). Most assays use citrate-treated plasma, but some assays, such as that for fibrinopeptide A, might require a special anticoagulant mixture. Second, the initial blood flow from a vein (usually 3–5 mL) should be discarded because it may contain procoagulant factors generated by the venipuncture. Third, the tourniquet time should be as short as possible to prevent activation of coagulation and fibrinolysis systems by hemostasis. Fourth, the blood should be processed as quickly as possible at 4 °C. An exception is blood destined for factor VII assays, in which it is known that cold activation can occur.

FIBRINOGEN

Although not strongly related to plasma lipids or dietary fats, the most widely studied coagulation factor in epidemiologic studies has been fibrinogen. Several forms of type 1 assays have been used, including clot-rate assays based on the method of Clauss (2), with mechanical endpoints or optical endpoints; clot-weight methods in which a clot is formed, removed from the plasma, and weighed (3, 4); and a variety of other methods, such as nephelometry after specific precipitation of fibrinogen. In all cases, fibrinogen has been shown to be an independent risk factor, so it seems likely that the method does not play a major role in determining whether plasma concentrations of this protein have important epidemiologic associations. Only in thrombolytic therapy or advanced disseminated intravascular coagulation, in which large quantities of intermediate thrombin-generated fragments are present, would the method be important, and these conditions are not generally applicable to studies of dietary intervention or epidemiology.

FACTOR VII

Factor VII has also been studied widely and has been shown to be related to plasma lipid concentrations (5–10) and dietary fat intake (11–18). Most studies used a traditional clotting assay, in which both preformed factor VIIa and the bulk of the

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### TABLE 1

Measurements of coagulation and fibrinolysis

<table>
<thead>
<tr>
<th>Description</th>
<th>Screening assay</th>
<th>Type 1 assay</th>
<th>Type 2 assay</th>
<th>Type 3 assay</th>
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<tbody>
<tr>
<td>Methods</td>
<td>Clot-based or clot lysis–based assays</td>
<td>Assays that estimate the circulating concentration of individual zymogen substrate and inhibitor concentrations</td>
<td>Assays that measure molecular species formed as a result of in vivo enzyme activity</td>
<td>Assays that directly measure plasma enzymes</td>
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<tr>
<td>Examples</td>
<td>Activated partial thromboplastin time, prothrombin time, euglobulin clot lysis assay</td>
<td>Fibrinogen; factor V, VII, VIII, etc; plasminogen activator inhibitor 1</td>
<td>Prothrombin fragment 1.2, fibrin fragment D-dimer, fibrinopeptide A, thrombin-antithrombin complex</td>
<td>Immunooassays</td>
</tr>
<tr>
<td>Advantages</td>
<td>Detects major abnormalities, easy to perform, no special blood collection</td>
<td>Relatively easy to perform, reproducible and sensitive, usually no special collection is required</td>
<td>Results reflect in vivo enzymatic activity, reproducible and sensitive</td>
<td>Specialized assays, often using components from chromogen or immunologic assay systems or both</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>No information on specific factors, difficult to standardize, not sensitive</td>
<td>Some assays require special collection, minor changes are difficult to interpret</td>
<td>Most require special collection or processing, collection artifacts are difficult to avoid, difficult to standardize, effect of endogenous enzyme inhibitors and clearance rates complicate results</td>
<td>Research-level assays, effect of endogenous enzyme inhibitors and clearance rates complicate results, most require special collection or processing, collection artifacts are difficult to avoid</td>
</tr>
</tbody>
</table>

1 From reference 1.

zymogen factor VII contribute to the final answer. The relative contribution of each form has not been rigorously established.

However, there are many variations on the traditional assay as well as several other types of factor VII assays. There is reason to believe that the type of factor VII assay may well play a role in determining the answers obtained. We (19) recently reviewed factor VII assays and several issues are clear: 1) variation in virtually all of the individual components of a traditional one-stage factor VII clot-rate-based assay (factor VIIc assay) can contribute to the final answer, 2) factor VIIc values are relatively weakly related to concentrations of factor VII antigen (in nine studies, R = 0.58–0.87, with most 0.65–0.75), and 3) probably the most important component of a factor VII assay for standardization purposes is the thromboplastin that is chosen, although the factor VII–deficient substrate plasma may also play a role.

A factor VII–phospholipid complex was reported in plasma (18). Our results (19) and those of others (20), however, indicate that this is likely an artifact of how the assay was performed; there is little evidence for such a complex at this time.

The nature of the components used in a factor VIIc assay have a large influence on the results in postprandial dietary studies. In a recent study by Silveira et al (17), the change in several factor VII–related variables was assessed. After a meal, factor VII antigen and factor VII amidolytic activity dropped slightly; factor VIIc measured with an assay using human brain thromboplastin rose slightly, ∼1.1-fold; factor VIIc measured with an assay using Thrombotest (bovine brain thromboplastin with adsorbed bovine plasma, Nyegaard and Co, Oslo, Norway) rose ∼1.8-fold; and factor VIIc measured with a more standard bovine brain thromboplastin rose ∼2.2-fold. All the factor VIIc assays used human factor VII–deficient plasma as the substrate plasma. Factor VIIc assays with bovine thromboplastin are reported to be essentially measurements of factor VIIa concentrations (ie, type 3 assays), being relatively insensitive to factor VII per se (21). Silveira et al (17) believe that their results indicate that alimentary lipemia activates factor VII to factor VIIa, and they report a variety of possible mechanisms to support this hypothesis, including the possibility that, as reported by Mitropoulos et al (22), large lipoprotein particles may express a surface suitable for activation of the contact system through the action of lipoprotein lipase. This would result in factor VIIa generation through factor XIIa activity (16, 22).

All of the epidemiologic work done by Meade, Miller, and Mitropoulos and colleagues in the Northwick Park Heart Study and in their dietary intervention studies has been done with use of still another variant, in which the substrate plasma is bovine plasma adsorbed to remove all vitamin K–dependent clotting factors, with human prothrombin and factors IX and X added back (3, 14–16, 22). The thromboplastin is also nonhuman in origin. The strong relation of factor VIIc to ischemic heart disease noted in these studies has not been confirmed in several other studies with different factor VIIc methods (10, 23). Whether this reflects different assay specificities [the English group commented that the lack of protein C may be important, making their assay more sensitive to factor VIIa concentrations than an all-human assay (24)], populations, sample handling [eg, factor VII will variably autoactivate in the cold (25)], or endpoint definitions for disease remains to be established. The Northwick Park study is the only major epidemiologic study of factor VII that used samples from nonfasting subjects.
ASSAYS FOR THROMBOSIS FACTORS

Recently, a different type 3 assay for factor VIIa was reported by Morrissey et al. (26). This assay makes use of a recombinant soluble tissue factor molecule in the thromboplastin. Under these conditions it is believed that only factor VIIa will register in the assay, because factor VII is not converted to factor VIIa. This assay has become commercially available and more will soon be known about how it responds to dietary fat intake. From the arguments given above, one would anticipate factor VIIa being a sensitive marker of postprandial lipemia and possibly sensitive to longer-term activation of factor VII as well.

The major factor VII inhibitor is tissue factor pathway inhibitor (TFPI, formerly called LACI and EPI). This key coagulation regulator is found in four compartments in the vasculature: in platelets, bound to the heparan sulfate moieties of the endothelium, bound to plasma lipoproteins, and free in solution. Heparin administration can increase the TFPI concentration in plasma severalfold. TFPI is primarily assayed by a relatively complex type 1 assay that measures the ability of plasma to specifically inhibit the factor VIIa–mediated formation of factor Xa (27). Antibodies do exist and immunoassays are possible, but not widely used.

SCREENING ASSAYS AND OTHER FACTOR ASSAYS

Several screening assays, such as the activated partial thromboplastin time and the euglobulin clot lysis time (ECT) assays, have seen limited application in epidemiologic and interventional studies. For example, the ECT assay was used in the Northwick Park Heart Study as a potential cardiovascular disease risk factor (3, 28). However, these assays are not widely used.

Also, little use has been made of other factor assays as potential risk-factor measurements or as diet-modifiable factors. Factor VIII was assayed in several epidemiologic studies, using a traditional one-stage clot-rate assay based on factor VIII–deficient plasma (10, 29, 30). von Willebrand factor has also been used to some extent (29), and there is one epidemiologic study of factor X concentrations, again using a one-stage clot-rate assay (31).

We (32) and others (33) have reported that all commonly measured vitamin K–dependent factors (factors II, VII, IX, and X; protein C; and protein S), not just factor VII, exhibit a relatively high degree of correlation with plasma lipid concentrations. However, this relation has been explored to the greatest extent with factor VII. Little is known about the postprandial changes of these other factors.

FIBRINOLYTIC FACTORS

Unfortunately, little is known about the relation of the fibrinolytic factors to lipids and lipid metabolism. Of the fibrinolytic factors, plasminogen activator inhibitor 1 (PAI-1) has been studied the most. Several studies show that plasma PAI-1 concentrations are associated with plasma triacylglycerol concentrations (34–36). This relation has been linked to the so-called insulin resistance syndrome (IRS; also known as syndrome X) and it has been suggested that insulin concentrations play a major role in regulating PAI-1 concentrations (37, 38). It has also been suggested that it is not insulin per se but rather the other sequelae of IRS (eg, hypertriglyceridemia) that play the regulating role (39). PAI-1 is also an acute-phase reactant and it has been clearly established that the well-known post-trauma hypofibrinolytic state is most likely caused by increased PAI-1 (40).

Whatever the mechanism supporting the association of PAI-1 and lipids, it is likely that the type of PAI-1 assay chosen will be important to the result. PAI-1 resides in the blood in several forms: free and active; free and inactive (latent); complexed to the enzyme it inhibits, t-plasminogen activator (also inactive); and possibly in other degraded or complexed forms. An enzymatic type 1 assay might appear to be most appropriate, but unfortunately the PAI-1 enzymatic assays are not sensitive and not completely specific for PAI-1. We have used extensively an immunologic type 1 assay for PAI-1, which is based on antibodies that detect free (active and latent) but not complexed PAI-1. The specificity of the assay should be well understood before it is used in a clinical study because the mechanism responsible for the association of PAI-1 with hypertriglyceridemia is unknown.

The only other plasma component that might be considered a fibrinolytic factor is lipoprotein(a) [Lp(a)] because of its ability, at least in vitro, to inhibit fibrinolytic complex formation (41). Assays for Lp(a) are currently in a state of confusion because few assays have been even partially characterized as to their sensitivity and specificity for the various forms of Lp(a) in plasma. Genetic heterogeneity, potential complexation and degradation, and the underlying lipoprotein metabolism all contribute to make an extraordinarily complex situation. It seems likely that the assay method chosen for Lp(a) will have potentially important effects on any diet intervention.

TYPE 2 ASSAYS

Little is known about the association of type 2 measurements such as of prothrombin fragment 1.2, fibrinopeptide A, or plasmin-α-2-antiplasmin complex concentrations with either lipid concentrations or dietary intervention (long or short term). These assays are only now beginning to be done in epidemiologic studies and have not been used to any great extent in diet studies. In general, most researchers use commercially available immunoassay kits, although some have made their own assays (42, 43). There is little standardization, and values obtained with a kit from one manufacturer (or research lab) compared with those obtained from another are often different in absolute values [eg, compare normal ranges for prothrombin fragment 1.2 concentrations as reported for the Dade Kit (Baxter Diagnostics, Inc, Miami) and the Enzygnost Kit (Behring Diagnostics, San Jose, CA)], although these values may correlate well.

FUTURE DIRECTIONS

Testing for coagulation and fibrinolysis factors in dietary and epidemiologic studies could benefit from advances in several areas. First, clot-based assays are still widely used, yet it seems likely that despite best efforts, other components of the plasma besides the factor of interest probably contribute to the results of the assay. Also, because the activating components are a
source of irreproducibility, these assays would benefit from better defined and standardized reagents.

Second, development work will continue on assays for the products of the in situ reactions (type 2 assays), such as prothrombin fragment 1.2 and fibrinopeptide A. For example, few assays currently exist that look at the reactions that occur on the endothelial cells, such as protein C activation, or reactions that occur on monocytes.

Third, at some point we will need to have provocative assays, the equivalents of an oral glucose tolerance test, to determine how sensitive the system is to a procoagulant stimulus.

Fourth, new genetic assays (eg, of PAI-1<sub>4G→5G</sub> or factor V<sub>Leiden</sub>) will improve our understanding of gene-gene and gene-environment interactions. This will become more and more important for understanding how complex systems such as lipid metabolism and coagulation-fibrinolysis interact.

REFERENCES

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