**Measurement of soluble fibrin monomer-fibrinogen complex in plasmas derived from patients with various underlying clinical situations**

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**Summary**

We previously reported a monoclonal antibody named IF-43 that specifically recognizes thrombin-modified fibrinogen (desAA- and desAABB-fibrin monomer) bound with fibrinogen or other D₁ domain-containing plasmic fragments such as fragments X,Y, and DJ, but not intact fibrinogen or cross-linked fibrin degradation products (XDP). Here, we tentatively named such complexes, soluble fibrin monomer (FM) -fibrinogen complex.

By utilizing IF-43, we have developed a kit to measure soluble FM-fibrinogen complex and compared the profiles with those of two established molecular markers for thrombo-embolic disorders: i.e. the thrombin-antithrombin complex (TAT) and the D-dimer in plasma of patients who underwent surgery without any thrombo-embolic complications. The result indicated that soluble FM-fibrinogen complex is a distinct entity from the two established molecular markers. We have also attempted to observe their profiles in patients with the disseminated intravascular coagulation syndrome (DIC). Although the profiles of soluble FM-fibrinogen complex in individual patients appeared to vary from one patient to the other, the plasma level of soluble FM-fibrinogen complex was found to be increased at the initial phase of disseminated intravascular coagulation syndrome. Thus, the soluble FM-fibrinogen complex may serve as an independent molecular marker for the detection of thrombin generation and the diagnosis of thrombosis. The soluble FM-fibrinogen complex may also serve as a risk factor for thrombosis, because it may precipitate as insoluble complexes beyond its threshold in plasma, or when it is modified by thrombin.

**Keywords**

Soluble fibrin monomer-fibrinogen complex, surgery, DIC

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**Introduction**

When thrombin is generated at the final step of blood coagulation, it cleaves a pair of fibrinopeptide A (FPA) located in the central E domain, converts fibrinogen to fibrin monomer (desAA-FM lacking only paired FPA’s, or type I fibrin) and exposes a pair of polymerization sites called the “A” sites or the α-chain knobs (1, 2). Each polymerization site “A” or α-chain knob specifically binds with its complementary site called the “a” site or the γ-chain knob residing in the D domain of another type I fibrin or fibrinogen molecules. Activation by thrombin is not necessary for exposure of the “a” polymerization site in the D domain (3, 4).

When sufficient amounts of thrombin are generated, and type I fibrin molecules are produced at the loci where the vascular integrity has been impaired, the type I fibrin molecules...
bind with one another in a highly ordered fashion, release another pair of fibrinopeptide B (FPB’s) therefore yielding des-AABB fibrin or type II fibrin, and form insoluble fibrin clots that function as hemostatic thrombi.

On the other hand, when limited amounts of type I fibrin are produced in the presence of an excess amount of fibrinogen, the type I fibrin molecules form complexes with fibrinogen and exist as soluble protein complexes (5-7). We define this sort of complex as the “soluble fibrin monomer (FM)-fibrinogen complex”.

We previously reported a monoclonal antibody termed IF-43, the epitope of which was located on the α-chain remnant corresponding to Aα (52-78) residues in the E domain of fibrin monomer (8). However, this epitope was found to be cryptic in the E domain of an acid-solubilized single FM molecule, but is exposed on the surface of the E domain, when FM molecule binds with fibrinogen molecules via the E-D binding sites. In an in vitro study, all the D1 domain-containing fibrinogen derivatives were shown to substitute the fibrinogen molecule, such as plasmic fragments X, Y and D1 derived from fibrinogen and FM molecules, as described previously (8).

In this paper, we describe a latex aggregation assay for the measurement of soluble FM-fibrinogen complex developed by utilizing IF-43, and discuss the behaviour of soluble FM-fibrinogen complex with special reference to those of other established molecular markers for intravascular thrombus formation such as the thrombin-antithrombin complex (TA T) and the D-dimer in plasmas derived from patients who underwent surgery without any thrombo-embolic complications. We also discuss the profiles in plasma from patients with the disseminated intravascular coagulation syndrome (DIC).

### Materials and methods

#### The latex aggregation assay
The latex aggregation assay was established using IF-43 conjugated to latex beads, and plasma samples derived from patients were tested for soluble FM-fibrinogen complex as previously described with minor modifications (8). Briefly, 3 μl aliquots of test materials were mixed with 150 μl of buffer, and the mixture was made up to 303 μl by the addition of 150 μl of latex beads suspension. The rate of aggregate formation was assessed by reading the absorbance at 800 nm (A800) at 17-second intervals for 5 minutes on an automatic analyzer 7170 (Hitachi Medical Corp., Tokyo, Japan) based on a latex photometric immunoassay with near infrared turbidity and expressed in terms of delta A800 as a function of the concentration of acid-solubilized type II fibrin in the test materials. The system was found to be able to measure desAABB-FM spiked to normal plasma up to 80 μg/ml (Fig. 1).

For the measurement of the D-dimer and TA T, LPIAACE D-dimer and LPIAACE TA T (both from Iatron Laboratories Inc., Tokyo, Japan) were used on an automatic analyzer LPIA (Mitsubishi Chemical Corp., Tokyo, Japan).

#### Plasma samples derived from patients
Nine volumes of blood were collected into a plastic syringe containing one volume of 3.8% sodium citrate by clean venipuncture from the patient, and the plasma was separated by centrifugation for 30 minutes at 3,000 rpm and 22°C. The plasmas were kept frozen at -70°C until use. Prior to the test, the plasmas were thawed quickly without forming bubbles in a water bath at 37°C and kept in the water bath for at least 30 minutes to assure the temperature of the plasmas had been brought to 37°C. The concentration of soluble FM-fibrinogen complex was expressed in terms of the amount of des ABB-FM added to normal human plasma, as described previously (8).

#### Absorption study with JIF-23-conjugated latex beads
The test plasmas were mixed with JIF-23-conjugated latex beads and kept at 37°C for 1 h, and then the latex beads were removed by centrifugation for 15 min at 10,000 rpm and 10°C. Then the plasmas were measured for soluble FM-fibrinogen complex and the D-dimer.

### Results

#### Profile of soluble FM-fibrinogen complex in the plasma of patients after surgery without any thrombo-embolic complications
In order to see whether soluble FM-fibrinogen complex is a discrete entity from other established molecular markers such as TA T and the D-dimer, we investigated the behavior of soluble FM-fibrinogen complex in plasmas derived from 11 patients...
who had undergone abdominal surgery, and compared the profile with those of TAT and the D-dimer.

The concentration of soluble FM-fibrinogen complex did not change significantly during the initial stage after surgery (1 - 2hr), but it increased significantly thereafter until day 3, and then decreased gradually reaching a plateau thereafter (uppermost panel, Fig. 2). On the other hand, TAT increased soon after surgery, remained elevated on day 1, and then decreased over a period from days 2 through 10. The concentration of TAT was found to return to the preoperative level on day 14 (middle panel, Fig. 2).

On the contrary, the D-dimer increased only slightly after surgery and remained at low levels during the first 3 days, but it became significantly increased from days 5 to 10. The level
remained higher than the preoperative level even on day 14 (lowest panel, Fig. 2).

Thus, the profiles of these three parameters related to intravascular thrombus formation are apparently distinct from one another, most likely reflecting different clinical aspects related to the formation of hemostatic fibrin clots and their resolution, and also their clearance from the blood circulation.

Assessment of soluble FM-fibrinogen complex in plasmas derived from the patients

Correlation between the concentrations of soluble FM-fibrinogen complex and the D-dimer

When the concentration of soluble FM-fibrinogen complex in plasmas from patients with a variety of diseases was plotted as a function of the concentration of D-dimer, virtually no correlation was noted between these two fibrinogen-derived molecular species (Fig. 3).

The level of soluble FM-fibrinogen complex in the plasma after depletion of the D-dimer

To confirm that IF-43 would not react with the D-dimer in the plasma samples, patient’s plasma was absorbed with JIF-23-conjugated latex beads, and then measured for soluble FM-fibrinogen complex and the D-dimer. The antibody JIF-23 was shown to recognize the amino-terminal disulfide-linked structure of plasmic D species (9). Although substantial amounts of D-dimer were removed from the test plasmas, on average more than 98.9%, the concentration of soluble FM-fibrinogen complex was not affected with an average recovery rate of 94.6 % (Table. 1).

Discussion

In this paper, we have described a latex aggregation test directed to the detection of soluble FM-fibrinogen complex.

Using this assay, we were able to show that soluble FM-fibrinogen complex was a distinct entity as a molecular marker for thrombin generation and successive fibrinogen to fibrin conversion in the circulating blood from two other established molecular markers, TAT and the D-dimer.

Although the mechanism of TAT formation has not been precisely elucidated in disease conditions e.g. DIC and thrombosis, the thrombin molecules generated at the loci may be released from the phospholipid bilayer, where the blood coagulation took place, and captured quickly by the antithrombin molecule bound to heparan sulfate on the vascular endothelial cell surface. As is widely accepted, the affinity of antithrombin molecule with heparan sulfate would be reduced when bound with thrombin. Consequently, the complex termed as TAT would be released from the vascular endothelial cell surfaces into the blood circulation. Such molecular interactions must take place in conjunction with thrombin formation, and thus TAT could be detected at an early stage of thrombin formation.

The thrombin-mediated fibrinogen to fibrin conversion may also take place soon after thrombin generation in the blood circulation, but it may not proceed as fast as the neutralization of thrombin by antithrombin bound to heparan sulfate on the endothelial cells, shown to be nearly instantaneous (10).

Although no information has so far been provided regarding the clearance rates of TAT and soluble FM-fibrinogen complex in the circulating blood in these clinical situations, we showed

| Table 1: The level of the D-dimer and soluble FM-fibrinogen complex remaining in plasma after depletion of the D-dimer with JIF-23. |
|---|---|---|---|
| No. | D-dimer (µg/mL) | solubl FM-fibrinogen complex (µg/mL) |
| | A | B | C |
| 1 | 56.8 | 0.2 | 2.6 | 2.9 | 3.7 |
| 2 | 20.9 | 0.1 | 30.3 | 28.7 | 30.9 |
| 3 | 5.7 | 0.0 | 10.7 | 12.0 | 12.2 |
| 4 | 29.2 | 0.2 | 66.3 | 64.1 | 68.6 |
| 5 | 11.0 | 0.2 | 18.3 | 18.3 | 20.7 |
| 6 | 50.9 | 0.1 | 17.1 | 13.9 | 20.0 |
| 7 | 13.4 | 0.1 | 5.1 | 4.8 | 4.9 |
| 8 | 45.7 | 0.1 | 59.5 | 68.8 | 67.3 |
| 9 | 12.2 | 0.1 | 59.3 | 48.6 | 63.9 |
| 10 | 11.5 | 0.0 | 45.3 | 39.9 | 47.3 |
| 11 | 3.7 | 0.3 | 27.4 | 26.8 | 28.5 |
| 12 | 20.6 | 0.1 | 12.2 | 10.6 | 12.8 |
| 13 | 35.1 | 0.3 | 21.1 | 18.0 | 18.8 |
| 14 | 17.2 | 0.3 | 7.7 | 5.9 | 5.9 |
| 15 | 9.7 | 0.2 | 4.3 | 4.1 | 4.7 |
| 16 | 11.8 | 0.1 | 16.0 | 15.1 | 17.1 |

A: before absorption; B: after absorption

C: absorption with non-immunized mouse antibody as control
that the appearance of soluble FM-fibrinogen complex was obviously delayed compared with that of TAT in plasmas derived from patients who had undergone abdominal surgery (uppermost and middle, Fig. 2).

Small amounts of D-dimer may also be produced in conjunction with the formation of initial hemostatic fibrin clots that involve tissue-type plasminogen activator (t-PA) present in plasma. However, compared with these molecular markers directly related to thrombin generation, the appearance of D-dimer may well be further delayed, until the phase of capillary blood vessel formation and release of vascular plasminogen activator or t-PA therefrom (lowest panel, Fig. 2). Indeed, the appearance of the D-dimer must be preceded by a series of varieties of molecular interactions for the formation of cross-linked fibrin clots, activation of plasminogen on the fibrin fibers by t-PA released from newly formed capillary cells (11, 12), and plasmin-mediated digestion of the cross-linked fibrin (13-15).

Studies on these profiles in patients with DIC have been ongoing in our laboratory, but as the number of samples tested is still limited, we have not discussed them in this paper. However, the profile of soluble FM-fibrinogen complex in plasmas of patients with DIC or in those derived from patients who had developed DIC during observation seems to support our presumption that soluble FM-fibrinogen complex is a distinct entity from other established parameters, in particular the D-dimer in patients with DIC as well.

Although the epitope of the antibody, IF-43, resides most probably in the carboxyl terminal segment of the α-chain remnant corresponding to the Αα (52-78) residues in the E domain of FM (8), this α-chain segment appears to be buried in the E domain that is tightly bound to the two cross-linked D domains of the D-dimer. Indeed, the amino-terminal regions of the three subunit polypeptides in the fibrin E domain possessing the “A” polymerization sites and the Ββ (15-42) residues were shown to be securely protected against plasmin, and therefore, the E domain that has lost the two polymerization sites could be produced only at the very late stage of plasmic digestion of cross-linked fibrin, fragment E3 (16). Thus, the epitope for IF-43 is thought to be buried in the cross-linked fibrin and their plasmic digests collectively termed as the D-dimer. Consequently, the epitope in the D-dimer would not be accessible to the antibody. The fact that IF-43 was able to distinguish soluble FM-fibrinogen complex from the D-dimer in plasmas seems to be compatible with these findings.

To support this hypothesis, we found virtually no correlation between the concentrations of soluble FM-fibrinogen complex and the D-dimer in a large number of plasma samples derived from patients with various diseases (Fig. 3). In addition, the absorption study with JIF-23-conjugated latex beads supports the idea that the molecular species recognized by IF-43 was distinguishable from the D-dimer recognized by JIF-23 (Table 1).

Also, in a study conducted by Francis et al. utilizing our antibody, IF-43, no significant correlation was observed between soluble FM-fibrinogen complex and the D-dimer (17). Of particular interest is that soluble FM-fibrinogen complex may serve as a risk factor for thrombosis, because it can be further modified by thrombin and becomes precipitable within the vasculature. Therefore, the clinical significance of soluble FM-fibrinogen complex ought to be independently appreciated from other fibrinogen-derived molecular markers in clinical situations related to intravascular thrombus formation including thrombosis and DIC.

References