Analysis of Fibrinogen Degradation Product in Severe Liver Disorders by Immunoblotting

MASAO TANAKA and TAKESHI OHKITA

Nagoya National Hospital, Hematological Disease Center, Nagoya 460

TANAKA, M. and OHKITA, T. Analysis of Fibrinogen Degradation Product in Severe Liver Disorders by Immunoblotting. Tohoku J. exp. Med., 1987, 153 (3), 179-187 — Increased serum fibrinogen degradation product (FDP) in liver cirrhosis and hepatic carcinoma as measured by latex agglutination was analyzed by means of SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-fibrinogen antibody. The antibody used in this study reacts with fibrinogen, fragment X, Y, D-D, D and E. The validity of this technique was confirmed by the analysis of the serum samples from patients with definite diagnosis of disseminated intravascular coagulation. Serum samples of 14 patients out of 18 with elevated FDP values and severe liver diseases were shown to contain no plasmic digest of fibrin or fibrinogen. By using the SDS-gel electrophoresis after disulfide bond reduction, seven serum samples from these 14 patients, who were shown to have no plasmic digest in serum, were found to contain unclottable fibrinogen retained in sera, while the remaining seven samples were revealed to have fibrin monomer in their sera. Four serum samples from the 18 patients were shown to have plasmic digest of fibrinogen, but these patients had additional diseases leading to intravascular coagulation. —— fibrinogen degradation product; liver diseases; immunoblotting

The clotting time of a mixture of plasma and thrombin is often abnormally long in liver cirrhosis and may be increased in other hepatic disorders and in obstructive jaundice (Ratnoff 1954; Jim 1957; Green et al. 1976; Lane et al. 1977). In a few instances, the delay in clotting has been related to either marked decreases or increases in the concentration of fibrinogen in plasma. In most instances, however, other mechanisms appear to be responsible for an abnormally long thrombin time in cirrhosis of liver. A growing number of reports have recorded the presence of functionally abnormal variants of fibrinogen in patients with hepatic disorders. The variants are recognized because the fibrinogen extracted from the plasma coagulates abnormally slowly upon the addition of thrombin. This acquired dysfibrinogenemia was first found in cirrhosis of liver (Ratnoff 1954) and has since been described in association with hepatic carcinoma (von Felten et al. 1969) and some severe acute hepatitis (Soria et al. 1970). On

Received May 22, 1987; accepted for publication August 28, 1987.

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the other hand, we sometimes encounter with the elevated FDP levels in patients with severe liver disease (Merskey et al. 1966; Gallus et al. 1972). FDP in these sera, measured with latex agglutination, may represent unclottable fibrinogen retained in sera, incompletely aggregated fibrin monomer, degradation product by the action of plasmin or other proteases upon fibrin or complex of fibrin monomers with fibrinogen (Van DeWater et al. 1986). Most current test for fibrin split product or FDP dose not distinguish among these possibilities.

In this paper, we attempted to characterize the elevated FDP in severe hepatic disorders by using immunoblotting technique and clarify the nature of FDP in these disorders.

**Materials and Methods**

*Patients samples.* In order to establish the sensitivity and specificity of the immunoblotting procedure, we initially studied the sera from the patients with definite diagnosis of disseminated intravascular coagulation (DIC). The diagnostic criteria for DIC were the presence of decreased platelet counts, prolonged prothrombin time, the low levels of plasma fibrinogen and elevated serum FDP level as measured by latex agglutination. After the sensitivity and specificity of the immunoblotting procedure was established, we then studied the serum samples from patients with liver cirrhosis and hepatic carcinoma. For this purpose, the medical records of 202 consecutive patients with severe liver disease, who were seen and treated at Nagoya National Hospital between January 1983 and June 1986, were reviewed. The diagnosis of liver cirrhosis was based on the long standing jaundice, repeated episode of ascites, presence of esophageal varices and abnormal liver function. The diagnosis of hepatic carcinoma was made mainly by the findings of the space occupying lesion on computed tomography of the liver. In addition to the laboratory findings of liver disease, the other clinical relevant data, including thrombosis and bleeding as well as the presence of coincident conditions predisposing to DIC (infection, secondary malignancy and pregnancy) were studied. Out of 202 records, 18 cases with severe liver diseases associated with elevated FDP (higher than 20 \( \mu \)g/ml) were found.

*Blood collection.* Blood samples (10 ml) were routinely collected into citrate (0.1 ml, 1.4 M trisodium citrate) and epsilon aminocaproic acid (EACA, 0.2 M in final concentration) and centrifuged at 2,300 \( \times \) g for 15 min at 4°C to prepare plasma. To prepare serum, blood was collected in glass tubes containing EACA (0.2 M in final concentration), soy bean trypsin inhibitor (1,800 \( \mu \)g/ml) and thrombin (20 \( \mu \)g/ml) and allowed to clot for 2 hr at 37°C. Patients samples were stored at -20°C prior to study.

*Coagulation study.* Prothrombin time was estimated by the standard method using a fibrinometer (Biggs and MacFarlane 1976). Fibrinogen was determined as thrombin clottable protein according to the method of Ratnoff and Menzie (1951). Serum FDP level was measured using the Thrombo-Wellco Test (Burrough Wellcome Co., Research Triangle Park, NC, USA) (Allington 1971).

*Preparation of standard fibrin degradation product.* A 10 ml volume of fibrinogen (Kabi, Stockholm, Sweden), dissolved in water to a final concentration of 10 mg/ml, was added to 70 ml of 0.15 M sodium chloride containing 0.05 M Tris-HCl buffer (pH 7.4). Human factor XIII (Green Cross Co., Tokyo) and calcium chloride were added to the final concentration of 12 \( \mu \)g/ml and 0.025 M, respectively. Clotting was started by adding human thrombin (Sigma, St. Louis, MO, USA) to a final concentration of 0.025 u/ml and the solution was incubated at 37°C for 4 hr.

Plasminogen (Sigma) was activated for 1 hr at room temperature with urokinase (Green Cross Co.). The reaction mixture consisted of 5 mg plasminogen, 200 Ploug units of
urokinase, 0.1 M Tris-HCl and 0.15 M NaCl, pH 7.4 in a volume of 1 ml. Plasmin activity was determined by measuring the rate of increase of absorbance at 247 nm of a 1 mM solution of p-tosyl-l-arginine-methyl ester hydrochloride (Sigma) in 0.1 M Tris and 0.15 M NaCl buffer (pH 7.4) as described for trypsin. The preparation of plasmic degradation products from cross-linked fibrin was performed according to the method of Francis et al. (1979) at an enzyme-substrate ratio of $8 \times 10^{-2}$ CTA units plasmin per milligram fibrin and a digestion time of 4 hr. Fragment D and E were prepared with Pevikon block electrophoresis by the method of Marder et al. (1969).

**Gel electrophoresis and immunoblotting.** Serum samples (2.5 μl) were prepared for electrophoresis by diluting 1:20 in a solution of 25 mM Tris-HCl (pH 7.0) containing 1.7% sodium dodecyl sulfate (SDS), bromphenol blue (0.001%) and 10% glycerin and incubated at 100°C for 5 min. SDS-polyacrylamide gel (7.5%) electrophoresis of the serum samples was carried out by the method of Laemmli (1970). Electroblot transfer of separated proteins was adapted from Burnette (1981). The transfer was accomplished at 6 V/cm for at least 8 hr at 4-8°C. As electrode buffer, 30 mM Tris-Base, 190 mM glycine and 2% (v/v) methanol were used.

Immediately after transfer, the nitrocellulose sheet was immersed in phosphate buffered saline (PBS) containing 1% (v/v) gelatin and 0.5% (v/v) Nonidet P-40 and incubated for 90 min with mixing. The sheet was then transferred to PBS containing 0.2% gelatin and 0.5% Tween-20 and rabbit-antihuman fibrinogen (Hoechst, Hoechst, FRG) diluted 1:50 (final volume 10 ml) and incubated with mixing for 3 hr at room temperature. The sheet was rinsed in PBS containing 0.2% gelatin and 0.1% Tween-20 for 60 min. This was repeated once, followed by immersion in 10 ml of the same solution containing alkaline-phosphatase-conjugated anti-rabbit IgG (Hoechst), diluted 1:25 for 1 hr at room temperature. The nitrocellulose paper was rinsed again, as described before, then briefly blotted with filter paper and stained for alkaline-phosphatase.

Molecular weights of polypeptide chains were determined by comparison with standard myosin (200,000), β-galactosidase (116,000), phosphorylase B (92,500), bovine serum albumin (66,000) and ovalbumin (45,000). All of these standard molecular weight markers were obtained from BioRad Chemical (Richmond, CA, USA).

**Plasmic digest of serum and disulfide bond reduction.** Plasmin was added to serum to a final concentration of 0.15 CTA U/ml and the incubation started at 37°C. Digestion was terminated after 3 hr by the addition of soy bean trypsin inhibitor. Disulfide bond reduction was done by mixing the 200 μl aliquots of plasmic digest with 0.3 ml of 3.75% SDS, 0.38 M sucrose, 0.025% EDTA, 5% β-mercaptoethanol in 0.1 M boric acid and heating at 60°C for 2 hr.

**Results**

In the experiments to test the sensitivity of this immunoblotting methods, it was determined that when the plasma fibrinogen concentration was 40 μg/ml, the fibrinogen band was readily apparent after transfer. FDP levels in the range of 5 to 80 μg/ml were also easily visualized. In an attempt to test the specificity of this immunoblotting technique, we initially studied the sera from DIC patients. The pattern of immunoblotting experiments with anti-human fibrinogen on sera from patients with definite diagnosis of DIC is shown in Fig. 1.

In this figure, lanes 1 to 9 were serum samples of DIC and lane 10 and 11 were purified fragment E and D, respectively, that were used as standard. Lane 12 was the controlled plasmic digests of purified cross-linked fibrin that was also used as standard. In this lane, there can be seen high molecular weight fragments of
cross-linked fibrin (molecular weight greater than that of fibrinogen, i.e., fragments XY 391,000, DXD 461,000, YXD 500,000), smaller cross-linked fragment DY that migrates in fragment X region and fragment D-dimer clearly showing that the fibrin had been covalently cross-linked. Smaller fragment in which the cross-link had been removed (Y and trace D) and fragment E are also shown here. In serum samples from patients with DIC, fragment X and Y are clearly seen and fragment D-dimer are detected on immunoblotting, indicating the plasmic digestion of cross-linked fibrin in these patients. This staining technique was applied to the study of serum samples of patients with severe liver diseases.

As shown in Fig. 2, and Table 1, 14 samples (patient 1-14) out of 18 did not show any detectable bands of plasmic digest of fibrinogen or fibrin. In these patients, early high molecular weight substances (uncloitable fibrinogen or fibrin monomer) were found without any evidence of plasmic degradation product in spite of the fact that they had apparently high FDP values as measured by latex agglutination. The platelet count and fibrinogen levels in plasma were variable. Thrombin time was prolonged in most patients studied. Apparent FDP levels
were not correlated with the platelet counts, thrombin time or fibrinogen concentration. In order to distinguish whether the high molecular weight substance observed in sera of liver disease is unclottable fibrinogen retained in serum or fibrin monomers, the sera were, after plasma digestion and the reduction of disulfide bonds, analyzed by SDS-gradient (5-15%) gel electrophoresis, followed by immunoblotting. The pattern of immunoblotting of the sera is illustrated in Fig. 3. In half of the sera out of 14 serum samples tested, the Aα, Bβ, and γ chains of fibrinogen were shown, while in remaining half of the serum samples, γ-γ, Bβ and β chains of cross-linked fibrin were demonstrated, indicating the presence of fibrin monomer in the sera of these patients. In contrast to the 14 patients (patient 1-14 in Table 1) with liver diseases who only had evidences of fibrin monomer or unclottable fibrinogen in their sera, four patients (patient 15-18 in Table 1) with liver disease and increased apparent FDP level exhibited the presence of plasma digest of cross-linked fibrin in their sera which was shown on immunoblotting. However, each of these 4 patients had an additional disease independently associated with DIC (Table 1).

DISCUSSION

We have shown in this paper that plasma degradation of fibrinogen and/or fibrin does not occur frequently in patients with liver diseases who have elevated

Fig. 2. Immunoblotting of serum samples from patients with liver cirrhosis. Standard preparation of 10 μg of fibrin split product (lane 7 and 8) was compared with the electrophoretic pattern of the sera of patients with liver cirrhosis (diluted 1:20). Lanes 1-4 correspond to serum samples from patients 1-4 listed in Table 1, and lanes 5 and 6 correspond to those from patients 13 and 14 in Table 1. SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described in Materials and Methods.
FDP levels measured by the usual laboratory method. The apparently elevated FDP levels in plasma of these patients may be attributed to the acquired dysfibrinogenemia, resulting in the uncleotted fibrinogen or fibrin monomer retained in the plasma. A small number of patients with severe liver disease had evidence of plasmin degradation of fibrinogen, but these patients had the additional disorders leading to intravascular coagulation.

The original case report of the occurrence of the abnormal fibrinogen in a patient with hepatitis (Soria et al. 1968) described an increase in the sialic acid content and decrease in D-galactose levels in the fibrinogen molecule. The further works by von Felten et al. (1969), Soria et al. (1970) and Brodsky et al. (1970) suggested either synthesis of an abnormal molecule or the release of a precursor of fibrinogen from necrotic liver cells. The finding by Green et al. (1976) of an improvement in fibrin polymerization in the plasma of patients with liver disease when excess calcium was added provided additional evidence in favor of a defect of fibrin monomer aggregation. The defect of fibrin polymerization appears an important finding which can be demonstrated easily in a large proportion of patients with severe parenchymatous liver disease. It is conceivable that the plasma is deficient in some as yet unknown factor necessary for aggregation (Ratnoff 1954; Jim 1957). With regard to the incidence of increased

<table>
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<th>Liver disease</th>
<th>Secondary Diagnosis</th>
<th>FDP (μg/ml)</th>
<th>Fibrinogen (mg/100 ml)</th>
<th>Prothrombin time (sec)</th>
<th>Platelet (×10⁴/μl)</th>
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<tr>
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<tr>
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levels in cirrhosis, Merskey et al. (1966) found nine out of 21 patients with hepatic necrosis or cirrhosis had elevated FDP levels as measured by the tanned red cell hemagglutination inhibition immunoassay. However, Marongiu et al. (1985) reported normal levels of fibrinogen and FDP in all 26 cirrhotic patients. Increased serum FDP levels in liver disease were reported by other investigators (Bergström et al. 1960; Gallus et al. 1972; Hillenbrand et al. 1974), but the incidence of increased FDP in this disorder was not uniform among the literature. Moreover, no attempt has been made so far to characterize the increased FDP in cirrhosis.

In this study, we have shown that 14 out of 18 cirrhotic or hepatic cancer patients with increased FDP levels had no evidence of fibrinogen or fibrin degradation by plasmin, because plasmic digest (fragment X, Y, D, E and D-D) were not detected in the sera by SDS-polyacrylamide gel electrophoresis and immunoblotting. In seven out of these 14 of these patients, unclottable fibrinogen retained in serum was shown and in the remaining 7 patients, γ-γ dimers of cross-linked fibrin were identified by dissolving the cross-linked fibrin
with disulfide-bond reduction.

On the other hand, four patients with increased serum FDP values had evidence of plasmic digests of fibrin which were identified by SDS-gel electrophoresis followed by immunoblotting. These four patients who had plasmic digests were not different from 14 patients with no evidence of plasmic degradation in terms of the severity of liver disease, findings of hemostatic test or serum FDP levels.

However, these four patients had the additional disorders leading to intravascular coagulation.

In conclusion, considerable number of patients with severe liver cirrhosis and hepatic carcinoma show elevated serum FDP levels, but these apparent FDP values, as measured by Latex agglutination, represent unclottable fibrinogen or cross-linked fibrin monomer, unless the patients have additional associated diseases which cause intravascular coagulation.

Acknowledgment

This study was supported by the Grant-in-Aid from the Ministry of Health and Welfare of Japan.

References


