ORIGINAL ARTICLE
Discrepancy between Fibrinogen Concentrations Determined by Clotting Rate and Clottability Assays during the Acute-Phase Reaction
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(Received 22 February 2000 by Editor H. Arnesen; revised/accepted 7 August 2000)

Abstract
Assays based on clotting rate are commonly used as a routine method for determining the fibrinogen concentration in plasma. However, little is known about the influence of the acute-phase reaction on this assay. In order to disclose discrepancies between the fibrinogen concentrations obtained by a clotting rate assay (as described by Clauss) and a reference assay for total clottable protein (according to Jacobsson), we compared the fibrinogen concentrations determined by these two methods in plasma samples collected preoperatively and on postoperative days 1, 3, and 5 in patients undergoing major elective surgery. The HMW (High Molecular Weight)-, LMW- and LMW0-fibrinogen fractions of the patient samples were also determined. In preoperative samples, good agreement between the two assays was found. In samples collected on postoperative days 1 and 3, the fibrinogen concentrations measured with the clotting rate assay were significantly higher than the concentrations measured with the total clottable protein assay (p = 0.015 on both days). SDS-gel electrophoresis showed an increase in the median HMW-fraction from 69.7% (range 64.3–70.4) in preoperative samples to 85.8% (80.7–87.6) in samples drawn on day 3. The difference between fibrinogen concentrations obtained by the two methods was significantly correlated to the HMW-fraction of the samples (r = 0.81, p < 0.0001). We conclude that during an acute-phase reaction, fibrinogen concentrations obtained by a clotting rate assay (as described by Clauss) are significantly higher than those measured by a total clottable protein assay (according to Jacobsson). The difference between the two methods correlates well with the relative HMW-fraction, indicating that the increase in HMW-fibrinogen is the main contributor to the observed discrepancy. © 2000 Elsevier Science Ltd. All rights reserved.

Key Words: Fibrinogen; High molecular weight fibrinogen; Acute-phase reaction; SDS-gel electrophoresis

Fibrinogen is a dimeric glycoprotein consisting of 3 pairs of polypeptide chains (Aα, Bβ, and γ). Under normal conditions, less than 70% of the fibrinogen molecules have both their Aα-chains intact (high molecular weight [HMW]-fibrinogen, MW=340 kD). The remaining molecules have lost the C-terminal end of one low molecular weight LMW-fibrinogen, MW=300 kD or both very low molecular weight LMW-fibrinogen, MW=280 kD Aα-
chains, resulting in impaired fibrin polymerization [1,2].

During acute-phase conditions (such as major surgery, acute myocardial infarction, and inflammatory conditions), the fibrinogen concentration in plasma rises rapidly, mainly due to increased synthesis of HMW-fibrinogen [3,4]. Thereafter, the increased HMW-fraction gradually decreases toward normal, whereas the total fibrinogen concentration remains elevated for a longer period of time.

Many techniques have been applied to quantify the fibrinogen concentration in plasma. The total clottable protein method described by Jacobsson is often considered to be a reference method [10]. For routine use, however, assays based on the rate of plasma clotting after adding thrombin are frequently employed [5]. Other methods, like fibrinogen precipitation by heating to 56°C [6,7] or by adding salts [8], or immunological assays based on polyclonal antibodies [7,9] are less commonly used. Clotting rate assays and the clottability methods have demonstrated good correlation over a wide range of fibrinogen concentrations [6,11,12,13]. However, details concerning patient selection and time schedule for blood sampling have not been presented in these studies. Therefore, knowledge about the influence of an acute-phase reaction on the clotting rate assay is limited.

The aim of the present investigation was to 1) examine whether the fibrinogen concentrations determined by the routinely employed clotting rate assay (according to Clauss) deviated from the fibrinogen concentration obtained by the reference method (as described by Jacobsson) during an acute-phase response, and 2) determine whether a discrepancy between the methods could be explained by the alterations in the HMW-fraction.

1. Materials and Methods

1.1. Patients

Seven patients about to undergo elective surgery were studied (4 men, 3 women, median age 66 years). Three patients were undergoing total hip joint replacement, two had an explorative laparotomy, and two patients were undergoing hemicolectomy. All patients had normal preoperative electrolyte concentrations. No patients experienced renal failure prior to or following the operation, and no patients suffered from paraproteinemias. All patients were given intravenous infusions (median of 6 L clear fluids, range 4–10 L, and 600 mL of packed red blood cells, range 0–1.2 L) preoperative and during the first postoperative day.

Blood samples were collected the day before the operation and thereafter on postoperative days 1, 3, and 5.

1.2. Plasma Samples

Blood samples for the clottability and the clotting rate assays were collected into Vacutainers® (Becton-Dickinson, Cedex, France) tubes containing Na-citrate 0.13 mol/L in a ratio of 1 volume Na-citrate to 9 volumes blood. Blood samples for SDS-gel electrophoresis were collected into Vacutainers® containing 15% EDTA in a ratio of 1 volume Na-citrate to 9 volumes blood. Blood samples for SDS-gel electrophoresis were collected into Vacutainers® containing 15% EDTA in a ratio of 1 volume EDTA to 83 volumes of blood. Blood samples for SDS-gel electrophoresis were collected into Vacutainers® containing 15% EDTA in a ratio of 1 volume EDTA to 83 volumes of blood. Plasma was prepared by centrifugation at 2000 × g for 15 minutes at 4°C within 1 hour after collection, and stored in small aliquots at −70°C. The aliquots were thawed at 37°C for 10 minutes before use.

1.3. Reagents

- Urea (Merck, Darmstadt, Germany)
- Bovine thrombin (Thrombostat®, Parke-Davis, USA)

1.4. Buffers

- Sodium barbital buffer, pH 7.35 [14]
- Phosphate buffer containing EDTA. Na₂-EDTA (final concentration 0.0125 mol/L) was added to 0.13 mol/L phosphate buffer with pH 6.1.
- Sample gel- and running buffers for SDS 3% polyacrylamide–0.5% agarose gel electrophoresis were prepared as described by Weinstein and Deykin [15].

1.5. Fibrinogen Assays

The clotting rate assay was performed according to Clauss [5]. 200 µL of a solution containing 1
volume citrated plasma and 9 volumes sodium barbital buffer were incubated at 37 °C for 2 minutes. 100 μL thrombin 100 NIH U/mL was added, and the clotting time was determined. If the clotting time was shorter than 7 seconds, a higher dilution of plasma was used. If the clotting time exceeded 15 seconds, a lower plasma-dilution was used. The fibrinogen concentrations were read from a calibration curve made from eight serial dilutions ranging from 1/5 to 1/30 of pooled plasma from 35 healthy blood-donors. Each sample was analyzed four times, and the mean fibrinogen concentration calculated. The clottability method was used to determine the fibrinogen concentration in the pooled plasma. The intra-assay coefficient of variation for the clottability assay was 1.5% at a fibrinogen concentration of 3.6g/L(n = 10) and 1.6% at a concentration of 4.7g/L(n = 10). To test the correlation between the two assays, the fibrinogen concentrations of 28 healthy students (16 men, 12 women; median age 23 years) were determined with both methods. A good agreement between the two methods was found (Figure 1 and Table 1).

1.6. Preparation of Fibrin for SDS-Gel Electrophoresis

EDTA-plasma (0.4 mL) was added to 0.8 mL EDTA-phosphate and 0.4 mL thrombin 30 NIH U/mL. After clotting for 1.5 hours at room temperature, the clot was synerised, washed in 0.9% saline for 10 minutes, and then dissolved in SDS-sample buffer. The fibrin was diluted to a concentration of 0.25 mg/mL and incubated at 60°C for 30 minutes, allowing the fibrin to dissolve completely.

1.7. Electrophoretic Procedures

Gels (3% polyacrylamid/0.5% agarose) were prepared as described by Weinstein and Deykin [15]. The electrophoretic procedure was performed on a MiniProtean II® (BioRad, Richmond, CA, USA).

Portions (7 μL) of the samples were applied to each well and electrophoresed for 1.5 hours at 25 V (15 minutes), 75 V (30 minutes), and 125 V (45 minutes). The gels were Coomassie-stained (Fig-
ure 2), and the fibrinogen fractions quantified by densitometric scanning using Kodak image station 440 CF with Kodak 1D image analysis software. The intra-assay coefficient of variation for the electrophoresis and densitometric scanning was 1.8\%(n = 10).

1.8. Statistical Methods

The Wilcoxon paired signed rank test was used to test the difference between the fibrinogen concentrations obtained by the two methods used. The correlation between the HMW-fraction and the discrepancy between the fibrinogen concentrations obtained by the clotting rate assay and the clottability assay in plasma samples collected on days 0, 1, 3, and 5 was calculated using the Spearman’s rank correlation test. \( P \) values below 0.05 were regarded statistically significant (two tailed).

2. Results

No significant differences in fibrinogen concentration were found using clotting rate and the clottability assays in preoperative patient samples: The medians were 2.85 g/L (range 2.45–3.30) with the Clauss method and 2.75 g/L (2.38–3.36) with the Jacobsson assay (Figure 3). On the first postoperative day, there was a statistically non-significant increase in fibrinogen concentration measured with the clotting rate assay, whereas a non-significant fall in fibrinogen concentration was found with the clottability assay. The concentration was significantly higher with the clotting rate assay than the clottability assay on day 1\((p = 0.015)\).

On postoperative day 3, a statistically significant increase in fibrinogen concentration was observed with both assays compared to day 1. Furthermore, the concentration obtained by the clotting rate assay was significantly higher than the one found with the clottability assay: 5.5 g/L (range 5.04–6.9) with the Clauss assay and 4.93 g/L (4.24–5.6) with the Jacobsson assay \((p = 0.015)\). On the fifth postoperative day, no further increase in the fibrinogen concentration occurred, with either the clotting rate or the clottability method. There was no statistically significant difference between the two methods at this time.

2.1. SDS-Gel Electrophoresis

Preoperative samples from seven patients showed a median HMW-fibrinogen fraction of 69.7\% (range 64.3–70.4), LMW-fibrinogen 26.2\% (24.2–27.1), and LMW\(_0\)-fibrinogen 6.14\% (3.5–7.9) (Figure 4). A statistically significant increase in the HMW fraction was observed in all patients on the first and third postoperative days: 80.6\% (76–83.1) \((p = 0.01)\) and 85.8\% (80.7–87.6) \((p = 0.03)\) of the total fibrinogen concentration on days 1 and 3, respectively. The highest HMW-fibrinogen fraction was observed on postoperative day 3 in all patients. On postoperative day 5 the median HMW-fibrinogen fraction was 79\% (range 74.2–
81.3). This was still significantly higher than on day 0 \(p = 0.03\). The relative LMW- and LMW'-fibrinogen fractions decreased correspondingly on both days (Figure 4).

Furthermore, a highly significant correlation between the HMW-fraction and the discrepancy in fibrinogen concentration obtained by the two assays was found \(r = 0.81, p < 0.0001\).

### 3. Discussion

In the present study, higher fibrinogen values were obtained by the clotting rate method (Clauss) than with the clottability method (Jacobsson) following major elective surgery. The clotting rate assay showed significantly higher fibrinogen concentrations than the clottability assay in all postoperative samples collected on days 1 and 3. The differences correlated well with the observed increase in HMW-fibrinogen fraction. The greatest HMW-fraction was observed on day 3, and coincided with the largest discrepancy between the two tested fibrinogen assays.

Several mechanisms may have contributed to the observed discrepancy between the two methods. The clotting rate assay is carried out by adding a large amount of thrombin (final concentration 33 NIH U/mL) to plasma diluted 1/10–1/20 fold. The cleavage of fibrinopeptide A (FPA) thus occurs very rapidly, resulting in a clotting time mainly dependent on the fibrinogen concentration and the polymerization properties of the fibrin monomers formed. Since it has been demonstrated that monomers formed from HMW-fibrinogen polymerize more rapidly than monomers formed from LMW-fibrinogen [1], this difference may constitute the main contributor to the observed discrepancy between the two methods for quantification of fibrinogen applied in the present study.

Acute phase conditions have also been shown to affect the degree of phosphorylation of fibrinogen [16]. During acute-phase conditions, such as following major surgery, the degree of phosphorylation has been shown to increase from the normal range of 25–30% to more than 50% within 24 hours [17]. Conflicting results have been reported regarding the effect of phosphorylation of FPA on the release rate of FPA by thrombin [18–21]. However, a more effective cleavage of FPAp than of FPA would probably have a negligible effect on the clotting rate assay used in the present study, since concentrated thrombin is added to very diluted plasma. Furthermore, the highest fraction of phosphorylated fibrinogen has been found within the first day after surgery [17], whereas the discrepancy between the two methods in our study was greatest three days after surgery.

All patients in our study received intravenous infusions. A dilution of plasma proteins including fibrinogen would, however, be expected to affect both assays to the same extent and not change the ratio between fibrinogen concentrations obtained by the two assays. Following major surgery, temporary changes in plasma concentrations of proteins other than fibrinogen, as well as low molecular substances, such as urea and electrolytes, take place. Such changes could theoretically be of sufficient magnitude to affect the fibrin polymerization rate, and thus the results obtained by the clotting rate assay in our study. Due to the 1/10–1/20 fold dilution of the plasma samples before assaying, however, the effect of such changes would be negligible.

In addition to methodological aspects, the present observations demonstrate the more rapid polymerization and aggregation of HMW-fibrin monomers, reflected by the higher fibrinogen
concentration measured by the clotting rate method. This increased polymerization rate may contribute to the high risk of venous thrombosis observed in acute-phase situations. In addition, it has recently been shown that an elevated HMW-fibrinogen concentration is associated with an increased risk of ischemic complications in patients presenting with an acute myocardial infarction [22].

An increased fibrinogen concentration is a strong predictor of atherosclerotic disease [23, 24, 25, 26]. However, it is controversial whether fibrinogen plays a causal role in the development of atherosclerosis or is simply a marker of the low-grade inflammation in atherosclerotic lesions. It is not known whether this inflammatory reaction may increase the HMW-fibrinogen fraction in these patients, and thus influence the fibrinogen concentration determined by a clotting rate assay. In order to achieve better insight in this matter, it would be of interest to study the qualitative properties of fibrinogen in patients with atherosclerotic disease.

References


