Summary

A soluble fibrin (SF) preparation has been developed as a potential standard by the Scientific and Standardization Committee for use in assays evaluating in vitro preparations and patient plasma samples. The SF standard was prepared by reaction of factor XIII-free fibrinogen with thrombin, followed by neutralization with hirudin and solubilization of the fibrin in acetic acid. As characterized by SDS-PAGE, the polypeptide chain structure shows the anticipated loss of fibrinopeptides and lack of ε or α chain crosslinking. The standard was added to pooled normal plasma at concentrations from 12.5 μg/ml to 340 μg/ml and tested with four commercially available assays based on immunologic reactions using ELISA or latex agglutination or on t-PA cofactor activity for plasminogen to plasmin conversion. Absolute “soluble fibrin” concentrations were calculated using the manufacturers’ calibrators and showed distinct dose-response relationships for each assay. Expression of the results following log-transformation produced a series of parallel lines, indicating that this SF preparation can serve as a standard, effectively normalizing the disparate proprietary internal calibrators currently used for each assay.

Introduction

Following reaction of fibrinogen with thrombin, fibrinopeptides A and B (FPA, FPB) are liberated, and fibrin monomers are formed that may circulate in plasma as soluble fibrin (SF) or polymerize into an insoluble fibrin deposit. Assays for FPA have been developed (1) and applied to characterization of hypercoagulable states (2), but the short half-life of FPA combined with the high sensitivity of the assay and potential artefactual elevation during venipuncture and in vitro plasma processing has curtailed its wide application (3). SF represents another molecular species resulting from the thrombin-fibrinogen reaction, and is potentially a useful marker of hypercoagulability with clinical application in the diagnosis and management of thrombotic disease. SF is not, however, a homogeneous molecular species, as different molecular species may have variable cleavage of FPA or FPB, and fibrin monomers can form complexes with fibrinogen, other fibrin monomers or degradation products of fibrinogen or fibrin. Further heterogeneity results from variable crosslinking of SF and fibrin degradation products by factor XIII.

Several methods for measuring SF in plasma have been developed, including assays based on the production of circulating fibrin polymers demonstrated by agarose gel electrophoresis (4), or quantitation of crosslinked fibrin polymers (5). Commercial assays based on immunologic reactions of antibodies directed against fibrin neo-epitopes exposed by thrombin action on fibrinogen (6-9) or on the fibrin-mediated acceleration of tissue plasminogen activator-induced conversion of plasminogen to plasmin (10, 11) have also been developed. Some have been evaluated in patients with thrombotic disorders, including venous thromboembolic disease (12-16), myocardial infarction (17, 18), DIC (19, 20) and complicated pregnancies (21, 22). However, despite studies that compare assays in a given group of patients (23-25), the comparison of results with one assay to those with another is hampered by the use of different proprietary calibrators for each assay. Because of the importance of SF as a marker of in vivo thrombin activity and its potential clinical value, the Subcommittee on DIC of the Scientific and Standardization Committee initiated a review of available SF assays, the first goal of which was to develop a uniform SF standard that could serve as a foundation for rational study design and quantitation of in vitro and in vivo test results. It was planned that the preparation would be made available to companies producing assay kits to aid in calibration. After initial discussion at SSC meetings in Jerusalem (1995), Barcelona (1996) and Florence (1997), a proposal in Ljubljana (1998) to adopt the material described herein as a standard for SF measurement was approved. Details of preparation of this SF standard and its quantitative reaction with selected assays follows.

Materials and Methods

Plasma preparation. After obtaining informed consent, 30 ml of blood was drawn into sodium citrate (0.38% final concentration) byatraumatic venipuncture from 40 normal healthy volunteer laboratory personnel. Plasma was prepared by centrifugation at 3,000 × g at 4 °C for 30 min, pooled, aliquoted and frozen at –80 °C. Tests were performed within six months of collection on freshly thawed samples.

Preparation of soluble fibrin. Lyophilized fibrinogen depleted of plasminogen and fibronectin was purchased from Enzyme Research (Lafayette, IN). The plasminogen content as measured by chromogenic assay with S2251 (H-D-valyl-L-leucyl-L-lysine-p-nitroaniline di-hydrochloride) (26) was <0.05 CU/ml, and the fibronectin content by ELISA assay (Biomedical Technologies Inc., Stoughton, MA) was < 0.1 μg/ml. Factor XIII in the preparation was inactivated by dialysis of reconstituted fibrinogen against 3.3 M urea containing 0.2 M epsilon aminocaproic acid for 18 h at 25 °C, and the fibrinogen was subsequently dialyzed against 0.02 M Tris buffer, pH 7.6 containing 0.15 M sodium chloride and 5mM EDTA. Factor XIII activity was assessed by analysis of γ chain crosslinking following clotting with thrombin (Fig. 1). SF was
prepared by a modification of the method of Soe et al. (9). Briefly, fibrinogen (11.2 mg/ml) was clotted with human thrombin (Calbiochem-Novabiochem, La Jolla, CA) at 37°C for two hours, after which hirudin (Sigma, St. Louis, MO) (2.5 U/ml final) was added to inactivate remaining thrombin. Supernatant fluid was removed by compression and absorption with cheese-cloth, and the clot was incubated in 0.02 M acetic acid (5 mg/ml) overnight at 37°C with gentle agitation. Small amounts of residual thrombus were removed by centrifugation at 10,000 × g for 20 min, and the SF concentration was determined by measuring optical density at 280 nm. The polypeptide chain composition of fibrinogen and fibrin was determined by electrophoresis in SDS–7% polyacrylamide gels.

Soluble fibrin assay. SF in 0.02 M acetic acid was diluted in pooled plasma containing hirudin (1 U/ml) to final concentrations of 12.5–340 μg/ml. Relevant descriptive information on the four assays tested against the SF standard are noted in Table 1.

Test A (Enzymun-test FM®, Boehringer Mannheim, Germany) is an enzyme-linked immunosorbent assay (ELISA) using an antibody directed against the α chain N-terminal residues 17-23 for both the biotinylated capture and the peroxidase-labeled tag antibodies (27). For this assay, 20 μl of test plasma is incubated with 60 μl of 5.33 M potassium thiocyanate, 0.02M sodium phosphate, pH 7.3 for 30 min to dissociate noncovalent complexes. One ml of biotinylated capture antibody solution (1.3 μg/ml biotinylated antibody, 0.1 M potassium phosphate, 5 mg/ml bovine serum albumin 0.5 μg/ml Tween 20, pH 7.0) is incubated with the plasma for 30 min in streptavidin-coated tubes. After washing with 4.3 mM sodium chloride solution, 1 ml of the tag antibody peroxidase-labeled solution is added to the tubes, incubated for 30 min and washed. Chromogenic reagent (0.95 mg/ml 2,2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) is added, incubated for 30 min, and optical density is measured at 422 nm. Concentration is based on comparison with a standard curve obtained with lyophilized calibrator consisting of plasma supplemented with des-AA fibrin provided by the manufacturer, using an automated system provided by and applied according to manufacturer’s instruction (6).

Test B (Fibrinostika® Soluble Fibrin, Organon Teknika, The Netherlands) is an ELISA in which capture antibody is directed against a neo-epitope on residues 312-324 of the fibrin γ chain (7). The tag antibody is a horseradish peroxidase-conjugated antibody directed against the carboxy-terminal α chain appendage (8). Plasma samples are diluted 1:5 in phosphate buffer prewarmed to 37°C, and 100 μl of the diluted sample is added to micro-ELISA strips coated with the capture antibody, then incubated at 37°C for 90 min. After washing, 100 μl of horseradish peroxidase-conjugated tag antibody is added to the wells and incubated at 37°C for 60 min. A volume of 100 μl chromogenic substrate (tetramethylbenzidine in urea peroxidase) is added and incubated for 30 min at 25°C. The reaction is terminated by 100 μl of 1 M sulfuric acid, and optical density is measured at 450 nm. SF concentration is derived from a standard curve prepared with calibrators based on thrombin-mediated conversion of fibrinogen to SF provided by the manufacturer.

Test C (Coatest® soluble fibrin, Chromogenix, Sweden) is a chromogenic assay based on SF acceleration of t-PA-mediated conversion of plasminogen to plasmin (10). A solution containing antibody against anti-plasmin is added to the wells in microwell strips which are precoated with plasminogen, recombinant t-PA and a plasmin-sensitive chromogenic substrate for 2-4 min prior to testing. Plasma samples are diluted 1:21 in 0.1 M Tris-acetate buffer, pH 7.9 containing 0.025% Tween 80 and incubated in the microwell strips. After incubation for 15 min at 20°C, absorbance is measured at 490 and at 405 nm. The difference in absorbance at 405 and 490 nm is subtracted from the control supplied by the manufacturer, and SF concentration is derived by a standard curve, also provided by the manufacturer.

Test D (Iatron Laboratories, Chiba, Japan) is an agglutination assay using latex beads coated with an antibody directed against an epitope on residues 17-78 of the fibrin α chain, as described by Soe et al. (9). An aliquot of 10 μl of plasma is mixed with 40 μl of 0.4% (w/v) latex bead suspension and added to 350 μl of 0.05 M Tris buffer, pH 8.0. Aggregation is measured by absorbance at 950 nm in proportion to the aggregation using an SF calibrator of acid-solubilized des-AABB fibrin provided by the manufacturer.

### Results

The polypeptide chain composition of a representative sample of SF is shown in comparison with fibrinogen, non-crosslinked fibrin and crosslinked fibrin in Fig. 1. SF and non-crosslinked fibrin both show α and β chains of slightly greater mobility than the fibrinogen Aα and Bβ chains, due to cleavage of FPA and FPB, and monomeric γ chains are present without evidence of γ-γ dimmer formation. Crosslinked fibrin shows γ-γ dimers, no γ chain monomers and decreased monomeric α chains with large molecular weight α chain polymers. The results indicate that SF is homogeneous and devoid of γ or α chain crosslinking, consistent with the purification process designed to inactivate factor XIII.

The SF preparation was added to pooled normal plasma at final concentrations of 12.5 μg/ml to 340 μg/ml and tested with four commercially available assays for SF (Fig. 2). The lower limit of sensitivity for SF was similar for all assays (12.5-25 μg/ml), and all showed increasing reactivity within the range tested, but the dose-response curves were distinctly different. Test A showed a minimal increase in optical density at concentrations of SF up to 100 μg/ml, followed by a roughly linear increasing response at concentrations between 100 μg/ml and 340 μg/ml. Test B produced a sigmoidal curve from 12.5 μg/ml to 340 μg/ml, whereas Test C had a linear increase from the detection limit of 12.5 μg/ml up to 200 μg/ml, with a decreasing slope up to 340 μg/ml. Results with test D were linear over the full range of concentrations up to 340 μg/ml.
To normalize these disparate and generally non-linear results, which used distinctly different assay endpoints, the data were log-transformed for direct comparisons (Fig. 3). The concentrations between 25-200 μg/ml were used as all assays resulted in a linear relationship between SF concentration and test results within this range. The test for parallelism was negative (p = 0.41) by analysis of covariance supporting the parallel line hypothesis. The second step in the analysis was to fit a model assuming parallel lines. The value for R² was very high (98%) thus the values are very close to linear.

**Discussion**

Assays for SF are based on reactions of monoclonal antibodies against distinct epitopes or on functional properties of fibrin that influence interactions between plasminogen and t-PA, and different molecular species of SF could either exaggerate or minimize assay reactivity. The lack of concordance between results with patient samples using different assays (23-25) may relate to these differences in assay specificities, but they are also complicated by the use of disparate internal calibrations for which no description is provided. A reference standard for SF would allow better interassay comparisons and would facilitate clinical interpretation. In this report we describe a preparation of SF and its assessment in four commercially available assays developed for measurement in plasma. Gel electrophoretic analysis of subunit polypeptide chains demonstrate that it is a homogeneous preparation of noncrosslinked fibrin (Fig. 1). All four assays were reactive with the SF preparation, with comparable sensitivity and concentration-dependent parallel linear results after log transformation of the data within a specified range (Fig. 3). Since the absolute results are based on each manufacturer's distinct internal SF calibrator, the SF preparation described here is shown to be a reasonable standard for different SF assays.

Other preparations of purified SF or treated plasma might also be considered for use as standards. For example, timed exposure of plasma fibrinogen to a low concentration of thrombin followed by hirudin neutralization would generate SF in plasma. Such a preparation has the advantage of mimicking pathologic conditions in which SF is formed in a plasma milieu, but would be difficult to standardize, and multiple molecular species would result due to variable fibrinopeptide cleavage and crosslinking. Also, ancrod cleavage of FPA (but not FPB) from purified fibrinogen or plasma could be used to generate noncrosslinked fibrin. This has the advantage of lack of crosslinking capacity and improved solubility, but the disadvantage is that FPB cleavage occurs physiologically with thrombin action and could generate molecular species with different reactivities.

The SF preparation described here is prepared using factor XIII inactivated fibrinogen, and it is therefore not crosslinked, although formation of crosslinked species after addition to plasma is possible. It has an advantage over a standard incorporating whole plasma (28), which would be more likely to undergo *in vitro* change upon storage, freezing and thawing than non-crosslinked purified preparations that are frozen or lyophilized in buffer. The homogeneity of the preparation described here does not mimic completely the complex derivatives resulting from *in vivo* fibrin formation in patients that may include crosslinked species and macromolecular complexes (29). It is likely that various assay approaches will react differently to degradation products of SF and degradation products of fibrin and fibrinogen, thereby adding variably to assay results. However, the principal use of

*Fig. 2* Soluble fibrin results using four commercially-available assays. Panels A-D show data for tests summarized in Table 1, with pooled normal plasma containing the SF preparation at the indicated final concentrations.
our SF preparation is to standardize SF assays, thereby permitting one to compare results between assays and to improve characterization of products in patient samples. For this purpose, the SF preparation provides the required properties of a homogeneous, well-defined molecular structure, with comparable reactivity with at least four assay techniques. The next step in developing this as an international standard will be a collaborative study involving several laboratories to determine inter- and intralaboratory variation, reproducibility of the preparation and its stability.

References