Effect of Streptokinase on Fibrinogen and Related Proteins in Plasma

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We investigated the effect of streptokinase on determination of fibrinogen and related clottable proteins in an effort to assess the relative reliability of these determinations in monitoring patients being treated with streptokinase. Citrated plasma was incubated with and without streptokinase, then assayed for fibrinogen (I), fibrin(ogen) degradation products (II), total clottable protein (III), and plasminogen (IV). Values for I decreased rapidly. Values for III and IV generally paralleled those for I, values for III lacked adequate sensitivity. II, low initially, increased to 80 mg/L in 1–2 h. Soybean trypsin inhibitor effectively stabilized all these constituents (except IV) in streptokinase-treated plasma, and we recommend that this (or some other such) inhibitor of fibrinolysis be used routinely when specimens are collected for these determinations. Data are presented that suggest that transient polymers are formed, the clinical significance of which is not known.

Additional Keyphrases: monitoring therapy • clotting • plasminogen • fibrin degradation products • disorders of hemostasis

In recent years the use of enzymes as fibrinolytic and thrombolytic agents for treating patients in a variety of clinical circumstances—including deep-vein thrombosis, peripheral-artery occlusion, pulmonary embolism, and myocardial infarction (1–4) —has generated much interest. Streptokinase (SK, streptococcal fibrinolysin) and urokinase (plasminogen activator, EC 3.4.21.31) have been the agents most widely used to date (5) although tissue plasminogen activator seems promising for the future (6). Because urokinase is more expensive, streptokinase is probably somewhat more popular. As with many therapeutic modalities, however, effective management relies heavily on laboratory determinations to assess the adequacy of the therapy on the one hand and to prevent toxicity (i.e., serious hemorrhage) due to excessive concentrations on the other hand.

Fibrinogen and thrombin-time determinations are two of the laboratory tests most frequently used to monitor such patients. The thrombin time is a rapid and sensitive screening test. It depends primarily on the concentration of fibrinogen and fibrin degradation products in plasma but is only semiquantitative. Because fibrinogen can now be quantified with automated instrumentation, fibrinogen measurement alone frequently suffices. The availability of synthetic substrates and automated methods for assay of plasminogen has made this determination an attractive adjunct to fibrinogen quantification.

Although the validity of measuring fibrinogen and plasminogen has been adequately demonstrated for patients with certain classes of hemostatic disorders, the reliability of these tests in regulating dosages during thrombolytic therapy to achieve the necessary "systemic" lytic state (1) is not as well established. Moreover, the effect of therapy with streptokinase on the laboratory determination of fibrinogen and related proteins has not been investigated in detail, although brief reports have been published recently (7–9). These reports indicated that discrepant values were obtained when fibrinogen was measured with the DuPont aco method and the Dade Data-Fi method. We therefore studied the quantitative effect of streptokinase addition to plasma on the analytical determination not only of fibrinogen but also of fibrin(ogen) degradation products, total clottable protein, and plasminogen. We suggest possible ways of increasing the reliability of these assays.

Materials and Methods

Materials

Soybean trypsin inhibitor (cat. no. T9008) was obtained from Sigma Chemical Co., St. Louis, MO 63178. Streptokinase ("Streptase"; Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ 08876) was stored at −70 °C in 0.1-mL aliquots (100 000 int. units/mL). All other chemicals were reagent grade unless otherwise noted.

The human plasma was unused blood-bank plasma, obtained for this study within five days of donation and stored for 4 °C preserved with citrate/dextrose/adamnin buffer anticoagulant. These samples, which were from three donors with no recent streptococcal infection, were not combined; the data from each were equivalent. The various plasma pools with different amounts of soybean trypsin inhibitor and SK were prepared within 10 days for the various experiments except as noted. To minimize the amount of time between addition of SK and the actual analytical determinations, plasma pools were prepared with or without inhibitor in various concentrations (0–2.5 mg/mL). At zero time, various amounts of a working solution of SK were added and aliquots were withdrawn at specified times for analyses. The working solution of SK was prepared by reconstituting a 10 g/L stock solution of SK with phosphate buffer (0.1 mol/L, pH 7.0) to give a final concentration of 100 int. units/mL.

Methods

Fibrinogen and plasminogen were determined in a DuPont aco II analyzer according to the manufacturer's suggested protocols. The composition of the reagent packs and optimization studies have been previously described (10). For calibration we used a five-point standard curve generated with the use of DuPont fibrinogen standards in concentrations of 650, 1770, 2890, 5030, and 7170 mg/L. Fibrinogen was also determined in a MLA 750 analyzer (Medical Laboratory Automation, Inc., Mount Vernon, NY 10550) with use of Dade (American Dade Division of American Hospital Supply Corp., Miami, FL 33172) "Data-F" fibrinogen reagents. The method was calibrated by using three Dade fibrinogen standards in the range between 500 and 4500 mg/L.

Fibrin(ogen) degradation products were measured by use of the Thrombo-Wellcote test latex particle agglutination procedure (11).

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Total clottable protein was determined by a modification of the method of Saleem et al. (12). Plasma (0.4 mL) was added to a small flask containing 12 mL of isotonic saline (NaCl, 8.5 g/L), 0.4 mL of phosphate buffer (0.2 mol/L, pH 6.4), and 0.6 mL of CaCl₂ (a 10 g/L solution of the anhydrous salt). After mixing, the solution was allowed to stand at room temperature for 15 min. Fibrin was then removed by winding it onto a glass rod. After rinsing and blotting the rod, the fibrin was dissolved by placing the rod in a tube containing 2 mL of a 30 g/L solution of NaOH and leaving the tube in a boiling water bath for 10 min. An aliquot was then removed for protein quantification by the biuret method (13).

For immunoelectrophoresis we used agarose plates (Corning Medical and Scientific, Corning, NY 14831) and antibody to fibrinogen (Cooper Biomedical, Inc., Malvern, PA 19355).

Results

It has been previously reported that discordant results were obtained when samples from patients being treated with streptokinase were assayed for fibrinogen by the DuPont axl method and the Dade Data-Fi method (7-9). The axl-derived values were consistently higher (>5000 mg/L), those by the Dade method were low (<1500 mg/L).

The etiology of the discrepancy was not determined, however. Figure 1 shows results of an experiment in which we added streptokinase to plasma and assayed for fibrinogen, plasminogen, total clottable protein, and fibrinogen degradation products. Fibrinogen was measured in both the DuPont axl and the MLA 750 (using Data-Fi reagents). As can be readily seen, values for all these constituents were grossly abnormal within 30 min of SK addition. The control plasma to which no SK was added gave mean values for fibrinogen (axl) of 2275 mg/L, for fibrinogen (Dade) of 1850 mg/L, for total clottable protein of 1838 mg/L, for fibrinogen degradation products of 10 mg/L, and for plasminogen of 87.1% of a normal plasma pool. Values for fibrinogen immediately after streptokinase addition were 10% higher than those obtained for the control plasma to which no streptokinase was added, when both were analyzed by the Dade method. Nevertheless, SK caused the fibrinogen values to decrease to less than 6% of the control in 1 h.

Values with the Dade method declined from an initial value of 70% of control to less than 50% over the same time period. The finding that DuPont values were consistently higher than Dade values at any time less than 30 min after addition of SK is consistent with the previously reported discrepancies between the two methods. Interestingly, however, the later axl values are significantly lower than those by the Dade method. SK has no direct effect on the DuPont fibrinogen method because, when SK was added to serum, no fibrinogen was detected. Fibrinogen values increased rapidly to a constant value of 50 mg/L. Total clottable protein could not be quantified, even at the earliest time when SK was present, because a cohesive fibrin clot could not be formed. At zero time and at 30 min there was visible turbidity, owing to fibrinogen polymerization, but the polymer could not be recovered on a glass rod as required by the method. Presumably this is also the reason that the value for total clottable protein is less for control plasma than for fibrinogen. Plasminogen values decreased rapidly, from 76% of control initially to 33% after 2 h.

Since certain protease inhibitors are known to inhibit the action of plasmin (formed by SK activation of plasminogen) on fibrinogen (14), we investigated the potential stabilizing effect of soybean trypsin inhibitor on fibrinogen and related polypeptides in SK-treated plasma. Figure 2 summarizes the effect of this inhibitor on fibrinogen, fibrinogen degradation products, total clottable protein, and plasminogen in plasma to which SK has been added: it effectively stabilized these analytes in all samples containing SK, except for plasminogen. Fibrinogen values are slightly higher by the DuPont method than by the Dade method because slightly different calibrators are used in the two methods. This difference is reflected in differences in mean values for the control plasma when analyzed with the two systems (i.e., 2345 mg/L (DuPont) vs 2200 mg/L (Dade)). To check for any direct effect of the inhibitor on fibrinogen determinations, it was also added to control plasma. By the DuPont method, the mean value (±SD) for four replicates was 2345 (13) mg/L without inhibition and 2263 (91) mg/L with it. Corresponding results by the Dade method were 2200 (178) and 2225 (50) mg/L. Evidently this inhibitor has no significant effect on fibrinogen measurements by either method. These results indicate what would be expected as a result of the use of soybean trypsin inhibitor in plasma samples and suggest that its use would be very effective in preserving samples from patients on therapy with SK.
In the case of plasminogen, soybean trypsin inhibitor is known to inhibit the action of plasmin on fibrinogen, and that it does not prevent the formation of the SK–plasminogen activator complex, which is formed at the expense of free plasminogen. Moreover, in the DuPont plasminogen incubation, residual plasminogen is known to be used and so it detects free and “complexed” plasminogen as well as plasmin. However, soybean trypsin inhibitor inhibits free plasmin, thus only residual plasminogen is detected.

In an attempt to investigate the higher values obtained for fibrinogen by the DuPont method at early times in the incubation, we repeated the experiment summarized in Figure 1 but took samples at 1, 2, 5, 15, and 30 min for analysis. The results (Figure 3) clearly demonstrate that there is a significant but transient increase in values for fibrinogen when samples are analyzed by the DuPont method within the first 5 min after exposure to SK. The other methods show a consistent decrease with time. To determine if immunologically active fibrinogen was present throughout the experiment, we also analyzed aliquots of each specimen by immunoelectrophoresis, using antibodies to fibrinogen. All specimens showed the characteristic fibrinogen arcs (data not shown), but we could discern no quantitative differences. Because antibodies to fibrinogen cross react with fibrinogen degradation products to various extents, it was impossible to say how much intact fibrinogen remained at the end of 30 min.

It should be noted that, even with extreme care to analyze samples as quickly as possible after introducing SK, plasminogen had decreased by nearly 50% of its initial value (98.6%, not shown) within 1 min of the addition of SK. The difference in fibrinogen values by the DuPont and Dade methods at 1–2 min is similar to the difference in control values for the two methods and reflects a slight difference in methods themselves, as discussed above.

Discussion

Although thrombolytic therapy with streptokinase has been demonstrated to be of clinical value in the treatment of deep vein thrombosis, pulmonary embolism, peripheral artery occlusion, and myocardial infarction, the value of fibrinogen estimates made with the DuPont aco in monitoring these patients has recently been questioned (7–9). Our data on normal plasma to which streptokinase is added indicate that there is indeed a discrepancy between values obtained by the DuPont method and the Dade method. Moreover, the direction of the bias for plasma which has been exposed to streptokinase for less than approximately 20 min is the same as has been reported (i.e., DuPont > Dade). But it should be remembered that the direction and magnitude of the bias is time dependent. Estimates of total clottable protein seem to parallel fibrinogen values; however, owing to difficulties in accomplishing complete fibrin recovery, the analytical sensitivity of this technique is poor. This problem may be due in part to the effect of fibrinogen degradation products on the formation of a stable clot as well as the effect of the low fibrinogen concentration in most of the samples. Determinations of fibrinogen degradation products seem to be of some value but are only semiquantitative. It is somewhat surprising nonetheless that values do not exceed 80 mg/L even after samples have been exposed to streptokinase for 2 h.

An equally important concern is whether the time-dependent decay demonstrated for both methods in our in vitro study reflects the decay time of fibrinogen in vivo. Because the concentration of fibrinogen in plasma samples can be stabilized by use of the inhibitor (Figure 2), our data suggest that the in vitro decay would lead to an underestimate of fibrinogen by both methods if samples are not handled appropriately. This problem can be obviated by assaying promptly after the specimen is withdrawn or by inhibiting fibrinogen degradation by use of a suitable inhibitor such as soybean trypsin inhibitor. In addition, the use of this inhibitor eliminates the discrepancy between values for fibrinogen by the DuPont method and by the Dade method. The only bias remaining in our data is the inherent bias between the two methods, which exists for all patients. Because analysis of samples without delay presents major logistical problems in most circumstances and because analytical biases are eliminated with use of the inhibitor, we propose its use for preserving all samples collected from streptokinase-therapy patients when fibrinogen and related constituents (except plasminogen) are to be measured. We are currently investigating the efficacy of this approach with clinical samples and the results should be the subject of a subsequent report. Plasminogen values as measured in the DuPont aco are not stabilized by the addition of soybean trypsin inhibitor, and because of this such values are probably less useful for monitoring patients undergoing streptokinase therapy. Other anti-fibrinolytic agents, such as e-amino caproic acid, that directly inhibit plasmin as well as plasminogen activity may be useful in stabilizing plasminogen but this premise has not been investigated.

With regard to the etiology of the transient increase in apparent fibrinogen measured by the DuPont aco (Figure 3), we believe that this arises from the production of an intermediate "clottable" Fragment X (17) by the action of plasmin on fibrinogen. This hypothesis is based on the fact that proteases such as trypsin and plasmin are known to cleave the Bβ chain of fibrinogen between arginine 14 and glycine 15 and between arginine 42 and alanine 43 in addition to sites further along the Bβ chain (16). These cleavages give rise to "early" Fragment X's (17). Thrombin cleaves the Bβ chain only at position 14 to give rise to fibrinopeptide B. Because cleavage of fibrinogen by plasmin can therefore give rise to fibrinopeptide B as well as larger fragments, it is not surprising that some of the products of plasmin-derived cleavage should form polymers. It should be noted that selective cleavage of the Bβ chain of fibrinogen with the use of copperhead (Agkistrodon contortrix) snake venom cleaves the chain only at position 14 and gives rise to aggregates (18). However, these polymers are not as stable as non-crosslinked fibrin, particularly at temperatures exceeding 25 °C (19). Figure 4 summarizes the proposed model for formation of the unstable Fragment X. In
the "clottable" Fragment X molecules there is incomplete removal of fibrinopeptides A or partial cleavage of the Bβ chain past residue 14, or both, and they do not form stable polymers above 25 °C. In both the DuPont acc and the MLA 750 analyzer turbidity is measured at 37 °C, a temperature at which these polymers are unstable. However, in the DuPont method Dextran T10 is incorporated to increase the turbidity of fibrin polymers (10, 20). We therefore suggest that, as a consequence, this transient polymer is detected by the DuPont method while use of the MLA 750 analyzer with Dade reagents does not stabilize these polymers and they remain undetected.

Immunoelectrophoresis with anti-fibrinogen antibodies was not helpful in further elucidating this process because they have significant and variable cross-reactivity with Fragment X's. At the time of our preliminary report of these findings (21), additional data were presented by Walker and Pope, using immunofixation electrophoresis, that supported the same concept (8). The clinical significance of these transient polymers is not understood and we do not know if they effectively participate in thrombogenesis in vivo.

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References

14. Ref. 5, p 1032.