Inhibition of Human Platelet Aggregation by Plasmin Digests of Human and Bovine Fibrinogen Preparations: Role of Contaminating Factor VIII-related Material

By D. E. Culasso, M. B. Donati, G. de Gaetano, J. Vermylen, and M. Verstraete

Preparations of human fibrinogen, digested by plasmin, inhibited ADP-induced platelet aggregation; the inhibitory activity was confined to the small dialyzable fragments accumulating during the degradation. Purified large molecular weight fragments D and E had no effect on ADP-induced aggregation, but fragment E inhibited thrombin-induced aggregation. Extensively degraded bovine fibrinogen preparations also inhibited platelet aggregation by ADP. Both human and bovine fibrinogen preparations were contaminated with factor VIII-related material (factor VIII-related antigen and factor VIII procoagulant activity, respectively); separation of factor VIII-related material from human or bovine fibrinogen by gel chromatography and subsequent plasmin digestion of the fractions revealed that the inhibitory activity was mainly linked to digested factor VIII-related material. This inhibitory activity was dialyzable. The effect of fibrinogen digests on platelet aggregation should therefore be reconsidered.

For many years inhibition of platelet aggregation by fibrinogen degradation products has been reported (for review, see reference 22). Fibrinogen at an early stage of degradation was found to be the most effective inhibitor by some investigators,9,10 whereas others suggested that mainly the small dialyzable fragments accumulating during proteolysis of fibrinogen by plasmin inhibit ADP-induced platelet aggregation.11,13,18

In the present paper the effect of human and bovine fibrinogen digests on platelet aggregation has been studied. The role of factor VIII-related material, which was found to be present in both human and bovine fibrinogen preparations, has also been considered.

Materials and Methods

Purified human and bovine fibrinogen were a gift of Kabi (Stockholm). Both preparations were more than 95% clottable. Solutions of 1% (w/v) clottable protein were prepared in bidistilled water adjusted to pH 7.4 with diluted NaOH and were incubated at 37°C with either human plasmin,2 at a final concentration of 0.2 CTA U/ml, or buffer. At different time intervals, aprotinin (Trasylol, Bayer, Leverkusen) was added at a final concentration of 500 kallikrein inhibitor U/ml. The anticoagulant activity of the digests was measured by the prolongation of the thrombin time of 1/1 (v/v) mixtures of digests and normal citrated plasma. The stages of digestion were defined as described by Stachurska et al.18: the digest obtained at an early stage of proteolysis, having maximal anticoagulant effect, was referred to as “early products” (about 20 min proteolysis under

From the Laboratory of Blood Coagulation, Medical Research Department, Academisch Ziekenhuis St. Rafael, University of Leuven, Leuven, Belgium.

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Address for reprints: Professor M. Verstraete, Katholieke Universiteit te Leuven, Kapucijnenvoer, 35-3000 Leuven, Belgium.

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the above mentioned conditions); "late products" corresponded to a later stage of proteolysis (about 120 min), when the anticlotting activity had reached a stable level, whereas "very late products" were obtained by exhaustive digestion of fibrinogen for 24 hr. The digests and the fibrinogen solutions incubated with buffer alone for identical periods of time were then dialyzed overnight at room temperature against 30 volumes of bidistilled water, adjusted to pH 7.4. Both the contents of the dialysis bag and the fluid outside were finally concentrated by lyophilization. For platelet aggregation studies the lyophilized materials were dissolved in Michaelis buffer, pH 7.3 (Stago, Paris), at a protein concentration of 5%. Protein concentration was determined by comparing at 280 nm the light absorbance of the samples with a reference curve obtained with purified human fibrinogen.

Purified D and E fragments from human fibrinogen were obtained by DEAE-cellulose chromatography of an extensively degraded fibrinogenolysate as described previously. Human factor VIII-related antigen was quantitated by electrophoresis through antibody-containing gel, using a specific rabbit antibody (Nordic, Antwerpen). A reference curve was constructed by serial dilution of a normal human cryoprecipitate and the result expressed in units per milliliter (1 U corresponding to the height of a rocket obtained with 1 U/ml procoagulant activity). Gel chromatography of fibrinogen preparations was performed as described previously, using 0.03 M Tris, pH 7.0, as eluting buffer; fibrinogen-related antigen and factor VIII-related antigen were determined on the eluted fractions of human fibrinogen; fibrinogen content and factor VIII procoagulant activity were determined on the eluted fractions of bovine fibrinogen.

Separation of platelets from plasma proteins was obtained by gel filtration of PRP through Sepharose 2 B (Pharmacia, Uppsala). Platelet aggregation was followed in a Born aggregometer as already described: human citrated platelet-rich plasma (PRP, 300,000 platelets per μl) or gel-filtered platelets were pre-incubated for 3 min at 37°C with the test sample before the addition of adenosine-5′-diphosphate (ADP, 2.10⁻⁶ M, final concentration) or bovine thrombin (0.5 NIH U/ml, final concentration).

RESULTS

Effect of Human Fibrinogen Digests on Human Platelet Aggregation

Column B of Fig. 1 shows the inhibition of ADP-induced platelet aggregation by human fibrinogen digests at different stages of degradation (early, late, very late). Inhibition was found with all the digests, but was strongest in the presence of very late products and had almost completely disappeared after

![Fig. 1. Effect of human fibrinogen degradation products (FDP) before and after dialysis on ADP-induced aggregation of PRP. Top series, early FDP; middle series, late FDP; lower series, very late FDP. Column A, fibrinogen incubated with buffer; column B, FDP before dialysis; column C, FDP after dialysis; column D, FDP recovered outside the dialysis bag.](image-url)
Fig. 2. Effect of purified human fragments D and E on thrombin-induced aggregation of gel-filtered platelets. Curve A, control; curve B, fragment D (0.35 mg/ml, final concentration); curve C, fragment E (0.34 mg/ml, final concentration). The arrow indicates the moment of addition of thrombin.

dialysis of the digests (column C); a very strong inhibitory activity was recovered in the dialysis fluids (column D). Plasmin and aprotinin alone had no effect in the system: human fibrinogen solutions, incubated at 37°C with buffer (instead of plasmin) during the time intervals corresponding, respectively, to early, late, and very late stages, did not exhibit any inhibitory activity (column A).

D and E fragments, purified from very late digests of human fibrinogen, did not inhibit ADP-induced aggregation in platelet-rich plasma; in contrast, when aggregation of gel-filtered human platelets was elicited by thrombin, inhibition was observed in the presence of fragment E, whereas fragment D, at the same protein concentration, was devoid of inhibitory activity (Fig. 2).

Human fibrinogen Kabi contains 0.12 U of factor VIII-related antigen per milligram clottable protein; in contrast, no factor VIII procoagulant activity could be measured. As plasmin digests of human factor VIII preparations inhibit platelet aggregation by ADP,6 we investigated whether digests of human fibrinogen, separated from factor VIII-related antigen by gel chromatography, still are able to inhibit platelet aggregation. Figure 3 shows that these digests are much less inhibitory than those obtained from nonchromatographed fibrinogen; the digest of the fraction containing factor VIII-related antigen, on the contrary, has a potent inhibitory effect despite its minute protein content. The fibrinogen fraction clotted normally before but was incoagulable after plasmin proteolysis.

Effect of Bovine Fibrinogen Digests on Human Platelet Aggregation

Bovine fibrinogen preparations have an aggregating activity on human platelets, which still is present in "early" and "late" digests, but disappears completely upon extensive degradation.3 Moreover, "very late" digests develop an inhibitory activity on human platelet aggregation, which, as with human fibrinogen digests, disappears after dialysis and is almost completely recovered in the dialysis fluid. Since in separate experiments3 bovine fibrinogen preparations were found to contain factor VIII procoagulant activity (0.25 U per mg clottable protein), we have investigated whether the inhibitory activity mentioned above was due to digestion of factor VIII.
Upon gel chromatography through 6% agarose of bovine fibrinogen preparations, a first peak was obtained, which contained high molecular weight factor VIII and was completely devoid of fibrinogen or fibrinogen-like material, and a second peak containing all the clottable protein and minor amounts of small molecular weight factor VIII.5 Both fractions, after extensive degradation by plasmin, were capable of inhibiting platelet aggregation by ADP (Fig. 4). The digest of the fraction containing high molecular weight factor VIII was a rela-
Fig. 5. Effect of a digest of high molecular weight bovine factor VIII, before and after dialysis, on ADP-induced aggregation of human PRP. Curve A, buffer; curve B, before dialysis; curve C, after dialysis. The arrow indicates the moment of addition of ADP.

tively much more potent inhibitor, since the protein content of this fraction was at least 200 times lower than that of the fibrinogen fraction. Dialysis completely abolished the aggregation-inhibiting activity of both digests (as shown, for the bovine high molecular weight factor VIII digest, in Fig. 5).

DISCUSSION

This study confirms the inhibition of the first phase of ADP-induced platelet aggregation by plasmin digests of human fibrinogen preparations. The inhibitory activity, present at all the stages of degradation, was confined to the dialyzable fraction of the digests, a finding in agreement with the reports of Larrieu et al. and Stachurska et al. Although the “very late products” appeared to be the most potent inhibitors, we could not find an important difference between the products at different stages of degradation, as was also observed by Niewiarowski et al. The absence of inhibition by fragments D and E, isolated from very late digests, confirms that only the small dialyzable fragments accumulating during proteolysis are inhibitory.

The effects of purified fragments D and E on thrombin-induced aggregation suggest that fragment E may compete with the platelet substrate(s) for thrombin. Indeed, in clotting systems, this fragment is a competitive inhibitor of the action of thrombin on fibrinogen, whereas fragment D acts primarily by inhibiting fibrin polymerization.

The human fibrinogen preparation used in this and several of the previous studies is contaminated with factor VIII-related antigen; the plasmin digest of fibrinogen, separated from this antigen by chromatography, still inhibits aggregation although to a much lesser degree than the digest of nonchromatographed fibrinogen. On the contrary, as already demonstrated, plasmin digests of human factor VIII preparations are very potent inhibitors of ADP-induced platelet aggregation; the fraction containing factor VIII-related antigen accounts for most of the inhibitory activity present in digests of human fibrinogen Kabi.
The effect of human fibrinogen digests on platelet aggregation should therefore be reconsidered.

The effect of bovine fibrinogen digests on platelet aggregation also was studied; as these aggregate human platelets even at the stage of "late digests," only "very late digests," devoid of aggregating activity, could be evaluated; these inhibited ADP-induced platelet aggregation. We have recently shown that bovine fibrinogen preparations are contaminated with factor VIII procoagulant activity, most of which is linked to a high molecular weight molecule; the latter, in fact, is responsible for platelet aggregation and can be completely separated from fibrinogen by gel chromatography. Digestion by plasmin of high molecular weight factor VIII leads to progressive disappearance of the aggregating activity and to development of an inhibitory effect on ADP-induced platelet aggregation. This activity is abolished by dialysis. The lysate of the fibrinogen fraction, obtained after removal of high molecular weight factor VIII by gel chromatography, still inhibited ADP-induced platelet aggregation but was, on a protein basis, 200 times less active than the high molecular weight factor VIII digest; moreover, as the fibrinogen fraction still contains some low molecular weight factor VIII procoagulant activity, the evidence that bovine fibrinogen degradation products inhibit platelet aggregation is not conclusive.

Solum et al. have reported that tryptic proteolysates of human serum also exert an inhibitory effect on platelet aggregation. Normal human serum does not contain fibrinogen-related antigen but has the same amount of factor VIII-related antigen as plasma; degradation products of the latter molecule could account, at least in part, for the inhibitory activity found in tryptic digests of human serum.

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