Unique Pattern of Fibrinogen Cleavage by Human Leukocyte Proteases

By Sophie B. Bilezikian and Hymie L. Nossel

An extract of human leukocytes was prepared which had marked fibrinogenolytic activity, as determined by its effect upon the thrombin clotting time of plasminogen-free fibrinogen. When incubated with a 1/10 dilution of the extract of 10⁶ leukocytes, fibrinogen became incoagulable within 30 min. The activity of the leukocyte extract was unaffected by 0.2 M epsilon-amino caproic acid and minimally affected by 100 U/ml of trasylol, but it was completely inhibited by 0.1 mg/ml soybean trypsin inhibitor and by 1% plasma. The degradation products of leukocyte protease-treated fibrinogen were clearly different from those of plasmin-treated fibrinogen when examined by polyacrylamide gel electrophoresis. The pattern of cleavage of the amino terminal ends of the Aα and Bβ chains of fibrinogen by the leukocyte extract was determined using radioimmunoassays specific for fibrinopeptides A and B. The fibrinopeptides themselves were not cleaved, but slightly larger dialyzable fragments containing the peptides were cleaved from the amino terminal ends of the Aα and Bβ chains. These larger fragments were clearly distinguished from the fibrinopeptides themselves by immunchemical means. The pattern of release of the fibrinopeptide-containing segments by the leukocyte proteases was different from the patterns described for thrombin, plasmin, trypsin, and certain snake venoms. The results suggest that the specificity of the leukocyte proteases for fibrinogen is unique, and provide a technique for further study of the role of these enzymes in normal and abnormal fibrinogenolysis.

Enzymatic conversion of fibrinogen to fibrin monomer by thrombin is a central feature of blood coagulation. Leukocytes may affect this process in several ways. They have both procoagulant and anticoagulant activity and may potentially either accelerate or retard the generation of thrombin. In addition, proteases from leukocytes may affect fibrinogen directly. A number of neutral proteases distinct from plasmin have been described in leukocytes which are capable of cleaving fibrinogen and fibrin at physiologic pH. The neutral proteases of leukocytes include elastases and chymotrypsin-like proteases.

It has been suggested that leukocyte proteases function in the normal degradation of fibrin as well as in the pathologic proteolysis of fibrinogen and fibrin. Supporting evidence obtained by electron microscopy indicates that leukocytes can ingest fibrin and alter its morphological appearance. Furthermore, leukocyte proteases can degrade fibrinogen into unclottable fragments.
which interfere with normal clot formation. All three chains of fibrinogen are
affected by these enzymes, but there is little specific information about how
the molecule is degraded. Such information might provide a way to identify the
action of the leukocyte enzymes in vivo and to clarify their role in physiologic
and pathologic processes.

In these experiments, an extract of human leukocytes was prepared which
degraded fibrinogen so that it was no longer clottable by thrombin. The fibrin-
gen degradation products produced by the leukocyte extract were analyzed by
polyacrylamide gel electrophoresis and found to be completely different from
those produced by plasmin. These observations confirmed those of Plow and
Edgington. In addition, the patterns of cleavage of the amino terminal ends
of the $\alpha A$ and $\beta B$ chains of fibrinogen by the leukocyte extract were determined
using specific radioimmunoassays for fibrinopeptides A and B. These patterns
were distinct from those produced by thrombin, plasmin, and trypsin.

**MATERIALS AND METHODS**

*Preparation of Leukocytes*

Leukocytes were separated from the venous blood of normal donors by dextran sedimentation
and osmotic lysis of red cells followed by differential centrifugation. The procedure was car-
pried out at 4°C using plastic syringes, tubing, pipettes, and containers and sterile water (Abbott
Laboratories, North Chicago, Ill.) for buffer solutions. The cells were washed five times with
0.1 $M$ NaCl buffered with 0.05 $M$ sodium phosphate (PBS) at pH 7.4 and suspended in PBS at
10$^8$/ml. Wright's stained smears of these preparations revealed no red cells and rare platelets.
There were 80%, 90%, neutrophils, 5%, 15%, lymphocytes, and occasional monocytes, eosinophils,
and basophils. Crude leukocyte extracts were prepared by disrupting the cells by freezing and
thawing eight times. The cellular debris was removed by centrifugation at 3000 $g$ for 30 min and
the supernatant solution was stored at −80°C.

*Thrombin Clotting Time*

The fibrinogenolytic activity of the leukocyte extract was determined by its effect upon the
thrombin clotting time of fibrinogen using plasminogen-free fibrinogen (the gift of Dr. E. J.
Harfenist, Department of Medicine, Columbia University College of Physicians and Surgeons)
at 1.25 mg/ml and bovine thrombin (Parke-Davis and Co., Detroit, Mich.) at 10 U/ml in PBS.
The fibrinogen had been passed over lysine sepharose, and on polyacrylamide gel electrophoresis
showed no evidence of degradation after incubation for 48 hr at 37°C with streptokinase. The
leukocyte extract was used at a dilution of 1/10 or 1/20 as required to render the fibrinogen
uncottable within 30 min.

*Polyacrylamide Gel Electrophoresis*

Polyacrylamide gel electrophoresis in 0.1%, dodecyl sulfate was used to compare the patterns
of degradation of fibrinogen produced by the leukocyte enzymes to those produced by plasmin.
Fibrinogen (10 mg/ml) in 0.1 $M$ Tris-HCl, pH 7.4, was incubated at 37°C with the leukocyte ex-
tract or with plasmin (A.B. Kabi, Stockholm, Sweden). Each enzyme was used at a concentration
such that the fibrinogen was still clottable at 30 min but unclottable at 1 hr. At selected times,
aliquots of each sample were removed and added to an equal volume of 10 $M$ urea in 2%, dodecyl
sulfate, with and without 0.1 $M$ dithiothreitol. Then 50 $\mu$g of unreduced sample or 75 $\mu$g of
reduced sample were applied, respectively, to 5%, or 7%, polyacrylamide gels, electrophoresed
at 7.5 mA/gel, and stained with Coomassie blue.
Cleavage of the Amino Terminal Ends of the Aα and Bβ Chains of Fibrinogen by the Leukocyte Proteases

Experiments were carried out at 37°C in 0.1 M NaCl buffered with 0.05 M Tris HCl, pH 7.4. One-ml samples of plasminogen-free fibrinogen at a final concentration of 1 x 10^{-6} M were incubated for selected times with a 1/10 final dilution of the leukocyte extract. The reaction was stopped and unreacted fibrinogen was removed by ethanol precipitation and centrifugation. Dialysates of the supernatant solution were assayed for fibrinopeptides A and B (FPA and FPB) both before and after treatment with human thrombin as previously described. FPA was assayed with antiserum R2, which was specific for FPA and which cross-reacted poorly with fibrinogen or with larger fragments from the amino terminal end of the Aα chain containing the A peptide. Similarly, FPB was assayed with antiserum RB22, which detected FPB but which had limited cross-reactivity with fibrinogen and with fragments from the amino terminal end of the Bβ chain containing the B peptide.

RESULTS

The crude extract of human leukocytes markedly prolonged the thrombin clotting time of plasminogen-free fibrinogen (Fig. 1). Fibrinogen was incoagulable after 30 min of incubation with a 1/10 or higher dilution of the extract of 10^8 cells. The activity of the leukocyte extract was unaffected by 0.2 M epsilon-amino caproic acid (EACA) and only slightly affected by 100 U/ml of trasylol (Fig. 1). In contrast, the activity was almost completely inhibited by 0.1 mg/ml soybean trypsin inhibitor and by 1% plasma (Fig. 2). The inhibition of the leukocyte fibrinolytic activity by plasma was extremely rapid and appeared to be complete within 1 min (Fig. 3).

The degradation products of fibrinogen produced by the leukocyte extract were compared to those produced by plasmin using polyacrylamide gel electrophoresis (Fig. 4). Plasmin treatment of fibrinogen resulted in the appearance of fragments X, Y, D, and E over 24 hr. Treatment of fibrinogen with the leukocyte extract yielded a large fragment of approximately the same size as fragment X, but it persisted for 6 hr and was still faintly visible at 24 hr (Fig. 4). The reduced samples of plasmin-treated and leukocyte protease-treated fibrinogen were compared to those produced by plasmin using polyacrylamide gel electrophoresis (Fig. 4). Plasmin treatment of fibrinogen resulted in the appearance of fragments X, Y, D, and E over 24 hr. Treatment of fibrinogen with the leukocyte extract yielded a large fragment of approximately the same size as fragment X, but it persisted for 6 hr and was still faintly visible at 24 hr (Fig. 4).

Fig. 1. Thrombin clotting time of fibrinogen incubated with leukocyte extract (●), and with leukocyte extract in the presence of 0.2 M EACA (×), or of 100 Units/ml trasylol (○).
Fig. 2. Thrombin clotting time of fibrinogen incubated with leukocyte extract (e), and with leukocyte extract in the presence of 0.1 mg/ml SBTI (x), or of 1% plasma (c).

Fig. 3. Inactivation of leukocyte extract by plasma. After incubation with PBS (e) or with 5% plasma (c) for the indicated times, the leukocyte extract was added to fibrinogen. After 30 min, the thrombin clotting time of the fibrinogen was determined.

Fig. 4. Electrophoresis on 5% polyacrylamide of fibrinogen degradation products. The left gel of each pair shows plasmin-treated fibrinogen, and the right gel shows leukocyte protease-treated fibrinogen for the same period of incubation. Fragments X, Y, D, and E and approximate molecular weights are indicated.
FIBRINOGEN CLEAVAGE

Fig. 5. Electrophoresis on 7.5% polyacrylamide of fibrinogen degradation products after reduction with dithiothreitol. The Aα, Bβ, and γ chains of the control sample are shown. The left gel of each pair shows plasmin-treated fibrinogen, and the right gel shows leukocyte protease-treated fibrinogen for the same period of incubation.

Fibrinogen also showed different patterns of degradation (Fig. 5). The γ chain of fibrinogen was relatively resistant to plasmin degradation, while all three of the constituent Aα, Bβ, and γ chains were degraded by the leukocyte extract within 6 hr (Fig. 5).

To study the patterns of cleavage of the amino terminal ends of the Aα and Bβ chains by the leukocyte proteases, dialysates of protease-treated fibrinogen were tested using radioimmunoassays specific for FPA and FPB. The results suggested that the leukocyte enzymes did not cleave the fibrinopeptides themselves but released slightly larger dialysable FPA- and FPB-containing fragments. Thus, when the dialysates were tested directly, very small quantities of FPA and FPB immunoreactivity were detected (Fig. 6). However, when the dialysates were tested after treatment with thrombin, significant and increasing quantities of both fibrinopeptides were detected with increasing incubation.

Fig. 6. Cleavage of fibrinopeptide-containing fragments by the leukocyte proteases. Fibrinogen was incubated with the leukocyte extract for the indicated times and then precipitated and dialyzed. (A) FPA immunoreactivity in dialysates before (c) and after (e) thrombin treatment. (B) FPB immunoreactivity in dialysates before (c) and after (e) thrombin treatment.
times (Fig. 6). Within 2 hr, all of the available FPA (2 moles/mole of fibrinogen) had been released in the form of FPA-containing fragments (Fig. 6). Quantitative recovery of FPB was not obtained even after 24 hr; in fact, the 24-hr sample contained much lower FPB immunoreactivity. The release of FPA- and FPB-containing fragments by the leukocyte extract was unaffected by 0.2 M EACA (Table 1).

DISCUSSION

These results confirm that human leukocytes contain proteases capable of acting on fibrinogen. The proteolytic extract described in these experiments degraded fibrinogen so that it was no longer clottable by thrombin. Fibrinogenolysis was unaffected by 0.2 M EACA, suggesting that the major protease in the extract was not plasmin and confirming the observations of Plow and Edgington. The activity of the leukocyte extract was slightly inhibited by trasylol and markedly inhibited by soybean trypsin inhibitor and by dilute plasma; the latter agent having a very rapid action.

It is possible that a major component of the extract may be granulocyte elastase. This enzyme, which is known to be fibrinogenolytic, is inhibited by soybean trypsin inhibitor and partially inhibited by trasylol. Granulocyte elastase is also inhibited by the plasma protease inhibitors, α1-antitrypsin and α2-macroglobulin. The rate of inhibition of the enzyme by these agents has not been studied.

Although the fibrinogenolytic activity of leukocytes is established, there is uncertainty about the relative contributions of plasmin and other enzymes to this activity. In these experiments, the degradation of fibrinogen by the leukocyte extract was examined both by electrophoretic and by immunochemical techniques, and in both cases the observed cleavage patterns were distinct from those produced by plasmin. Polyacrylamide gel electrophoresis of plasmin-treated fibrinogen revealed fragments X, Y, D, and E. In contrast, gels of leukocyte protease-treated fibrinogen revealed a completely different pattern, which was characterized by the prolonged persistence of a large fragment (Fig. 4). These results are also in agreement with those of Plow and Edgington. On the reduced samples of plasmin-treated fibrinogen, the γ chain was relatively resistant to plasmin degradation, as has previously been reported by Mosesson et al. In contrast, all three of the constituent chains of fibrinogen were degraded by the leukocyte enzymes within 6 hr (Fig. 5). Plow and Edgington reported an even more rapid disappearance of these chains. This slight discrepancy could be due to differences in the relative concentrations of the various enzymes in the leukocyte extract, perhaps reflecting differences between individual donors.

Table 1. Effect of 0.2 M EACA Upon the Cleavage of FPA- and FPB-containing Fragments by Leukocyte Proteases*

<table>
<thead>
<tr>
<th>Fragments (pmoles)</th>
<th>With 0.2 M EACA</th>
</tr>
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<tbody>
<tr>
<td>FPA containing</td>
<td>1146</td>
</tr>
<tr>
<td>FPB containing</td>
<td>316</td>
</tr>
</tbody>
</table>

*Incubation for 1 hr at 37°C.
FIBRINOGEN CLEAVAGE

The leukocyte extract could also be distinguished from thrombin, plasmin, and trypsin based upon its pattern of cleavage of the amino terminal ends of the Aα and Bβ chains of fibrinogen. Dialysates of leukocyte protease–treated fibrinogen, when tested directly, had very little FPA or FPB immunoreactivity. After treatment of the dialysates with thrombin, significant amounts of FPA and FPB were detected, indicating the presence of fibrinopeptide-containing fragments in these samples. Interpretation of these findings is based on studies of the specificity of the R2 antiserum to FPA. These studies indicated that thrombin treatment of FPA produced no increase in immunoreactivity, whereas thrombin treatment of larger polypeptides containing the FPA sequence resulted in an approximately 100-fold increase in immunoreactivity. These larger polypeptides included Aα 1-23 and Aα 1-51.23 Hence the present finding that thrombin treatment produces an approximately 100-fold increase in FPA immunoreactivity indicates that the leukocyte extract does not cleave the Arg (Aα 16)-Gly (Aα 17) on the Aα chain of fibrinogen and suggests that the cleavage site may be at or internal to Arg (Aα 23)-His (Aα 24).

There is insufficient information about the specificity of the FPB antiserum RB22 to make similar deductions for the cleavage site on the Bβ chain. Because of the marked increase in FPB immunoreactivity following thrombin treatment (Fig. 6), it is suggested by analogy that Arg (Bβ 14)-Gly (Bβ 15) is not cleaved and that the cleavage site is internal to that bond. The fragments which are released are small enough to be dialyzable (molecular weight is probably less than 6000) but larger than the fibrinopeptides themselves. The lack of quantitative recovery of FPB even after prolonged incubation probably reflects further proteolytic degradation of the FPB-containing fragment(s).

This action of the leukocyte proteases upon fibrinogen is in marked contrast to that of thrombin, plasmin, and trypsin. Both thrombin and trypsin cleave the fibrinopeptides directly.32,33 Plasmin cleaves a fragment from the Bβ chain containing FPB, but cleaves no FPA-containing material until the terminal stages of its action.34,35 The unique pattern of cleavage of fibrinogen by the leukocyte proteases has provided a potential method of identifying the action of these enzymes in vivo, by measuring FPA and FPB immunoreactivity in clinical blood samples before and after thrombin treatment. Such studies may provide information about the contribution of the leukocyte enzymes to physiologic and pathologic fibrinogenolysis.

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