Effects of Porcine Plasmin on the Coagulation and Fibrinolytic Systems in Humans

By Ulla Hedner, Lilian Johansson, and Inga Marie Nilsson

Pig plasmin (Lysofibrin) was given to 11 patients with phlebographically verified venous thrombosis, 2 of whom were treated two and three times, respectively. The effect on coagulation and fibrinolytic parameters was studied. The platelet count, Owren’s P&P (prothrombin plus factors VII and X), plasminogen, factor XIII, and antithrombin III did not change during the treatment. All patients developed a proteolytic activity demonstrable on both unheated and heated fibrin plates. The fibrinogen decreased successively to very low levels, and parallel to this an increase in fibrin/fibrinogen degradation products was found. The factor VIII and factor V activities decreased immediately after each Lysofibrin infusion but normalized rapidly again. The factor VIII molecule, however, retained its reactivity to rabbit antiserum against factor VIII. Immediately after the plasmin infusion a decrease of both α2-macroglobulin (α2-M) and the rapidly reacting α2-antiplasmin was observed. α2-M decreased successively and in several of the patients values were unmeasurable for a period of some days. A complex formation between pig plasmin and the α2-antiplasmin was demonstrated in crossed immunoelectrophoresis. The complexes were rapidly cleared from the circulation. No interaction between the pig plasmin and the inhibitor of the plasminogen activation, α1-antitrypsin or inter-α-inhibitor, was found.

Fibrinolytic Agents have been used for more than 10 yr in the treatment of thromboembolic disease. The agent most commonly employed is streptokinase (SK).1,5 SK is, however, antigenic and gives rise to high titers of SK antibodies prohibiting repeated treatment for at least one-half year.6,7 To avoid this side effect, human plasmin has been tried with a certain degree of success.8,10 Good clinical results have also been obtained with highly purified activator-free pig plasmin.11 Pig plasmin has been given repeatedly without any allergic problems.12

This paper reports the effect of infusion of highly purified pig plasmin (Lysofibrin, Novo, Copenhagen) on the coagulation and fibrinolytic systems.

MATERIALS AND METHODS

Clinical Material

Eleven patients with phlebographically verified deep venous thrombosis (DVT) were given Lysofibrin. The patients elected for the Lysofibrin treatment had no other known diseases and no known focus for potential bleeding such as tumors, cerebral hemorrhage, recent surgical trauma, or any bleeding disorders. One of the men, 44 yr old, who had had DVT for at least 1 mo was treated three times at 3 4-wk intervals. Another man, aged 34, with DVT was treated twice with Lysofibrin at a 3-wk interval. The patients gave informed consent for this treatment.

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Fig. 1. Design of the study. Dose of Lysofibrin was 30 Novo U/kg body weight each time. All 11 patients received three doses, and two of the patients received a fourth dose. One patient was given a second Lysofibrin treatment for 2 days 3 wk later and another patient received a second and a third treatment at 3-4-wk intervals.

Plasmin

Porcine plasmin prepared by trypsin activation of highly purified pig plasminogen (Lysofibrin) was used. After activation the trypsin excess was eliminated by a fractionated precipitation procedure. The plasmin was then further purified by gel filtration and lyophilized. It was standardized with a caseinolytic method and contained 20-25 Novo U/mg nitrogen. (1 Novo unit is defined as the amount of enzyme producing an increase in optical density at 275 nm of 1.0 in a caseinolytic system with a digestion time of 20 min; it corresponds to 3.5 CTA units.) The plasmin was stabilized by addition of L-lysine (7.3 mg/1 Novo unit plasmin) and was supplied lyophilized in vials containing 1000 Novo units plasmin.

Plasmin Treatment (Fig. 1)

All patients were tested for primary allergy to pig plasmin (0.2 ml Lysofibrin solution, 1 Novo U/ml s.c.) before treatment was started.

An initial dose of 30 Novo U/kg body weight was given intravenously during 90 min (dose 1). A plasmin solution containing 4 Novo U/ml was used. After the initial dose an equal amount of plasmin was given as a maintenance dose intravenously during 5-6 hr, corresponding to 6-7 Novo U/kg body weight/hr (dose 2). A corresponding maintenance dose was also given the following day (dose 3). Two patients also received such a dose on the third day (dose 4). The Lysofibrin treatment was followed by heparin and dicoumarol treatment.

Blood for examination of the coagulation and fibrinolytic factors was obtained before (sample I) and immediately after the initial dose (sample II), and after the first maintenance dose (sample III). Blood samples were also obtained before (sample IV) and after the plasmin maintenance dose on days 2 (sample V) and 3 (samples VI and VII).

Collection of Blood

Citrated plasma was collected using the silicone technique as described previously. Serum was prepared as described by Paraskevass et al. Blood for determination of fibrin/fibrinogen degradation products (FDP) was collected in the presence of thrombin, e-aminocaproic acid, and serum prepared as described previously. Both citrated plasma and serum were stored at -60°C and all samples were tested simultaneously. Pooled citrated plasma or serum from 25-40 apparently healthy volunteers was used as a standard.

Coagulation Studies

The following determinations were made: platelet count; activated partial thromboplastin time; factor VIII activity of citrated plasma collected in the presence of Trasylol, 250 KIU/ml (VIII:C); factor VIII related antigen (VIIIIR:Ag); Owren's P&P (prothrombin plus factors VII and X);
Table 1. Values for Various Coagulation and Fibrinolytic Components Obtained Before Treatment (Sample I) and After Second Plasmin Dose (Sample III) in 11 Treated Patients

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<th>Pt. No.</th>
<th>Sample No.</th>
<th>VIII:C (%)</th>
<th>VIIIIR:Ag (%)</th>
<th>Factor V (%)</th>
<th>Fibrin plate (sq mm)</th>
<th>Fibrinogen (g/liter)</th>
<th>Factor XIII (%)</th>
<th>Plasminogen (FSF U/ml)</th>
<th>a2-M (%)</th>
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Normal: 60-160 60-160 80-120 80-120 0-70 0 2.0-4.0 14-30 80-120 80-120 75-122 <5

Fibrinolytic Studies

The following determinations were made: euglobulin clot lysis time (the euglobulin fraction at pH 5.9); fibrin, by the plate method using both unheated and heated plates (both citrated plasma and resuspended euglobulin fraction were tested); fibrinogen; and plasminogen. The a2-macroglobulin (a2-M) was determined both immunonechemically by the rocket method of Laurell using a monospecific antiserum (Behringwerke, Marburg, Germany), and by an esterolytic method. The a1-antitrypsin (a1-AT) and the inter-a-trypsin inhibitor (I-a-I) were studied immunonechemically with monospecific commercial antisera (Behringwerke, Marburg, Germany). The inhibitors of the plasminogen activation were determined immunonechemically with the rocket technique.

The antiserum used was raised in rabbits against the inhibitor of plasminogen activation as described previously. This antiserum was made monospecific by absorption with haptoglobin and ceruloplasmin as described by Hedner. In addition the a2-antiplasmin described by Mullertz and by Collen et al. was determined with a specific antiserum against this inhibitor prepared and kindly supplied by Dr. Collen, Leuven, Belgium. This antiserum was raised in rabbits against a plasmin inhibitor complex produced by activating human plasma plasminogen by addition of SK. The antiserum was then subjected to Sepharose-coupled plasminogen to neutralize the antibodies directed against plasmin. The rocket method of Laurell using the antiserum in a dilution at 1/100 was used.

The crossed immunoelectrophoresis technique according to Ganrot was used to study the various inhibitors during plasmin treatment.

RESULTS

Results are summarized in Table 1 and Fig. 2. Platelet count, P&P, plasminogen, factor XIII and antithrombin III never changed during treatment.

All patients developed a proteolytic activity after administration of the plasmin, although the degree of proteolysis varied. The proteolytic activity was...
Fig. 2. See opposite page.
demonstrable on fibrin plates, both unheated (range of sample III: 100–263 sq mm) and heated (range of sample III: 18–113 sq mm). The percentage increase ranged between 20% and 94% on unheated plates. The fibrinogen levels decreased successively to unmeasurable levels or levels of about 1.0 g/liter (range of sample III: 0–1.7 g/liter) immediately after the first or second day's maintenance plasmin dose. Parallel to the fibrinogen decrease, an increase in FDP was found. After the first day's maintenance dose values of 22–6600 µg/ml were obtained.

Immediately after the initial dose the VIII:C decreased markedly in all the patients, and after the maintenance dose (sample III) no or almost no measurable VIII:C was found (1.75%–8%) in five of the patients. The other five tested ranged between 20% and 87%. This corresponded to a percentage decrease varying between 40% and 99%. The levels rapidly normalized again, and about 18 hr after the latest plasmin dose VIII:C levels near the original ones were found. This pattern was repeated after every plasmin infusion. In one patient VIII:C never decreased below 61%. The VIIIIR:Ag showed a completely different pattern. A successive rise (from 70%–308% for sample I up to 400%) throughout the observation period was noted in most patients. After the first day's maintenance dose of plasmin (sample III) a decrease in factor V to 40%–60% was noted in six of the patients. In the other five patients only a slight decrease was observed; 18 hr later, the level was almost normal but decreased again to about 40%–70% after the second day's maintenance dose. It was again normal on the following day.

A successive decrease of α2-M was found during the treatment period. The values obtained by the two methods used correlated well with each other. In crossed immunoelectrophoresis decreased peaks were seen parallel to the findings with the other methods. The level of α2-M decreased to less than 10% after the first (sample III), or second day's maintenance dose (sample V) and remained at this level throughout the observation period in most of the patients. The range of sample III levels was 2%–64%, corresponding to a percentage decrease of 99% to 32%. The α2-antiplasmin determined with the specific antiserum of Collen showed a successive decrease and reached the lowest value (15%–30%) after the second maintenance dose (sample V). In crossed immuno-electrophoresis using the antiserum of Collen (diluted 1/100), two precipitates with different electrophoretic mobilities were seen after the initial Lysofibrin dose (Fig. 3). The demonstrated immunologic identity indicated complex formation between the antiplasmin and plasmin. The complex formation could be found after every Lysofibrin dose but disappeared rapidly again and could not be seen 18 hr after the plasmin was given. The inhibitors of the plasminogen activation did not change significantly during the treatment. Unchanged patterns of the α1-AT and Iα-I were seen in crossed immunoelectrophoresis throughout the treatment period.

Fig. 2. Effect of Lysofibrin on various clotting and fibrinolytic parameters in a patient with a DVT given four doses of Lysofibrin. Lysofibrin doses are given in Novo units (A) P&P, factor V (upper panel), and factor VIII (lower panel). (B) Fibrinolytic parameters FDP, fibrinogen (upper panel), α2-M, antithrombin III (center panel), and fibrinolytic activity on fibrin plates (lower panel).
DISCUSSION

Porcine plasmin (Lysofibrin) has been reported to have a good lytic effect on venous thrombi.\textsuperscript{11} Porcine plasmin has never been found to give rise to antibodies when given to humans.\textsuperscript{12,29} Our experience is in accord with these findings, as no side reactions were seen with repeated plasmin treatment in two patients. The effects of the agent on the various coagulation and fibrinolytic factors have, however, not been thoroughly investigated. In this study Lysofibrin has been proved capable of inducing a proteolytic activity with fibrinogenolysis resulting in high levels of FDP.

In our study the $\alpha_2$-M decreased during the plasmin treatment successively immediately after the initial dose. Crossed immunoelectrophoresis using antiserum against $\alpha_2$-M showed no complex formation. This did not, however, exclude complex formation between pig plasmin and $\alpha_2$-M because of the similarity in migration between free and complexed $\alpha_2$-M.\textsuperscript{30} On the contrary, the pronounced decrease of $\alpha_2$-M strongly indicates a complex formation with rapid elimination of $\alpha_2$-M–plasmin complexes. Such an enhanced elimination of complexed $\alpha_2$-M was demonstrated previously.\textsuperscript{31} The circulating $\alpha_2$-M did not normalize until 5–7 days after the treatment with plasmin had been stopped, in accord with the earlier finding of a rather slow synthesis of this protein.\textsuperscript{13,34}

After administration of pig plasmin the fast $\alpha_2$-antiplasmin described by Mülertz\textsuperscript{26} and by Collen et al.\textsuperscript{27} immediately reacted with plasmin, as reflected in the rapid complex formation found on crossed immunoelectrophoresis. However, 12 hr after the plasmin infusion the complexes had disappeared and most of the antiplasmin level was restored (Fig. 3). This finding suggests a quick turnover of the antiplasmin–plasmin complexes and a synthesis rate much faster than that of $\alpha_2$-M of the protein.

No interaction was found between pig plasmin and the $\alpha_1$-AT, I-α-I, or the inhibitor of the plasminogen activation. As for $\alpha_1$-AT, no such interaction was found by Niléhn and Ganrot\textsuperscript{33} during SK therapy. As during urokinase treatment,\textsuperscript{35} no antithrombin III was consumed during Lysofibrin treatment.

The plasmin infusions were immediately followed by a substantial fall of factor VIII activity. This finding is in agreement with that of McKee et al.\textsuperscript{36} that digestion of purified factor VIII by plasmin results in loss of activity. About 18 hr after the plasmin infusion a large amount of functioning factor VIII had reappeared in the circulation, suggesting a synthesis rate of factor VIII well within
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that found for other proteins. However the decrease in factor VIII activity was not accompanied by a decrease in the VIII:Ag. On the contrary, a small but consistent successive increase was observed during the course of treatment. This result could have been due to an overestimation of the factor VIII protein in the rocket method as a result of a partial degradation by plasmin providing a factor VIII molecule of a lower molecular weight and a faster migration rate in the gel. Such a changed migration pattern was found by Henriksson and Holmberg in studies in vitro of plasmin degradation of factor VIII. It was also shown by McKee et al. that the plasmin-inactivated factor VIII molecule is still able to induce platelet aggregation by ristocetin, thus indicating that the plasmin digestion affects only the procoagulant activity of the molecule. In our study it was shown that the antigenic properties of the molecule also were unaffected by plasmin digestion, thus underlining the discrepancy between biologic and immunochemical assays.

In summary, infusion of pig plasmin (Lysofibrin) was followed by the appearance of proteolytic activity in the circulation, resulting in fibrinogen degradation and the formation of FDP. It was also shown that pig plasmin bound α2-M and α2-antiplasmin, but did not influence the inhibitors of plasminogen activation, α1-AT or I-α-I. Rapidly eliminated complexes between pig plasmin and α2-antiplasmin could be demonstrated. The degradation and inactivation of factor VIII by plasmin was confirmed. It was also shown that the inactive factor VIII molecule still reacted with a heterologous antiserum against human factor VIII.

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