On the Mechanism of Thrombolysis by Plasmin

By Clara M. Ambrus, M.D., Ph.D., Nathan Back, D.Sc., and Julian L. Ambrus, M.D., Ph.D.

On theoretical grounds, the high level of circulating antiplasmin in man was considered an objection to the clinical use of plasmin. Norman postulated that normal human plasma contains at least 30 times the amount of antiplasmin necessary to neutralize the plasmin which could be formed by activating all circulating plasminogen. Nevertheless, in controlled clinical studies plasmin proved to be an effective clot-dissolving agent in a variety of thrombo-embolic conditions. Since the therapeutic dose of plasmin in these studies was insufficient to neutralize all circulating inhibitors, its mode of action remained an intriguing question.

Several investigators have reported the high affinity of plasmin to fibrin and suggested that the enzyme, once in high concentration on the clot, is not susceptible to inhibition by circulating antiplasmin. Others suggested that plasmin is not responsible for thrombolysis per se, but attributed therapeutic effects to its ability to activate plasminogen trapped into the clot.

It was found in our laboratory, that after an infusion of labeled plasmin into dogs, fibrinolytic activity of the blood rapidly decreases, while radioactivity persists in the circulation for a long time. The question arose whether in these experiments, circulating radioactivity does not represent a plasmin-antiplasmin complex which is continuously releasing plasmin to the clot, thus slowly dissolving it. Dissolution of experimental clots occur long after completion of the plasmin infusion at a time when there is no more circulating fibrinolytic activity. These findings agree well with the delayed clot lysis observed in patients after a plasmin infusion.

In vitro it was shown that a plasmin-antiplasmin complex with no caseinolytic activity may still be fibrinolytic due to the effective competition of fibrin with antiplasmin for plasmin.

If the conclusions drawn from the above experiments are correct, one should be able to lyse clots introduced into experimental animals after the injected plasmin is no longer circulating in the free form. Moreover, in vivo thrombolysis should be obtainable with proteolytically inactive plasmin-antiplasmin complexes formed in vitro. The experiments described in this paper were designed to investigate these questions.

Methods

Mongrel dogs of either sex and of 10 to 12 Kg. body weight were used. Clots of purified, labeled or unlabeled human fibrin were produced in the femoral and jugular veins as previously described.

Human fibrinogen was purified and labeled with as previously reported.

Lysis of clots as expressed by disappearance of radioactivity in the thrombosed area, was continuously recorded with the aid of a scintillation counter, radiation rate meter, and Esterline recorder.

Human plasminogen was prepared according to the method of and activated with streptokinase (SK).

Fbek ion III of human plasma used as starting material was kindly supplied by Dr. James Pert, American National Red Cross Blood Program through E. R. Squibb & Co.

Streptokinase was kindly supplied by Dr. J. M. Ruegg, Lederle Co., Pearl River, New York.
TABLE 1
Lysis of Clots Formed in Vivo, Kept in the Circulation for One Hour, Then Removed and Incubated in Saline at 37 C.

<table>
<thead>
<tr>
<th>Dog number</th>
<th>Time limits of presence of clot in circulation after beginning infusion (hours)</th>
<th>Plasmin dose RPMI U./Kg. I.V. in 15 minutes</th>
<th>Preinfusion level of antiplasmin RPMI U./100 ml plasma</th>
<th>Fibrinolytic activity while clot in circulation, RPMI U./100 ml plasma</th>
<th>% decrease within 72 hours in weight of clot during in vitro incubation at 37 C.</th>
<th>Change in fibrinogen level</th>
</tr>
</thead>
<tbody>
<tr>
<td>4, 5</td>
<td>0-3</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6, 7</td>
<td>1-2</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1-2</td>
<td>4910</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*F. = trace; 24-hour lysis time.

Clots of human fibrin were formed in three major veins (jugular and femoral veins) of dogs according to the technique described previously. Two of these clots were excluded from the circulation by constricting ligatures. Plasmin or saline was infused intravenously in a 15-minute period. The clot which was left open to the circulation was removed one hour after the beginning of the infusion, weighed, and incubated in saline at 37 C. Simultaneously, constricting ligatures proximal and distal to the second clot were released, the clot exposed to the circulation for one hour, then removed and incubated in saline. In turn, the third clot was opened to the circulation, and after one hour was removed and placed in saline at 37 C. as the previous one. Results of this experiment are shown in table 1. Fifteen or 30 RPMI U. (units)/Kg. plasmin infused in a 15-minute period produced plasma fibrinolytic activity in two out of five dogs. The euglobulin fractions of the plasma samples, however, consistently showed fibrinolytic activity.

Results

Clots of human fibrinogen was assayed as described previously and expressed in RPMI units. One RPMI unit of plasmin is defined as the activity able to lyse in two minutes 0.6 ml. of a 0.3 per cent purified human fibrin clot, formed with 1 NIH unit of purified thrombin at 45 C. and pH 7.2. One antiplasmin unit is defined as the amount able to neutralize 1 RPMI unit of plasmin during an incubation period of 60 minutes at 28 C. The method of assay was previously described in detail. Caseinolytic assay of plasmin activity was performed according to the method of Mülertz.

To neutralize plasmin activity in vitro, human plasma was used with high antiplasmin activity. In previous in vitro experiments, purified antiplasmin preparations were used. There are, however, several proteins with antiplasmin activity in normal human plasma. In order to approximate physiological conditions as far as possible, we have preferred in the present experiments to employ whole human plasma instead of purified antiplasmin preparations. Ability of plasmin preparations to activate further amounts of plasminogen (activator activity), and plasminogen activity were determined by methods described earlier. Assays of plasmin, plasminogen, and activator were also performed on the euglobulin fraction of the plasma of experimental animals.
one animal which had measurable free fibrinolytic activity in the plasma had a very low initial antiplasmin level. The plasma of this animal did not form a clot upon the addition of thrombin, probably because of a combination of decrease in fibrinogen level and rapid fibrinolysis. In the other animals, the fibrinogen level did not change during the course of the experiment. Nevertheless, most of the clots removed from these dogs exhibited considerable lysis during the 72 hours in vitro incubation. Many of the clots that completely lysed did so already after 24 hours. Clots removed from control animals showed minimal weight change during in vitro incubation.

A second series of experiments was designed to investigate whether a plasmin-antiplasmin complex, proteolytically inactive in vitro, would have fibrinolytic activity under in vivo conditions. Thus, labeled fibrin clots were formed in the major veins of dogs, and radioactivity over the clots was measured as described previously. The amount of antiplasmin-rich human plasma was determined, which will abolish the caseinolytic activity of 1 RPMI unit of SK activated human plasmin during a one-hour incubation at 28 C. At that time, fibrinolytic activity of the mixture was too low to be quantitatively expressed in our assay, but gave a lysis time of five hours which is well below 0.01 RPMI U./ml of the mixture. This complex was also tested for activator activity against the serum of the recipient dogs and the euglobulin fractions prepared from the recipient animal's plasma. No activator activity was found for either of these systems.

Groups of dogs were treated with intravenous infusions of 15-minute duration of the following:

Group I: saline; group II: antiplasmin alone in the same amount used in the plasmin-antiplasmin complex preparation (given to dogs of group V); group III: SK in the same quantity (120 Christensen U./Kg. body weight) used in preparing the plasmin preparation (given to animals of groups IV and V); group IV: 15 RPMI U./Kg. of SK-plasmin (a preparation high in activator activity); and group V: 15 RPMI U./Kg. of SK-plasmin in complex form with sufficient antiplasmin to abolish caseinolytic activity. Table 2 shows the results of this experiment.

Infusion of saline (group I) or antiplasmin (group II) alone resulted in little change in the radioactivity of the clots. Statistical analysis revealed no difference between these groups. In dogs receiving plasmin (group IV) or plasmin-antiplasmin complex (group V), clots showed significant and comparable lysis in 24 hours (P < 0.05 compared to groups I and II). Animals infused with SK alone (group III) showed less lysis of clots than that obtained with plasmin infusions. The level of significance of the difference between lysis obtained in group III versus that in groups IV and V was 0.10 > P > 0.05.

Discussion

In the first series of experiments, clots were in contact with the circulation of the dog
for only one hour. During this time, only 4 out of 15 clots were exposed to "free" fibrinolytic activity as measured in whole plasma; yet, all clots lysed to a considerable extent during subsequent in vitro incubation. Plasmin, therefore, must have been present in the bloodstream in a neutral form, most likely complexed to antiplasmin. This complex must have persisted for at least two to three hours after completion of the infusion, and the fibrin clots must have been able to remove and adsorb plasmin from this circulating complex. Since antiplasmins do not precipitate with the euglobulin fraction of plasma, fibrinolytic activity obtained in this fraction should include most plasmin reversibly complexed by antiplasmin. Indeed, plasma samples with no "free" plasmin activity showed considerable plasmin activity in the euglobulin fraction as shown in table 1, representing the amount of plasmin activity of the circulating plasmin-antiplasmin complex. The euglobulin fraction of plasma of normal dogs had a low level of plasmin activity which may explain the small degree of lysis obtained in clots from control animals.

The fact that in vitro lysis of the clots occurred very slowly—in a matter of many hours—indicates that the amount of enzyme removed was small in proportion to the fibrin in the clot and that the enzyme was relatively stable. Since SK-plasmin loses activity very fast even at room temperature, stability at 37 C. in this case may reflect the protective effect of fibrin on plasmin, as reported by others.

Since plasmin lysed clots to a much greater extent than its content of SK infused alone, it is obvious that lysis in these instances is not due to an "endogeneous activation" of plasminogen by the activators. On the other hand, plasmin-antiplasmin complex with no caseinolytic and plasminogen activator activity lysed clots as well as plasmin alone. Lysis, therefore, must be attributed to the capacity of the clots to remove effectively and adsorb plasmin from the complex.

SK-activated-plasmin is a potent proteolytic enzyme with a wide range of substrates. Its specificity toward fibrin under in vitro conditions is not of a high degree. If plasmin, as suggested, is indeed continuously formed under physiological conditions, it is imperative to have a mechanism which regulates its substrate specificity toward fibrin. Norman stated that at least one of the antiplasmins binds plasmin in a reversible form. This reversible antiplasmin may well be the carrier and physiological regulator of plasmin specificity.

Summary

I23-labeled purified human fibrin clots were introduced into the circulation of dogs at various time intervals after an infusion of human plasmin. One hour later, the clots were removed and incubated in saline at 37 C. In most cases, at the time when these clots were in the circulation, there was no significant "free" plasmin activity demonstrable in the blood. Nevertheless, the clots removed from these animals exhibited in vitro lysis upon incubation. Plasmin-antiplasmin complexes were formed in vitro with no demonstrable caseinolytic activity. These complexes were as effective in dissolving fibrin clots in vivo as the same amounts of plasmin were alone. The activator (streptokinase) alone used to activate plasminogen to plasmin was significantly less effective than streptokinase-activated-plasmin, alone or in complex form with antiplasmin. These experiments were interpreted as pointing toward the ability of fibrin to compete effectively with antiplasmin for plasmin. Thus, antiplasmin may serve as a transport form or reservoir of plasmin, releasing it when fibrin clots are available, but protecting other plasma proteins from its proteolytic effect. Other phenomena apparently confirming this hypothesis have been mentioned.

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References


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