Stability of Fibrin Contiguous to Intima of Veins

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Activators capable of bringing about conversion of a proenzyme of blood, profibrinolysin (plasminogen), to the active proteolytic enzyme, fibrinolysin (plasmin), have been reported to be present in the tissues of veins. Astrup and coworkers isolated a tissue activator from veins which promoted an in vitro activation of profibrinolysin. Studies in vivo by Kwaan et al. have indicated the presence of fibrinolytic activity in blood taken from vessels subjected to a variety of stimuli. More recently, Todd, using histochemical techniques, has observed that fibrin undergoes dissolution when brought into intimate contact with ultra-thin slices of vein.

Although the evidence cited indicates that the walls of blood vessels contain an activator of profibrinolysin, only semiquantitation of the activity has been possible by the published methods. The technique used in the present study permits quantitation on a relative basis, and its application to the veins of dogs furnishes additional evidence supporting the concept that the vasculature itself supplies at least one of the fibrinolytic factors which are involved in the dissolution of intravascular blood clots. The experiments were designed to measure and compare by chemical methods (1) the stability of fibrin in contact with surfaces other than the intima of veins and (2) the stability of the same preparation of fibrin when maintained in close contact with the intima of veins.

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Methods

Bovine fibrinogen (99 per cent clottable) was prepared by the freeze-thaw method of Ware et al. The solution was stored in 0.5-ml. aliquots at -20 C. Immediately before use, an aliquot was thawed at 37 C. and diluted with imidazole buffer, pH 7.25 (prepared as directed by Mertz and Owen), and distilled water to give a final protein concentration of 0.2 per cent in a solution equivalent in ionic strength to 0.9 per cent NaCl. The Folin-Gocalten reagent was prepared immediately before use by dilution of one part of the commercial reagent (Anderson Laboratories, Fort Worth) with five parts of distilled water. The tyrosine standard was 0.1 N solution of HCl containing 0.1 mg. of L-tyrosine per ml.; this was frozen in 0.5-ml. aliquots and stored at -20 C. The biuret reagent was prepared essentially as directed by Weichselbaum; lyophilized horse serum (obtained from Cappel Laboratories, West Chester, Pennsylvania) served as a standard for the biuret test. When reconstituted, the solution contained 7.3 Gm. per cent protein (micro-Kjeldahl).

The thrombin solution (approximately 100 NIH units per ml.) was prepared by dissolving the contents of 1 vial of Thrombin Topical in 25 ml. of 0.9 per cent NaCl and bringing the volume to 20 ml. by the addition of glycerol, U.S.P. Although the thrombin used in these experiments has been found to contain some profibrinolysin, as well as other impurities, it is doubtful that it could have significantly altered either the quantitative or qualitative results in this series of experiments. The same amount of thrombin was used in all experiments, and when the veins were absent, unless urokinase was added to the systems, no fibrinolysis occurred.

The bovine fibrinolysin, prepared by the method of Loomis, contained 1.16 Loomis units per mg. Immediately before use, 20 mg. of the dry powder were dissolved in 1 ml. of 0.066 M, pH 7.7 phosphate buffer. The concentration of fibrinolysin in the buffered solution was adjusted by the addition of 0.9 per cent NaCl to bring the lysis of a

*Thrombin Topical (bovine) and bovine fibrinolysin were supplied through the kindness of C. J. Campbell, R. E. Maxwell, and E. C. Loomis of Parke, Davis and Company.
standard clot to 10 minutes at 37 C. To form the clot, 0.2 ml. of 0.2 per cent fibrinogen and 0.2 ml. of the buffered solution of fibrinolytic in a 75-mm. by 8-mm. I.D. test tube were mixed by stirring with a glass rod which had been dipped in a solution of thrombin. The concentration of the thrombin solution was 1,250 N.I.H. units per ml. When a rod 2 mm. in diameter was dipped in the thrombin solution to a depth of 1 cm. and then introduced in the solution to be clotted, approximately 2 N.I.H. units of thrombin were transferred. The time required for lysis of the clot was determined by the till-tube method.11

The epsilon-aminoacaproic acid (EACA) solution was prepared by dissolving crystalline epsilon-aminoacaproic acid* in pH 7.7 phosphate buffer to a final molarity of 0.006.

The prokinase, stored in 1-ml aliquots at -20 C., contained approximately 1,000 units per mg. of protein; the activator concentrate was prepared by the method of Coelander and Guest.12

PREPARATION OF VEINS

External jugular veins were obtained from healthy mongrel dogs of approximately 14 Kg. average weight. The animals were anesthetized with pentobarbital sodium administered intravenously in a single injection of approximately 30 mg. per Kg. body weight. After exposure of the veins, the adventitia was removed carefully in situ. Ties were placed on the veins as far apart as possible and the segment of vein between the ties excised. Each excised vein was carefully washed before they were fastened to the stainless steel rods. Though the veins had been carefully washed before they were fastened to the stainless steel rods, the amount of hemoglobin released was insufficient to account for the difference to be described, and its release appears to have had no measurable effect on the generation of fibrinolytic activity when the intimal surface of a vein was exposed to a fibrin clot.

MEASUREMENT OF PROTEOLYTIC ACTIVITY

Rods carrying the everted veins were submerged in a solution containing 4.2 ml. of phosphate buffer, pH 6.4, and 0.7 ml. of 0.2 per cent bovine fibrinogen. Thirty minutes after the addition of 100 units of thrombin in 0.1 ml. of solution, the fibrin was wound onto the intimal surface of the vein; the "clotted vein" was transferred to 5.0 ml. of phosphate buffer, pH 7.7, and incubated at 37 C. At the beginning of the incubation and at the end of five hours, 0.5-ml. aliquots of the incubation medium were removed and their content of soluble Folin-Ciocalteu reactive substances determined.

The amount of soluble Folin-Ciocalteu reactive substances released into the incubation medium over a period of five hours was taken as a measure of fibrinolytic activity. A 0.5-ml. portion of the incubation mixture was mixed with 1.5 ml. of 0.1 N NaOH and placed in a boiling water bath for 30 minutes. After cooling, 3.0 ml. of 15 per cent Na2CO3 and 1.0 ml. of diluted Folin-Ciocalteu reagent were added to each tube and the contents stirred. The blank consisted of 2.0 ml. of 0.1 N NaOH, 3.0 ml. of 15 per cent Na2CO3, and 1.0 ml. of Folin-Ciocalteu reagent. Thirty minutes were allowed for the development of the color which was read on a Klett-Summerson colorimeter with a red (620-nm) filter.

The release of protein, in addition to soluble Folin-Ciocalteu reactive material, was measured by the quantitative biuret procedure. Some hemoglobin was also leached from the veins, even though the veins had been carefully washed before they were fastened to the stainless steel rods. The amount of hemoglobin released was measured by the method of Mills and Randall.13 It was found that the amount was uniformly 10 to 15 per cent of the total Folin-Ciocalteu reactive material which was released during the five hours of incubation. The amount of hemoglobin released was insufficient to account for the differences to be described, and its release appears to have had no measurable effect on the generation of fibrinolytic activity when the intimal surface of a vein was exposed to a fibrin clot.

STATISTICAL ANALYSIS

The probability that in a given experiment, the data obtained from one situation were from a different population than data obtained from a contrasting or control phase of the same experiment was evaluated by the application of Student's t-test.

Experiments and Results

EXPERIMENT 1

To ascertain whether or not intima of veins possessed fibrinolytic activity, the following tests were performed: Jugular veins of 12 dogs were everted on rods and coated with a layer of fibrin (system A). Parallel incubation and analysis of the incubation fluids were performed with everted veins without fibrin...
TABLE 1
Lytic Activity of Isolated, Everted Dog Veins Against Bovine Fibrin

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>Mean ± S.E. (mg. tyrosine X 10)</th>
<th>Differences between systems and level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vein + clot (VC)</td>
<td>1.47 ± 0.12 (VC - C) (V - C)</td>
<td>(VC - V - C)</td>
</tr>
<tr>
<td>Clot only (C)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vein only (V)</td>
<td>0.80 ± 0.00 (system B)</td>
<td></td>
</tr>
</tbody>
</table>

EXPERIMENT 2
The presence of profibrinolysin in the fibrinogen and thrombin reagents was tested by mixing several 0.2-ml aliquots of 0.2 percent bovine fibrinogen with 0.2-ml portions of a solution containing from 2 to 4 units of urokinase dissolved in phosphate buffer. The clots resulting from the addition of approximately 2 N.I.H. units of Thrombin Topical and incubation at 37 C. were tested for lysis by the tilt-tube method.

Results: All clots containing added urokinase, an activator of profibrinolysin, lysed in less than 20 minutes, demonstrating that profibrinolysin was present in the fibrinogen and/or thrombin used as reagents in this experiment and in experiment 1. Thus, since no urokinase was added in experiment 1 and since fibrin without vein was stable (experiment 1 C), if activation of adsorbed profibrinolysin was responsible for the degradation of fibrin in experiment 1 A, the activator must have been supplied from the intima of the vein.

EXPERIMENT 3
The solubilization of fibrin in intimate contact with the endothelium of vein has been postulated to result from the action of the proteolytic enzyme, fibrinolysin. Since EACA is an inhibitor of both fibrinolysin and its activators, this substance was incorporated into the experimental systems; if, in the presence of EACA, fibrin in contact with the endothelium of veins was stabilized, indirect evidence would be obtained that the proteolysis was brought about by fibrinolysin. Experiments 1 A and 1 B were repeated; in addition, 6 X 10^{-3} M EACA was incorporated in one-half of the tubes containing the buffer.
at pH 7.7 in which vein plus fibrin and vein alone were incubated.

**Results:** The data are presented in table 2. In the presence of EACA, there was a significant reduction in the amount of Folin-Ciocalteu reactive material appearing in the soluble phase when the intima of a vein was in contact with fibrin. The appearance of Folin-Ciocalteu reactive material from vein alone was not significantly inhibited by EACA.

**EXPERIMENT 4**

The effect of EACA on the Folin-Ciocalteu test was evaluated in two ways:

A. An EACA solution (0.25 per cent) was mixed with the standard tyrosine solution and phosphate buffer to give a final concentration of 0.006 M EACA and 0.066 M phosphate buffer; the pH of the system was 7.7. A second tyrosine standard was prepared with all of its components at the same concentration as described above, except that EACA was omitted. Aliquots from the two systems were withdrawn and subjected to alkaline hydrolysis, and the Folin-Ciocalteu reactive substances were determined in the hydrolysates.

B. Mixtures containing (1) bovine fibrinogen, 0.1 per cent, and human urokinase and (2) bovine fibrinogen, 0.1 per cent, and bovine fibrinolysin were clotted with thrombin and incubated at 37 C. until the fibrin had completely disappeared. The lysed mixture in each case was divided into two parts; to one was added a 0.25 per cent aqueous solution of EACA in an amount sufficient to bring the concentration of EACA in the lysed mixture to 0.006 M. To the remaining part of the lysed mixture, a volume of distilled water equal to that which accompanied the EACA was added. The resulting samples in duplicate were subjected to hydrolysis and analyzed for tyrosine by the Folin-Ciocalteu technique.

**Results:** The amount of color which developed in the presence of EACA (six different experimental pairs) was the same as that which developed in its absence. Thus, EACA appears to have no effect on the determina-
tion of tyrosine, regardless of whether or not urokinase and fibrinolysin were also present.

**EXPERIMENT 5**

To test the possibility that the agents responsible for the apparent dissolution of fibrin were liberated from the vein into the soluble phase of the incubation mixture, two methods were used: (a) Fibrin wound onto a stainless steel rod and an everted vein on a rod were incubated at 37 C. side by side but not in physical contact in a 100 X 13 mm. test tube, the bottom of which had been molded in such a manner as to provide a ridge of glass between the lower ends of the two steel rods. (b) An everted vein on a rod was preincubated in buffer, pH 7.7, for five hours at 37 C. This vein and rod were then removed, and a stainless steel rod bearing a layer of fibrin was placed in the residual solution and incubated for an additional five hours.

**Results:** Decreased stability of fibrin was observed in both experimental situations, indicating that the agents responsible for dissolution of the fibrin had been released into the incubation medium. This was shown in experiment (a) by the liberation of greater amounts of Folin-Ciocalteu reactive material than from vein alone and in experiment (b) by a steady increase in Folin-Ciocalteu reactive material during the five-hour period in which fibrin was incubated in the medium which had previously been in contact with the intima of a vein. The number of such experiments was insufficient to permit statistical appraisal of the data.

**EXPERIMENT 6**

The adventitial surfaces of veins were tested for fibrinolytic activity by the same procedures used to test the intimal surfaces, except that the stainless steel rod occupied the lumen of the vein and fibrin was wound onto the perivascular surface.

**Results:** When the perivascular surface, essentially free of adventitia, was exposed to fibrin, the amount of Folin-Ciocalteu reactive substances released during the five-hour incubation was not measurably different from that found when only the perivascular surface, without fibrin, was present. These data indicate that the perivascular surface of jugular veins of dogs, under the experimental conditions imposed, does not supply either an activator of profibrinolysin or an active proteolytic enzyme capable of solubilizing fibrin.

**Discussion**

The experimental evidence supports the hypothesis that fibrinolysin, or activators, or both are present in or on the intimal surface of the jugular veins of the dog. The activity appears to be dependent on the presence of the intact surface since, in this laboratory, extracts and homogenates of veins have shown no measurable activity.

Although the evidence obtained through the use of EACA provides support for the hypothesis that the fibrinolytic enzyme system is involved in the dissolution of fibrin, information is not available regarding the behavior of chymotrypsin, as well as other unidentified agents, which might have a role in the decreased stability of fibrin. Trypsin, however, is not involved in a major way since EACA does not inhibit this enzyme.15

Kwaan et al.3-5 have suggested that veins are capable of releasing an activator or a fibrinolysin into the circulation and that the mechanism for the release is triggered by ischemia and anoxia. In the experiments described, the veins probably received an inadequate oxygen supply during handling and during incubation. Tagnon10 has also stated that anoxia is a stimulus to fibrinolysis.

Available evidence favors the hypothesis that the intima of veins releases an activator of profibrinolysin. Strong support for this concept is supplied by Todd's8 studies with heated and unheated fibrin plates. The data presented in this report are also consistent with the release of an activator of profibrinolysin from the vascular intima; in experiment 2, the presence of profibrinolysin in the fibrin, formed from the reaction of thrombin and fibrinogen, was demonstrated. Additional experiments are planned in which profibrinolysin-free fibrinogen and thrombin will be used.
Through the use of such reagents, it should be possible to demonstrate whether or not a fibrinolytic enzyme is released from the intima of veins. If a fibrinolytic enzyme is not released, the dissolution of contiguous fibrin must be mediated through activation of the proenzyme in association with the clot.

The experimental procedures described herein quantitate the stability of fibrin maintained in intimate contact with a large and relatively intact segment of vein. The activity in blood vessels from different regions in an organism can thus be compared, and changes which occur during various in vivo states may be estimated. Such studies are in progress.

Summary

A technique is described which employs an everted segment of canine jugular vein to study the effect of the intima on contiguous fibrin. Following eversion of the vein on a stainless steel rod of suitable dimensions, the vein was coated with a layer of fibrin by winding it onto the endothelial surface. The rate at which soluble Folin-Ciocalteu reactive material appeared in the incubated media of clots so prepared was significantly higher than that developed in systems containing either everted vein alone or fibrin alone. EACA suppressed the liberation of soluble tyrosine from contiguous fibrin but was without effect on the liberation of soluble Folin-Ciocalteu reactive material from the vein alone.

References

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