The origin of fibrin breakdown products and the interpretation of their appearance in the circulation

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SYNOPSIS It has been shown that the incubation of human plasma with urokinase at a concentration sufficient to cause rapid lysis of the clots formed on the addition of thrombin does not give rise to the production of measurable concentrations of non-clottable fibrinogen breakdown products. Also, breakdown products could not be detected in the course of experiments *in vivo* when urokinase was administered to monkeys and only in very low concentrations when a fibrinolytic state was induced by exercise in three healthy human volunteers. In contrast, high concentrations of breakdown products were found after thrombin infusion into monkeys.

It is concluded that circulating fibrinogen is not readily broken down into non-clottable products by the fibrinolytic enzymes, and that normal animals and healthy human subjects do not have substantial deposits of fibrin that are available for breakdown during a fibrinolytic episode. The presence of breakdown products in the circulation is therefore likely to be indicative of the fibrinolytic response to an initial coagulation event.

The appearance in the circulation of raised concentrations of non-clottable substances derived from fibrin or fibrinogen which are capable of agglutinating with anti-fibrinogen antiserum is widely regarded as an established feature of a fibrinolytic state in human subjects. Such an effect has been observed following exercise or the administration of adrenaline (Das, Allan, Woodfield, and Cash, 1967), in a variety of pathological conditions (Thomas *et al*, 1970), and, most dramatically, in patients with a defibrination syndrome (Ferreira and Murat, 1963; Merskey, Kleiner, and Johnson, 1966; Bonnar, Davidson, Pidgeon, McNicol, and Douglas, 1969).

Although the non-clottable products of fibrinolytic activity *in vivo* are commonly referred to as fibrin breakdown products, the nature of the available assays does not permit discrimination between polypeptides deriving from fibrin and those deriving from fibrinogen, and there is at present no clear evidence to establish their origin. The question is of some importance, for while the induction of a fibrinolytic state is clearly desirable in the treatment of thrombotic disease, fibrinogenolysis, if severe or prolonged, could well be disadvantageous.

The evidence as to whether breakdown products appear in the plasma of normal subjects as a result of the induction of a fibrinolytic state is equivocal.

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Studies of the effects of phenformin-ethyloestrenol combinations in normal subjects and in rheumatoid patients led Fearnley, Chakrabarti, and Evans (1969) to conclude that the raised concentrations of breakdown products that they found in both groups were the result of the proteolysis of fibrin deposits rather than of circulating fibrinogen. They also commented that the available evidence indicates that fibrinogenolysis is encountered only in states of pathological fibrinolytic activity. In contrast to Fearnley's observations, Gormsen and Vad (1970) were unable to detect increased breakdown products in the plasma of normal subjects whose fibrinolytic activity had been raised by the administration of phenformin and ethyloestrenol and by other agents, including nicotinic acid.

In this paper, evidence is presented which supports the view that the polypeptides measured as breakdown products in the serum following the stimulation of fibrinolysis are the products of fibrin breakdown, and that the breakdown of fibrinogen to non-clottable fragments is likely to be encountered only in exceptional circumstances.

Materials and Methods

Plasminogen-rich bovine fibrinogen was prepared by fractional precipitation from plasma fraction I

(Armour Laboratories, Eastbourne, England) according to a procedure described earlier (Gallimore and Shaw, 1967).

Thrombin (bovine, 5000 NIH units/ampoule) and urokinase (reference standard, 2340 Ploug units/ampoule) were obtained from Leo Laboratories, Hayes, Middlesex, England.

Soya-bean trypsin inhibitor, 3× crystallized, was purchased from California Biochemicals, Los Angeles, USA.

Fi-test (latex particles coated with antihuman fibrinogen antiserum) was supplied by Hyland Division, Travenol Laboratories, Inc, Costa Mesa, California, USA.

Tris-(hydroxymethyl)-methylamine (Tris) was obtained from BDH Ltd, Poole, Dorset, England.

6-Aminocaproic acid (EACA) was bought from Koch-Light Laboratories, Colnbrook, Bucks, England.

Sernylan (phencyclidine hydrochloride) was purchased from Parke, Davis & Company, Hounslow, London, England.

Tris-HCl buffer, pH 7.4, 0.1 M, was prepared by dissolving 12.1 g of the base in water, adjusting the pH to 7.4 with 1N-HCl and the volume to 1 litre.

Other reagents were of Analar grade.

RHESUS MONKEYS

Rhesus monkeys (*Macaca mulatta*) were young males, weighing 3.5 kg, purchased from Shamrock Farms (Great Britain) Ltd, Henfield, Sussex, England.

BLOOD AND PLASMA

Venous blood was withdrawn from human subjects into plastic syringes. For the collection of blood samples from monkeys, the animals were immobilized with Sernylan (4 mg/kg im). Blood was then withdrawn into a plastic syringe from a femoral vein. When sequential blood samples were required, the animal was cannulated via a saphenous vein; a slow intravenous drip was instituted in order to prevent clotting within the cannula, and blood was withdrawn through a three-way tap by a two-syringe technique. After withdrawal, blood was immediately transferred to a plastic container standing in crushed ice.

When plasma was required, the blood was mixed with 1/9 volume of aqueous trisodium citrate dihydrate (3.8% w/v); platelet-poor plasma was then obtained by centrifugation at 3000 g for 20 minutes.

DILUTE BLOOD CLOT LYSIS

The assays of dilute blood clot lysis times were performed by adding 0.2 ml of blood (immediately after collection) to 1.8 ml of 0.12 M sodium acetate

solution adjusted to pH 7.4 containing 2.5 units of thrombin; trisodium citrate dihydrate (360 µg/ml) was included in the diluent used with human blood. After mixing the samples were incubated at 37°, and the clot lysis times were measured; all estimations were performed in duplicate. Details of the procedure are given by Gallimore and Shaw (1969).

FIBRIN BREAKDOWN PRODUCTS

The estimation of fibrin breakdown products in blood was performed by the following modification of a method of Merskey *et al* (1966). Samples of blood, each of 0.9 ml, were transferred to tubes containing 0.1 ml of a solution of soya bean trypsin inhibitor (50 µg) and thrombin (2.5 units) in Tris-HCl buffer. After standing for two hr at room temperature and at +2° overnight, the clots were centrifuged and the sera collected. For the estimation of fibrin breakdown products, serial dilutions of the sera from 1/2 to 1/3200 were prepared in Tris-HCl buffer. Approximately 20 µl of each solution was introduced into a capillary tube together with 20 µl of Fi-test reagent; the tubes were then rocked for five min to mix the contents, and were inspected for latex aggregation. From the maximum dilution of the serum producing aggregation it is possible to calculate the concentration of fibrin breakdown products by comparison with standard dilutions of human and monkey plasma samples containing known amounts of fibrinogen. The quantitative expression of the results of the assay rests on the unsubstantiated assumption that fibrinogen and fibrin breakdown products cause equal aggregation at equal concentrations. Thus, although valid comparisons can be made between serum samples from the same species, some doubt exists as to the absolute values assigned to fibrin breakdown product concentrations. A similar procedure for determining fibrin breakdown products has recently been described in some detail by Melliger (1970).

Experimental

BREAKDOWN OF PLASMA CLOTS AND OF PLASMA FIBRINOGEN BY UROKINASE

To 0.30 ml aliquots of human or Rhesus monkey plasma were added various amounts of urokinase dissolved in Tris-HCl buffer, and sufficient of the same buffer to bring the volume to 0.45 ml. Clots were formed by the addition of 0.05 ml of thrombin solution (200 u/ml); these were incubated at 37° and the lysis times were recorded. Further 0.30 ml portions of the same plasmas were then incubated at 37° for various times with the same amounts of urokinase, the total volume again being made to

Human Plasma				Rhesus Monkey Plasma				Bovine Fibrinogen ¹			
Urokinase Added (units)	Clot Lysis Time (min)	Plasma Incubation Time (min)	Clotting Time (sec)	Urokinase Added (units)	Clot Lysis Time (min)	Plasma Incubation Time (min)	Clotting Time (sec)	Urokinase Added (units)	Clot Lysis Time (min)	Fibrinogen Solution Incubation Time (min)	Clotting Time (sec)
16	23.1	10	<20	45	9.0	10	<20	3	19.2	10	<20
		20	<20			20	<20			20	>60 ²
		60	<20			60	<20			30	N.C.
40	8.2	10	<20	75	6.0	10	<20	5	14.8	5	<20
		20	<20			20	<20			10	<20
		60	~30 ³			60	~20			20	N.C.
80	4.5	10	<20	150	4.0	10	<20	10	10.0	5	<20
		20	<20 ³			20	<20			10	30 ³
		60	~60 ³			60	~20			20	N.C.

Table I Comparison of the rates of proteolysis of fibrin and of fibrinogen by combinations of urokinase and plasminogen in plasma and in purified systems

¹Very similar results were subsequently obtained in similar assays with plasminogen-rich human fibrinogen

²Weak clot

N.C. = No clot

0.45 ml with Tris-HCl buffer. The capacity of these samples to clot on the addition of 0.05 ml of the thrombin solution was then observed, clotting times being recorded, and any marked abnormality of clot strength noted. The results of these experiments are given in Table I.

BREAKDOWN OF FIBRIN AND FIBRINOGEN BY UROKINASE

Fibrin clots were prepared from plasminogen-rich bovine fibrinogen and the urokinase concentrations needed to bring about their lysis in about 10, 15, and 20 min were determined. Clots were prepared in Tris-HCl buffer containing 0.1 ml of 1% (w/v) fibrinogen and 5 units of thrombin in a total volume of 0.5 ml; various amounts of urokinase were included and the exact clot lysis times were measured at 37°. The breakdown of plasminogen-rich fibrinogen by similar concentrations of urokinase was next studied. Tubes containing 0.1 ml of the same 1% fibrinogen solution in a total volume of 0.45 ml were incubated at 37° with various amounts of urokinase. By observing the capacity of the incubated samples to clot when 0.05 ml of a thrombin solution was added, the breakdown of fibrinogen could be detected. It was found that concentrations of urokinase sufficient to cause the lysis of a fibrin clot under these circumstances could, in the absence of thrombin, bring about the breakdown of fibrinogen to non-clottable fragments at a similar rate. These results are also summarized in Table I.

DEVELOPMENT OF FIBRIN BREAKDOWN PRODUCTS FOLLOWING THE INCUBATION OF PLASMA CLOTS AND OF UNCLOTTED PLASMA WITH UROKINASE

To 4 × 0.60 ml portions of citrated human plasma

was added 0.14 ml of a solution of urokinase in buffer (400 u/ml); they were then clotted with 0.1 ml of a solution of thrombin (200 u/ml). The clots were incubated at 37° and clot lysis occurred in approximately 16 min; the tubes were transferred to an ice bath after 7.5, 15, 30, and 60 min. Immediately after removal of each tube from the incubator, 0.1 ml of a solution of soya bean trypsin inhibitor (0.5 mg/ml in 0.5 M EACA) was added and any residual clot was synergized. After storage overnight at +2°, traces of fibrin were removed and the serum samples were assayed for the content of fibrin breakdown products. In the same experiment, 3.0 ml of plasma was mixed with 0.7 ml of the same urokinase solution and was incubated at 37°. Portions (0.6 ml) were transferred at 7.5, 15, 30, and 60 min to tubes each containing 0.1 ml of a solution of thrombin, soya bean trypsin inhibitor, and EACA. Clotting was allowed to continue for 60 min at room temperature; the fibrin was then removed and the

Incubation Time (min)	Human Plasma + Urokinase		Monkey Plasma + Urokinase	
	FBP (μg/ml) Produced by		FBP (μg/ml) Produced by	
	Plasma Clots ¹	Non-clotted Plasma	Plasma Clots ¹	Non-clotted Plasma
7.5	<2	<2	<2	<2
15	500	<2	200	<2
30	>1000	<2	>1000	<2
60	>1000	<2	200 ²	<2

Table II Development of fibrin breakdown products following incubation of human and monkey plasma and plasma clots with urokinase

¹Clots lysed in 16 min

²Clots lysed in 14 min

³Poor reaction with Fi-test

serum samples were allowed to stand overnight at +2°. After removal of any further traces of fibrin, the sera were assayed for fibrin breakdown products.

A similar experiment was performed with citrated monkey plasma: 0.08 ml of a urokinase solution (750 u/ml) was added to each aliquot of plasma (0.6 ml), giving rise to a clot lysis time of approximately 14 min, and 0.4 ml of the same solution was added to 3.0 ml of plasma in the second part of the experiment. In all other particulars the experiments with human and monkey plasmas were identical. The results of both are given in Table II.

HUMAN EXERCISE STUDY

Blood samples were collected from three male subjects 5 min before, and 2, 30, and 60 min after vigorous exercise on a stationary bicycle, lasting six minutes. The subjects, all aged approximately 40 years, were rested for 45 min before the experiment, and between the collection of the last three blood samples. The blood was transferred to plastic containers in an ice bath; duplicate samples were then quickly removed for the dilute blood clot lysis assay and 0.9 ml portions were added to 0.1 ml of soya bean trypsin inhibitor-thrombin solution in order to provide serum for the estimations of fibrin breakdown products. The results of these assays are shown

in Table III. They show that a marked increase in fibrinolytic activity in these subjects is accompanied by only a very small and transient rise in fibrin breakdown products.

STUDIES IN MONKEYS

Administration of urokinase

Two Rhesus monkeys both weighing approximately 3.2 kg were tranquillized with Sernylan and cannulated. Blood samples were removed at intervals before and after the rapid intravenous administration of urokinase dissolved in sterile isotonic saline. One monkey received 12000 units of urokinase (approx. 3580 u/kg), the other 23400 units (approx 7650 u/kg). Blood samples were tested for fibrinolytic activity (dilute blood clot lysis time assay) and serum samples were prepared in the presence of soya bean trypsin inhibitor for the estimation of concentrations of fibrin breakdown products. The results of the assays are given in Table IV, and clearly show the development of a pronounced 'fibrinolytic' state without the appearance of fibrin breakdown products in the blood.

Administration of thrombin

Two Rhesus monkeys (4.4 and 3.0 kg) were tranquillized with Sernylan and cannulated, and an

Time before (-) or after (+) Exercise (min)	Subject Number					
	1		2		3	
	DBCLT (min)	FBP (µg/ml)	DBCLT (min)	FBP (µg/ml)	DBCLT (min)	FBP (µg/ml)
- 5	240	3	450	3	203	<1
+ 2	52	6	143	6	31	2
+ 30	162	3	378	3	133	1
+ 60	225	3	355	3	180	1

Table III Effect of exercise on fibrinolysis (DBCLT) and on serum FBP concentration in three adult human subjects

Experiment 1: Urokinase: 3600 u/kg			Experiment 2: Urokinase: 7600 u/kg		
Time before (-) or after (+) Urokinase Infusion (min)	DBCLT (min)	FBP (µg/ml)	Time before (-) or after (+) Urokinase Infusion (min)	DBCLT (min)	FBP (µg/ml)
- 36	>300	<2	- 25	190	<2
- 8	>300	<2	- 11	250	<2
+ 10	49	<2	+ 2	8	<2
+ 36	48	<2	+ 10	20	<2
+ 65	45	<2	+ 29	54	<2
+ 125	270	<2	+ 56	26	<2

Table IV Administration in vivo of urokinase to monkeys: DBCLT and FBP assays

Time before (-) or after (+) Thrombin Infusion (min)	- 10	+ 1	+ 10	+ 30	+ 60	+ 90
Serum concentration of FBP (µg/ml)	0	0	450	450	600	600
Experiment 1	0	0	600	600	600	600
Experiment 2	0	0	600	600	600	600

Table V Administration in vivo of thrombin to monkeys (200 u/kg): serum FBP concentration

intravenous saline drip was set up. Blood samples were removed at intervals before and after the administration of thrombin (200 u/kg), which was infused over a period of about three minutes. Of each blood sample, 0.9 ml was added to 0.1 ml of soya bean trypsin inhibitor-thrombin solution and the resulting serum was collected and assayed for concentration of fibrin breakdown products. The results (Table V) show the presence of fibrin breakdown products in the serum of both animals, in high concentration.

Discussion

The results of the *in vitro* experiments described in this paper (Tables I-III) demonstrate that the addition of a plasminogen activator to plasma, in sufficient quantity to give rise to the rapid lysis of the clot which forms when coagulation has taken place, does not cause substantial breakdown of the fibrinogen in the unclotted plasma. This observation, which was equally valid with human and monkey plasma, accords with the findings of Niléhn (1967) who investigated the streptokinase-induced breakdown of fibrinogen and fibrin in human plasma.

The results in Table I show that the clottability of monkey plasma was not affected, and that of human plasma only slightly impaired, when these plasmas were incubated with urokinase for periods several times longer than those needed for clot lysis to take place following clot formation by the addition of thrombin. This observation is substantiated by the results in Table II, which show that no breakdown products could be detected when plasma was incubated with urokinase, in contrast to the situation where the serum from plasma clotted in the presence of the same amount of urokinase was assayed. In this situation little or no breakdown product appeared until clot lysis was well advanced: at and after this stage, high concentrations were detected in both the human and monkey systems, the end point of the titrations with Fi-test reagent being at similar dilutions to that with untreated plasma. With the clot digests from monkey plasma, however, it was noticed that the titre of breakdown products appeared to be reduced after prolonged incubation, suggesting that the lower molecular weight polypeptides released from monkey fibrin do not react so efficiently with the Fi-test reagent as does the parent fibrinogen molecule. That the protection of plasma fibrinogen from breakdown by combinations of plasminogen and urokinase is related to the presence of inhibitors of fibrinogenolysis, and not simply to structural differences between the two substrates (fibrinogen and fibrin) was indicated by their equally rapid proteolysis in purified systems where the inhibitors were absent (Table I).

However, the possibility cannot be entirely discounted that small amounts of fibrinogen breakdown products, in insufficient concentration to modify the rate of clot formation, might be incorporated into the clot structure and thus escape detection in the serum.

The relevance of this evidence *in vitro* to the situation *in vivo* is obvious. It was anticipated that the development of a fibrinolytic state *in vivo* by the release of plasminogen activator into the circulation would not give rise to the destruction of fibrinogen or to the appearance of breakdown products in the blood, unless the lytic condition was accompanied or preceded by fibrin deposition. This inference was of course based on the assumption that urokinase behaves in a similar manner to the intrinsic plasminogen activator in the blood. Apart from the observations of Fearnley *et al* (1969), high serum concentrations of breakdown products have not been encountered in response to the induction of a fibrinolytic state in healthy subjects (see, *eg*, Das *et al*, 1967; Gormsen and Vad, 1970) in conditions where the blood has been allowed to clot in the presence of a protease inhibitor. However, elevated levels of fibrin breakdown products have been recognized in conditions where fibrin deposition is believed to have occurred, such as rheumatoid arthritis, myocardial infarction, and most markedly in the consumption coagulopathies that may develop with obstetrical complications (Ferreira and Murat, 1963; Bonnar *et al*, 1969). Our results with human volunteers (Table III) support the findings of Das and of Gormsen and show that the production of a vigorous fibrinolytic state in normal subjects, indicated by a fall in dilute blood clot lysis time to less than a third of the resting value, is accompanied by the appearance of only a very small (2-3 µg/ml) and short-lived release of breakdown products. Even this small amount of breakdown product may be a consequence of coagulation occurring as the primary response to exercise (Kontinen, 1968) rather than of the lysis of pre-existing fibrin deposits. A similarly pronounced fibrinolytic state in monkeys induced by infusion of urokinase also failed to liberate measurable amounts of breakdown products (Table IV), although in these animals the fibrinolytic state was of longer duration than that produced in the human subjects.

The capacity of the procedure to detect fibrin breakdown products in the blood samples taken from monkeys was amply confirmed by the response evoked to the infusion of thrombin. The results (Table V) show that in these circumstances intravascular coagulation was rapidly followed by the appearance of high concentrations of circulating

fibrin breakdown products in the blood. It has been established from other experiments with ^{125}I -labelled fibrinogen that infusion of thrombin into monkeys as described above causes pronounced defibrination and the rapid reappearance of labelled material in the blood.

Two main inferences may be drawn from these observations: first that the provision of sufficient urokinase to activate the fibrinolytic system *in vitro* or *in vivo* does not lead to the breakdown of a significant amount of plasma fibrinogen, and second, that normal animals and healthy human subjects have little or no intravascular or other accessible fibrin that can be used as a substrate by proteolytic enzymes developed during fibrinolytic episodes.

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