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(Citation)
The Kobe journal of the medical sciences,14(4):281-291

(Issue Date)
1968-12

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100489079

Kobe J. Med. Sci. 14, 281-291, December 1968

FACTORS CONTROLLING COUPLING IN CONTACT-FIBRINOLYSIS

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Indexing Words
fibrinolysis; Hageman factor; heparin; anticoagulant

Yoshinori FUNAHARA, Sumio NAKAMURA, and Shosuke OKAMOTO. *Factors Controlling Coupling in Contact-Fibrinolysis.* Kobe J. Med. Sci. **14**, 281-291, December 1968——Evidence is presented for the activation of the fibrinolytic system by a contact system through two pathways, one dependent on the absence of calcium ions and the other independent of the presence or absence of calcium ions. Dog blood exposed to contact with kaolin was used in the investigations, and the fibrin plate method (standard and heated) employed for fibrinolytic enzyme assay. Decalcified plasma or serum exhibited fibrinolytic activity after exposure to kaolin; but heparinized plasma, in both the case of absence and presence of calcium ions, did not show fibrinolytic activity after exposure to kaolin. Further, among serum samples from the twenty-five dogs investigated only five exhibited fibrinolytic activity after exposure to kaolin. However, serum which was unaffected by kaolin alone did exhibit fibrinolytic activity when EDTA, in sufficient quantity to combine with all calcium ions, was added prior to the exposure to kaolin: the EDTA serum did not show any activity in the case of its recalcification prior to exposure to kaolin. Nevertheless, addition of calcium ions to serum exhibiting kaolin-induced fibrinolytic activity had no suppressing effect on such fibrinolysis; and even when calcium ions were added to this type of serum prior to exposure to kaolin fibrinolytic activity was seen. In both the case of addition of calcium ions and of addition of heparin, streptokinase activation of the fibrinolytic system, and the activity of the fibrinolytic enzyme, were not suppressed. These results strongly suggest the possibility that two pathways exist between the contact system and the fibrinolytic system, and that heparin can block both routes.

INTRODUCTION

The role of the Hageman factor in the blood clotting system has been investigated by many workers since the publication of Ratnoff and Colopy's report in 1955[17]. Margolis (1958, 1960)[11,12] and V. Eisen (1964)[8] have shown that the Hageman factor plays an important part in the release of plasma kinin. In relation to the fibrinolytic system, the action of the Hageman factor has also been studied by several authors following the findings of Niewiarowski and Prou-Wartelle (1959)[14], viz. that exposure of intact plasma to a foreign surface produces a marked

Received for publication October 10, 1966
Authors' names in Japanese : 船原芳範　中村寿美雄　岡本彰祐

enhancement of fibrinolysis so long as the Hageman factor level of the studied plasma is normal: this effect was not seen in plasma deficient in Hageman factor. Similar results were obtained by Iatridis and Ferguson (1961)[5] with the Hageman trait plasma. Further, these same authors (1962)[6] noticed that the contact factors, when activated, caused an increase in plasminogen activator in the euglobulin fraction. Murakami and Ongi confirmed these results in 1965[18], and Takada et al. (1966)[21] reported that the proactivator of plasminogen prepared from human plasma was converted to activator by contact factor action.

The present authors' work was carried out on the blood of dogs, in which kaolin clotting was unusually rapid. Kaolin-fibrinolytic coupling was readily observed in decalcified conditions, but was not constantly found in the case of recalcified serum. Pursuing these studies further finally leads to the conclusion that there exist two pathways between the fibrinolytic system and the contact system, and that one pathway is stable and inhibited by calcium ions while the other is unstable and uninhibited by calcium ions.

MATERIALS AND METHODS

Fibrinogen: Bovine fibrinogen obtained from Armour Pharmaceutical Company, Illinois, U. S. A. was used.

Thrombin: Bovine thrombin topical obtained from Mochida Pharmaceutical Company, Tokyo, Japan was used. The thrombin was dissolved in borate saline buffer (pH 7.4) at a concentration of 20u per millilitre.

Dog blood samples:

a) Citrated plasma: Whole blood was collected in a dry siliconized syringe and mixed with sodium citrate (3.8% solution) in the proportion of nine volumes of blood to one volume of citrate. The plasma was obtained by centrifuging the mixture at 4°C (using siliconized centrifuge tubes). Platelet-rich plasma was obtained by centrifugation at 1,000 r. p. m. for 5 minutes, and platelet-poor plasma by centrifugation at 3,000 r. p. m. for 30 minutes.

b) Oxalated plasma: Nine parts of blood were mixed with one part of 1.34% (w/v) sodium oxalate and the mixture subjected to centrifugation under similar conditions to the above.

c) EDTA plasma: Nine parts of blood were mixed with one part of 50mM EDTA solution and the mixture subjected to centrifugation under similar conditions to the above.

d) Serum: Whole blood was collected in a siliconized centrifuge tube, and the serum obtained by centrifugation at 3,000 r. p. m. for 10 minutes after an incubation of 20 minutes at 30°C.

e) Euglobulin fraction: A one millilitre sample of blood was added to 19 millilitres of distilled water and the pH adjusted to 5.2 with 1% acetic acid solution. The euglobulin fraction was separated by centrifugation and then redissolved in one millilitre of borate saline buffer (pH 7.4). All these procedures were carried out at 4°C.

Determination of fibrinolytic activity by the fibrin plate method: Astrup's standard fibrin plate method[1] and Lassen's heated fibrin plate method[9] were

used in the determination of fibrinolytic activities and in the investigation of the effects of epsilon-amino n-caproic acid (EACA)[15] and trans-form aminomethylcyclohexane carboxylic acid (t-AMCHA).[16]

Experimental procedures in kaolin activation: Kaolin activation experiments were performed on twenty-five dogs. Blood samples were obtained from the femoral artery after anaesthesia with sodium pentabarbiturate. Kaolin was added to the plasma or serum to reach a final concentration of 10 milligrams per millilitre. These mixtures were incubated for 10 minutes at 37°C and then centrifuged at 3,000 r. p. m. for 10 minutes. The fibrinolytic activities of the supernatants were measured by the heated and standard fibrin plate methods.

RESULTS

I. Fibrinolytic activity of plasma and serum exposed to kaolin.

Decalcified plasma (citrated, oxalated and EDTA plasmas) previously exposed to contact with kaolin showed fibrinolytic activity on both standard and heated fibrin plates. Heparinized plasma, decalcified and undecalcified, showed no fibrinolytic activity after exposure to kaolin.

The response of serum samples to exposure to kaolin was variable: in five cases of the twenty-five dogs investigated fibrinolytic activity (of the same order as that of decalcified plasma) was exhibited, but in the remaining twenty cases no activity was observed.

In all control samples, i. e. those not exposed to kaolin, no fibrinolytic activity was shown on standard or heated fibrin plates.

These results are summarized in quantitative form in Table 1 below.

No differences were observed in the fibrinolytic activities of platelet-rich and platelet-poor samples in this series of experiments.

Table 1. Kaolin exposure and the fibrinolytic activities of serum and treated plasma (25 dogs). (Figures represent fibrinolysed areas of plates, in square millimetres: expressed as means plus or minus their standard deviations.)

	Not exposed to kaolin		Exposed to kaolin	
	Standard plate	Heated plate	Standard plate	Heated plate
Citrated plasma	0	0	90±10	71±9
Oxalated plasma	0	0	90±10	71±9
EDTA plasma	0	0	90±10	71±9
Heparinized plasma (decalcified)	0	0	0	0
Serum	0	0	90±10 (five cases)	71±9 (five cases)
			0 (twenty cases)	0 (twenty cases)

II. Fibrinolytic activity of euglobulin fractions of plasma and serum exposed to kaolin.

The euglobulin fraction of kaolin-exposed, decalcified plasma showed fibrinolytic activity of almost the same level as kaolin-exposed, decalcified plasma. Furthermore, the euglobulin fraction of kaolin-exposed, heparinized plasma also exhibited this same level of activity.

In the case of the euglobulin fraction of kaolin-exposed serum, a similar level of activity was again observed.

These activities were completely suppressed by 10^{-2} molar concentrations of EACA or 10^{-3} molar concentrations of t-AMCHA.

Each of the euglobulin fractions of plasma and serum unexposed to contact with kaolin exhibited a similar level of activity to its corresponding kaolin-exposed fraction.

These results are summarized in quantitative form in Table 2 below.

Table 2. Kaolin exposure and the fibrinolytic activities of euglobulin fractions of serum and treated plasma (25 dogs). (Figures represent fibrinolysed areas of plates, in square millimetres : expressed as means plus or minus their standard deviations.)

Euglobulin fraction of :	Not exposed to kaolin		Exposed to kaolin	
	Standard plate	Heated plate	Standard plate	Heated plate
Citrated plasma	81±6	64±7	81±6	64±7
Oxalated plasma	81±6	64±7	81±6	64±7
EDTA plasma	81±6	64±7	81±6	64±7
Heparinized plasma (decalcified)	81±6	64±7	81±6	64±7
Serum	81±6	64±7	81±6	64±7

III. Effects of calcium ions and EDTA on fibrinolytic activity of serum and plasma induced by kaolin.

In order to elucidate the action of calcium ions on fibrinolysis induced by kaolin, plasma and serum samples were taken from two dogs (in one case from an individual whose serum had not shown fibrinolytic activity after exposure to kaolin (Dog 1), and in the other from one whose serum had shown fibrinolytic activity after exposure to kaolin (Dog 2)).

In Dog 1, when EDTA (final concentration 5 mM) was added prior to exposure to kaolin, the serum came to show fibrinolytic activity: the same level of activity

was exhibited by kaolin-exposed EDTA plasma. However, neither EDTA serum nor EDTA plasma showed fibrinolytic activity in the case of recalcification (final calcium ion concentration 10 mM) before exposure to kaolin.

These results are summarized in quantitative form in Table 3-a below.

Table 3-a. Effects of calcium ions and EDTA on fibrinolytic activity of serum and plasma induced by kaolin (Dog 1). (Figures represent fibrinolysed areas of plates, in square millimetres: expressed as means plus or minus their standard deviations.)

	Not exposed to kaolin		Exposed to kaolin	
	Standard plate	Heated plate	Standard plate	Heated plate
Serum	0	0	0	0
Serum with EDTA added (final conc. 5 mM)	0	0	90±10	71±9
EDTA serum with Ca ions added (final conc. 10 mM)	0	0	0	0
Plasma with EDTA added (final conc. 5 mM)	0	0	90±10	71±9
EDTA plasma with Ca ions added (final conc. 10 mM)	0	0	0	0

Table 3-b. Effects of calcium ions and EDTA on fibrinolytic activity of serum and plasma induced by kaolin (Dog 2). (Figures represent fibrinolysed areas of plates, in square millimetres: expressed as means plus or minus their standard deviations.)

	Not exposed to kaolin		Exposed to kaolin	
	Standard plate	Heated plate	Standard plate	Heated plate
Serum	0	0	90±10	71±9
Serum with EDTA added (final conc. 5 mM)	0	0	90±10	71±9
EDTA serum with Ca ions added (final conc. 10 mM)	0	0	90±10	71±9
Plasma with EDTA added (final conc. 5 mM)	0	0	90±10	71±9
EDTA plasma with Ca ions added (final conc. 10 mM)	0	0	90±10	71±9
EDTA plasma with Ca ions added (final conc. 25 mM)	0	0	90±10	71±9

Further, when calcium ions (final concentration 10 mM) were add to EDTA serum or EDTA plasma after exposure to kaolin, the fibrinolytic activity induced by kaolin was not suppressed.

All euglobulin fraction samples prepared from treated and untreated serum and plasma of Dog 1 showed fibrinolytic activity ($81\pm8\,mm^2$ on standard plates, and $71\pm9\,mm^2$ on heated plates).

Addition of magnesium ions (final concentrations 10 mM, 25 mM) to EDTA serum or plasma, both before and after contact with kaolin, had no suppressing effect on the fibrinolytic activity.

In Dog 2, addition of EDTA (final concentrations 5 mM) with and without subsequent addition of calcium ions (final concentrations 0, 10, 25 mM) had no effect on the fibrinolysis induced by contact with kaolin, i. e. the activities were equal to that of kaolin-exposed serum.

These results are summarized in quantitative form in Table 3-b.

As discussed elsewhere, these results indicate the existence of two pathways between the contact system and the fibrinolytic system, one of which is blocked by calcium ions. Preliminary data suggest that the pathway unaffected by the presence or absence of calcium ions ceases to exist when the serum has been allowed to stand for about three hours at room temperature, whereas the pathway blocked by calcium ions remains unaffected.

IV. Effect of calcium ions and heparin on fibrinolysis induced by streptokinase.

The results described in Section III suggest that calcium ions under certain conditions may suppress the activation process of the fibrinolytic system by kaolin.

Table 4. Effect of calcium ions and heparin on fibrinolysis induced by streptokinase (using euglobulin fractions prepared from dried human plasma).

Final concentration of calcium ions (mM)	Fibrinolytic activity (mm^2) (Heated plates)
0	100
10 (added after SK)	100
15 (added after SK)	100
25 (added after SK)	100
10 (added before SK)	100
25 (added before SK)	100
Final concentration of heparin (u/ml)	
5 (added after SK)	100
10 (added after SK)	100
5 (added before SK)	100
10 (added before SK)	100

The following experiments were designed to discover the effect of calcium ions and heparin on the activation process of the fibrinolytic system by streptokinase.

Various concentrations of calcium ions and heparin were added to euglobulin fractions prepared from dried human plasma, both before and after the addition of streptokinase.

It was found that calcium ions of concentrations up to 25 mM and heparin of concentrations up to 10u/ml had no suppressing effect on the fibrinolysis induced by streptokinase. The results are summarized in quantitative form in Table 4.

DISCUSSION

Results obtained in the present work showed that dog blood which had been exposed to contact with kaolin under certain conditions came to exhibit high fibrinolytic activity. This observation posed a crucial question: Dose kaolin directly activate the fibrinolytic system or is the process an indirect one, a consequence of the activation of the contact system?

On the basis of evidence presented by Iatridis and Ferguson (1961, 1962)[5,6] V. Eisen (1964),[3] Holemans and Roberts (1964),[4] Murakami and Ongi (1965)[13] and Takada et al. (1966),[21] which indicated that the fibrinolytic system is activated by active Hageman factor (or contact product), it seemed possible that the activation of the fibrinolytic system by kaolin might indeed be indirect. The results given above (Tables 3, 4) suggest that in fact two routes may exist between the contact system and the fibrinolytic system, i. e. a stable pathway which is blocked by calcium ions and an unstable pathway which is not blocked by calcium ions.

Although the physiological functions of these pathways remain in doubt, there seems to be justification in speculating that the pathway not inhibited by calcium ions may remain active under normal physiological conditions, allowing the development of a local fibrinolytic state and so the removal of fibrin from a restricted area. On the other hand, the pathway blocked by calcium ions would seem to be active only under pathological conditions, allowing the development of a systemic fibrinolytic state.

Heparin is believed to be a substance capable of effectively blocking both these routes. The results given in Tables 1, 2, and 4, viz. that heparin prevents activation of the fibrinolytic system by kaolin but is ineffective in inhibiting activation by streptokinase, suggest that heparin blocks either the pathways between the contact system and the fibrinolytic system, or the activation process of the contact system itself. Furthermore, von Kaulla and McDonald (1958)[23] have reported that heparin has no influence on fibrinolysis induced by urokinase. According to our preliminary studies and Bloom's data (1962),[2] it is tentatively held that heparin acts to block the pathways which connect the contact and fibrinolytic systems. However, further research is necessary to satisfactorily elucidate the true mode of action of heparin in relation to these systems.

It was proposed by Iatridis and Ferguson (1961, 1962),[5,6] Murakami and Ongi (1965)[13] and Takada et al. (1966)[21] that in the process of activation of the

fibrinolytic system active Hageman factor may activate human proactivator to activator. Solution of the question as to whether such a route could be related to the calcium ion-inhibited pathway or not must again depend on further critical studies.

It was observed that in the case of the use of ballotini (glass beads) or celite in place of kaolin only weak fibrinolytic activity resulted. Concerning the reasons for kaolin's possession of a stronger power to induce fibrinolysis in comparison with other substances activating the contact system, Soulier and Prou-Wartelle (1960)[19] suggested that kaolin adsorbs clotting factors other than the contact factors, but that celite, being a less powerful activator of the contact system, does not adsorb such additional factors. Moreover, Jobling and Petersen (1914)[7,8] have reported that adsorption of serum proteases inhibitors by various materials (such as kaolin, starch, paste, agar and bacteria) results in demonstrable proteolytic activity of serum. Possession of ability to adsorb many factors may thus be an important element in the activation of the fibrinolytic system.

Decalcified plasma handled with non-siliconized glassware showed fibrinolytic activity on standard fibrin plates only, the contact system activated by exposure to glass being able to activate the fibrinolytic system of such plates. The euglobulin fraction of plasma handled with siliconized glassware showed fibrinolytic activity on both heated and standard fibrin plates, the level of activity being of the same order as that in kaolin-exposed decalcified plasma.

Ratnoff and Conley (1951)[17] have presented evidence indicating that the calcium content of euglobulin fractions prepared from native plasma is one-tenth to one-twentieth of that normally present in whole plasma. When the concentration of calcium ions was raised to the level of normal whole blood, clotting occurred promptly in siliconized tubes. Furthers, Tocantins et al. (1951)[22] and Speer et al. (1965)[20] have demonstrated that anti-Hageman factor is found in the supernatant resulting from euglobulin precipitation. These data strongly suggest that the procedure used to obtain euglobulin fractions, even with siliconized glassware, may activate the Hageman factor and produce conditions favourable for activation of the fibrinolytic system. Thus, dilute blood clot lysis time (MacFarlane, 1937),[10] which has been thought to be a sensitive method for measuring fibrinolytic activity, may perhaps reflect fibrinolysis induced by Hageman factor which has been previously activated as a result of the blood dilution.

SUMMARY

1. Plasma or serum alone, without exposure to kalin, showed no fibrinolytic activity on standard or heated fibrin plates.

2. Decalcified plasma or serum after contact with kaolin came to show fibrinolytic activity.

3. Heparinized plasma, in both the absence and presence of calcium ions, showed no fibrinolytic activity even after exposure to kaolin.

4. Of the twenty-five dogs examined, in five cases only did the serum exhibit activity after contact with kaolin.

5. Serum not showing fibrinolytic activity after exposure to kaolin did exhibit such activity with addition of EDTA (final concentration 5 mM) before the exposure to kaolin. EDTA serum showed no activity when it was recalcified (final concentration 10 mM) before exposure to kaolin.

6. Addition of calcium ions to serum showing fibrinolytic activity after contact with kaolin had no suppressing effect on the fibrinolysis even if the addition was made prior to the exposure to kaolin.

7. Neither calcium ions (final concentration 25 mM) nor heparin (final concentration 10u/ml) inhibited activation of the fibrinolytic system by streptokinase or the activity of the fibrinolytic enzyme (experiments with human euglobulin fraction).

8. Euglobulin fractions obtained from all serum and plasma samples showed approximately the same level of fibrinolytic activity.

9. Such fibrinolytic activities were completely inhibited by a 10^{-2} molar concentration of EACA or a 10^{-3} molar concentration of t-AMCHA.

10. In conclusion it is suggested that two pathways exist between the contact system and the fibrinolytic system (one blocked by calcium ions and the other unaffected by the presence or absence of calcium ions), and that both these routes are inhibited by heparin.

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