The effect of fibrin clots and clot-bound thrombin on the development of platelet procoagulant activity

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The Effect of Fibrin Clots and Clot-Bound Thrombin on the Development of Platelet Procoagulant Activity

Rachana Kumar, Suzette Béguin, H. Coenraad Hemker

From the Department of Biochemistry, Faculty of Medicine and Cardiovascular Research Institute, University of Limburg, Maastricht, The Netherlands

Summary

We tested different types of clot for their ability to provoke procoagulant activity in platelets: normal clots from platelet poor plasma (des AABB- or fibrin II clots), similar clots in which the adsorbed thrombin has been inhibited by hirudin, and clots obtained by the action of two snake venom enzymes that release only fibrinopeptide A (des AA- or fibrin I clots). Analogous clots from fibrinogen solutions were also tested.

In platelet rich plasma (PRP), where platelet coagulant phospholipids (PCP) are rate limiting for thrombin generation, the addition of any type of clot enhances the generation of thrombin thus it induces the appearance of PCP. Clots containing active adsorbed thrombin are the most potent ones in this respect. Lactate dehydrogenase (LDH) levels do not increase in the course of the thrombin generation so the platelets are not damaged in the process. Non-centrifugable PCP could be demonstrated to appear during the process, so the production of procoagulant phospholipid microparticles must be part of the mechanism. Membrane transbilayer phosphatidyl serine movement (flip-flop) can not be demonstrated in PRP as the activated platelets are caught in the emerging clot.

In order to demonstrate flip-flop, we tried to investigate the influence of clots on washed platelets. However, contrary to platelets in a plasma milieu, isolated platelets are damaged by fibrin clots, especially in the presence of thrombin, as can be judged from the appearance of LDH.

We conclude that, in PRP, clots induce the appearance of PCP from platelets by vesiculation, possibly accompanied by flip-flop and that thrombin accelerates the process but is not an absolute requirement.

Introduction

It is becoming increasingly evident that primary haemostasis through platelet activation and aggregation and secondary haemostasis through clotting are processes that amplify each other in all types of haemostatic and thrombotic events (see ref. 1 for a review). A powerful resonance loop establishes itself at a site of vessel injury because thrombin very effectively activates platelets and activated platelets accelerate thrombin generation by exposing a procoagulant phospholipid surface (2-4) and releasing platelet factor V (5-7). Thus, the formation of thrombin and platelet activation are interdependent processes and it is not easy to dissociate the role of one from another.

In the process of clotting of fibrinogen by thrombin, a certain amount of thrombin is adsorbed on the fibrin (8-11). Clot-bound thrombin still exhibits catalytic activity since it has been reported to catalyze the release of FPA from fibrinogen (12-14), to hydrolyse chromogenic substrates (13) and to shorten the coagulation time of blood and plasma (14-16). The fibrin clot thus behaves as a reservoir of enzymatically-active thrombin, more significantly so as the inhibition characteristics of bound thrombin by plasma antithrombins have been demonstrated to be much different from fluid phase thrombin, notably that this thrombin is insensitive to antithrombin (11, 12, 15). There also arises the question, whether the adsorbed thrombin still could function in the thrombin-mediated feedback reactions that are so typical for the coagulation mechanism. Recently we have reported (17, 18) that clot-bound thrombin could indeed further enhance thrombin formation by sustaining the activation of plasma cofactor proteins factor V and VIII. We have also observed that in platelet rich plasma (PRP) clots significantly lower the lag phase prior to the burst of thrombin generation, indicating that platelets are activated by clots.

The present article investigates the influence of clots produced by thrombin and non-thrombin enzymes on the stimulation of platelets. It has been shown that the major platelet surface receptor GPIb/IIia (αIIBβ3, integrin) is activated by a variety of extracellular matrices including fibrin (19) leading to highly adherent behaviour of the platelets. Clots may also induce the platelet release reaction (14). Because there are several forms of platelet activation that do not lead to the exposure of procoagulant surfaces (20), it remains to be seen how the procoagulant behaviour of the platelets is affected by clots and clot bound thrombin. To this end, in the work presented here, we have studied the effect of different types of clots on the generation of platelet prothrombin converting activity in platelet rich plasma and in suspensions of washed platelets and compared it with the effect of well known platelet activators such as Ca²⁺-ionophore A23187 or IIa and collagen, alone or in combination.

Materials and Methods

Materials

Fatty acid free bovine serum albumin (BSA), ovalbumin (grade V) and Calcium ionophore, A23187 were purchased from Sigma (St. Louis, MO). Horse tendon collagen (type I) was from Hormon Chemie (Munich, Germany). Chromogenic substrate used for thrombin was S2238: H-D-Phe-Pip-Arg-pNA,2HCl. Purified fibrinogen was obtained from Kordia (Leiden, The Netherlands). Reptilase was supplied by Laboratories Stago (Asnieres, France) and Agihal, a purified fraction of Agkistrodon halys halys snake venom, that splits fibrinopeptide A from fibrinogen was obtained from Prof. L. Yukelson (Tashkent, Uzbekistan). Recombinant hirudin (Knoll AG) was a kind gift of Dr. W. Hornberger.
Plasma

PRP for thrombin generation experiments was obtained by centrifugation at 250 g, 15°C, 10 min of freshly drawn citrated blood (1 volume trisodium citrate 0.13 M to 9 volumes blood) of a healthy donor. The platelet count was adjusted to 3*10^9/ml with homologous platelet poor plasma (PPP) obtained by double centrifugation of PRP at 1000 g, 15°C, 10 min. PRP for the measurement of generation of platelet procoagulant activity was obtained from blood taken 5:1 on ACD (183 mM glucose, 80 mM trisodium citrate, 52 mM Citric acid) centrifuged under the same conditions as described above. The platelet count in this case was adjusted to 1*10^9/ml.

Platelet Preparations

Six volumes of blood drawn from healthy donors by venipuncture was collected on one volume of an anticoagulant mixture consisting of 42 mM EDTA, 42 mM trisodium citrate, 25 mM NaOH (pH 6.6). The blood was centrifuged for 15 min at 250 g and the platelet pellet was resuspended in 19 volumes buffer (pH 6.6) consisting of 25 mM HEPES, 136 mM NaCl, 1.7 mM MgCl2, 25 mM glucose, 0.5% BSA and 1 volume anticoagulant mixture (see above). Platelets were thus washed twice centrifuging at 500 g for 15 min and were finally resuspended in 1 volume Hepes buffer (pH 6.6) and 4 volumes of same Hepes buffer (pH 7.5). The platelet concentrations were determined using a Coulter counter. All the platelet handling was done at room temperature.

Proteins

Human prothrombin was purified according to Discipio et al. (21). Thrombin was purified from prothrombin after activation with factor Xa as described by Rosing et al. (22). The thrombin concentration was determined by active site titration according to Chase and Shore (23). Bovine factor Xa was prepared from factor X, using RVV-X according to method of Fujikawa et al. (24). Factor Xa concentrations were calculated after active site titration according to Smith (25). Bovine factor V was purified by the procedure described by Dahlback (26) and Suzuki et al. (27) with minor modifications as explained in ref. 28. Factor Va was prepared by activation of factor V with thrombin and concentrations of Va were determined kinetically as described by Lindhout et al. (29).

Methods

Preparation of Clots

Plasma clots: PPP (250 μl for one clot) was coagulated by recalcification to a final concentration of 23 mM CaCl2 in a glass tube and incubated at 37°C for 1/3 h. The clot was wound on a plastic spatula, dried and incubated in (human) serum for 10 min. The clot was then extensively washed in 2 ml of Buffer A that was changed three times in order to eliminate any soluble material trapped in the clot. That no unbound thrombin remained with the clot was checked by the absence of amidolytic activity in the last washing buffer on 52238.

Purified fibrinogen clots were prepared from 250 μl of 3 mg/ml fibrinogen solution in 150 mM NaCl by adding CaCl2 (23 mM) and 1/50 volume of Reptilase or Agital solution or, in some cases, 30 mM Na2HPO4. Extensive washing of the clots was practiced in all the cases. Non-crosslinked fibrin clots i.e. des AA fibrin obtained by the action of snake venom that only release fibrinopeptide A have been referred to as fibrin I clots while des AABB fibrin clots (cross linked) are designated as fibrin II clots.

Measurement of Thrombin Generation in PRP

This was done as previously described (30). Briefly, 240 μl of PRP was diluted with 60 μl of Buffer A (50 mM Tris HCl, 100 mM NaCl, 0.5% BSA; pH 7.35) and coagulation was triggered with 60 μl CaCl2 (Final concentration 16.7 mM). The clots were added to the reaction mixture 90 s after triggering coagulation. The reaction mixture was continuously stirred by a small spatula and 10 μl subsamples were drawn at equally spaced intervals and diluted in prewarmed (37°C) cuvettes containing 490 μl of Buffer B (Buffer A with 20 mM EDTA, pH 7.9) containing 200 μM S2238. The reaction was stopped after 2 min by adding 300 μl of 1 M Citric acid. As soon as the reaction mixture coagulated, the clot was wound on the spatula and discarded. From the rate of change of optical density measured at 405 nm, the amidolytic activity was calculated by comparing with a standard calibration curve made with active site - titrated human α-thrombin.

Measurement of Platelet Prothrombin Converting Activity

In washed platelet suspensions: The reaction mixture consisted of 343 μl of washed platelet suspension with platelet count 0.5*10^9/ml to which 7 μl of 100 mM CaCl2 was added. The mixture was magnetically stirred at 37°C at 350 rev/min with teflon coated stirring bars. A stirring assembly was used which allowed to stir 7 reaction mixtures simultaneously at 37°C. After 3 min prewarming, platelet activation was initiated by adding a platelet stimulator. The generation of platelet prothrombin-converting activity was determined by transferring a 10 μl sample from the platelet activation mixture to 240 μl of an assay mixture containing 3 mM factor Va, 5 nM factor Xa and 1 μM prothrombin in 25 mM HEPES (pH 7.7), 175 mM NaCl, 5 mg BSA/ml, 2 mM CaCl2. A reference prothrombinase blank was measured by transferring a 10 μl Hepes buffer pH 6.6 aliquot to the prothrombinase mixture instead of the platelet activation mixture. The formation of thrombin was stopped after 1 min by transferring 10 μl aliquots to cuvettes (at 37°C) with 990 μl buffer (50 mM Tris, 175 mM NaCl, 0.5 mg/ml ovalbumin, 20 mM EDTA; pH 7.9) containing 335 μM S2238. From the absorbancy change recorded at 405 nm, the amount of thrombin formed is calculated via a calibration curve made with known amounts of thrombin. The time course of generation of platelet prothrombin – converting activity was then determined by samples taken from the platelet activation mixture at various time intervals. Controls with non-stimulated platelets were always run in parallel. Finally, the amount of thrombin formed in the prothrombinase blank was subtracted from all the readings.

In PRP: 100 μl of PRP, 1:6.7 diluted in Tris buffer A (see above) was incubated for 5 min with or without clots. 50 μl subsample from PRP was transferred to 100 μl of an assay mixture containing 0.45 nM factor Xa, 10.5 nM factor Va, 3 μM Prothrombin and 12 nM Ca2+ in Tris buffer A. At 4 min, 10 μl of subsample was taken to the cuvette containing 465 μl of Tris buffer B (see above). The amount of thrombin formed was calculated from the absorbancy change measurement at 405 nm after the addition of 25 μl of S2238 (4 mM).

Release of Platelet Lactate Dehydrogenase (LDH)

Samples were assayed for the release of platelet LDH by using a commercially available kit (Boehringer, Mannheim). 200 μl PRP or the activated platelet suspension was centrifuged at 9000 g for 3 min in an Eppendorf centrifuge. 5 μl of the supernatant was added to a cuvette with 125 μl of a buffer containing 51.7 mM Phosphate buffer (pH 7.5), 0.19 mM NADH and 0.6 mM Pyruvate. The activity of LDH was calculated from the absorbancy change at 334 nm. The total LDH content of the platelets was determined in a platelet suspension that was sonicated for 1 min.

Results

The Influence of Clox on Thrombin Generation in PRP

In recalcified PRP, there is a prolonged lag phase prior to the thrombin burst. Lag times preceding thrombin generation in a plasma milieu have been shown to be caused by the time required for a) factor V and/or VIII activation and b) for the generation of a platelet procoagulant...
zymes such as reptilase and Agihal, also shorten the lag phase in PRP.
The presence of thrombin, clots generated by thrombin-like venom en-
PRP (Fig. 1). Surprisingly, this effect is not critically dependent upon
considerably shortens the lag time that precedes the thrombin burst in
appearance of procoagulant phospholipids on/from the platelets is rate
shown previously that in PRP, under the conditions used here, the
physical interaction between clots and platelets is a prerequisite for the
s10ns.

The prothrombin-converting activities induced by plasma clots and
other agonists (IIa, collagen, IIa plus collagen and A23187) are sum-
marized in Table 1.

Under the conditions chosen in this experiment the capacity of the
various activators to induce platelet procoagulant activity is in the or-
der: A23187 >3 clots >IIa plus collagen >collagen >2 clots >1 clot
=IIa =no activator.

To check whether the generation of platelet procoagulant activity
should be attributed to the clot itself or to a factor released from clots
the control experiments presented in Table 2 were performed. Clots in-
cubated with buffer in the absence of platelets do not induce any pro-
thrombinase activity. This means that clots do not release a factor that
on itself stimulates prothrombin activation. The supernatant of clots in-
cubated with buffer in the absence of platelets do not induce any pro-

Another essential feature of clot-induced platelet procoagulant
activity is the requirement for stirring during the incubation of platelets
with the clot. A similar requirement has been reported for the genera-
tion of platelet prothrombin-converting activity by thrombin, collagen
and A25187 (4, 31).

In our earlier work (18) it has been attempted to quantify the amount
of thrombin adsorbed to the clot by the technique of comparing the
thrombin potential, the area under the thrombin time curve in the pre-

Since Agihal and reptilase-clots (fibrin I clots) have been shown to be
unable to promote factor V or VIII activation (18), this confirms the
rate limiting role of platelet procoagulant phospholipids. This strongly
suggests a direct effect of fibrin on the generation of platelet procoagu-
ant activity. To test this hypothesis, we have studied the influence of
fibrin clots on the generation of platelet procoagulant activity under
conditions where no thrombin could be formed, i.e. in suspensions of
washed platelets.

The Effect of Plasma Clots on the Prothrombin-converting Activity
of Washed Platelets

Plasma clots were added to a washed platelet suspension and after
different time intervals aliquots were drawn and transferred to assay
mixtures containing prothrombin, factor Xa, factor Va and Ca²⁺ ions, in
which procoagulant lipid was the rate limiting component of prothrom-
binase. In this way the time course of generation of procoagulant
activity (i.e. prothrombinase activity) in the platelet suspension was
followed. The ability of clots to induce platelet procoagulant activity
was compared with that of the established platelet activators, thrombin
and the ionophore A23187 (Fig. 2). The incubation of platelets with
clots results in an immediate appearance of procoagulant activity that
linearly increases in time and that is proportional with the number of
clots present in the platelet suspension. Platelets incubated with one
plasma clot exhibit a 3-fold higher prothrombin-converting activity
than control platelets. Activation of platelets with 30 nM thrombin cau-
ses approximately the same effect as one clot. The stimulation by 3
plasma clots results in a linear increase that within one hour attains the
level of 60% of the maximum procoagulant activity reached in platelet
suspensions activated with the Ca²⁺ ionophore A23187. The initial rate
of generation of procoagulant activity by the ionophore is, however,
much higher than that obtained in the presence of clots.

The prothrombin-converting activities induced by plasma clots and
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Table 1 Platelet procoagulant activity provoked by different triggers in washed platelet suspensions

<table>
<thead>
<tr>
<th>Trigger</th>
<th>Level at t = 60 (nM)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>23 ± 5 (n = 13)</td>
<td>17-33</td>
</tr>
<tr>
<td>Thrombin</td>
<td>66 ± 21 (n = 6)</td>
<td>34-95</td>
</tr>
<tr>
<td>Collagen</td>
<td>99 ± 46 (n = 3)</td>
<td>62-165</td>
</tr>
<tr>
<td>Thromb + Coll, 1 clot</td>
<td>138 ± 20 (n = 3)</td>
<td>118-158</td>
</tr>
<tr>
<td>2 clots</td>
<td>72 ± 20 (n = 6)</td>
<td>47-101</td>
</tr>
<tr>
<td>3 clots</td>
<td>91 ± 38 (n = 6)</td>
<td>70-168</td>
</tr>
<tr>
<td>A23187</td>
<td>286 ± 68 (n = 13)</td>
<td>84-450</td>
</tr>
<tr>
<td>A23187</td>
<td>414 ± 70 (n = 12)</td>
<td>394-536</td>
</tr>
</tbody>
</table>

The amount of IIa formed (nM) after 60 min of platelet activation in the absence or presence of various stimulators. The results are presented as Avg. ± SD (number of times the experiment is done). To express, the donor dependent scatter the range obtained is also given. Experimental conditions are the same as in legend of Fig. 2. The concentrations of stimulants were: IIa (30 nM), Collagen (10 μg/ml), IIa/collagen (1 nM IIa/10 μg/ml collagen), A23187 (4 μM). Fibrin II clots have been used.

Table 2 Requirements for the generation of prothrombin-converting activity in platelet-clot suspensions

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reaction mixture</th>
<th>Prothrombin-converting activity (nM/IIa/min)</th>
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<tr>
<td>1</td>
<td>Buffer</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>Supernatant of clot suspension</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>Platelets</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>Platelets + supernatant of 3 clots</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>5</td>
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<td>286 ± 68</td>
</tr>
</tbody>
</table>

Experimental set up was the same as for Fig. 2. In experiments 1 and 3 the reaction mixture consisted of Hepes buffer (pH 6.6, see materials and methods) and platelet suspension respectively. In experiment 2, the plasma clots were incubated in buffer in the absence of platelets. For experiment 4, the plasma clots were incubated in buffer for 30 minutes. The clots were taken out and platelet suspension was added. Finally in experiment 5, the prothrombinase activity was measured when plasma clots were incubated with platelet suspension. In all the experiments 2 mM Ca²⁺ was added. The results are expressed as Average ± SD (n = 5 for first 4 experiments and n = 13 for experiment 5).

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Effect of Clots that do not Contain Thrombin

The experiments reported above were performed with clots that are known to contain bound thrombin (i.e. uninhibited fibrin II clots). The experiments in Fig. 1 show that clots obtained by the action of snake venom enzymes on purified fibrinogen (fibrin I clots) are also able to generate platelet procoagulant activity. Figure 3 shows a time course of generation of prothrombin-converting activity in platelet suspensions in which fibrin I clots were used as platelet agonists. To ensure that venoms by themselves do not influence the reaction, Agihal and Reptilase were added to the platelet activation mixture in 1/50 volume (as for obtaining the clots) and prothrombinase activity was measured (Inset graph, Fig. 3). With fibrin I clots, a modest but significant increase of the prothrombin-converting activity was seen, that was, however much lower than that of fibrin II clots. This is indicative for a role of both fibrin (I or II) and clot-bound thrombin in the generation of procoagulant activity in washed platelet suspensions.

The importance of clot-bound thrombin was further tested by determining the effect of plasma clots that were treated with an inhibitor of thrombin. To this end plasma clots (fibrin II clots) were incubated with 1 μM of hirudin for about 2 h, blotted dry and transferred to the platelet suspension without washing. In this way a small fraction of the hirudin is transferred to the platelet activation mixture, in order to ensure complete inhibition of thrombin bound to the clot. Since in our prothrombinase assay the platelet suspension is subsampled to a mixture with prothrombinase components, the hirudin that is carried over is diluted many times. We determined that the amount of hirudin carried over was less than 1 nM in the final solution which will be too low to inhibit the thrombin formed in the assay mixture. However, to confirm this a control experiment was performed in which 30 nM hirudin was added to the platelet mix containing three plasma clots. The time course of generation of platelet prothrombin converting activity was determined by drawing aliquots from the mixture and transferring them to the prothrombinase mix. By dilution just over 1 nM hirudin reaches the final solution in this way. The amount of thrombin measured after 60 min of platelet activation was 326 nM which is above the mean of 13 experiments without hirudin and within the mean ± 1 SD range (Table I).

Clot associated thrombin activity before and after hirudin treatment was determined by measuring the antithrombin activity on S2238. With three untreated fibrin II clots the thrombin measured after 15 min of in-
Fig. 4 Effect of addition of IIa to fibrin I clots and hirudin treated clots. Washed platelet suspension was activated by (!) Fibrin I clots and (A) plasma clots with inhibited IIa (treated with 1 µM Hirudin) for 60 minutes, after which 5 nM IIa was added to the platelet activation mixture. The control platelets (O) were without stimulator till 60 minutes and then 5 nM IIa was added to them. The platelet procoagulant activity was measured as described in the legend of Fig. 2. The solid symbols represent the continuations of the same curves after thrombin addition.

The Effect of Thrombin on the Prothrombin-converting Activity of Platelet-clot Suspensions

Washed platelets were incubated for 60 min in the presence of clots that did not contain active thrombin (fibrin I clots or hirudin-treated plasma clots) prior to the addition of 5 nM thrombin. Figure 4 shows that the addition of thrombin to platelet suspensions that contain clots causes the platelet procoagulant activity to rise steeply to levels that even exceed those obtained with the Ca-ionophore A23187. Such a rise does not occur when thrombin is added to control platelets incubated without clots. In these experiments the levels of prothrombin-converting activity are much higher than those obtained with untreated plasma clots. This indicates that the synergistic effect of free thrombin in the generation of fibrin-induced platelet procoagulant activity is much more pronounced than that of clot-bound thrombin.

Leakage of Lactate Dehydrogenase

Platelet procoagulant activity can result from a transbilayer transport of phosphatidylserine to the outside of the intact membrane (flip-flop), by the shedding of microparticles or by disruption of the cell membrane. As a measure of membrane disruption we used the release of cytoplasmic lactate dehydrogenase. To estimate the contribution of membrane damage to the generation of procoagulant activity we plotted the latter as a function of the release of LDH (Fig. 5). It is seen that the combination of thrombin with clots (of any type) causes procoagulant activity that is accompanied by substantial release of LDH, as is, a fortiori, the activity produced by sonication. Thrombin plus collagen, or the Ca-ionophore cause a release of LDH that, for the same amount of procoagulant activity, is only 20% of maximal. So the platelet procoagulant activity induced by clots in washed platelets is lysis associated.

This imposed LDH measurements in PRP during thrombin generation. The LDH level measured in PPP was 102 U/l, in sonicated PRP the level was 594 U/ml. However in PRP, not significant rise in LDH was obtained (Fig. 6). Knowing the experimental variation of the individual measurements, this means that less than 1% of the platelets had shed its LDH during the clotting of PRP.

Table 3  Comparison of activities evoked by various clots in washed platelet suspensions

<table>
<thead>
<tr>
<th>Agonist</th>
<th>% Procoagulant activity (after 60 min of platelet activation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma clots</td>
<td>100 ± 9.8</td>
</tr>
<tr>
<td>Fibrinogen + IIa (30 nM) clots</td>
<td>98 ± 10.2</td>
</tr>
<tr>
<td>Plasma clots treated Hirudin (1 µM)</td>
<td>26 ± 4.6</td>
</tr>
<tr>
<td>Fibrin I clots</td>
<td>21 ± 4.8</td>
</tr>
<tr>
<td>None</td>
<td>8 ± 1.1</td>
</tr>
</tbody>
</table>

Experimental set up is the same as for Fig. 2. The various clots were obtained as described under methods. For the purpose of comparison the prothrombinase activity induced by plasma clot has been taken as 100% value. The data obtained for 9 donors is expressed as Average ± SEM.
activate factors V and VIII (18). In the course of these experiments it was carried for PPP incubated with clots and PRP without clots served as controls. 

Values obtained after 4 min of activation. Measurements simultaneous-ly allowed in subsamples that were transferred to a prothrombinase mixture to PRP and the generation of platelet procoagulant activity was followed in subsamples that were transferred to a prothrombinase mixture devoid of phospholipids, as used for washed platelets. Table 4 lists the values obtained after 4 min of activation. Measurements simultaneously carried for PPP incubated with clots and PRP without clots served as controls.

**Discussion**

We previously reported that thrombin bound to fibrin is capable to activate factors V and VIII (18). In the course of these experiments it appeared that fibrin clots, even if they did not contain thrombin, foster thrombin generation in platelet rich plasma (PRP). In this article was further investigated the mechanism behind this phenomenon.

Platelets can assist in thrombin generation by exposing procoagulant phospholipid and by releasing factor V. Under our experimental conditions, i.e. in 2.3 diluted normal PRP, factor V is present in excess and procoagulant lipids are rate limiting (30). Therefore the release of factor V does not contribute significantly to the thrombin generating capacity of PRP but the availability of procoagulant phospholipid from platelets does. So, if fibrin clots enhance thrombin generation in PRP, these clots must induce the exposure of such phospholipids.

Platelet procoagulant phospholipid activity, also called platelet factor 3 (PF3), is dependent upon the exposure of negatively charged phospholipids, chiefly phosphatidylserine (PS). In the resting platelet, PS is almost exclusively located in the cytoplasmic layer of the cell membrane. Upon activation by thrombin or collagen, and especially if both activators are present, PS is transferred from the inner to the outer monolayer of the intact membrane (3). In this process platelets also shed procoagulant phospholipid particles (32, 33). PS also gets exposed when the platelet membrane is disrupted by methods such as freeze thawing or sonication. Such extreme situations however do not occur in vivo and their biological significance seems limited.

Our results show that fibrin clots of any type provoke platelet procoagulant activity in PRP as well as in washed platelet suspensions. Notably non cross-linked fibrin I clots, obtained with snake venom enzymes that only release fibrinopeptide A, still exhibit activity. In isolated platelets the presence of thrombin is necessary for a maximal effect (Table 3). This may be thrombin already adsorbed to the clot. In plasma, where explosive thrombin generation necessarily follows the appearance of platelet procoagulant activity, it is hard to distinguish between the effect of fibrin alone and that of fibrin plus thrombin.

Contrary to what could be expected from its effect on the isolated platelets (3), collagen has been reported not to enhance thrombin generation in PRP (30). The obvious difference between the two situations is that in PRP fibrin is formed by the thrombin generated. We therefore wanted to explore the possibility that fibrin might replace collagen as an inducer of the flip-flop reaction. However, when isolated platelets are stirred with clots, especially if thrombin is present too, they disrupt, as can be judged from their shedding LDH (Fig. 5). This experimental setup therefore is not suitable for the demonstration of flip-flop, and the experiments are inconclusive. In contrast, the induction by clots of procoagulant activity in platelets in PRP is not accompanied by the shedding of LDH. This means that the procoagulant activity is due to PS at the outside of non-disrupted platelets and/or of shedded microparticles. We suppose that the isolation of platelets renders them more vulnerable than platelets in PRP are. We are searching for conditions in which isolated platelets can be incubated with fibrin and thrombin without shedding LDH but thus far we did not find any. For the moment we have no data that make us revise our original hypothesis that fibrin in clotting plasma can play a similar role as collagen does in suspensions of isolated platelets.

Our observations broach the question of in how far platelet disruption may be an integral part of the physiological haemostatic reaction. It is not uncommon for cell death to contribute to physiological functions (cf. holocrine secretion) (34) and disrupted platelets are a common phenomenon in EM pictures of the haemostatic plug (35).

In Table 1 we observe a large variation in platelet procoagulant activity. This is only partly due to random experimental error. In fact the experimental variation when calculated from experiments carried out on the same platelet suspension under identical conditions was less than 8%. The remainder of the variation must be ascribed to variation of
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