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On the Clot-Promoting Activity of Human Platelets in a One-Stage Prothrombinase Assay

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Abstract. The procoagulant activity of activated platelets in a one-stage prothrombinase assay is reevaluated. It is shown that the apparent procoagulant activity of platelets activated by ADP or collagen can be explained by minor cell lysis accompanying platelet activation. The reduction in clotting time observed with thrombin activated platelets can be explained by a combined effect of minor cell lysis and release and activation of factor V from the platelets. Platelets stimulated by ionophore A23187 or by the combined action of collagen plus thrombin show a much shorter clotting time than can be accounted for by minor platelet lysis or release and activation of factor V from the platelets.

The results with this clotting assay essentially confirm previous observations [*Bevers* et al.: Eur. J. Biochem. *122:* 429–436, 1982] using a spectrophotometric method with highly purified coagulation factors and a chromogenic substrate to measure the rate of thrombin formation with activated platelets.

Introduction

The ability of activated platelets to reduce the clotting time in coagulation assays that are sensitive to procoagulant phospholipids has been well established [1–3]. However, clotting assays in the presence of platelets are known to be sensitive to low percentages of cell lysis [3], and to release and activation of factor V from the platelets [4]. We have recently shown, using a chromogenic substrate assay with highly purified coagulation factors, that platelets stimulated by the combined action of collagen plus thrombin strongly increase the rate of factor X and prothrombin activation, as compared to nonac-

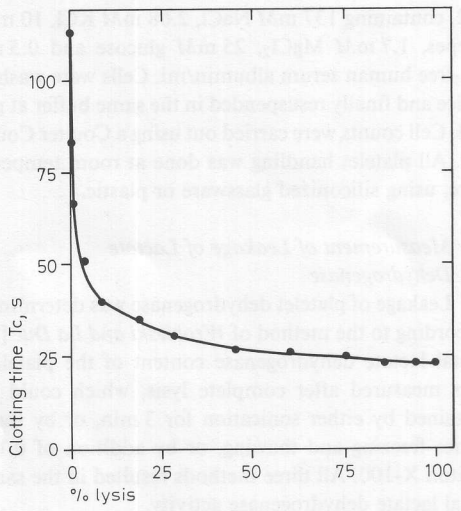

Fig. 1. Clotting time of sonicated platelets as a function of percentage lysis produced by sonication. Clotting was measured in the absence of added factor Va (see text). The longest clotting time shown (114 s) corresponds to 0.4% of lysis; non sonicated platelets had a clotting time of 178 s. Data are shown for one representative platelet preparation.

Results

Figure 1 shows the relationship between the clotting time (in the absence of excess added factor Va) and the amount of platelet lysis. Platelet lysis was induced by sonication of washed platelet suspensions for various time periods at a minimal output of the sonifier. Lysis, measured by the activity of lactate dehydrogenase in the supernatant, was expressed as a percentage of this enzyme activity in a completely lysed platelet preparation. Addition of intact platelets to the assay system hardly affects the clotting time (see also table I) although this might differ from one platelet preparation to another, depending on the amount of lysed platelets already present in the nonsonicated preparation. Trace amounts of lysed platelets induce a significant decrease in clotting time. A nonlinear relationship between clotting time and platelet lysis is obtained. The decrease in clotting time produced by 1% lysis in the platelets is more than half of that observed with a completely lysed platelet preparation. A similar nonlinear relationship (with shorter clotting times) was obtained in the presence of an excess of exogenously added factor Va (compare table I).

The effect of a 10-min activation period with different agonists on the procoagulant activity of intact washed human platelets is given in table I. Platelets contain factor V which can be released and activated as a result of the action of some platelet agonists [13–15]. Because factor Va is a cofactor in the activation of prothrombin, this might interfere with the coagulation assay. Therefore, the assay was carried out either in the absence or in the presence of exogenously added excess of factor Va. As a result of the addition of factor Va, the rate of thrombin formation will be increased leading to a decrease in clotting time. Also, the blank clotting time with buffer instead of platelets is significantly lowered in the presence of added factor Va. Addition of nonstimulated platelets hardly affects the blank clotting time, either with or without added factor Va in the assay. Since minor amounts of platelet lysis strongly shorten the clotting time and some platelet lysis accompanies platelet activation, it will be clear that for an evaluation of the clot-promoting effect of activated platelets a control for the contribution of lysed cells in the assay is an essential requirement.

When platelets stimulated by ADP or collagen are used in the clotting assay (either in the presence or absence of exogenously added

Table I. Percentage of lysis in and clotting time of sonicated and activated platelets

Sonication time, or platelet activator (final concentration)	Lysis %	Clotting time, s	
		absence of excess factor Va	presence of excess factor Va
Buffer	–	193	115
Platelets (not activated or sonicated)	–	189	98
1 s sonication	1.7	79	49
3 s sonication	5.1	57	38
6 s sonication	11.9	44	31
10 s sonication	19.2	38	27
12 s sonication	24.9	35	25
3 min sonication	100	23	18
ADP (10 μ*M*)	1.1	90	54
Collagen (10 μg/ml)	1.9	76	48
Thrombin (2 n*M*)	1.7	57	49
Collagen + thrombin	1.6	35	29
A23187 (3 μ*M*)	2.7	27	23

Due to minor variations in the amount of lysis, data are given for one representative platelet preparation. In total, 17 platelet preparations were tested that with two exceptions all showed a similar behavior.

factor Va), the reduction in clotting time closely corresponds to that of sonicated preparations that show the same amount of lysis as produced during the activation procedure. Similar results were obtained with platelets activated with serotonin, epinephrine or kaolin (data not shown). Without added factor Va in the clotting assay, thrombin-activated platelets show a shorter clotting time than can be accounted for by lysis, but this discrepancy disappears in the presence of an excess of exogenous factor Va. This strongly suggests that in the absence of added factor Va the reduction in clotting time is not only caused by minor cell lysis (some 1.7%) but also by release and activation of factor Va. It should be noted here that according to the data of *Chesney* et al. [15], 0.1 ml of platelets (10^8 cells/ml) used in the assay can contribute approximately 1 unit of factor V when this factor is fully released and activated into factor Va. The small amount of thrombin used to activate the platelets does not significantly affect the clotting time in this assay system. In contrast to the platelet activations described above, platelets stimulated by A23187 or by the combined action of collagen plus thrombin produce a much shorter clotting time (both in the absence or presence of added factor Va) than can be accounted for by minor cell lysis and/or release and activation of factor V from the platelets.

Discussion

The clotting time observed in a one-stage prothrombinase assay using Russell's viper venom will be dependent on the availability of negatively charged procoagulant phospholipids for the formation of the prothrombinase complex [16]. In the platelet plasma membrane, the negatively charged phospholipids are almost exclusively present in the inner leaflet of the membrane [17, 18]. This explains why a preparation of intact non-stimulated platelets does not significantly shorten the clotting time. Platelet damage, leading to the exposure of the inner surface of the plasma membrane, thereby offering a negatively charged phospholipid surface for the formation of the prothrombinase complex, results in a decrease in clotting time.

Lowering of the clotting time by the addition of activated platelets in the coagulation assay can have at least three different explanations: (i) release and activation of factor V from the platelet α-granules since the plasma mixture offered in the coagulation assay normally does not contain an excess of this factor and hence is sensitive to the addition of factor Va; (ii) exposure of the negatively charged inner surface of the platelet plasma membrane as a result of lysis caused by the activation procedure; (iii) exposure of negatively charged phospholipids at the platelet outer surface as a result of rearrangements in the plasma membrane caused by the agonist-receptor interaction. The data presented here show that the decrease in clotting time caused by ADP or collagen-activated platelets can be explained by platelet lysis. It should be emphasized, however, that lysis was measured by the activity of the cytoplasmic enzyme lactate dehydrogenase in the platelet supernatant after activation. Therefore, it cannot be excluded that the presence of this enzyme in the supernatant is a result of a transient leakage and does not necessarily reflect platelet damage and exposure of the inner surface of the plasma membrane. However, the close correlation in clotting time between platelets lysed by sonication and platelet lysis produced by certain agonists strongly suggests that percentage leakage of lactate dehydrogenase during platelet activation in fact reflects percentage of cell damage.

The effect of release and activation of factor V from the platelet granules can be of importance only in those cases where platelets are activated by thrombin or a combination of another agonist with thrombin. This can be seen in table I comparing the reduction in clotting time between platelets activated with collagen and platelets activated with thrombin. In the absence of exogenously added extra factor Va the assay will be sensitive to factor Va from the platelets. Although activation by collagen results in a similar percentage of lysis as activation by thrombin, the clotting time observed with thrombin-activated platelets is shorter than that observed with collagen-activated platelets. This can be explained by the release and activation of factor V from the platelets which occurs upon activation with thrombin. (Collagen only induces release but not activation of factor V.) When an excess of factor Va is added to the assay, the difference in clotting time between collagen- and thrombin-activated platelets disappears, and equals that of sonicated preparations showing the same amount of lysis.

Addition of platelets activated by a combination of collagen plus thrombin produces an even further reduction in clotting time, which cannot be explained by either cell lysis or release and activation of factor V. It has

been shown previously that this activation procedure exposes negatively charged phospholipids (particularly phosphatidylserine) at the cell outer surface, as a result of transbilayer movement of phospholipids by a process not involving lysis of the cells [5]. It is suggested that this process is in fact mainly responsible for the observed reduction of the clotting time with platelets activated by collagen plus thrombin. A similar explanation can be given for platelets activated by ionophore A23187.

It should be mentioned that the concentrations of the agonists used were all sufficient to produce optimal release and aggregation, i.e no increase in α-granula or dense body release or in the extent of aggregation was observed upon increasing the agonist concentration. Also, the effect of the combined action of collagen plus thrombin could not be evoked by using higher thrombin concentrations to activate the platelets, or combinations of thrombin with other agonists.

The data obtained with this clotting assay are in close agreement with those obtained using an assay system with purified coagulation factors and a chromogenic substrate to measure the rate of thrombin formation in the presence of activated platelets [5]. Although care must be taken with the interpretation of clotting times with respect to lysis of the platelets or release and activation of factor V, the assay described here is suitable to determine whether or not negatively charged phospholipids become exposed at the platelet outer surface as a result of activation by a platelet agonist.

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