Thrombin generation and secretion of platelet Factor 4 during blood clotting.

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J Clin Invest. 1978;61(4):1102-1106. https://doi.org/10.1172/JCI109010.

Research Article

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Thrombin Generation and Secretion of Platelet Factor 4 during Blood Clotting

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ABSTRACT We have studied the platelet release reaction and thrombin generation during the spontaneous clotting of whole blood in vitro. Both thrombin formation and secretion of platelet Factor 4 were detected at least 12 min before clotting (clotting time, 22–26 min). Initially, at low thrombin concentrations (2–5 ng/ml), there is a small increase in plasma platelet Factor 4 (<1% of the amount present in serum). This is followed by a gradual increase in both platelet Factor 4 and thrombin concentrations over a 12 to 20-min interval. Finally, 5 min before clotting, there is a rapid increase in both thrombin generation and platelet secretion.

Thus, we have shown that the release of platelet Factor 4 is a prolonged reaction and the extent to which it occurs parallels thrombin generation. It is only when thrombin concentrations are high (45–90 ng/ml)—during the period of clot formation—that the major part of platelet Factor 4 secretion occurs. Release of platelet Factor 4, like fibrin formation, occurs in the last step of in vitro coagulation.

INTRODUCTION

Platelets have been assigned a dual role in hemostasis. Initially, after vascular injury, a platelet aggregate or plug is formed on the subendothelial surface. During plug formation, platelets undergo changes that result in the exposure of a phospholipid surface on which Factors X and II are converted to their active forms (1). Several recent observations indicate that the interaction of platelets with clotting factors during thrombin formation is more complex than initially postulated. Osterud et al. (2) have shown that platelets contain an activated form of Factor V and have postulated that its release may be important in prothrombin activation. Experiments by Miletich et al. (3) demonstrate that the rate of thrombin generation is 10 to 100-fold faster when platelets are used in place of phospholipid and Factor V in the activation of prothrombin by Factor Xa.

Once thrombin is formed, it stimulates platelet secretion and aggregation (4). Some of the initial steps in this interaction have been characterized in experiments using purified thrombin and washed platelets. Thrombin binds specifically and reversibly to high affinity receptors on the platelet surface (5–8). There is good evidence to suggest that binding must occur before secretion can take place (8, 9). Although secretion does not occur if the active site of thrombin is blocked (4), the lack of thrombin turnover and the requirement for a threshold concentration of thrombin before release can occur (10) suggest that the thrombin-induced release is not a simple enzymatic process.

Our experiments were designed to study the interaction of platelets with thrombin in whole blood. Because we had previously demonstrated that thrombin is present early in clotting (11), we were interested in seeing whether or not there was evidence for an effect of thrombin on platelets at a similar time period. We found that the time-course of secretion of a platelet protein, platelet Factor 4 (PF_4)[1] parallels closely the generation of thrombin during in vitro blood clotting.

METHODS

Chemicals and radioisotopes were purchased from commercial sources as defined (11–14). Prostaglandin E_1 (PGE_1)

Received for publication 19 December 1977 and in revised form 23 January 1978.

[1] *Abbreviations used in this paper*: PF_4, platelet Factor 4; dbcAMP, $N^6O^{2'}$-dibutyryl adenosine 3′,5′ monophosphate cyclic AMP; PGE_1, prostaglandin E_1.

 J. Clin. Invest. © *The American Society for Clinical Investigation, Inc., 0021-9738/78/0401-1102 $1.00*

was generously provided by Dr. John Pike, Upjohn Co., Kalamazoo, Mich. Cohn fraction III paste and plasminogen-free fibrinogen used in these studies were provided by the American Red Cross National Fractionation Center with the partial support of the National Institutes of Health grant HL 13881. Outdated platelets for PF_4 purification were obtained from the American Red Cross, Tulsa, Okla. and Wichita, Kans., and the Wadley Institutes of Molecular Medicine, Dallas, Tex.

PF_4 secretion and thrombin generation in clotting blood. Venipuncture was performed on normal volunteers using 16 gauge needles. The first 5–10 ml was discarded, then 40 ml was collected rapidly into new polypropylene tubes and allowed to clot at room temperature (20°–24°C). Samples were removed at regular intervals, anticoagulated with 0.1 vol. of 3.8% trisodium citrate and assayed for either PF_4 or thrombin. To blood samples that were assayed for PF_4, 100 mM $N^6O^{2'}$-dibutyryl adenosine 3',5' monophosphate cyclic AMP (dbcAMP) (final concentration 1 mM) and 1 mg/ml PGE_1 in ethanol (final concentration 0.0028 mM) were added immediately. Aliquots assayed for thrombin were treated as described (11). All samples were kept on ice before centrifugation at 10,000 *g* for 10 min at 4°C. Plasma to be assayed for PF_4 was stored at −20°C, then shipped to San Antonio packed in dry ice. It had previously been determined that there was no difference in PF_4 concentration when measured in either fresh or frozen-thawed plasma.

To determine to what extent platelets might secrete PF_4, apart from that induced by clotting, 80 ml of blood was drawn and divided into two aliquots. One was immediately anticoagulated with either trisodium citrate (final concentration 0.38%), or 0.25 M EDTA (final concentration, 0.0058 M). The other was allowed to clot. Samples were removed at regular intervals, processed as described above, and assayed for PF_4 and thrombin.

Effect of dbcAMP and PGE_1 on thrombin-induced PF_4 secretion. Blood was drawn from normal volunteers using a 19-gauge needle. Samples were then immediately anticoagulated with 3.8% trisodium citrate (final concentration 0.38%), and divided into two equal portions. To one fraction, dbcAMP and PGE_1 were added in the concentrations as described for the clotting experiments. Samples of 2.0 ml were removed and thrombin, final concentrations of 0.4–5.0 U/ml was added. The other portion was treated identically except that neither dbcAMP nor PGE_1 were added. The samples were incubated at room temperature for 10 min, then centrifuged at 10,000 *g* for 10 min at 4°C, and the plasma removed for assay.

Thrombin radioimmunoassay. The techniques for human thrombin purification (12) and radioimmunoassay (11) have been published previously. The thrombin was assayed as described (13) and had a 3,200 U/mg sp act and was homogeneous based on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

PF_4 radioimmunoassay. The methods for PF_4 purification (14) and radioimmunoassay (15) have been described. The interassay control was 7.3±1.8 ng. There was a 10–15% coefficient of variation when the same sample was run in the same assay.

RESULTS

Secretion of PF_4 in clotting blood. When freshly drawn blood was allowed to clot spontaneously in polypropylene tubes, the following observations were made: First, PF_4 secretion was initiated as early as 3 min after venipuncture and as long as 25 min before visible clotting (Fig. 1A). The precise time of onset was variable among normal subjects. In some experiments, PF_4 secretion was not detected for as long as 10 min after venipuncture. In all instances there was at least a 12-min interval between initiation of PF_4 secretion and the time of clotting (clotting times in these experiments ranged from 22–26 min). The base-line PF_4 values tended to be higher than other published series, however, much larger volumes of blood were drawn and there was a longer interval before anticoagulant and platelet inhibitors were added. Second, once initiated, the secretory process occurred over a prolonged period (15–20 min) rather than as a rapid burst. Third, PF_4 secretion appeared to occur in at least two stages. Initially small amounts of secretion proceeded slowly for several minutes during which time there was at least one lag period with little or no increase in PF_4 concentration. This was of variable duration, lasting as long as 8 min. Then, in the second stage, there was a secretory burst over 4–6 min with at least a 20-fold increase in PF_4 concentration. This occurred just before the time of clotting.

Correlation of thrombin generation with PF_4 secretion. When prothrombin activation, as measured by thrombin radioimmunoassay (11), was compared with PF_4 secretion, certain similarities were seen (Fig. 1B). As described (11), small amounts of thrombin (2–5 ng/ml) appeared early after venipuncture (Fig. 1B). In most experiments, thrombin generation either preceded or occurred at the time of the first increase in PF_4. In some experiments, the initial secretion of PF_4 was observed before an increment in thrombin concentration could be detected. After the initial appearance of thrombin, a lag period in further prothrombin activation occurred (Fig. 1B) similar to that which took place in PF_4 secretion. A slow increase in thrombin concentration followed, again similar to that observed in PF_4 secretion. In all cases, if a delay in thrombin formation was observed, a delay was also seen in PF_4 secretion. Shortly before clotting, a marked increase in both thrombin and PF_4 concentrations was noted (Fig. 1A and B). At the time of clotting, when the thrombin concentration was 70–100 ng/ml, PF_4 in serum was 200 to 1,000-fold higher than base-line values.

To determine the extent to which PF_4 might be secreted from platelets apart from that induced by thrombin, PF_4 and thrombin levels were determined on blood samples which were obtained simultaneously and either anticoagulated immediately or allowed to clot. When the blood was anticoagulated immediately, the plasma PF_4 concentration did not rise until 10–14 min after venipuncture, irrespective of the anticoagulant (trisodium citrate or sodium EDTA, two experiments each). The PF_4 concentration then gradually increased with incubation over the next

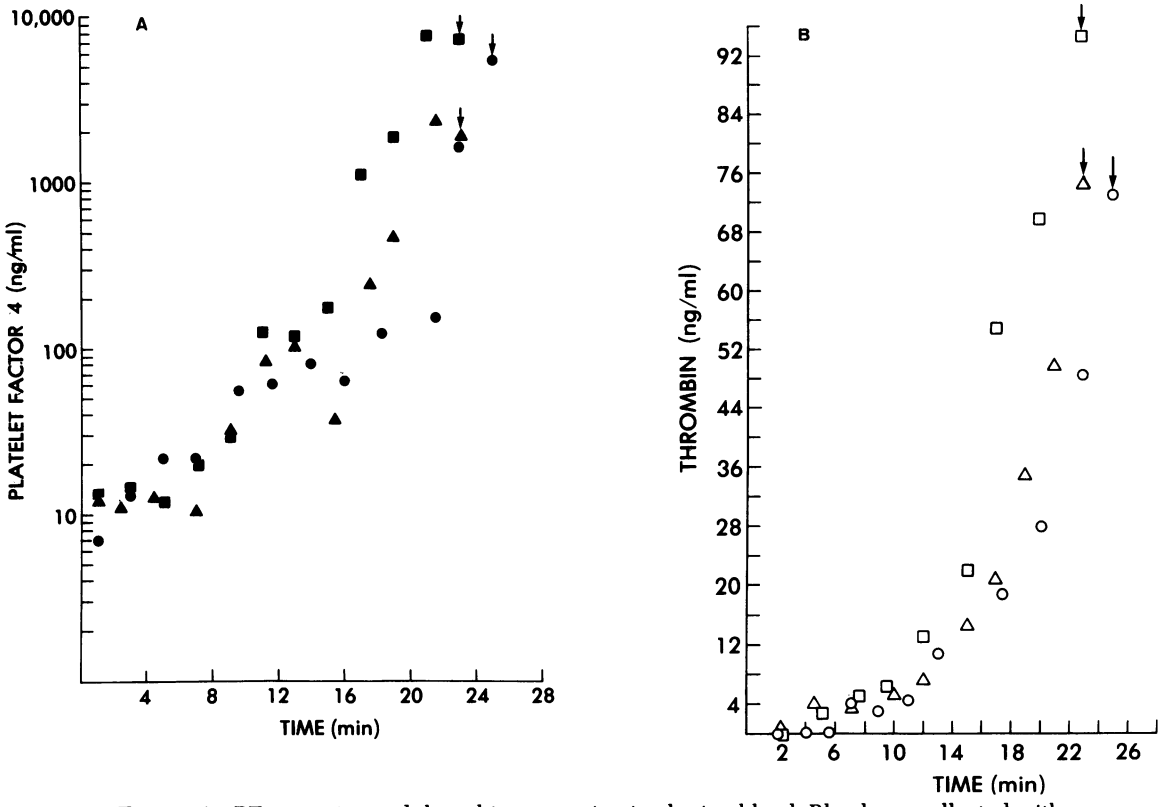

FIGURE 1 PF_4 secretion and thrombin generation in clotting blood. Blood was collected with a 16-gauge needle and allowed to clot at room temperature in a polypropylene tube. Serial aliquots were removed and plasma assayed for (A) PF_4 (closed symbols), or (B) thrombin (open symbols). The results are based on blood obtained from three normal individuals. The arrows represent the time at which clotting occurred.

10–15 min with final levels of 260–360 ng/ml (<5% of the PF_4 present in serum). One possible explanation for this increase in PF_4 concentration is that incubation in anticoagulants is injurious to the platelets and results in secretion. Although we could detect no thrombin generation, a small amount may have occurred and induced secretion.

Net PF_4 secretion was determined by subtracting the PF_4 values for each time point measured in anticoagulated blood from values observed at the same time points in simultaneously obtained clotting blood. As shown in Fig. 2, ≅2 min after the first detectable thrombin generation and 10 min after venipuncture, there is a progressive rise in PF_4 concentrations. The initial PF_4 concentrations in these experiments—both clotting and anticoagulated specimens—were higher (39–125 ng/ml) than our normal base-line values. This appears to be related to the longer time necessary to collect the large volume of blood used in these experiments.

Inhibition of thrombin-induced secretion by dbcAMP and PGE_1. To study PF_4 secretion during clotting, it was necessary to demonstrate that platelet secretion subsequent to collection of samples at each time point could be inhibited. Otherwise, the PF_4 concentration would represent additional secretion that might occur as the samples were prepared for radioimmunoassay. Although a small amount of secretion was seen when thrombin at concentrations of 0.8 U/ml (250 ng/ml) or less was added to whole blood containing dbcAMP and PGE_1, this represented <5% of the PF_4 secreted when these inhibitors were not present (Table I). At higher thrombin concentrations, i.e. 1.6 U/ml, the PF_4 concentration rose to 967 ng/ml. Still, this represents only 13% of the PF_4 secreted without inhibitors. At 3.2 U/ml and higher, inhibition is less effective with 25–50% of normal secretion.

DISCUSSION

We have studied platelet secretion in clotting whole blood that is unperturbed by anticoagulants or centrifugation before stimulation. Previously, we demonstrated that small amounts of thrombin are formed early in clotting, before a visible clot appears (11). We hypothesized that this thrombin could be a stimulus for the platelet release reaction. Our present observa-

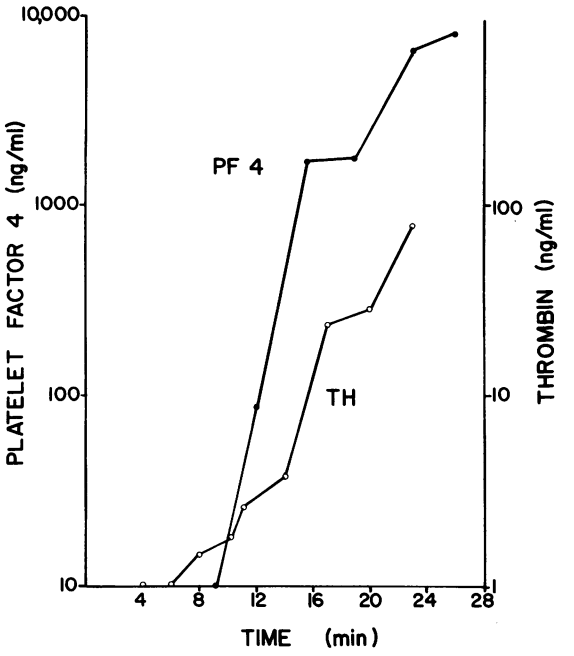

FIGURE 2 PF_4 secretion and thrombin generation in anticoagulated and clotting blood. Blood was collected with a 16-gauge needle and distributed equally into two polypropylene tubes. One was anticoagulated immediately with 0.1 volume of 3.8% trisodium citrate and the other allowed to clot. (Clotting time 23 min.) Serial samples were removed and assayed for PF_4 or thrombin. Net PF_4 secretion (●) calculated by subtracting PF_4 values in the anticoagulated blood from values obtained at corresponding times in clotting blood; thrombin (○) in clotting blood.

tion that secretion of PF_4 also begins at least 12 min before clotting and is blocked in anticoagulated blood supports this hypothesis. Over 90% of the release of PF_4, however, occurs during the last 4–5 min of clotting. Platelet secretion, on the other hand, does not appear necessary for the initial appearance of thrombin. Low levels of PF_4 were always present at the earliest time points. Whether this is due to platelet activation during blood collection and handling has not been determined. There are several possible explanations for the observation that in some experiments PF_4 secretion occurred before thrombin generation could be detected. The radioimmunoassay may not have detected a small change in thrombin concentration which may have occurred at this earliest stage of blood clotting or thrombin bound to platelets may not have been detected in the radioimmunoassay. Alternatively, there may have been a stimulus for this initial PF_4 secretion other than thrombin. A third possibility is that centrifugation of blood samples alone resulted in the release of small amounts of PF_4.

TABLE I
Effect of dbcAMP and PGE_1 on Thrombin-Induced Platelet Secretion

Thrombin final concentration		PF_4	
		A	B
U/ml	*μg/ml*	*ng/ml*	
0		35	61
0.4	0.125	124	3,260
0.8	0.25	197	4,170
1.6	0.5	967	7,533
3.2	1.0	3,870	8,150
5	1.56	1,818	6,917

Blood was collected into 0.1 vol of 3.8% trisodium citrate and divided into two portions, "A" and "B." To fraction A, dbcAMP (final concentration 1 mM) and PGE_1 (final concentration 0.0028 mM) were added. Then, thrombin was added to 2.0-ml aliquots from each fraction and the samples placed on ice. After 10 min, the samples were centrifuged and the plasma removed for PF_4 assay.

The long duration of secretion can be explained by the previous observation that the extent of platelet secretion is dependent on the thrombin concentration (10). One possible explanation for the time-course observed, therefore, is that early on, the concentration of thrombin is just high enough to induce partial secretion, but only much later are concentrations sufficiently high to induce complete secretion. Alternatively, there may be different populations of platelets with different sensitivities to thrombin: a small population which is very sensitive and secretes at low thrombin concentrations, and larger populations that secrete later and at higher thrombin concentrations. Our data do not allow us to distinguish between these possibilities at this time. Nor do we know the effects of the secretion of adenosine diphosphate or the prostaglandin metabolites on this process. It may be that once a critical extracellular concentration of either of these substances is reached, secretion is greatly accelerated.

It has been reported that when thrombin is added to citrated platelet-rich plasma or whole blood, the concentration necessary to induce platelet secretion is 100-fold higher than that required to cleave fibrinopeptide A from fibrinogen (16). This observation may be misleading in that it suggests secretion might occur much later than fibrin formation or clotting. These experiments, however, may not correspond to what happens physiologically. When platelet secretion is stimulated by endogenous thrombin, the concentration on the platelet surface is probably much higher than that of plasma because that is where prothrombin activation presumably occurs (3). Furthermore, there is evidence to suggest that activated clotting factors may be protected from inhibition by antithrombin III when bound to a phospholipid surface (17, 18).

We do not know whether the secretory pattern that we have observed for PF_4 under these conditions

is different from that of other platelet intracellular constituents. It has been suggested that antiheparin activity is located in the alpha granules of platelets (19, 20). Holmsen et al. (21) have reported that higher concentrations of thrombin are needed for alpha granule release than for dense body secretion. Also, it has been reported that release of antiheparin activity is slower than that of serotonin when washed platelets are stimulated to secrete (22). If this is the case, one would expect secretion to occur earlier or be completed more rapidly than we observed for PF_4. However, the differences observed in these experiments may be related to the types of assays used to detect release rather than to different secretory time courses. Thus, a radioassay was used to measure serotonin release and functional assays to measure alpha granule release (22). It may be that the latter are not as sensitive as the former and do not detect low levels present in the early stages of secretion.

The precise role of platelet secretion in prothrombin activation is unclear. Miletich et al. (3) have shown that platelets have specific receptors for Factor Xa which appear on their surface only after secretion takes place. The receptor has characteristics similar to Factor V. The rapid formation of thrombin shortly before clotting, therefore, may be the result of increased binding of Factor Xa to Factor V secreted from platelets at this time.

We have developed a method for studying the interaction of platelets and coagulation factors which will allow further characterization of this process in normal and pathologic blood clotting. In the latter case, this may improve our understanding of the specific consequences of clotting factor deficiencies or platelet defects on coagulation.

ACKNOWLEDGMENTS

We wish to thank Linda S. Krentz for her expert technical assistance.

This work was supported by grants from the National Institutes of Health (HL 21403) and the Medical Research Service, Veterans Administration.

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