Clot Retraction Facilitates Clot Lysis

By Roger C. Carroll, Jonathan M. Gerrard, and James M. Gilliam

Platelet facilitation of clot lysis was studied using the dilute clot lysis assay, a standardized assay for fibrinolysis shown to correlate with the development of postoperative deep vein thrombosis. Clots prepared from dilute platelet poor plasma showed prolonged clot lysis when compared with clots prepared in a similar fashion from dilute platelet rich plasma. Since in the presence of platelets clot retraction or contraction occurred, we evaluated a possible direct contribution of retraction to clot lysis. Dilute platelet poor plasma clots were compacted by centrifugation, to a similar extent as that achieved during clot retraction in dilute platelet rich plasma. These clots now lysed at a rate that approached that seen with dilute platelet rich plasma clots. Using an alternate approach, dilute platelet rich plasma clots were treated with cytochalasin B to prevent clot retraction. Such clots now showed prolonged lysis similar to that seen with dilute platelet poor plasma. The prolonged lysis of cytochalasin B treated dilute platelet rich plasma clots was corrected by artificial compaction of the clots. The results suggest that clot retraction markedly facilitates clot lysis, and shows that a major role of platelets to facilitate clot lysis is the effect of these cells to cause clot retraction.

MATERIALS AND METHODS
Preparation of Dilute Platelet Rich Plasma and Dilute Platelet Poor Plasma

Venous blood was withdrawn from the antecubital vein of normal donors after obtaining informed consent and immediately diluted with Fearnley’s phosphate pH 7.4 buffer (45 mM sodium phosphate, 5 mM potassium phosphate) at 4°C in a ratio of one part blood to nine parts buffer. Dilute platelet rich plasma (dPRP) was obtained by centrifugation of the diluted whole blood at 250 g for 8 min at 4°C in an IEC clinical centrifuge model 428, Damon/IEC Division (Needham Hts, Mass.). Dilute platelet poor plasma (dPPP) was then prepared by additional centrifugation of the dPRP for 10 min at 5000 g in a Beckman model L-5-50 Ultracentrifuge (Beckman Instruments Inc., Palo Alto, Calif.) using a SW-25 swinging bucket rotor at 4°C. These centrifugations were done with the brake off. Platelet hemocytometer counts of dPRP ranged from 20,000 to 30,000/μl while dPPP counts were 100–300/μl unless otherwise noted.

Clot Lysis Assay

125I-fibrinogen (human) (0.1–0.2 μCi) obtained from Abbott Laboratories (North Chicago, Ill.), (50–100 μCi/mg), was freshly thawed and centrifuged in a Beckman Microfuge B at 10,000 g for 5 min at room temperature to remove aggregated material. The supernatant 125I-fibrinogen was added to dPRP and dPPP samples and aliquots (0.5 ml) of the labeled dPRP or dPPP were added at 4°C to 1.5 ml microfuge tubes without caps, (Sarstedt BB411-1500 obtained from Bolab Incorporated, Derey, N.H.), containing 0.1 ml of 20 U/ml bovine thrombin, (Parke-Davis & Co., Detroit, Mich.) in Fearnley’s buffer, immediately mixed by stirring with a small spatula, and allowed to clot for 30 min at 4°C before transfer to a 37°C bath.

After incubating the sample tubes in the 37°C bath for 30 min, they were transferred to a 37°C warm room maintained at 100% humidity to prevent evaporation. The sample tubes were positioned in a disposable collection plate (Gilmor Medical Electronics, Middleton, Wis.), modified to accommodate the microfuge tubes by drilling out the collection well bottoms. The collection plate containing the microfuge tubes was placed on a Gilmor microfractionator (Gilmor Medical Electronics, Middleton, Wis.). An auxiliary timer, (the control unit from another fraction collection, Model 327, Instrumentation Specialities Co., Lincoln, Neb.), controlled a flow valve on the tubing connecting the microfractionator to a reservoir of 1% glutaraldehyde in Fearnley’s phosphate buffer. The timing sequence set on the auxiliary timer was initiated by turning on the device that opened the flow valve to allow a number of drops of

From the Departments of Biochemistry and Pathology, University of Oklahoma, Oklahoma City, and Northeastern Ohio University, College of Medicine, Rootstown, Ohio.

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Address reprint requests to Dr. Roger C. Carroll, University of Oklahoma, Oklahoma City, Okla.

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fixative equal to 0.55 ml to fall over a period of 60 sec into the first clot sample tube. The microfication was set to advance to the next tube after a set number of drops was counted. The event marker circuit on the microfication was used to signal the auxiliary timer to begin timing the delay interval between tubes and closing the flow valve. After the delay interval the cycle repeated until all 80 tubes in the collection plate were fixed at the selected time intervals. To confirm that the reaction was stopped, unfixed samples were immediately centrifuged and the lysis values compared with samples fixed and centrifuged at the end of the time course. These two sets of samples gave identical lysis curves, a result that has been repeated several times. At the end of the time course, insoluble material associated with the fibrin clot was separated from the soluble fibrin products by centrifugation for 5 min at 10,000 g, at room temperature in a Beckman microfuge B. A 0.5 ml aliquot of the supernatant was then withdrawn for counting released 125I-fibrin degradation products. Aliquots of unclotted dPRP and dPPP were also counted to determine total 125I-counts in the sample. Background unincorporated 125I-fibrinogen counts (10%-20% of total counts) were determined on samples taken just before initiating lysis by transfer to 37°C. The percent lysis was then calculated by dividing the supernatant counts minus the background counts in each sample by the total counts minus the background counts and multiplying by 100.

Compaction of the Fibrin Clot

To compact the fibrin network artificially, sample microfuge tubes containing formed dPPP or dPRP clots were centrifuged 2 min at 10,000 g in a Beckman microfuge B at room temperature, 10-20 min after transferring to the 37°C bath.

Cytochalasin B Treatment

To evaluate the influence of cytochalasin B, this agent was added to the dPRP or dPPP 10 min before stirring with thrombin. The cytochalasin B at a final concentration of 10 µg/ml was added in ethanol to a volume not exceeding 2 µl/ml and controls receiving ethanol alone were run in parallel.

Beta-Thromboglobulin and Platelet Factor IV Release

A commercial radioimmunoassay kit purchased from Amersham (Arlington Heights, Ill.) was used to measure released beta-thromboglobulin as a marker for platelet alphagranule release.10 These determinations were done on unlabeled and fixed dPRP clot samples. Following centrifugation of the samples for 2 min at 10,000 g at 4°C, aliquots of supernatants were immediately withdrawn and frozen for later radioimmunoassay. Two control samples included an aliquot of unclotted dPRP supernatant that was assayed for beta-thromboglobulin levels before addition of thrombin (usually 1%-2%) and an aliquot that was freeze-thawed and directly assayed for total betathromboglobulin levels. A similar procedure was used to measure platelet factor IV as another marker for granule release.10

Preparation of Releasate From Gel Filtered Platelets

Platelet rich plasma was prepared from acid citrate-dextrose anticoagulated whole blood as previously described.11 Gel filtered platelets were prepared by applying 5 ml of citrated platelet rich plasma to a Sepharose 2B (Sigma, St. Louis, Mo.) column (2.5 cm × 20 cm) equilibrated and eluted at room temperature with a modified Fearnley’s phosphate buffer pH7.4 containing 0.05 mM calcium chloride, 0.1 mM magnesium chloride, 10 mM glucose, and 1 mg/ml bovine serum albumin. The platelets (100,000-150,000/µl) were activated at 37°C with stirring by either 50 µg/ml arachidonic acid or 10 µM A23187 and after 3 min the platelets pelleted by centrifugation for 10 min at 5000 g in a Beckman model L5-50 Ultracentrifuge using a SW-25 swinging bucket rotor at 4°C. The supernatant containing the releasate was collected by syringe and gave residual platelet counts of 200-400/µl.

RESULTS

Mechanical compaction of the fibrin network of dilute platelet poor plasma clots by centrifugation markedly accelerated lysis of such clots (Fig. 1), so that 50% lysis occurred in about 5 hr instead of the 11 hr seen in the uncompacted dPPP clot. The degree of compaction achieved by centrifugation as determined by weighing the clot and unexpressed plasma approached that seen when dPRP clots were fully retracted (Table 1). Furthermore, the rate of lysis seen with compacted dPPP clot approached the rate of lysis seen with dPRP, where 50% lysis occurred in 3.2 hr. Compaction of dPRP clots had little effect on the lysis curve, except to slightly accelerate lysis in the first 2.5 hr. Similar results were obtained with three different normal volunteers.

To rule out the possibility of a residual platelet contribution to the lysis of compacted dPPP clots, a
Table 1.

<table>
<thead>
<tr>
<th>Time After Transfer to 37°C (min)</th>
<th>Weight of Unexpressed Plasma</th>
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<tbody>
<tr>
<td></td>
<td>Uncompacted dPRP clot</td>
</tr>
<tr>
<td>0</td>
<td>600 mg ± 10 mg</td>
</tr>
<tr>
<td>10</td>
<td>520 mg ± 10 mg</td>
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<tr>
<td>30</td>
<td>322 mg ± 2 mg</td>
</tr>
<tr>
<td>60</td>
<td>86 mg ± 4 mg</td>
</tr>
<tr>
<td>90</td>
<td>10 mg ± 5 mg</td>
</tr>
<tr>
<td>210</td>
<td>5 mg ± 2 mg</td>
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Triplicate clot samples for each set of determination were weighed after carefully removing the expressed plasma by transfer pipet. The weight of the microfuge tube was determined by removing the clot and weighing the tube after wiping dry with a tissue paper and the weight of the clot determined by subtracting the tube weight from the total weight of the tube plus the clot.

sample of dPPP containing 250 platelets/μl was centrifuged a second time at 5000 g for 10 min at 4°C, and was assayed before and after compaction. The platelet count was reduced to 30 platelets/μl. This twice centrifuged dPPP sample behaved identically to once centrifuged dPPP with respect to both compacted and uncompacted samples. We also determined that the dPPP used in these experiments was substantially free of platelet releasate by assay of beta-thromboglobulin as a marker of platelet granule secretion. The amount of beta-thromboglobulin was 3%–6% ± 3% of the total found in freeze-thawed dPRP.

In order to further evaluate the effect of platelet releasate on dilute clot lysis we prepared dPPP from dPRP that had been previously treated with 10 mM 2-deoxyglucose and 5 μg/ml antimycin A for 30 min at 37°C. The clot lysis of this dPPP is very similar to dPPP prepared without metabolic inhibitors as shown in Fig. 2. These conditions have been shown to metabolically deplete the platelets so that they are incapable of active release of granule contents.15 The dPPP still contained 2%–4% ± 2% of the amount of platelet factor IV found in freeze-thawed dPRP. The residual platelets (200–250/μl) would account for 1%–2% of the platelet factor IV found but within the error limits of the assay it is difficult to completely rule out the presence of 1%–2% releasate in the dPPP. To determine what effects substantial amounts of soluble platelet releasate might have on clot lysis we added platelet releasate obtained from gel filtered platelets as described in Materials and Methods, to give platelet factor IV levels equivalent to freeze-thawed dPRP. The clot lysis of these samples after compaction of the clots was compared to compacted control dPPP samples without releasate (Fig. 3). As seen from the data the presence of releasate added before clotting the samples slightly slows the rate of lysis so that 50% lysis occurred approximately 1–2 hr later.

The results obtained by compaction of dPPP clots to concentrate the fibrin network strongly suggest that platelets promote clot lysis by mechanically concen-
The degree of compaction, or as a result of the effect of cytochalasin B, did not affect thrombin induced platelet secretion since both cytochalasin B treated and untreated platelets released 70%-80% of their beta-thromboglobulin content within 10 min after transfer to the 37°C bath.

**DISCUSSION**

Previous results have shown that platelets make an important contribution to clot lysis. Inhibition of dilute platelet rich plasma clot lysis by cytochalasin B, and restoration of lysis with compaction by centrifugation. Blood from a normal volunteer was used to prepare dPRP and dPPP as described in Materials and Methods. After labeling the samples with 0.1 to 0.2 μCi/ml 125I-fibrinogen the dPPP was divided into two 20-ml samples. To one dPPP sample was added 0.04 ml of 6 mg/ml cytochalasin B in ethanol to give a final concentration of 10 μg/ml while the control dPPP sample received 0.04 ml ethanol alone. After a further 10 min incubation at 4°C the samples were clotted with 0.1 ml thrombin (20 U/ml) in microfuge tubes as before. After 30 min at 4°C, sample tubes were transferred to a 37°C bath and after an additional 10 min at 37°C, the indicated sample clots were compacted by centrifugation for 2 min at 10,000 g. Fibrinolysis was stopped in individual sample tubes at the indicated time points by addition of 0.55 ml of 1% glutaraldehyde in Fearnley's buffer and at completion of the time course undegraded fibrin was pelleted and supernatant aliquots counted for 125I-fibrin degradation products. The lysis curves shown are for (a) dPRP, (b) dPPP + 10 μg/ml cytochalasin B, (c) dPRP + 10 μg/ml cytochalasin B, compacted by centrifugation.
addition of soluble platelet releasate to dPPP caused only a slight inhibition of clot lysis.

Confirmation of this important role of clot retraction in clot lysis was obtained in experiments using the agent cytochalasin B that interferes with the interaction of platelet contractile proteins actin, actin binding protein and alpha actinin. Cytochalasin B completely inhibited clot retraction and prolonged clot lysis until it approached that seen in dPPP. The effect of cytochalasin B could be corrected by artificial compaction. Cytochalasin B did not affect clot lysis by suppression of platelet granule secretion since release was similar in treated and untreated samples. The lack of effect of cytochalasin B on platelet secretion has been reported previously by others. 

Our results indicate that clot retraction makes a major contribution to clot lysis. The earlier suggestion that clot retraction might facilitate recanalization of thrombosed blood vessels therefore seems to be supported, with the new dimension added, that the contraction of the clot markedly facilitates the lysis required for complete opening of the thrombosed blood vessel. It will be important to study individuals with thrombotic disorders with long clot lysis times to see if their defect can be corrected by compaction suggesting defective clot retraction might contribute to a tendency to thrombosis in certain circumstances.

The mechanism by which concentration of the fibrin network results in enhanced lysis remains an area of investigation. However, a hypothesis compatible with these results is that retraction of the fibrin network increases the local concentration both of the fibrin and bound fibrinolytic factors. The result of this effect may be to directly enhance fibrinolysis. In addition, since fibrin can accelerate the rate of plasminogen to plasmin conversion, (the major fibrinolytic protease of plasma) by plasminogen activator, the high local concentration of fibrin may also promote clot lysis through this mechanism.

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REFERENCES


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