CONCISE REPORT

Sickled Erythrocytes Accelerate Clotting In Vitro: An Effect of Abnormal Membrane Lipid Asymmetry

By Danny Chiu, Bertram Lubin, Ben Roelofsen, and L. L. van Deenen

A membrane lipid abnormality induced by sickling and found as a permanent alteration in the irreversibly sickled cell (ISC) is the rearrangement of phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) from the inner to the exterior side of the lipid bilayer. Since PS can provide a catalytic surface for the binding of blood coagulation factors and thus can exhibit procoagulant activity, we investigated the influence of oxy and deoxy reversibly sickled cells (RSC) as well as ISC on clotting in vitro. Red blood cells (RBC), as the source of phospholipid, were added to platelet-poor citrated plasma containing Russell’s viper venom (RVV) and clotting time was measured after recalcification. The clotting time after addition of normal RBC and oxy-RSC was similar to the saline blank (100 sec). In contrast, both oxy-ISC and deoxy completely sickled RSC shortened clotting time by 30%. Using liposomes prepared with identical phospholipid composition to the outer lipid leaflet of either normal RBC, RSC or ISC clotting times similar to those with intact cells were achieved. Since the liposomes did not contain protein, accentuation of clotting appears to be related to abnormal phospholipid organization, in particular to the abnormal exposure of aminophospholipids on the outer surface of the membrane. This abnormality may contribute to the pathogenesis of the vaso-occlusive episode in sickle cell anemia.

ONE OF THE MOST prominent pathologic features of sickle cell anemia (SCA) is the occurrence of microvascular occlusion, which is responsible for such clinical manifestations as painful crises and organ damage. The mechanism of vaso-occlusion has generally been attributed to the abnormal shape and reduced deformability of the sickled erythrocytes. In addition, increased adhesion of sickle erythrocytes to vascular endothelium has also been suggested to play an important role. However, the concept that vascular occlusion is not entirely precipitated by sickle erythrocytes alone, but that thrombosis may also be of pathogenic significance in microvascular occlusive crises has also been gained acceptance.

The involvement of phospholipids in blood coagulation has long been recognized, and their role in blood coagulation is believed to provide a suitable lipid/water interface to interacting coagulation factors, thus guiding and accelerating some of the reactions leading to clot formation. This procoagulant activity is normally provided by activated platelets and is commonly referred to as platelet factor 3. The phospholipid organization in normal erythrocytes is such that it does not provide a suitable catalytic surface for coagulation factors and thus normal erythrocytes do not affect blood coagulation. However, since we have found that sickling is associated with an abnormal exposure of aminophospholipids on the outer surface of the erythrocytes, we investigated the effects of this membrane phospholipid abnormality on blood coagulation.

MATERIALS AND METHODS

Blood from sickle cell patients and normal individuals was collected in 0.1 M acid citrated buffer. To prepare platelet-poor plasma, erythrocyte-free plasma was centrifuged at 5000 g for 10 min and only the top two-thirds portion was collected as platelet-poor plasma (<1000 platelets/μL). To obtain subpopulations of erythrocytes, washed RBC were separated according to density into top, middle, and bottom fractions on discontinuous stractan gradients. Unsealed ghosts were prepared from washed RBC according to the method of Fairbank et al. Liposomes with specific phospholipid composition were prepared according to the method described by Zwaal et al.

The effect of RBC samples and of liposomes on blood coagulation was determined by a modified Russell’s viper venom (RVV) clotting assay. In essence, this assay system is a one-stage prothrombinase assay. Prothrombinase complex is composed of factor X, factor V, calcium ion, and phospholipids. Factor X can be activated in vivo by either intrinsic or extrinsic pathways. In RVV assay system, factor X is activated by an enzyme in RVV. Since the conversion of prothrombin to thrombin by prothrombinase is the last step in the coagulation cascade requiring phospholipid, we adapted this assay system to determine the effect of abnormal phospholipid organization on blood coagulation. In our RVV clotting assay, a 0.1-ml aliquot of citrated platelet-poor plasma was incubated at 37°C in a fibrometer. After 2 min of incubation, 30 μl of RVV solution containing approximately 12 ng of RVV (Wellcome Research Laboratories, Beckenham, England) was added to the plasma and the mixture was incubated for another 30 sec. A 0.1-ml aliquot of saline (serving as blank) or various dilutions of RBC samples, ghosts, or liposome preparations was then added to the plasma and
RESULTS

Figure 1 shows the effect of normal RBC, unfractionated sickle RBC and unsealed ghosts prepared from both normal and sickle RBC on RVV clotting time under fully oxygenated conditions. The RVV clotting time, which was approximately 100 sec for saline, is plotted against the logarithm of phospholipid concentration. RVV clotting assay involves the incubation of red cell or ghost samples with citrated platelet-poor plasma and RVV. Blood clotting was initiated by recalcification of the assay mixture, and RVV clotting time was determined by a fibrometer. The bars on the graph indicate the range of 5 separate experiments.

**Figure 1** Effect of normal RBC, sickle RBC, and leaky ghosts on RVV induced in vitro blood coagulation under fully oxygenated conditions. The RVV clotting time, which was approximately 100 sec for saline, is plotted against logarithm of phospholipid concentration. RVV clotting assay involves the incubation of red cell or ghost samples with citrated platelet-poor plasma and RVV. Blood clotting was initiated by recalcification of the assay mixture, and RVV clotting time was determined by a fibrometer. The bars on the graph indicate the range of 5 separate experiments.

RVV mixture, followed by addition of 0.1 ml of 25 mM CaCl₂ to initiate blood coagulation. The time required for the formation of a firm fibrin clot was determined. To determine the effect of sickling on RVV clotting time, RBC were first incubated under humidified 95% N₂/5% CO₂ for 1hr to induce sickling. The sickled RBC were then used in the RVV clotting assay as described above.

DISCUSSION

The data obtained in the present study demonstrate that unlike normal erythrocytes, which have no effect on RVV clotting time, erythrocytes from sickle cell patients accelerate RVV clotting time (Fig. 1). Our study also demonstrates that oxygenated RSC accelerate in

hemolysis in each assay mixture was similar and insignificant (<0.10%).

The effect of oxygenated ISC and RSC on RVV clotting time is shown in Fig. 2. Sickle erythrocytes were separated into ISC-poor and ISC-rich fractions using discontinuous stractan gradients. ISC-poor sickle RBC had very little effect on RVV clotting time under oxygenated conditions. However, RVV clotting time was greatly affected by ISC. As in the case of unfractionated sickle RBC, the shortening in RVV clotting time by ISC was dose dependent. At approximately 10% final hematocrit, ISC shortened RVV clotting time by more than 30%.

Although ISC-poor sickle erythrocytes had very little effect on RVV clotting time under oxygenated conditions, this same fraction of sickle erythrocytes shortened RVV clotting time to a similar degree as oxygenated ISC-rich fractions when the assay was carried out under deoxygenated conditions. The results of this experiment are shown in Table 1. At a 10% final hematocrit, neither oxygenated nor deoxygenated normal erythrocytes affected the RVV clotting time. Oxygenated ISC-poor sickle erythrocytes also had no effect on RVV clotting time. However, when ISC-poor cells were deoxygenated and completely sickled they shortened RVV clotting time by 30% at 10% final hematocrit.

To further delineate the relationship between shortened RVV clotting time and abnormal membrane phospholipid organization in sickled erythrocytes, we prepared liposomes with similar phospholipid composition as the outer leaflet of oxy RSC (PC 44%, PE 12%, SM 44%, and no PS) and that of deoxy RSC and ISC (PC 38%, PE 22%, SM 38%, and PS 2%) and determined the effect of such liposomes on RVV clotting time. The results of these experiments are shown in Fig. 3. Liposomes with identical phospholipid composition to the outer lipid leaflet of normal erythrocytes did not affect the RVV clotting time. In contrast, liposomes with phospholipid composition similar to the outer leaflet of sickled erythrocytes significantly shortened the RVV clotting time. Within the range of liposome concentration used in our experiment, the extent of shortening in RVV clotting time was directly related to the quantity of liposome added.
vitro blood coagulation (Fig. 2 and Table 1). This effect appears to be related to the abnormality of membrane phospholipids in sickle cells as supported by the results of our liposome experiment (Fig. 3).

Although erythrocytes are generally not considered to be important for hemostasis, the procoagulant activity of erythrocytes has been recognized for some time. Shinowara and Gollub reported that erythrocytes have thromboplastic activity. Quick et al. first noted that upon hemolysis, erythrocytes "release" a clotting factor that enhances blood coagulation. The coagulant activity exhibited by red cell hemolysate is now believed to be due to the exposure of procoagulant phospholipid within the lysed erythrocytes.

The main function of phospholipid in blood coagulation is to provide a catalytic surface on which various coagulation factors interact, thus accelerating the blood coagulation process. Among the four major phospholipids in red cell membrane, only PS in combinations with appropriate amounts of PC, PE, or SM, exhibits considerable procoagulant activity. Since PS molecules are confined to the cytoplasmic surface of intact normal erythrocytes and are unaccessible to plasma coagulation proteins, intact normal erythrocytes would not be expected to enhance coagulation. In contrast, once PS molecules on the erythrocyte membrane become accessible to plasma coagulation factors, as in the case of unsealed lysed erythrocytes or sickled erythrocytes, blood coagulation is accelerated (Fig. 1). The abnormal exposure of PS on the outer surface of sickled erythrocytes either alone or in combination with increased PE may behave similar to platelet factor 3 and thus upset the delicate balance between regulating hemostasis and avoiding thrombosis. This abnormality may impose a constant hypercoagulable state in sickle cell patients and may contribute to the frequent incidence of thrombotic episodes in these patients.

A causal relationship between abnormal exposure of procoagulant phospholipid on the outer surface of the red cell membrane and hypercoagulability may exist in paroxysmal nocturnal hemoglobinuria and hemo-

**Table 1. Effects of Sickling on RVV Clotting Time**

<table>
<thead>
<tr>
<th>Blood Sample</th>
<th>RVV Clotting Time (sec)</th>
<th>95%</th>
<th>95%</th>
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<tr>
<td></td>
<td></td>
<td>O2/5%</td>
<td>N2/5%</td>
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<tr>
<td>Normal erythrocytes (n = 5)</td>
<td>101 ± 4.4</td>
<td>100 ± 2.5</td>
<td></td>
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<tr>
<td>ISC-poor sickle erythrocytes (n = 5)</td>
<td>97 ± 3.9</td>
<td>72 ± 5.8</td>
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<td>Mean ± 1 SD.</td>
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lytic uremic syndrome. Abnormalities in the red cell membrane, hypercoagulability, and thrombosis are found in these two disorders. Although the contribution that a defect in the red cell membrane might have to the hypercoagulable state has not been defined, a similar causal relationship might exist in sickle cell anemia, thereby supporting a common pathologic mechanism.

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