Oxidation-Induced Changes in Microrheologic Properties of the Red Blood Cell Membrane

By Robert P. Hebbel, Andrew Leung, and Narla Mohandas

It has been hypothesized that some of the irreversible microrheologic abnormalities of sickle red blood cell (RBC) membranes could result from autoxidative perturbation. To model this possibility, we used micromechanical manipulation to examine the static extensional rigidity and inelastic or plastic behavior of normal RBCs exposed to phenazine methosulfate (PMS), an agent that generates superoxide from within the cell. In response to this stress, RBC membranes became stiff as evidenced by increasing extensional rigidity. At 50 μmol/L PMS they were as stiff as the membranes of most dense, dehydrated sickle RBCs; and at 25 μmol/L PMS the membranes were similar to somewhat less dense sickle RBCs. When examined for inelastic behavior, RBCs exposed to PMS even at 10 μmol/L showed hysteresis in loading and unloading phases of the curve relating aspiration length to suction pressure, and they developed membrane bumps that persisted after RBC release from the pipette. Examination of single cells in both isotonic and hypotonic buffers showed that the effect of PMS on RBC microrheology is not mediated by cellular dehydration. Independent confirmation of the membrane stiffening effect of PMS was obtained by ektacytometric analysis of resealed RBC ghosts, with sickle-like increases in membrane rigidity observed between 50 and 100 μmol/L PMS. The rigidity of these ghosts was partially ameliorated by exposure to a thiol reductant. In terms of biochemical abnormalities, treated RBCs became significantly different from control RBCs at 25 μmol/L PMS, at which point they just began to enter the sickle range for amounts of membrane thiol oxidation and membrane-associated heme. The sickle average was achieved at 50 μmol/L PMS (for thiol oxidation) to 100 μmol/L PMS (for membrane heme). Thus, micromolar concentrations of PMS induce abnormalities of membrane microrheology that closely mimic those of unmanipulated sickle RBCs while reproducing similar degrees of oxidative biochemical change. We conclude that membrane protein oxidation could explain existence of an irreversible component to the abnormal rheology of the sickle membrane.

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The clinical course of sickle cell anemia is punctuated by episodic vasoocclusive events caused by the abnormal microcirculatory behavior of sickle blood. This pathophysiology results, at least in part, from the presence of poorly deformable erythrocytes (RBCs) characterized by defects of cellular microrheology that are attributable to changes in both cell cytoplasm and membrane organization. The former, an increase in cytoplasmic viscosity due to cellular dehydration, is thought to be a dominant factor responsible for decreased deformability of sickle cells. However, recent microrheologic data also have implicated unusual membrane rigidity stemming from a reversible association of sickle hemoglobin with the membrane, as well as from irreversible changes in membrane skeletal protein organization.

For dense, dehydrated sickle RBCs, altered membrane rheology due to irreversible membrane changes is most evident in the persistence of membrane deformation after the removal of deforming forces (plastic flow) and the continued manifestation of abnormally increased extensional static rigidity despite normalization of cell hydration status. The biochemical and structural basis for these phenomena has not been delineated yet. Rank et al previously speculated that the abnormal thiol oxidation of cytoskeletal proteins in the sickle RBC membrane might account for this plastic behavior. More recently, it has been shown that there is a correlation between membrane thiol oxidation and membrane-associated heme in sickle RBCs. Importantly, the extent of both thiol oxidation and membrane-associated heme is highest in most dense sickle RBCs, the population of cells exhibiting the most marked abnormalities in membrane rheologic properties.

To determine whether irreversible rheologic abnormalities of sickle membranes could result from oxidation-induced changes in membrane organization, we have examined the microrheology of normal RBCs in which the excessive autooxidation of sickle RBCs is simulated by incubation with phenazine methosulfate, an agent that stimulates intracellular generation of superoxide.

Materials and Methods

Cell preparation. Fresh citrated blood (citrate:blood = 1:9) was obtained from volunteer normal donors. Without delay, RBCs were washed four times with PBSG (1 part 0.12 mol/L sodium phosphate buffer, 3 parts 0.154 mol/L NaCl, 1 g/L glucose, pH 7.4) with removal of buffy coat. To stimulate intracellular generation of superoxide, we incubated normal RBCs with various concentrations of phenazine methosulfate (PMS) at hematocrit 5% in PBSG for 60 minutes at 37°C, after which RBCs were thoroughly washed with PBSG. They were then used for biochemical assays, or they were suspended in PBS (10 mmol/L phosphate, pH 7.4, NaCl to 290 mOsmol/L) for micropipette studies.

Biochemical assays. Several parameters of oxidative damage were evaluated to monitor the effect of incubating RBCs with PMS. Amount of thiol oxidation in sodium dodecyl sulfate-solubilized

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membrane proteins was measured both by titrating reduced thiols using 2,2-dithiopyridine and by thiol-disulfide exchange chromatography. Amount of peroxidation was estimated by measurement of thiobarbituric acid reactive substances (TBARS), with correction for any contaminating chromogens as previously described. Amount of truly membrane-associated heme and heme protein was measured spectrophotometrically on inside-out membranes (IOM) solubilized in formic acid.

Microscopic observations. Micropipette aspiration technique was used to evaluate the contribution of oxidative changes in the RBC membrane to its rheologic properties. In this study we focus on the extensional properties of the membrane and on the recoverability of cell shape after deformation. Details of the micromechanical system and analysis used to measure RBC mechanical properties have been described previously.

Briefly, the membrane extensional rigidity (μ) was derived from observation of cell length (L) aspirated into a small micropipette (with radius Rp) in response to the increase in suction pressure (P). Analysis of this experiment has shown that μ is proportional to the derivative of the pressure with respect to length; μ = Rp² × dP/dL. Inelastic or plastic behavior of the membrane was evaluated by comparing RBC mechanical behavior during the progressive increase in aspiration pressure (loading phase) to the behavior observed when the aspiration pressure was slowly reduced (unloading phase). For a truly elastic material, the curves describing the force/deformation relationship for loading and unloading phases are very similar; ie, there is no hysteresis. In contrast, for materials that exhibit inelastic or plastic behavior, the loading and unloading curves are different, and hysteresis is evident. This can be illustrated dramatically by persistent, residual bumps on the cell surface after release of applied forces.

To determine whether altered membrane properties were due to changes in cell hydration state, we performed mechanical tests on PMS-treated RBCs in both isotonic buffer (290 mOsmol/L) and hypotonic buffer (210 mOsmol/L). For accurate comparison, the tests were performed on the same cell, which was transferred by micromanipulation from one chamber on the microscope stage containing isotonic buffer to an adjacent chamber containing hypotonic buffer. Transfers were accomplished by insertion of each cell into a large bore pipette that was drawn through the air-water interfaces.

We also quantified dynamic membrane deformability of normal and oxidized RBCs using the ektacytometer. For these measurements, resealed RBC ghost membranes were suspended in 3 ml of Stractan II (St Regis Paper Co, Tacoma, WA) (290 mOsmol/L, 22 cp, pH 7.4) and exposed to increasing shear stress as previously described. Analysis of the relationship between deformability index and shear stress provides a measure of membrane deformability. It should be noted that unlike the micropipette method, which generates an accurate measure of elastic modulus, the membrane deformability determined by ektacytometry is a complex property influenced by a number of material properties including elastic and bending moduli, as well as membrane viscosity and surface area.

RESULTS

Biochemical sequelae of oxidation. RBCs exposed to all concentrations of PMS retained normal morphology with smooth biconcave contour. However, the effect of PMS on membrane protein was evident in a diminished number of titratable reduced thiols in RBC ghosts as the PMS concentration increased (Fig 1). A comparable dose-response relationship was observed for thiol oxidation measured by a different technique, thiol-disulfide exchange chromatography (data not shown). In addition, the amount of membrane-associated heme increased progressively as PMS concentration was increased (Fig 1). Little change in these biochemical parameters was observed after exposure of RBCs to PMS up to 10 μmol/L, but both effects began to enter the sickle range (and became significantly different from control) at 25 μmol/L PMS. The average degree of biochemical alteration observed in native, unmanipulated sickle cell membranes was fully reproduced at 50 μmol/L PMS. The average degree of biochemical alteration observed in native, unmanipulated sickle cell membranes was consistently exceeded by the effect of PMS on RBCs (Fig 1).

Fig 1. Biochemical alterations induced by PMS. After exposure of normal RBCs to PMS at the indicated concentrations, the number of titratable reduced thiols (O) and the amount of generic heme on IOM (●) were determined in 3 and 4 experiments, respectively. The asterisk indicates values that have become significantly different from control RBCs. For comparison, thiol (n = 8) and IOM heme (n = 31) values previously obtained for unmanipulated sickle RBCs are shown at the far right. All data are shown as mean ± SD.

Micropipette study of normal and oxidized RBCs. Measured values for static extensional rigidity (μ) of normal and PMS-treated RBCs are shown in Table 1. RBCs exposed to PMS at concentrations up to 10 μmol/L remained similar to normal control cells, but at higher concentrations of PMS, the shear modulus deteriorated (μ increased) as dose escalated. Indeed, the shear modulus identified for normal RBCs exposed to 50 μmol/L PMS is comparable with that of dense, dehydrated sickle RBCs (previously found to have μ = 21 ± 7.8 × 10⁻³ dyne/cm²), and our result for RBCs exposed to 25
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Table 1. Static Extensional Rigidity of PMS-Treated RBCs

<table>
<thead>
<tr>
<th>PMS Dose (µmol/L)</th>
<th>Shear Modulus, µ (x 10^-9 dyn/cm)</th>
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<tr>
<td>Control</td>
<td>8.1 ± 1.7 (10)</td>
</tr>
<tr>
<td>2.5</td>
<td>9.4 ± 0.5 (5)</td>
</tr>
<tr>
<td>10</td>
<td>7.5 ± 0.8 (5)</td>
</tr>
<tr>
<td>25</td>
<td>11.1 ± 2.1 (5)</td>
</tr>
<tr>
<td>50</td>
<td>21.2 ± 6.3 (12)</td>
</tr>
<tr>
<td>100</td>
<td>53.5 ± 38.5 (12)</td>
</tr>
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</table>

Values shown as mean ±SD (number of cells examined). Results are here combined for two separate experiments using different normal donors.

µmol/L PMS is similar to that reported for somewhat less dense sickle RBCs.

During the micromechanical measurement of membrane extensional rigidity, we observed that the curves relating aspirated length to suction pressure described different functional behavior for normal and oxidized RBCs. For normal cells, the force/deformation relationship remained constant during increasing aspiration pressure (loading phase) and during decreasing aspiration pressure (unloading phase) (Fig 2A). This absence of hysteresis indicates truly elastic membrane behavior. In contrast, for RBCs treated with PMS, the curve describing aspirated length versus suction pressure was markedly different during loading and unloading phases (Fig 2B and C), indicating that oxidized membranes exhibit inelastic or plastic-like behavior.

This plastic-like behavior also was manifested by residual, persistent bumps observed on the RBC membrane surface after its release from the pipette following extensional deformation (Fig 3). Indeed, these residual bumps developed in every RBC that had been exposed to PMS at 10 µmol/L or higher concentrations, while none of the normal RBCs exhibited them. Interestingly, we thus found that oxidized cells exhibited plastic-like behavior at PMS concentrations that did not increase the shear modulus value. This is evident, for example, in that the curve for the representative RBC depicted in Fig 2B shows some hysteresis but preserves a normal slope (µ).

To critically evaluate the potential contribution of any cellular dehydration to the observed properties of oxidized RBCs, we studied the effect of increased hydration on their mechanical properties. The PMS-treated RBCs were first tested in isotonic buffer (290 mOsmol/L) and then in hypotonic buffer (210 mOsmol/L). Increased hydration in the latter buffer did not alter the shear modulus of the oxidized cells. For example, for one cell the shear modulus was 19 x 10^-9 dyn/cm in isotonic buffer and 20 x 10^-9 at 210 mOsmol/L. Examination of four other oxidized RBCs gave similar results. Likewise, swelling of oxidized RBCs did not alter their plastic behavior, so that residual bumps were seen on the membrane surface after RBC release from the pipette following extensional deformation even at 210 mOsmol/L (data not shown). Thus, we found no evidence that observed microrheologic properties of PMS-treated RBCs could be explained by any dehydrating effect of oxidant exposure.

Ektacytometric studies. Independent confirmation of the apparent membrane-stiffening effect of PMS was obtained by ektacytometric examination of resealed RBC ghosts. As summarized in Fig 4, membranes prepared from oxidized RBCs required much higher levels of applied shear stress to reach equivalent deformability index (DI), an effect that was magnified at progressively larger PMS concentrations. For example, membranes prepared from RBC exposed to 500 µmol/L PMS required an 8.5-fold higher shear stress...

Fig 2. Micropipette studies show the abnormal microrheology of PMS-treated RBCs. This plot shows L/RP (aspiration length normalized by pipette radius [RP]) as a function of membrane tension (DP × RP/2 in dynes/cm where DP is the aspiration pressure). The open circles (O) show the loading phase of the experiment, and the slope of the straight line fit shown here gives the modulus of extensional rigidity (µ) for the cell membrane. The closed circles (•) show the unloading phase of the aspiration experiment. The data shown here (a representative experiment) are for a control RBC (A), an RBC exposed to 10 µmol/L PMS with abnormal hysteresis but normal µ (B), and an RBC exposed to 50 µmol/L PMS with hysteresis and abnormal µ (C).
Fig 3. Residual bumps after release of oxidized RBCs from the micropipette indicate inelastic properties. These videomicrographs were taken of a normal RBC (A) and RBCs treated with PMS at 25 μmol/L (B) or 100 μmol/L (C). These persistent bumps on the RBC membrane were seen after release of every cell that had been exposed to PMS at 10 μmol/L or greater concentrations.

Fig 4. Ektacytometry confirms the membrane-stiffening effect of PMS. The relationship between deformability index (linear scale) and shear stress (log scale) provides a visual representation of the membrane rigidity shown by these studies performed on resealed RBC ghosts. Curves are shown for ghosts derived from control RBCs (○) and for RBCs treated with PMS at 25 μmol/L (△), 50 μmol/L (□), 100 μmol/L (△), 250 μmol/L (●), and 500 μmol/L (■). Data are plotted for the linear portion of the curves; at higher shear stress, the curves flatten and converge.

These studies have documented the effect of PMS-induced oxidation on RBC microrheologic properties. We chose PMS for this work because it uses cytoplasmic hydrogen donors to stimulate intracellular generation of superoxide,11 thus enabling us to reproduce the directionality of pathologic
it is particularly notable that the micromolar PMS concentrations used here result in membrane changes that closely mimic the abnormal microrheologic properties of dense, dehydrated sickle cells.2,4 That these abnormalities of oxidized RBC are not ameliorated by hypotonic buffer argues against an altered state of cell hydration as the cause of observed changes in the membrane rheologic properties and implies a direct role for structural alterations in the membrane.

It was previously suggested that the component of abnormal extensional rigidity and plastic-like behavior of the sickle RBC membrane that is not reversed by cellular rehydration might result from association of hemoglobin with the membrane and/or from oxidative changes in membrane protein organization.4,5 The present findings enable us to suggest that such effects might, indeed, account for the irreversible component of microrheologic abnormalities observed during studies of sickle cells. We still cannot definitively distinguish between effects of heme on the membrane and effects of oxidant per se; but, of course, in the context of oxidative pathobiology the roles of membrane heme and endogenous oxidant may be entwined inextricably.15,16 Nevertheless, it may be noted that we unequivocally influenced microrheology using only 10 μmol/L PMS, at which concentration there was minimal, if any, increase in membrane heme. Therefore, we favor the hypothesis that the observed adverse microrheologic effect is due to protein oxidation rather than heme per se. That DTT only partially ameliorates the PMS effect could simply reflect the participation of oxidative damage to other, nonthiol amino acids in this phenomenon. Because of the remarkably low effective concentration of PMS, its effect almost certainly is different from that induced by high concentrations of exogenous peroxide (formation of hemoglobin/spectrin adducts).17 Thus, the putative protein targets relevant to microrheologic alteration remain to be defined, and it is hoped that future studies will refine these observations further.

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REFERENCES


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