Microrheologic Investigation of Erythrocyte Deformability in Diabetes Mellitus


This study was undertaken to determine whether diabetes alters the viscoelastic properties of erythrocytes. The oldest and youngest 10% fractions of circulating red cells were separated by centrifugation of freshly drawn blood obtained from ten diabetics with disease of one to 20 years' duration and from an equal number of age- and sex-matched control subjects. Cells from each fraction were suspended in phosphate-buffered saline, and their rheologic behavior was examined in a rheoscope. The elongation of the cells, the percentage of cells that tank-treaded in response to shear stress, tank-treading frequency, and the rate of recovery of cell shape upon cessation of shear stress were determined in the oldest and youngest 10% of cells for diabetics as well as for controls. All four parameters were virtually identical for diabetics and controls. Additional aliquots of cells were taken for assessment of nonenzymatic glucosylation of hemoglobin and cell membrane protein. The absence of any measurable difference in rheologic behavior of cells from diabetic and control subjects, despite substantial differences in nonenzymatic glucosylation of hemoglobin and cell membrane proteins, suggests that the magnitude of glucosylation observed in these cellular constituents does not alter the viscoelastic properties of the cells. The implication of these observations is that microvascular complications of diabetes are not attributable to altered deformability of red cells.

THE MULTIFACTORIAL pathogenesis of vascular complications of diabetes is widely acknowledged. In addition to multiple metabolic and hormonal imbalances, a variety of hemorheologic perturbations also have been described and implicated in the pathogenesis of diabetic vascular diseases. Abnormalities in leukocytes, erythrocytes, and platelets, as well as in plasma proteins, have been reported by many investigators and have been reviewed recently by Jones and Peterson. Most of the abnormalities appear to be the consequence of insulin deficiency and/or hyperglycemia, since they can be reversed by correction of hyperglycemia.

Since red cells must change shape in order to pass through the capillary bed, considerable interest has been focused on the possibility that the diabetic state might impair red cell deformability (i.e., by nonenzymatic glucosylation of hemoglobin and/or membrane proteins) and that stiffened red cells might mechanically traumatize capillaries, thereby contributing to diabetic microangiopathy.

Both increased plasma viscosity and decreased erythrocyte deformability have been reported to contribute to elevated whole blood viscosity in diabetics and, furthermore, to depend critically on metabolic control. This dependence may explain the somewhat conflicting evidence found in recent literature concerning erythrocyte deformability. Using the filtration technique to gauge deformability in a comparative study, Barnes et al. found no significant difference between diabetic and control cells except in a subgroup of 14 diabetic patients with severe complications (retinopathy, nephropathy). Their results were in essential agreement with those of Schmid-Schönbein and Volger, who also used the filtration technique.

In another study employing the filtration technique, Juhan et al. found erythrocyte deformability to be lower and platelet aggregation higher than normal in uncontrolled insulin-dependent diabetics and that controlled insulin infusion reversed both of these abnormalities within 24 hours. These investigators further observed that deformability of normal erythrocytes was reduced by a 30-minute incubation in plasma from uncontrolled insulin-dependent diabetics but was normal in plasma from insulin-dependent diabetics controlled by 24-hour insulin infusion or with insulin added in vitro. Insulin also has been reported to significantly decrease the microviscosity of the membrane lipid layer in both diabetic and normal erythrocytes.

Finding an increase in pressure gradient necessary to cycle individual erythrocytes from diabetic blood through a glass capillary, relative to control cells, McMillan et al. concluded that there was a significant impairment of erythrocyte deformability in diabetes. These investigators also observed that diabetic erythrocytes took longer to recover their discoidal shape after ejection from a 4-μm glass capillary—a sign of increased shear viscosity in the cell membrane.
Although its relationship to membrane shear viscosity has not been defined, membrane microviscosity, determined by fluorescence depolarization, has been reported to be significantly higher in diabetic erythrocytes and to correlate primarily with fasting blood glucose.\(^7\) Using a microcapillary method similar to that of McMillan et al.,\(^6\) La Celle\(^8\) also examined diabetic erythrocytes, but found no evidence that deformability is abnormal in these cells or that membrane elasticity is altered. An earlier study in our laboratory, in which a microrheologic approach was taken, indicated decreased deformability in response to shear stress relative to control erythrocytes of comparable age.\(^9\) However, the presence of substantial numbers of cup-shaped cells among the oldest (10% fraction) diabetic erythrocytes raised questions about a possible osmotic artifact in that study.

The rate of doublet formation among erythrocytes scattered over a microscope slide has been proposed as a novel measure of erythrocyte deformability by McMillan et al.\(^{10,11}\) These investigators found the frequency and velocity of doublet formation to be markedly reduced in populations of diabetic cells relative to normal erythrocytes. These observations were interpreted as indicating an increased resistance to curvature change in the membrane of diabetic erythrocytes.

Sewchand et al.\(^12\) used micropipette manipulation to determine the viscoelastic properties of erythrocytes drawn from diabetics with retinopathy. They concluded that there was no significant abnormality in the elastic and viscous moduli of the erythrocyte membrane in diabetes.

The influence of glucosylated hemoglobin on the deformability of diabetic erythrocytes was investigated by Boudart et al.\(^13\) again by means of filtration.\(^13\) No correlation was found between relative filtration time and cell hemoglobin \(A_1\) (HbA\(_1\)) concentration, even though viscometry of membrane-free hemoglobin solutions revealed that glucosylated hemoglobin fractions had intrinsic viscosities about 2\(\frac{1}{2}\) times those of non-glucosylated fractions.

In none of the aforementioned studies was any attempt made to segregate diabetic erythrocytes by age. Only four groups of investigators\(^8,9,12\) have reported attempts to quantify deformability of diabetic erythrocytes by microrheologic techniques, ie, techniques in which deformations of individual cells are observed under controlled flow conditions, and their findings are not in agreement. The present study was designed to provide a definitive microrheologic characterization of deformability in the diabetic erythrocyte and at the same time to examine the dependence of deformability on cell age. We elected to carry out the study in the rheoscope, an instrument that permits microphotometric recording of the responses of individual erythrocytes to well-defined shear flows.\(^14\) Density-separated erythrocytes were subjected to graded levels of shear stress, and measurements were made of steady-state elongation, frequency of membrane rotation (tank treading), and the time course of shape recovery following instantaneous cessation of shearing. In parallel with these rheologic observations, several physical and chemical properties of the cells were assessed in a search for correlates of rheologic behavior. These properties included mean cell volume, mean corpuscular hemoglobin concentration, concentration of hemoglobin \(A_1\), membrane glucosylation, and cell content of lipid-soluble phosphorus. The latter was measured as an index of mean membrane surface area. In conjunction with the experiments on diabetic cells, corresponding experiments were conducted with normal erythrocytes paired with diabetic cells according to age and sex of donor and also cell age. The results obtained with the control erythrocytes, which specifically address intrapopulation effects of age, are reported in a preceding companion paper.\(^15\)

### MATERIALS AND METHODS

The materials and methods used in this study are exactly as detailed in the companion paper.\(^15\) For economy, those details are not repeated here. Tank-treading frequency measurements were performed as described in an earlier report.\(^14\) It is necessary here only to state that blood samples were taken from ten diabetic subjects (four males and six females), ranging in age from 21 to 67 years and with diabetes of one to 20 years' duration. Four of the subjects required insulin and six were treated with oral agents and diet. All were judged to be moderately to well-controlled metabolically. One of them had background retinopathy, and three of seven on whom muscle biopsies were performed had thickening of muscle capillary basement membranes. Volunteer control donors were matched by sex and age to the diabetics. Differences between diabetics and controls were tested for statistical significance using the Student's \(t\) test. Differences between the oldest and youngest circulating cells were assessed using a paired \(t\) test.

### RESULTS

As was observed in control cells,\(^15\) 100% of the young diabetic cells (top 10% fraction) were capable of tank-treading over the entire range of shear stress generated in the rheoscope (10 to 100 dynes/cm\(^2\)). It should be noted that this range covers approximately the physiologic range of shear stress likely to be experienced by individual red cells in the normal human circulation. Only in the old cell fraction were cells observed that could not be induced to tank tread. As shown in Fig 1, 84.0% ± 9.5% of these cells were tank-treading at the lowest stress (10 dynes/cm\(^2\)), and this percentage increased to 97.0% ± 2.9% at 100 dynes/cm\(^2\). Virtually all of these values fall within 1 SD of the mean (stippled area) for control cells.\(^15\)
ERYTHROCYTE DEFORMABILITY IN DIABETES

Figure 2 shows plots of projected length-width ratio vs applied shear stress for both young and old cell fractions. Again, most of the data points fall within 1 SD of the mean (stippled area) for corresponding data from control subjects. The same is true of the tank-treading frequency (TTF) data displayed in Fig 3. In this figure, the corresponding control data, represented by the stippled bands, are taken from Tran-Son-Tay's dissertation. Shape recovery time data for diabetic and control cells are compared in Table 1. Values for diabetics and controls are virtually identical, with the recovery times for older cells being significantly longer than those of the younger cells.

Results of the other physical and chemical assays performed are summarized in Table 2. The youngest and oldest cells showed statistically significant differences in every parameter assessed. The oldest and youngest diabetic cells were significantly smaller and contained less lipid-soluble phosphorus than corresponding cells from control subjects. Glucosylation of hemoglobin was significantly increased in the youngest as well as in the oldest circulating cells of diabetics compared to controls. On the other hand, while glucosylation of membrane protein was increased in old cells of diabetics relative to nondiabetics, differences between diabetics and nondiabetics were not statistically significant for the youngest 10% of cells. Mean cell hemoglobin concentration (MCHC) was slightly higher in the oldest 10% fraction of cells from diabetics than in corresponding cells from controls.

Correlation coefficients (r) for HbA1 and for membrane protein glucosylation vs recovery times and length/width (L/W) were not statistically significant for the youngest or for the oldest 10% fractions of cells from diabetics or for pooled data from diabetics and controls.

**DISCUSSION**

The rheoscopic observations summarized in Figs 1 through 3 and in Table 1 indicate that the rheologic behavior of the youngest and oldest circulating erythrocytes from diabetic subjects is virtually identical in every respect to that of control cells from nondiabetic subjects. Although the curves of steady-state elongation

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**Fig 1.** Percentage of "old" red cells tank treading vs applied shear stress. Plotted points are means and bars represent 1 SD. Numbers in parentheses indicate donors. All young cells (top 10% fraction) were tank treading at all levels of applied stress. The stippled band represents the mean ± 1 SD of corresponding data from control subjects (Fig 2, reference 15).

**Fig 2.** Projected length-width ratio of tank-treading cells as a function of applied shear stress. Plotted points are means computed from means of seven or ten donors (numbers in parentheses); bars represent 1 SD. The stippled band indicates the mean ± 1 SD for corresponding curves for the control cells (Fig 3, reference 15).

**Fig 3.** Tank-treading frequency as a function of applied shear rate. Plotted points are means, and bars represent 1 SD of mean TTF values from seven donors. Stippled bands indicate spread (mean ± SD) of corresponding data from control subjects.
Table 2. Physical and Chemical Properties of Young and Old Populations of Erythrocytes From Diabetic Subjects

<table>
<thead>
<tr>
<th></th>
<th>Young (Top 10%)</th>
<th>Old (Bottom 10%)</th>
<th>t</th>
<th>Significance Level (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cell volume (μm²)</td>
<td>91.71 ± 3.25§</td>
<td>90.07 ± 3.41†</td>
<td>3.18</td>
<td>&lt;.025</td>
</tr>
<tr>
<td>Mean cell hemoglobin concentration (g/dL RBCs)</td>
<td>32.37 ± 0.89</td>
<td>33.39 ± 0.63‡</td>
<td>2.57</td>
<td>&lt;.050</td>
</tr>
<tr>
<td>HbA₁ (percentage of total Hb)</td>
<td>7.99 ± 1.92‡</td>
<td>10.91 ± 2.27§</td>
<td>3.26</td>
<td>&lt;.010</td>
</tr>
<tr>
<td>Membrane glucosylation (nmol HMF/mg protein)</td>
<td>2.62 ± 1.28</td>
<td>5.88 ± 0.87†</td>
<td>8.54</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Lipid-soluble phosphorus (mg Pi per RBC × 10¹⁰)</td>
<td>3.60 ± 0.16‡</td>
<td>2.74 ± 0.16†</td>
<td>17.26</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

HMF, hydroxymethylfurfural.

*Entries represent mean ± SD calculated from the mean values of ten donors. Numbers in parentheses are corresponding data for cells from control subjects (reference 16, Table 1).

†Different from value for control subjects (in parentheses) P < .025.
‡Different from value for control subjects (in parentheses) P < .01.
§Different from value for control subjects (in parentheses) P < .005.

Young旧.

The data plotted in Fig 3 clearly indicate no significant difference between diabetic and control erythrocytes of comparable age. It is of interest to note here a principal conclusion of our earlier study of variance of TTF in normal cell populations, viz, that TTF measurements may be useful in distinguishing rheologically abnormal cell populations that differ from normal cells by at least 10%. In view of these insignificant differences in rheologic behavior of red cells from diabetics and control subjects, it would seem reasonable to conclude that none of the more substantive differences in membrane and hemoglobin glucosylation, cell volume, and lipid-soluble phosphorus content between diabetic and control cells significantly affects the viscoelastic properties of diabetic red cells. In particular, it appears that glucosylation of red cell constituents is of no consequence to the rheologic behavior of red cells.

These observations and conclusions are consistent with those of LaCelle and Sewchand et al., but are not in accord with those of Schmid-Schönbein, McMillan, and Juhan et al. It is of course possible that subtle differences in physical properties of diabetic and control cells might not be detected by the rheoscope. On the other hand, even if there are subtle differences in physical properties or rheologic behavior of diabetic red cells, it seems unlikely that they would contribute to capillary basement membrane thickening (CBMT), one of the well-characterized vascular complications of diabetes. If altered red cell deformability was a significant factor in the pathogenesis of CBMT, one would predict that the magnitude of CBMT should be comparable in virtually all tissues of diabetics. The finding that CBMT varies greatly in different skeletal muscle capillaries and is much more pronounced in the lower extremities than in trunk muscles of diabetics strongly suggests that local environmental factors are more important in the pathogenesis of CBMT than any changes in physical characteristics of red cells, which should affect capillaries in all tissues, and especially muscles, to the same extent. By the same logic, we would suggest that any other alterations in the macro- rheologic properties of blood, ie, viscosity of whole blood or plasma, in diabetics are unlikely to be of importance in the pathogenesis of CBMT. Lastly, in view of the very substantial differences in viscoelastic properties of the youngest and oldest circulating cells from normal subjects and from diabetics, and the role of the spleen in eliminating the oldest circulating cells on the basis of their impaired deformability, it is unlikely that diabetes could render cells any less deformable than the oldest circulating cells in normal subjects or, if it did, that such cells could remain in the circulation long enough to damage vessels. Consistent with this thesis, Peterson et al reported that the half-life of ⁵¹Cr-labeled red cells was shortened in diabetics and was lengthened by 15% when efforts to normalize blood glucose levels reduced hemoglobin A₁ levels from 10.1% before treatment to 5.6% after better control was achieved.

The possibility that we might not have observed red cell changes in diabetic subjects because the severity of diabetes in the population examined was unusually mild is also unlikely. HbA₁ values for the oldest 10% fraction of cells were approximately 14% and 15% in
two subjects, and the difference in membrane protein glucosylation for controls and diabetics was almost twofold, which is in good agreement with the differences reported by Miller et al.\textsuperscript{22} The actual range of membrane glucosylation values for the oldest 10% of circulating cells varied from a low of 1.82 (in a control subject with $t_c$ of 0.151 seconds) to 7.29 nmol HMF/mg protein (in a diabetic with $t_c$ of 0.176 seconds). Despite this fourfold range in membrane glucosylation, there was no association between membrane glucosylation and estimates of $t_c$, L/W, TTF, and percent of tank-treading cells. This wide range of membrane glucosylation values also implies a comparable spread in mean 24-hour blood glucose values that should encompass subjects at increased risk of developing vascular complications. Indeed, background retinopathy was present in one diabetic and muscle capillary basement membrane data available on seven diabetics demonstrated marked basement membrane thickening in two and early thickening in a third. Thus, four of the ten diabetics had evidence of microvascular disease.

It is of interest that while substantial differences in glucosylation of hemoglobin as well as membrane proteins were observed between the oldest 10% of cells from diabetics and controls, correspondence for the youngest 10% of cells was not as good. The difference in glucosylation of hemoglobin was appreciable and statistically significant in the youngest cells, while the difference in glucosylation of membrane proteins was minimal. The explanation for this apparent discrepancy may lie in differences in the specificity of the methods used to assess glucosylation of hemoglobin and membrane proteins. The high-pressure liquid chromatography (HPLC) method used to assess glucosylation of membrane protein is highly specific for ketoamine linkages, the stable end-product of the two-step nonenzymatic glucosylation reaction. An ionic exchange column chromatographic method was used to assess glucosylation of hemoglobin. In addition to the slowly formed stable end-product of the nonenzymatic glucosylation reaction, this method also measures the highly labile Schiff base intermediate. These highly dissociable Schiff base intermediates can form within a few hours after exposure to high blood glucose and likewise dissociate just as rapidly after exposure of cells to low glucose levels. The kinetics of formation and dissociation of the Schiff base intermediates are such that they are 60 times more likely to dissociate than to form the highly stable ketoamine end-product.\textsuperscript{23} The relative contents of these two glucose adducts in normal red cells are estimated at 0.4% and 4.0%, respectively,\textsuperscript{23} but would differ for young vs old circulating cells. Thus, the marked differences in blood glucose levels of diabetics and controls would be much more likely to lead to substantial differences in the content of labile Schiff bases (which would have been measured in hemoglobin) than in glucose bound in ketoamine linkage (which was measured in the membranes) during the relatively short life of the youngest 10% of circulating cells examined.

The slight decrease in lipid-soluble phosphorus content of diabetic red cells implies that they have less membrane surface area; however, this was not associated with any change in rheologic behavior. The smaller volume measured for diabetic red cells differs from the report of Davidson et al.\textsuperscript{24} that diabetic cells were very slightly larger than those of normal controls.

In contrast to the minimal differences, if any, in rheologic behavior of red cells from diabetics and controls, the differences in rheologic behavior of the young and old cells of both control and diabetic populations are striking. These results, together with the findings of substantial differences in the extent of glucosylation of membrane proteins of diabetic and nondiabetic subjects, suggest that membrane glucosylation is not an influential factor in the deformability of erythrocytes in diabetes. This interpretation is consistent with the conclusion of McMillan and Brooks\textsuperscript{25} that glucosylation of the membrane protein spectrin does not modify its function.

REFERENCES


Microrheologic investigation of erythrocyte deformability in diabetes mellitus

JR Williamson, RA Gardner, CW Boylan, GL Carroll, K Chang, JS Marvel, B Gonen, C Kilo, R Tran-Son-Tay and SP Sutera