Low-Frequency Submicron Fluctuations of Red Blood Cells in Diabetic Retinopathy

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Objective: To characterize cell membrane mechanical fluctuations of red blood cells (RBCs) in patients with diabetic retinopathy.

Methods: Point dark-field microscopy–based recordings of these local displacements of the cell membrane in human erythrocytes were compared between patients with severe proliferative diabetic retinopathy and healthy controls. The study was performed on discoid RBCs.

Results: The average of the maximal displacement amplitude in the diabetic patients was 13.9% ± 1.7% (236 ± 29 nm) compared with 18.7% ± 1.75% (318 ± 30 nm) for the controls (P<.001). The decrease of the RBCs’ average displacement amplitude was not correlated with the variation in negative curvature of the central area of discoid cells.

Conclusions: Microdisplacements of the cell membrane, which reflect the bending deformability of the RBCs, are directly connected with its efficiency in passing through capillaries narrower than its own diameter. These microdisplacements were significantly reduced in patients with severe diabetic retinopathy because of an increase in viscoelasticity of the cell membrane. Such reduced cell membrane microdisplacements, which reflect lower bending deformability of the RBC, reduce the ability of the cell to enter and pass through small capillaries, increasing tissue ischemia and consequently contributing to the development of diabetic retinopathy.


DIABETIC retinopathy is among the most common diabetic complications and one of the leading causes of vision loss and blindness in industrialized countries. Hemodynamic changes are believed to play an important role in its pathogenesis. However, the retinal blood flow abnormalities, the factors that mediate them, and their role in the development of end-stage retinopathy are still unclear.1-3 Focal areas of capillary closure and nonperfused capillaries appear to develop relatively early after the onset of diabetes. It was postulated that hypoxic and ischemic retinas adjacent to such areas release vasoactive agents and growth factors that increase blood flow and initiate vasoproliferative changes. Again, the exact mechanism responsible for the capillary closure and nonperfusion, and whether these changes are preceded and caused by blood flow changes, is not fully understood. Increased blood viscosity was found only in the deoxygenated blood of patients with diabetic retinopathy and not in healthy or diabetic patients without retinopathy.4 It also has been suggested that the ability of red blood cells (RBCs) to change their shape (deformability) is decreased in diabetes. Such an impairment of the RBCs’ deformability might be another contributing factor to reduction of blood flow in the capillaries.5-6

The anatomical capillary luminal diameter is between 2.5 and 9 µm,7,8 while the functional luminal diameter is 0.8 to 1.0 µm smaller.9 However, the average diameter of the discoid RBCs was reported by Weinstein9 to be about 8.07 ± 0.43 µm or 8.56 ± 0.21 µm, which is in agreement with our own measurements of 8.3 ± 0.4 µm (see the “Patients and Methods” section). Therefore, it is clear that even under normal conditions, the RBCs are subject to substantial morphological changes to facilitate their passage through the capillaries. In diabetic microangiopathy, the capillary diameter decreases even further because of thickening of the basal membrane and the accumulation of metabolites on the endothelial surface of the diabetic eye.10 Thus, a decrease in RBCs’ deformability may be an important factor in the sequence of events leading to diabetic retinopathy.

Previous studies that investigated erythrocyte deformability in diabetic patients11-23 yielded conflicting results. In most of these studies, only some aspects of RBC deformability were studied, and the investigations included only a small number of diabetic patients, with or without microcirculatory decompensation as evidenced by end-organ involvement.

In the present study, we investigated the deformability of RBCs in diabetic patients by means of a novel technique that measures...
PATIENTS AND METHODS

PATIENTS

Two groups of patients were included in the study. The first group included all consecutive patients (n = 12) with diabetic retinopathy who were scheduled for vitrectomy in the ophthalmology department of the Tel Aviv Medical Center, Tel Aviv, Israel, from September 1, 1995, through the following 6 weeks, because of an unresolved vitreous hemorrhage or a traction retinal detachment involving the macula. These patients were compared with a second group of patients (n = 9) who were of similar age, were nondiabetic, and attended the same ophthalmology department for cataract extraction. This group served as controls. Consent was obtained from all subjects after the nature of the procedure was carefully explained.

BLOOD WITHDRAWAL

A sample of 3 mL of venous blood was drawn from each patient in either EDTA (5 mmol/L) or heparin solution (15 U/mL) and immediately transported to the laboratory. Measurements were performed within 2 to 3 hours after blood withdrawal.

PREPARATION OF RBCs FOR DEFORMABILITY MEASUREMENT

Of the sample of the withdrawn blood, 50 to 100 µL was diluted in 1.5 mL of phosphate-buffered saline solution (PBS; 130-mmol/L sodium chloride, 10-mmol/L glucose, 5.5-mmol/L phosphate buffer [pH 7.4], 1-mg/mL bovine serum albumin) filtered through 0.2-µm pores (Millipore filters; Millipore Corporation, Bedford, Mass). The blood was then washed twice with PBS, followed by 2 successive centrifugations (1500 rpm, 2 minutes, 28°C). The Buffy coat and 10 to 20 µL of packed cells were gently removed. The RBC suspension, diluted in PBS, was then introduced into the experimental chamber consisted of 2 coverglasses separated by a distance of 0.2 mm. Preincubation of RBCs for 20 to 30 minutes in the chamber at 24°C to 27°C was performed to allow the cells to become attached to the coverglass. To avoid the effects of cell shrinkage that occur because of the fixation and dehydration of RBCs, the diameter of studied living RBC varied. For in vitro measurement of variations in the biconcavity of discocytes (RBCs of neonates) approaches 100% (Ic/Ir). The biconcavity of discoid RBC is the result of negative curvature in the central part of the RBC. Biconcavity is proportional to the ratio of the thickness of the peripheral cell rim to that of the central area of the discoid RBC. The light-scattering intensity of the RBC depends on the thickness of the central and peripheral areas of the cell. We measured the central area thickness by registering the intensity of light scattering from 1 µm² in the middle of the cell. The thickness of the peripheral rim was estimated as a maximal intensity of the light scattering by scanning the 1-µm² light spot in radial direction over the peripheral rim. The maximal value of light-scattering intensity corresponds to a middle position of the 1-µm² light spot on the cell rim at the plane of the RBC. The sensitivity of measurements of maximal intensity of scattered light by scanning of the light spot on the cell rim was about 10%, and the position of the light spot was controlled microscopically by magnification of ×1125. Thus, the biconcavity was determined as a ratio of the light-scattering intensities in the peripheral rim (Iₚ) and in the central area of the cell (I₉), normalized to the light intensity in the rim (1 – Iₚ/I₉)%.

The probability of null hypothesis (P) was calculated by means of the t test of significance between control and diabetic sample means.

MEASUREMENTS OF VARIATIONS IN THE BICONCAVITY OF DISCOID RBCs

The biconcavity of discoid RBC is the result of negative curvature in the central part of the RBC. Biconcavity is proportional to the ratio of the thickness of the peripheral cell rim to that of the central area of the discoid RBC. The light-scattering intensity of the RBC depends on the thickness of the central and peripheral areas of the cell. We measured the central area thickness by registering the intensity of light scattering from 1 µm² in the middle of the cell. The thickness of the peripheral rim was estimated as a maximal intensity of the light scattering by scanning the 1-µm² light spot in radial direction over the peripheral rim. The maximal value of light-scattering intensity corresponds to a middle position of the 1-µm² light spot on the cell rim at the plane of the RBC. The sensitivity of measurements of maximal intensity of scattered light by scanning of the light spot on the cell rim was about 10%, and the position of the light spot was controlled microscopically by magnification of ×1125. Thus, the biconcavity was determined as a ratio of the light-scattering intensities in the peripheral rim (Iₚ) and in the central area of the cell (I₉), normalized to the light intensity in the rim (1 – Iₚ/I₉)%.

The probability of null hypothesis (P) was calculated by means of the t test of significance between control and diabetic sample means.

The biconcavity of discoid RBC varied. For instance, when the biconcavity of flat discocytes is negligible (Iₚ ≈ I₉), neither excess of membrane area nor membrane negative curvature exists. However, the biconcavity of enlarged discocytes (RBCs of neonates) approaches 100% (Iₚ ≈ I₉).

STATISTICAL ANALYSIS

The probability of null hypothesis (P) was calculated by means of the t test of significance between control and diabetic sample means.
mechanical fluctuations of the cell membrane, which reflect the bending membrane deformability.25-28 These fluctuations consist of reversible fast local displacements (frequency range of 0.3–30 Hz) of the cell membrane and were observed in different types of cells.24,26 The similar values of the membrane-bending modulus (\(K_c = 2-3 \times 10^{-12} \text{erg}\)) were obtained from micropipette and mechanical fluctuation measurements.28,29 Cell membrane fluctuations (CMFs) were demonstrated to directly correlate with the filterability of RBCs30 and lymphoid cell lines.31 Thus, lower displacement amplitudes of the cell membrane would be expected to be associated with a decreased ability of RBCs to pass through blood capillaries and a consequently less efficient oxygen delivery to the tissues.

The purpose of the present study was to characterize CMFs in patients with severe proliferative diabetic retinopathy, to the extent that the patients were in need of vitrectomy for either an unresolved vitreous hemorrhage or a traction retinal detachment involving the macula. We chose this group of patients in particular because we assume that, in such severe end-organ involvement, reduced ability of the RBCs to pass through capillaries might be a contributing factor. Significant differences between nondiabetic and diabetic subjects, if they exist, are apt to be more pronounced in this group of patients than in those with less severe end-organ involvement.

**RESULTS**

We measured the local displacements of the cell membrane in biconcave discoid RBCs. Point dark-field microscopy–based recordings of these local displacements, in human erythrocytes, were measured in a group of 12 patients with severe proliferative diabetic retinopathy compared with those in 9 healthy controls.

The mean cell membrane mechanical fluctuations (\(|\Delta I/I|\)), measured at the outer edge of the RBC rim in patients with severe diabetic retinopathy, was 13.9% ± 1.7%, in comparison with 18.7% ± 1.75% in controls (\(P<.001\)) (Figure 1). Membrane displacement in control cells (318 nm) decreased to 236 nm in the RBCs of patients with severe proliferative diabetic retinopathy. No statistical difference was found between the coefficients of variation in diabetic and control groups (0.17 ± 0.05 and 0.15 ± 0.04, respectively; \(P = .45\)).

For blood taken with heparin from patients with severe diabetic retinopathy (\(n = 7\)), the average displacement amplitudes were 13.7% ± 0.88% vs 14.1% ± 2.5% in blood taken with EDTA (\(n = 5\)) (\(P = .73\)). For blood taken with heparin in controls (\(n = 4\)), the average displacement amplitudes were 17.8% ± 1.8% vs 19.3% ± 1.5% in blood taken with EDTA (\(n = 5\)) (\(P = .21\)). Thus, no difference in fluctuation amplitude was found when the anticoagulant was either heparin or EDTA (Figure 2).

The amplitude of flickering, measured as \(|\Delta I/I|\)% in the central region of the biconcave erythrocytes, was 14.8% ± 5.44% in the control group vs 13.4% ± 6.23% in the diabetic group (\(P = .61\)). Thus, the main decrease of membrane displacement amplitude in RBCs of patients with diabetic retinopathy was observed only in the cell’s periphery.

The amplitude of CMFs depends not only on the viscoelastic properties of the membrane and its skeleton but also on its curvature, ie, on cell shape.23 In our control group of 80 discocytes, the biconcavity of the discoid RBC varied in the range of \([1 - (I_c/I_r)]\)% = 10% to 80%. That is, the negative curvature of the cell membrane in the central part of the cell varied from cell to cell (Figure 3, top). However, the amplitude of CMF, \(|\Delta I/I|\), measured at the cell’s periphery, did not depend on biconcavity, \(\{1 - (I_c/I_r)\}\)% as can be seen from the fitting of the obtained data: \(|\Delta I/I|\)% = \(19.3% ± 1.9%\) − \(0.026 ± 0.027 \times [1 - (I_c/I_r)]\). A similar independence was observed among the 87 cells of diabetic patients, \(|\Delta I/I|\)% = \(11.7% ± 1.1%\) + \(0.02 ± 0.02\) \times \([1 - (I_c/I_r)]\) (Figure 3, bottom). Fluctuation amplitude extrapolated to biconcavity of 0% (ie, to a biconcavity of flat discoid RBCs) was 11.7% ± 1.1% in diabetic RBCs. This value is 1.6-fold smaller than the value obtained for control RBCs (19.3% ± 1.9%). This suggests that the observed decrease of fluctuation amplitude in diabetic cells is attributed to the increase of the viscoelasticity of the membrane-skeleton complex, rather than to the changes in RBC biconcavity.

It is also possible that in diabetes, the percentage of older, more dense RBCs is higher, and that these are the RBCs that are less deformable. A more dense, older RBC should be accompanied by an increase in light-scattering intensity, and thus there would be an inverse
dependence between $(\Delta I/I)\%$ and intensity of light scattering $(I)$. However, we did not find any correlation between $(\Delta I/I)\%$ and $I$ in either control or diabetic RBCs (Figure 4). Data of control and diabetic cells were fitted as $(\Delta I/I)\% = (19.9\% \pm 1.3\%) - (0.17 \pm 0.16) \times I$ and $(\Delta I/I)\% = (14.1\% \pm 1.1\%) \times (0.03 \pm 0.13) \times I$, respectively; i.e., the slope of dependence of $(\Delta I/I)\%$ on $I$ is negligible. That is, the CMF amplitude does not depend on the light-scattering intensity and thus is independent of possible changes in cell density. Accordingly, our conclusion is that the decrease in fluctuation amplitude cannot be attributed to possible enrichment in older, more dense cells in the diabetic RBC population of discoid RBCs.

**COMMENT**

Cell membrane fluctuations are driven by metabolic, adenosine triphosphate–dependent forces in addition to the thermal ones.\textsuperscript{25,27,32} This adenosine triphosphate dependence is demonstrated by the CMF registered on the cell edge. Our results show a reduction of 25.7% in the amplitude of membrane mechanical fluctuations in RBCs of diabetics with severe proliferative diabetic retinopathy relative to control RBCs from healthy donors. The observed decrease of CMF in RBCs of diabetic patients is found only at the cell rim and not in the central area of discocytes. We suggest that the metabolic component of CMF is decreased in RBCs of diabetic patients with severe diabetic retinopathy.

<table>
<thead>
<tr>
<th>Source, y</th>
<th>Patients</th>
<th>Solution</th>
<th>Method (Filter Pore Size)</th>
<th>Result</th>
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<tr>
<td>Schut et al,13 1993</td>
<td>DM with and without end-organ damage vs controls</td>
<td>PBS Filterability (5 µm) and ektacytometry</td>
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<td>MacRury et al,\textsuperscript{21} 1990</td>
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<td>Garnier et al,\textsuperscript{12} 1990</td>
<td>DM vs control</td>
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<td>Kikuchi et al,\textsuperscript{23} 1990</td>
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<tr>
<td>Stone et al,\textsuperscript{15} 1986</td>
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<td>Bareford et al,\textsuperscript{17} 1986</td>
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<tr>
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<td>Tillmann et al,\textsuperscript{19} 1981</td>
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<td>McMillan et al,\textsuperscript{14} 1978</td>
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<td>Schmid-Schonbein and Volger, 11 1976</td>
<td>DM</td>
<td>Plasma Filterability (5 µm)</td>
<td>Decreased deformability</td>
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* DM indicates diabetes mellitus; PBS, phosphate-buffered saline.
The most common technique for assessing RBC deformability uses filterability measurements. Three other, less popular techniques are ektacytometry, rheoscopy, and micropipette aspiration. Various studies that investigated RBC deformability in diabetic patients are summarized in the Table. The studies reviewed were only those that measured washed RBCs and do not include investigations that measured the filterability of whole blood. About one half of the studies demonstrated decreased RBC deformability, while the others did not find any change in filterability.

Thus, it is yet unknown whether changes in deformability of RBCs exist in diabetic patients with or without retinopathy.

In filtration studies, the RBC suspension (in PBS or in plasma) is passed through 3-µm- or 5-µm-diameter micropores. The 4 studies11,18,19,23 that used RBC suspension in plasma showed decreased filterability, while the 9 studies that used RBC suspension in PBS or Ringer solution found conflicting results. From these data, it is possible to contend that the external medium may have an effect on RBC filterability in diabetics.

Our results demonstrate that the decrease in CMFs in diabetics is not correlated with curvature changes of control or diabetic discoid RBCs (Figure 3), nor with an increase in cell density (Figure 4). Thus, the changes demonstrated in CMFs may be explained by an increase in the metabolic-dependent viscoelasticity of the membrane structure in diabetic RBCs. The increase in viscoelasticity of the cell membrane possibly consists of an increase in the membrane-bending rigidity25,27,29 and also of an increase of membrane microviscosity.34,35 Both can lead to plugging of capillaries by RBCs.

It is not clear whether the reduced deformability of RBCs reflects another “end-organ damage” resulting from a long-standing hyperglycemic state concomitant with retinopathy, or whether it is one of the factors directly responsible for tissue ischemia, which results in retinopathy. In either case, reduced RBC deformability might impede blood flow in the already disturbed microcirculation of diabetic patients, thereby contributing to the progression of retinopathy.

It is our impression that the finding of decreased CMFs of RBCs in patients with severe diabetic retinopathy is an important step in understanding this complicated and multifactorial disease state.

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