MORPHOLOGY AND PATHOMORPHOLOGY

Structural and Mechanical Characteristics of Erythrocyte Membranes in Patients with Type 2 Diabetes Mellitus

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The morphology and structure of erythrocyte cytoskeleton and local mechanical characteristics in patients with type 2 diabetes mellitus and donors were studied by atomic force microscopy. Poikilocytosis and anisocytosis, spatial reorganization of the cytoskeleton (loosening and condensation of the actin-spectrin network), and modification of local mechanical properties of erythrocytes were characteristic of diabetics. These results indicate significant heterogeneity of erythrocyte population in the patients, most likely due to the presence of erythrocytes of different age groups, which can promote the development of diabetes complications (angiopathy).

Key Words: *type 2 diabetes mellitus; erythrocytes; cytoskeleton; local mechanical characteristics; atomic force microscopy*

Oxidative stress in cells and tissues plays an important role in the pathogenesis of diabetes mellitus (DM). Oxidative stress manifests by increased levels of free radicals and LPO; it suppresses glycolysis, protein and nucleic acid production, and enzyme activities and promotes oxidation-phosphorylation uncoupling. The rate of free radical formation during oxidative stress surpasses the rate of their neutralization by the antioxidant system. Oxidative stress is regarded not only as the main mechanism of delayed complications of DM, but also as a factor underlying the development of DM. For example, disturbances of the cell antioxidant defense system are observed in patients with type 2 DM (DM-2) and their close relatives [3,7]. Hyperglycemia promotes hyperproduction of active oxygen and nitrogen forms, including superoxide anion radical, nitrogen monoxide, and peroxinitrite. Peroxinitrite (product of diffuse-controlled reaction of superoxide anion radical and nitrogen monoxide) is characterized by high chemical activity towards all biologically significant molecules and its life span is sufficient for diffusion to distances comparable with the cell size. Peroxinitrite is hypothesized to be one of the main factors in the development of DM complications, including angiopathies [4,8].

Erythrocyte dysfunction is a component of angiopathy development. Toxic effects of glucose on erythrocytes manifest in restructuring of the erythrocyte membranes, disorders in hemoglobin oxygen-binding activity, modification of mechanical characteristics of the membrane and cell in general. Our previous atomic force microscopy (AFM) studies showed that peroxinitrite modifies structural

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and mechanical characteristics of erythrocyte membrane and cytoskeleton and erythrocyte shape [1,2].

Here we studied structural and mechanical state of erythrocytes in patients with DM-2 by AFM.

MATERIALS AND METHODS

Group 1 (n=15; mean age 58.3±7.4 years) was formed from DM-2 patients followed up at Regional Clinical Endocrinology Center (Gomel). Control groups consisted of volunteers without DM-2 aged over 50 years (group 2; n=6; mean age 61.0±6.6 years) and under 25 years (group 3; n=3).

Capillary blood of DM-2 patients and donors was collected into a fine capillary. In parallel, blood sugar was measured on a BIOSEN-5030 biochemical analyzer. Cells for AFM were fixed in 1% glutaraldehyde. Erythrocytes were washed once in a buffer and twice in distilled water, placed onto slides, and dried.

The preparations were examined under an NT-206 atomic force microscope (MicroTestMachine) in the contact scanning mode using CSC38 needles (MicroMash). The topography, AFM console vertical deviation map, and lateral force map of the cell surface were recorded. Fractal analysis of lateral force maps was carried out for quantitative evaluation of cell surface structure (0.5×0.5 μ fragments were cut out from 1.5×1.5 μ maps with 256×256 pixels resolution). Fractal dimension (D_F) was calculated using SurfaceXplore 1.3.11 software (MicroTestMachine) by the Surface-Perimeter method, with surface division into 200 horizontal layers.

Mechanical properties of membrane sites were quantitatively evaluated by statistical force spectroscopy [2] on horizontal sites of the cell surface (convex parts of erythrocytes). The mechanical characteristics of experimental and control samples were compared by the apparent elasticity (E) modulus at probe depth of 10 nm. The data are presented as $E'=E/E_0$, where E_0 is selected mean value of apparent elasticity modulus of control samples (2D).

Seleced characteristics were compared using Student's t test for independent groups and Fisher's test.

RESULTS

Erythrocyte polymorphism (poikilocytosis and anisocytosis) is characteristic of patients with DM-2. A total of 62% normocytes (discocytes) were detected in the blood of DM-2 patients *vs.* 72% in donors. "Crest" erythrocytes, spherocytes, planocytes, macrocytes, and microcytes were detected among modified erythrocyte forms (Fig. 1).

Glutaraldehyde fixation of erythrocytes preserved the cytoskeleton structure. The surface of the dried fixed cell represented a typical two-dimensional reticular structure, better seen on lateral force maps. The lateral force values in each point of the cell surface (determined by torsion deformations of AFM console) were caused not only by the relief characteristics, but also by the resistance of the membrane-cytoskeleton system.

Erythrocyte cytoskeleton structure in patients with DM-2 was modified in comparison with erythrocyte cytoskeleton structure in donors of groups 2 and 3. Structures similar to those in donors were seen in erythrocytes of patients with DM-2 (Fig. 2, *a*, *b*); condensation (Fig. 2, *c*) and loosening of the cytoskeleton network were noted (Fig. 2, *d*). Fractal dimension of the lateral force maps was used for quantitative evaluation of the cytoskeleton structure (Table 1).

Group 1 was divided into 2 subgroups because of heterogeneous D_F dispersion in the group. The distribution of standard D_F deviation in DM-2 patients was multimodal with a clear-cut minimum at SD=0.125. This standard deviation value was selected as the boundary for the two subgroups. Differences in the dispersion in subgroup 2 were statisti-

TABLE 1. Fractional Dimension of Lateral Force Maps for Erythrocytes and Blood Sugar Level in Patients (M±m)

	Group	D_F	SD	Number of patients in sampling	Blood sugar, mmol/liter
1	subgroup 1 (SD<0.125)	2.859	0.089*	64 (5)	6.6±1.3+
	subgroup 2 (SD>0.125)	2.854	0.161	259 (10)	8.8±2.3+
2		2.848	0.107*	110 (6)	5.0±0.6
3		2.837	0.116*	56 (3)	4.6±0.6

Note. *p<0.005 compared to subgroup 2 (group 1) according to Fisher's test; +p<0.03 compared to group 2 (Student's t test for independent variables).

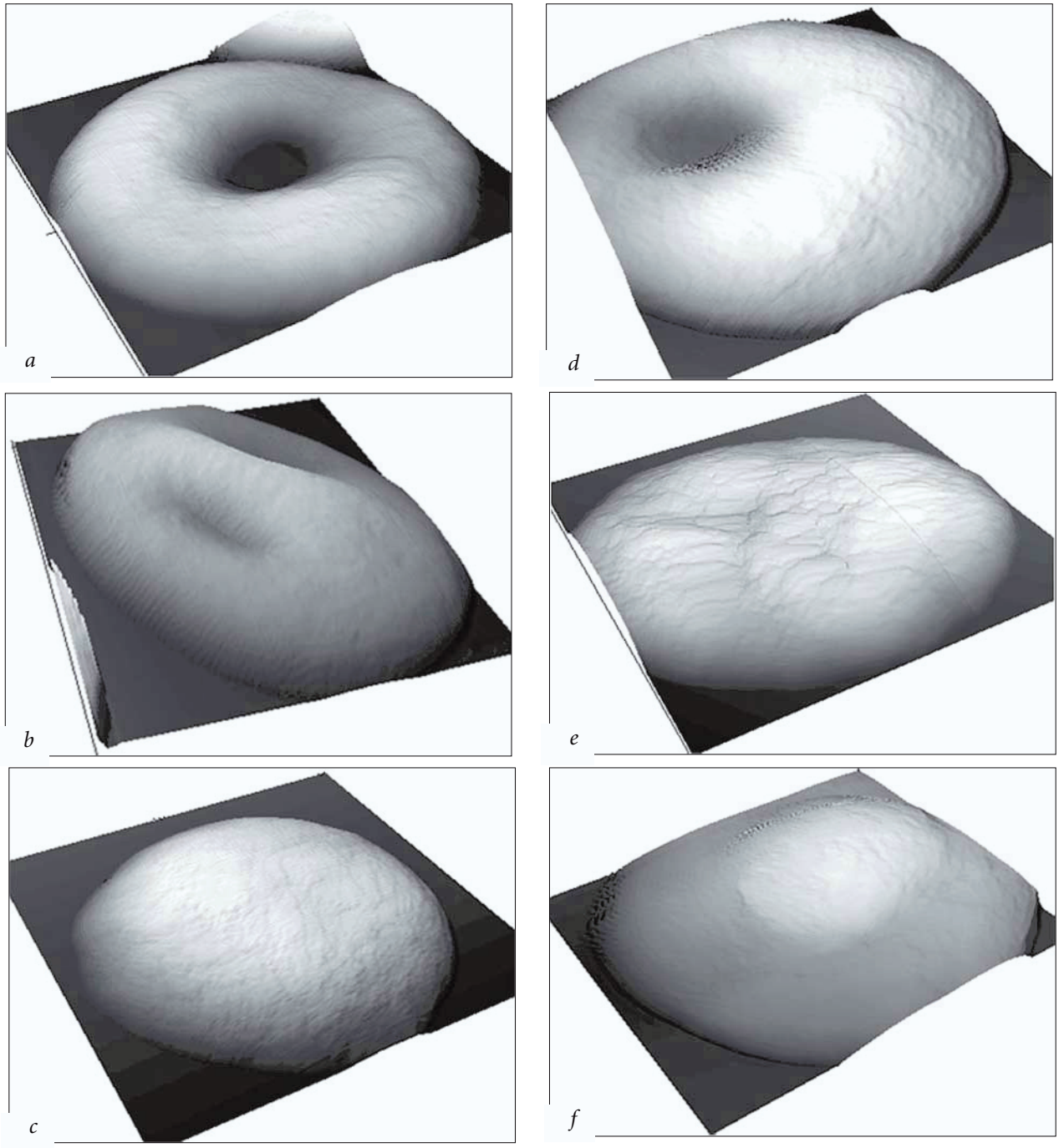

Fig. 1. Topography of some erythrocytes in the blood of DM-2 patients. *a*) discocyte, 6.9×6.9 μ image; *b*) "crest" erythrocyte (7.7×6.0 μ); *c*, *d*) spherocytes of different stages (6.7×6.7 μ and 5.8×5.8 μ); *e*) planocyte (9.5×9.5 μ); *f*) target-like erythrocyte (6.9×5.8 μ).

cally significant in comparison with subgroup 1 and groups 2 and 3 (Table 1). On the other hand, no statistically significant differences in D_F means were detected for all studied groups. Blood sugar levels in both subgroups of DM-2 patients were significantly higher than in the controls.

Elastic characteristics of erythrocytes are determined mainly by the elastic characteristics of the cytoskeleton. Modification of the cytoskeleton structure leads to modification of the elastic characteristics of erythrocytes. Distribution of the apparent local elasticity moduli (E) for DM-2 patients exhibited a trend to an increase of the mean value in comparison with E distribution for donor erythrocytes (Fig. 3). Similarly as with the fractal dimension, E dispersions of experimental group (DM-2) differed from the control (2D). The E value for erythrocytes from DM-2 patients shifted stronger

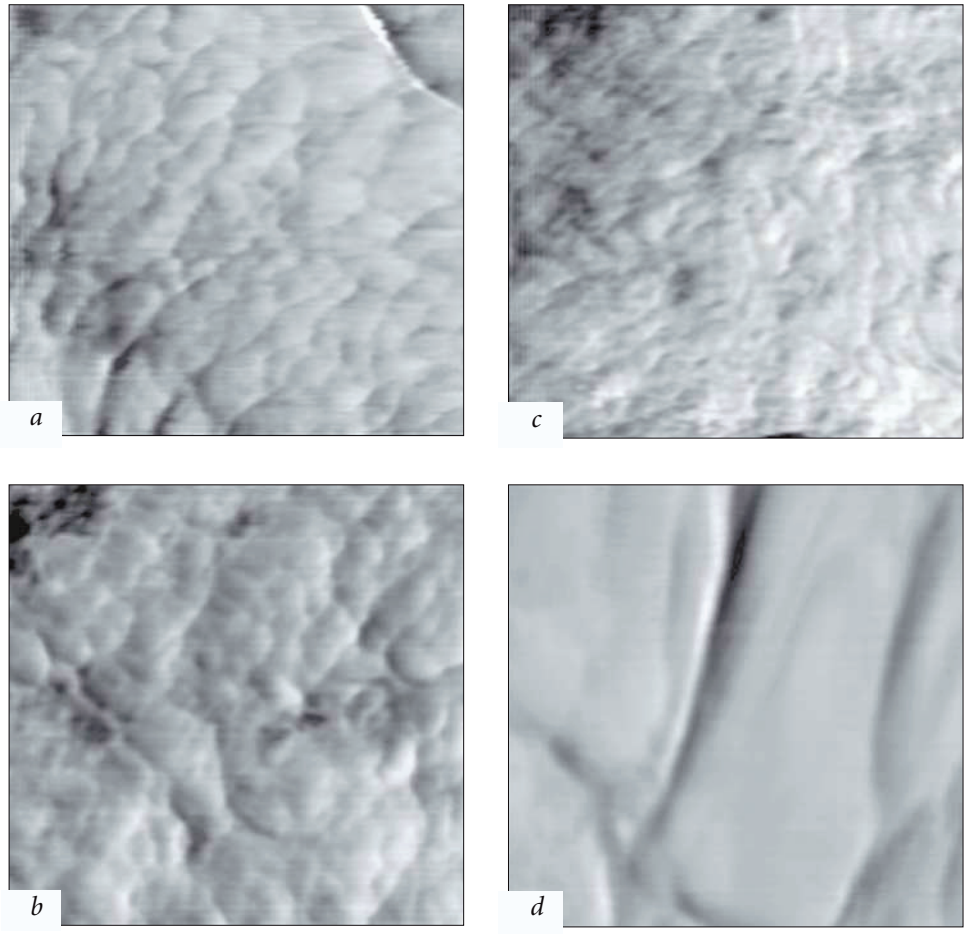

Fig. 2. Lateral force maps of erythrocyte membrane sites (1×1 μ) in group 2 donors (*a*) and patients with DM-2 (*b-d*).

from the mean towards higher or lower values in comparison with the E value in the control group. Hence, significant heterogeneity of the elastic properties is characteristic of erythrocytes of DM-2 patients.

Oxidative stress caused by peroxinitrite treatment of erythrocytes leads to modification of their membrane cytoskeleton structure [1]. The cytoskeleton network is condensed, which manifests in increased D_F value of the lateral force maps of erythrocyte membrane sites and local rigidity of erythrocytes. Changes in the cytoskeleton structure and lipid bilayer composition caused by oxidative processes lead to erythrocyte crenation.

Study of the morphology, structural and mechanical characteristics of erythrocytes in DM-2 patients showed that all observed modifications cannot be explained by oxidative stress alone. The cytoskeleton structure of some erythrocytes was really impaired and they exhibited high rigidity corresponding to their reaction to oxidative stress. Another part of patients' erythrocytes was characterized by lower D_F and lower values of apparent local elasticity modulus in comparison with the corresponding values in normal human erythrocytes. It is assumed that young erythrocytes are less rigid than old cells [5]. On the other hand, numerous immature erythrocytes (reticulocytes) are released into peripheral blood in DM-2. These cells contain some organelles (mitochondria, ribosomes, vacuoles, *etc.*) and are characterized by labile (restructuring) cytoskeleton [6,10]. It is hypothesized that these intracellular structures determine high total rigidity of reticulocytes during their filtration through small pores [9]. Unfortunately, there are no data on the local mechanical characteristics of membranes in these cells. That is why widening of the function density (D_F, E) of structural mechanical properties distribution in the erythrocyte population of patients with DM-2 can be explained by high percentage of old, young, and immature erythrocyte forms. Structural functional heterogeneity of erythrocyte population is also confirmed by pronounced polymorphism of erythrocytes.

Hence, AFM of individual erythrocytes detected higher variability of their structural mechanical characteristics in erythrocyte population of patients

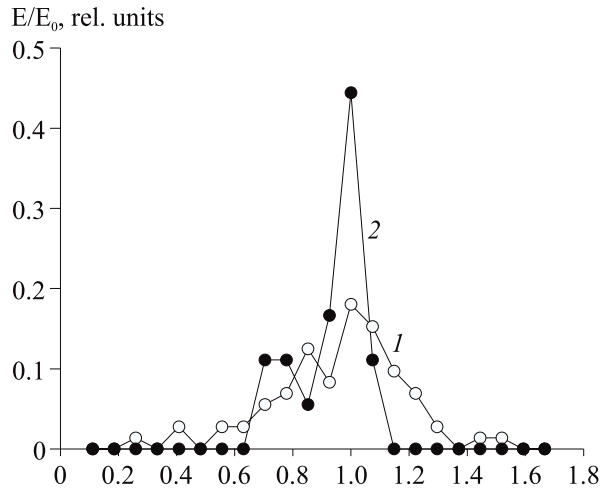

Fig. 3. Distribution of arbitrary elasticity moduli (E/E_0) of patient's erythrocytes (group 1; *1*) and donors (group 2; *2*). E is the value of erythrocyte apparent local elasticity modulus; E_0 is selected mean for apparent local elasticity modulus for group 2 donor erythrocytes.

with DM-2 in comparison with the control. Circulation of erythrocytes with heterogeneous structural mechanical properties can no doubt modify the vascular walls and be one of the causes of angiopathies. That is why restoration of normal erythropoiesis feedback is one of conditions for effective prevention of angiopathy in DM-2.

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