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Conformational change of membrane proteins leads to shape changes of red blood cells

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Abstract

High-resolution atomic force microscopy (AFM) allows a new insight into the surface of mammalian cells. Using the human red blood cell (RBC) as a model, we have demonstrated an important correlation between the conformation of membrane proteins measured from the external face of the cell and the cell shape.

Keywords: Red blood cell; Erythrocyte; Cell shape; Protein conformation; Atomic force microscopy

1. Introduction

Our current understanding of the mechanism of the shape change of a biological cell is based on the 'bilayer couple mechanism' developed by Sheets and Singer [1]. It assumes that an expansion of the inner or outer leaflet of the membrane relative to the other leads to changes of the double-layer-based curvature since the two leaflets cannot separate from each other due to their coupling by hydrophobic interactions. It is well known that e.g. red blood cells (RBCs) from various mammalian species can have different resting shapes [2]. The 'normal' discocyte is able to transform to either echinocytes or stomatocytes depending on a large variety of membrane and cytoplasmic parameters [3–5]. In addition, a spontaneous shape change occurs if the cells are in contact with a glass surface [6]. It has been demonstrated that an expansion of one of the leaflets can occur after the transversal redistribution of the membrane phospholipids or after an insertion of amphiphilic compounds into the membrane [3,7]. On the other hand, also a conformational change of integral membrane proteins could lead to an expansion of one leaflet of the membrane double layer relative to the other one and in turn result in a shape change. Such a possibility was speculated by Gimsa and Ried [8] interpreting the echinocytogenic effect of stilbene disulfonates (e.g. DIDS) and pyrimido-pyrimidines (e.g. dipyridamole), which are known inhibitors of the anion transport protein (band 3) in terms of a ligand-induced 'recruitment' of band 3 protein to an outward-oriented conformation [9]. However, no experimental data are available to support such an idea although other authors described a possible relationship of anion transport inhibition and RBC shape [10,11]. Therefore, the aim of the present study was to investigate whether the RBC shape depends on the conformation of the band 3 proteins, i.e. is affected by the change in the cross-sectional area of each band 3 monomer in the outer leaflet of the membrane. Another parameter assumed to affect the RBC shape per se is the transmembrane potential [12–14]. However, the effect of the transmembrane potential is controversial and a possible mechanism remains unclear [15]. Therefore, changes of the transmembrane potential were

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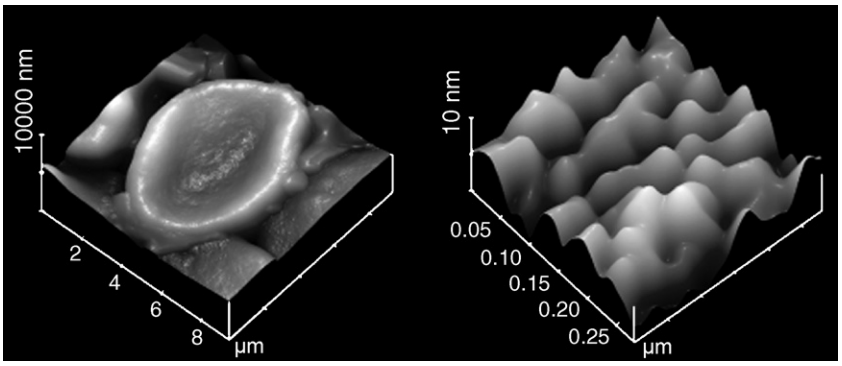

Fig. 1. Surface plot of a human red blood cell (discocyte) based on AFM image measured in air (humidity of 60%). Left: whole cell plot (8×8 µm^2), right: high-resolution plot (0.25×0.25 µm^2).

taken into consideration with respect to the obtained shape changes of the cells.

2. Material and methods

Using the Tapping mode of the AFM technique (Bioscope IV, Veeco Instr., Santa Barbara, USA), the surface topography of glutaraldehyde-fixed wet human RBCs within nanometer resolution has been analyzed. The fixation with glutaraldehyde (2.5% v/v) has been started immediately (within 1 min) after the cells were transferred into the various solutions used to obtain different cell shapes (experimental conditions see below). The fixation process took less than 1 min. Ultra sharp silicon tips with a length of 15–20 µm and an opening angle of less than 10° (Anfatec, Germany) have been used for the measurements. The applied force was kept constant for measurements of different cells and during a single cell scan. The globular structures above the non-deformed (by the applied force) lipid membrane have been registered. The lipid membrane determines the vertical zero level. An AFM image of a typical surface plot of a whole RBC (discocyte) and the corresponding high-resolution image are shown in Fig. 1.

The surface topography of the classical RBC shapes – discocytes, stomatocytes, and echinocytes – obtained by different experimental maneuvers has been investigated (Figs. 1–3). The RBC shape was monitored by light microscopy. Discocytes are formed in physiological (high) ionic strength solution (145 mM NaCl, 7.5 mM KCl, 10 mM glucose, 5.8 mM Na_2HPO_4/NaH_2PO_4, abbreviated as HIS solution) at pH 7.4. Addition of the band 3 inhibitor niflumic acid (NA, 10 µM) to this solution did not affect the cell shape. NA was used since it is known that it blocks the anion transport across the RBC membrane without changing the conformation of the transporter (band 3) [16]. Stomatocytes were obtained in HIS solution at low pH (5.6), in a low ionic strength solution (250 mM sucrose, 7.5 mM KCl, 10 mM glucose, 5.8 mM Na_2HPO_4/NaH_2PO_4, abbreviated as LIS solution) at pH 7.4, and after the addition of NA to the LIS solution at pH 7.4. A transformation from discocytes to echinocytes occurred in presence of the anion transport inhibitors DIDS (10 µM) and furosemide (FS, 10 µM) to the HIS as well as LIS solution. Both transport inhibitors are known to affect the conformation of the band 3 proteins [17–19]. Therefore, the inhibition of the anion transport seems not to be solely of importance for the RBC shape. Echinocytes were also formed immediately after the cells got in contact with a glass surface.

To take into consideration the transmembrane potential of the RBCs, the anion transport inhibitors (NA, DIDS, FS) were added to the LIS solutions before and after the RBCs were transferred from the HIS into the LIS solution. In the first case the transmembrane potential remains as low as in HIS solution (about −8 mV) whereas in the second case it increases to about +50 mV [20]. The cells were taken for investigation, including the fixation with glutaraldehyde (see above), immediately

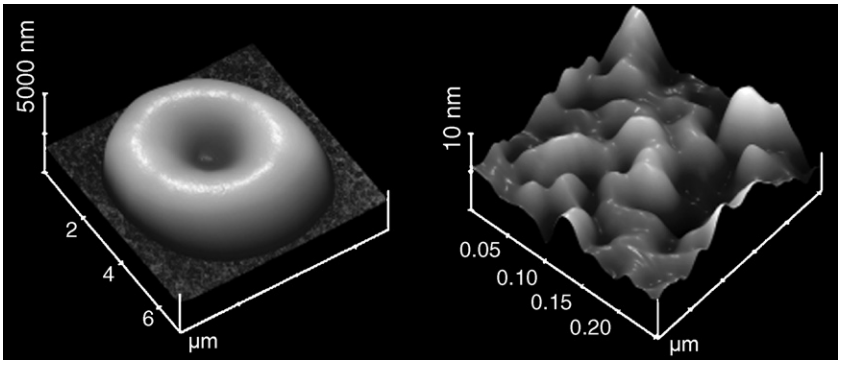

Fig. 2. Surface plot of a human red blood cell (stomatocyte) based on AFM image measured in air (humidity of 60%). Left: whole cell plot (6×6 µm^2), right: high-resolution plot (0.25×0.25 µm^2).

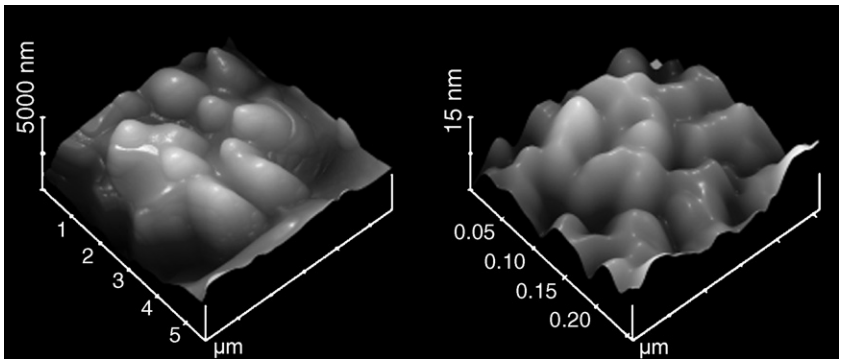

Fig. 3. Surface plot of a human red blood cell (echinocyte) based on AFM image measured in air (humidity of 60%). Left: whole cell plot (5×5 µm^2), right: high-resolution plot (0.25×0.25 µm^2).

(within 1 min) after the RBCs were transferred into the LIS solutions.

Neuraminidase (Nase) treatment (from *Vibrio cholerae*, Sigma-Aldrich, 1 U/ml, haematocrit 0.002%) was carried out as described in [21]. The incubation medium was the HIS solution which contained additionally 2 mM $CaCl_2$. The quality of the glycocalyx cleavage was estimated by determining the amount of released *N*-acetyl-neuraminic acid according to a protocol described in [22].

3. Results and discussion

Analyzing the volume of the part of the membrane proteins exposed to the external surface (measuring both their height and diameter), significant differences could be observed for the 3 classical RBC shapes. The partial protein volumes were calculated as a segment of a sphere using the equation: $V_m = h\pi/6(3r^2+h^2)$. Under all conditions leading to stomatocytes, the part of the protein volume at the external face is significantly decreased in comparison to 'normal' discocytes, whereas it is significantly increased for echinocytes. The corresponding mean values for all volumes of the external part of the proteins measured under conditions leading to stomatocytes, discocytes, and echinocytes are represented in Fig. 4. One should realize that the mean volumes of the three RBC shapes measured are significantly different (*t*-test, $p<0.01$) taking into account that the number of partial protein volumes analyzed is larger than 100 (about 20 proteins per cell image, and at least 4 cells for each condition leading to the different cell shapes have been analyzed). A more detailed analysis of the partial protein volume is presented in Fig. 5. It shows the frequency of the distribution of the protein volumes measured divided in groups that contain the number of proteins within 5-nm^3 intervals. Based on the mean values for the obtained volumes of the external part of the proteins (Fig. 4), their area at the extracellular surface was calculated. The area was calculated from the partial volume determination (and not directly from the measured protein diameter) since the vertical resolution of the AFM measurements is much higher (0.1 nm) in comparison to the horizontal resolution (1 nm). The values obtained for the protein area of stomatocytes, discocytes, and echinocytes are 69.5±3.6 nm^2, 77.6±8.5 nm^2, and 83.3±1.5 nm^2, respectively. These values are likely an overestimation because the membrane proteins are covered by structural constituents of the glycocalyx. The relative change of the

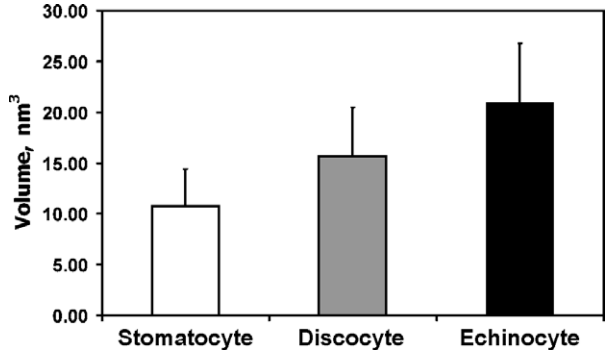

Fig. 4. Mean values for all obtained volumes of the external part of the membrane proteins under conditions leading to stomatocytes, discocytes, and echinocytes, The error bars represent S.E.M., $n>100$ for each shape, the values are significantly different, $p<0.01$.

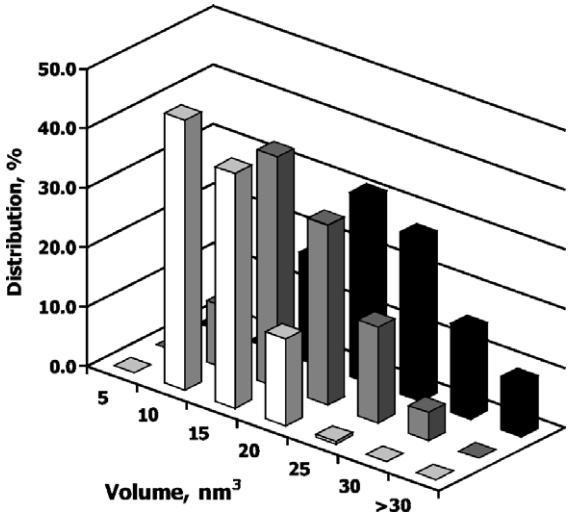

Fig. 5. Frequency of the distribution of the obtained volumes of the external part of the membrane proteins under conditions leading to stomatocytes (white columns), discocytes (grey columns), and echinocytes (black columns) (cf. Fig. 4) divided in groups that contain the number of proteins within 5-nm^3 intervals.

protein area should not be affected by the glycocalyx structure as long as no local changes of the glycocalyx density have to be assumed. However, to see whether the glycocalyx has an effect on the estimated values, experiments were carried out where the glycocalyx was cleaved by 60% using Nase. Surprisingly the mean value for the volume of the external part of the proteins measured for discocytes after Nase treatment was found to be higher in comparison to untreated cells (26.3 ± 9.9 nm^3, $n=83$ vs. 15.7 ± 4.8 nm^3, $n=108$). This result might be explained in two ways: (i) a change of the glycocalyx density, i.e. a change of the electrostatics of the cell surface, could lead to greater protrusion of the membrane proteins due to the change in repulsive forces, and/or (ii) the thickness of the glycocalyx layer removed by Nase was greater between the protein structures than the thickness on the top of them.

One has to take into consideration that the band 3 protein is the major integral protein of the RBC membrane existing in 10^6 copies per cell and, therefore, occupies a significant area of the membrane surface. It can be assumed that at least every second integral membrane protein in the RBC is a band 3 protein. The volume of solubilized band 3 protein as determined with the AFM technique has been reported in the literature [23]. The estimated value of the total band 3 volume is, however, more than 10 times larger than the value of the partial (external) volume determined in the present paper, suggesting that approx. 90% of the protein volume are embedded in the membrane and localized in the cytoplasm. The calculated values for the protein area at the extracellular surface of RBCs with different shapes shows that the external leaflet of the membrane is decreased by 10.4% or increased by 7.3% when the cells are transformed from discocytes to stomatocytes and from discocytes to echinocytes, respectively (the values have to be halved if one assumes that the measured proteins are dimers). For this calculation, a surface area of the RBC of 137 μm^2 was assumed [24] as well as that 50% of all proteins are band 3 proteins. It is generally proposed that a relative change of the area of the extracellular membrane leaflet of less than 1% relative to the inner one is enough to explain the changes in cell shape [1,25]. Therefore, our findings demonstrate that a conformational change of membrane proteins, especially the band 3 protein in the RBC membrane, can modify the relation of the area of the outer membrane leaflet relative to the inner one, which in turn can lead to a change of the overall shape of the cell.

Such a mechanism implies that the forces onto the lipid phase by a conformational change of the protein are altering the tension within the two membrane leaflets. It is interesting to note that the opposite effect, namely an alteration of the conformation of membrane proteins induced by an incorporation of lipophilic molecules into the membrane, has been described recently [26]. This opposite effect is of great importance to understand the mechanosensitivity of membrane proteins, especially ion channels. Therefore, the tension within the two membrane leaflets can influence the conformation of membrane proteins and vice versa. In general, both mechanisms described in the present paper as well as by Markin and Sachs [26], widen our view on the interaction of membrane proteins and lipids.

Interestingly, the change in the transmembrane potential of the cells caused by the addition of the transport inhibitors (NA, DIDS, and FS) to the LIS solutions after the cells were transferred into this LIS solution, compared with the situation where the cells were transferred after the inhibitors were added to the solution, did not result in shape changes. Therefore, the transmembrane potential does not seem to play a substantial role in RBC shape changes. However, it cannot be excluded completely that the inhibition of the band 3-mediated transport overrides the influence of the transmembrane potential on shape [13,27].

Acknowledgments

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