Variations of erythrocyte morphology in different pathologies

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Abstract. In our study we evaluated erythrocytic morphology in different pathologies which can modify flowing red cells. We followed the methodology proposed by Zipursky which allows a three-dimensional evaluation of the red cell and a classification according to the shapes observed through the optical microscope. We studied 150 subjects: 20 normal subjects, 58 patients suffering from vascular diseases, 40 affected by diabetes (type II) (10 without and 30 with vascular diseases), 22 patients with liver disease, 5 patients with monoclonal gammapathies and 5 dehydrated patients. Results show that in normal subjects bowls, which is the shape of the most deformable red cells, are more (55%) than discocytes (44%); the altered forms are only 1%. In vascular patients we noted a statistically significant increase of discocytes (60%). There are no significant differences between subjects affected by diabetes without vascular disease and normal subjects. In diabetics with vascular diseases there are more discocytes (57%) and some altered forms (3%). In patients suffering from chronic hepatitis a great increase (13%) in echinocytes and knizocytes was noticed, which suggests an alteration in the fluidity of the membrane. Our observations testify the importance of this simple methodology in focusing the morphological alterations which can be accounted for both by pathologies of the red cells and by changes in their metabolism.

Keywords: Erythrocyte morphology, vascular disease, diabetes, liver disease

1. Introduction

The principal parameters that regulate the deformability of erythrocytes are the cell’s internal viscosity, the surface area–volume ratio and the intrinsic viscoelastic and molecular properties of the membrane [17,22]. Both in the macrocirculation and microcirculation, there is a rheologically important phenomenon – the continuous rotation of the membrane about its liquid contents – on account of which the external shear stress is transmitted through the membrane to the cytoplasm. Thus, the lipid–protein planes of the membrane slide alternately forward and backward, while the cytoplasm flows in a circle inside the cell. Hence, one can understand why the microviscous properties of the cell membrane and of the cytoplasm determine the rheological behavior of the erythrocyte [4,13,28]. The molecular basis of the increase in rigidity of the erythrocyte membrane is connected mainly with alterations of the lipid composition – the cholesterol–phospholipid ratio changes in favor of the former. However, there are also other contributing factors, such as the polyunsaturated fatty acid content, the

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methylation of phosphatidylethanolamine, the increase of intracellular calcium, the phosphorylation of the proteins, the cross-links between the membrane proteins, and the peroxidation of the membrane lipids [9,24,31,32]. The study of erythrocyte morphology has always been of great interest since it can reveal the alterations underlying a worsening of the rheological condition of the blood, leading to a reduction of oxygen transport to the tissues [10]. Many methods have been proposed to measure the deformability of erythrocytes, but often their practical execution has been complicated and they have produced unsatisfactory results. Above all, we refer to the methods of filtration derived from the attempt to reconstruct in vitro the system of hematic circulation through a capillary network, though taking into account, with regard to the microcirculation, how very different the geometry of the filtering systems is [6]. The filtration of whole blood with polycarbonate filters, which expresses the deformability of the total blood and thus the mode of deformation of all its cellular elements, is therefore a parameter similar to the viscosity. The filtration of washed erythrocytes measures in a more specific way the deformability of the red blood cells [29,31]. However, the washing procedure profoundly alters the erythrocyte, both its metabolic and membrane characteristics, and this method does not consider the extraglobular factors which have a great importance in the determination of the deformability of the cell [12,23]. Our aim was to study the erythrocyte morphology in various pathological situations, which can provide important indications in both the hematological and hemorheological fields. For this purpose, we utilized a method proposed by Zipursky that is easily performed and inexpensive. It allows a three-dimensional assessment of the red blood cell and a classification based on its appearance in light microscopy [33].

2. Materials and methods

We studied 150 subjects: 20 normal persons (11 males and 9 females, mean age 51.3 ± 10.2 years); 58 vasculopathic patients (30 males and 28 females, mean age 62.5 ± 9.8 years); 40 patients with type 2 diabetes mellitus in metabolic balance, of whom 10 were without vasculopathic complications (5 males and 5 females, mean age 52.4 ± 11.3 years) and 30 were affected by vasculopathy (14 males and 16 females, mean age 58.6 ± 10.4 years); 22 patients with chronic hepatitis and hepatic cirrhosis (12 males and 8 females, mean age 50.7 ± 8.9 years); 5 patients with monoclonal gammopathy; and 5 dehydrated patients.

2.1. Preparation of the specimens

To 50 µl of venous blood, drawn with EDTA as anticoagulant, we added 0.5 ml of phosphate buffer (0.125 M) pH 7.3–7.4, immediately after sampling. To obtain fixation of the erythrocytes, we subsequently added 0.5 ml of 0.3% glutaraldehyde which had previously been integrated with the phosphate buffer with pH 7.3–7.4 and osmolarity of 290–310 mosm. The specimen, thus prepared, was mixed and left to rest for 5 min in order to obtain fixation. The specimen was prepared at room temperature immediately after the blood was drawn, since even brief waiting periods lead to the appearance of cell alterations and artifacts, the magnitude of these being proportional to the length of time. Cells which are fixed immediately after having been drawn up keep the shape which they had in circulation in a dynamic way.
2.2. Evaluation of the specimen

A drop of blood fixed with the glutaraldehyde was mixed with four drops of glycerol. Two drops of this preparation were then placed on a microscope slide and left to rest for at least 40 min. The erythrocytes could then be observed within a maximum time of 5–6 hours. The observation was performed with a light microscope with a 100 × immersion lens and the three-dimensional evaluation was carried out by varying the focus. In this way, the lens exerts a slight pressure on the liquid being observed, causing the complete rotation of the cell (moving in the viscous medium), which can thus be observed on all sides. The evaluation consisted of the observation of 100 erythrocytes per specimen and the ascertainment of the morphological composition (in percentages) according to the classification described by Bessis [1,2]. In normal subjects we have a great number of bowls, hemiconevate red cells derived from the distortion of discocytes in blood flow which, due to their deformability, change shape continuously; discocytes follow in percentage: their shape (similar to a biconcave lens) is more stable because they are characterized by a lesser degree of deformability. Pathological forms, which in normal subjects are present in lower numbers, are: knizocytes, with a slightly smaller diameter than discocytes and characterized by two or three concavities in the membrane caused by alterations in their lipidic component: they are reversible forms and are quite frequent in liver diseases. Echinocytes have on their surface from 10 to 30 spicules, regularly distributed, characterized by introflexions of the membrane caused by the modification of the surface/volume ratio: frequent in liver diseases and in dehydratation. Even less frequent forms are: dacrocyes, tear-shaped, which contain altered proteins and are typical in thalassaemia and in myelofibrosis; acanthocytes, with a fewer number of irregular spicules than echinocytes caused by irreversible lipidic alterations of their membrane; keratocytes, stirrup-shaped, with two spicules, present in some kinds of anaemia; schizocytes, resulted from red cell fragmentation, have different shapes. Other kinds of red cells, spherocytes, megalocytes, and immature erythrocytes, can be found in several kinds of anaemia (Fig. 1).

3. Results

Results are reported in Table 1. In the normal subjects, the bowl-shaped erythrocytes, which are the most deformable, represent the greatest proportion, reaching a mean of 55% of the total; the discocytes, cells that are still physiological but more rigid, represent 44%; the clearly altered cells, mainly echinocytes and knizocytes, do not exceed a mean value of 1%. In the vasculopathic patients vs. controls, there is a statistically significant increase of the discocytes to about 60%, while the rest are mainly bowl-shaped cells. We almost never observed a significant increase of the altered forms (Fig. 2). In the diabetics without vascular complications, there are no significant differences

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Diabetes</th>
<th>Diabetes with microangiopathy</th>
<th>Vascular disease</th>
<th>Liver disease</th>
<th>Dehydratation</th>
<th>Myeloma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bowls</td>
<td>55.4 ± 12.3</td>
<td>46.7 ± 26.8</td>
<td>40.8 ± 20.3</td>
<td>37.3 ± 21.5</td>
<td>56 ± 19.7</td>
<td>51 ± 14.4</td>
<td>60.3 ± 9.6</td>
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<tr>
<td>Discocytes</td>
<td>43.8 ± 12.6</td>
<td>52.4 ± 26.5</td>
<td>57.3 ± 22.5</td>
<td>61.1 ± 21.4</td>
<td>31.8 ± 15.9</td>
<td>38.6 ± 18.2</td>
<td>35.6 ± 5.5</td>
</tr>
<tr>
<td>Knizocytes</td>
<td>0.4 ± 0.73</td>
<td>0.66 ± 0.7</td>
<td>1.68 ± 2.63</td>
<td>1.29 ± 2.20</td>
<td>5.69 ± 6.03</td>
<td>6.33 ± 6.02</td>
<td>3 ± 4.35</td>
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<tr>
<td>Echinocytes</td>
<td>0.13 ± 0.35</td>
<td>0.11 ± 0.33</td>
<td>0.13 ± 0.44</td>
<td>0.08 ± 0.33</td>
<td>7.08 ± 23.23</td>
<td>3.1 ± 1.32</td>
<td>0.33 ± 0.57</td>
</tr>
<tr>
<td>Other forms</td>
<td>0.13 ± 0.51</td>
<td>0.14 ± 0.2</td>
<td>0.31 ± 0.60</td>
<td>0.50 ± 1.21</td>
<td>1 ± 2.57</td>
<td>1 ± 0.67</td>
<td>0.66 ± 0.57</td>
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*p < 0.05 Student’s t-test.
Fig. 1. Different shapes of red cell; all pictures taken by means of optical microscope Zeiss Axiomat through interference contrast (Nomarski).

Fig. 2. G.C. – 80 years. Peripheral obliterative arterial disease with angina pectoris. Discocytes 88% – bowls 12% (×50).

Fig. 3. M.S. – 82 years. Diabetes. Discocytes 50% – bowls 49% – echinocytes 1% (×50).

from the normal subjects (Fig. 3). In the diabetics with vasculopathy, as revealed both clinically and instrumentally, there is a significant increase of the discocytes to a mean of 57% and a modest increase of the altered forms (3%) (Fig. 4). In the chronic hepatopathy patients, we observe a marked increase
Fig. 4. P.G. – 70 years. Peripheral obliterative arterial disease with diabetes. Discocytes 70% – bowls 28% – knizocytes 2% (×50).

Fig. 5. M.A. – 66 years. Liver disease. Bowls 52% – discocytes 32% – echinocytes and knizocytes 16% (×50).

Fig. 6. M.P. – 78 years. Myeloma. Discocytes 40% – bowls 54% – knizocytes 6% (×50).

of the echinocytes (n.s.) and knizocytes ($p < 0.05$) vs. controls, which make up about 13% of the total and, in some situations, represent absolutely the largest percentage. These forms indicate the presence of an altered fluidity of the cell membrane (Fig. 5). In the groups with monoclonal gammopathy and
with dehydration, there is a certain increase (n.s.) of the altered forms, although it is not constant, which shows how the alterations of the blood, regarding on one hand the electrolytic balance and the osmolarity and on the other hand the presence of an abnormal quantity of gammaglobulin, can have a negative influence on the deformability of erythrocytes [16,27] (Fig. 6).

4. Discussion

Our observations testify to the usefulness of the method proposed by Zipursky. It permits a detailed evaluation of single erythrocytes similar to that obtained with the electron microscope, though with significantly less cost and with an extremely simple mode of execution. According to this method, the erythrocytes, fixed immediately after the blood sampling and observed in a three-dimensional manner in a viscous medium, undergo complete rotations and can thus be evaluated and studied on all sides [2,33]. The cells can effectively be considered close to reality, being identical to those observed with the electron microscope and to those photographed in vivo in human capillaries [1,14]. Of fundamental importance is the fact that the cells do not undergo alterations of the membrane, as instead occurs with the method of filtration of washed erythrocytes [6,12,29,31]. Therefore, the observations obtained with this method are extremely valuable compared with the traditional smear of peripheral blood, whose limitations, as is well-known, are constituted by a two-dimensional evaluation and by the presence of artifacts due to the mode of execution of the specimen and to its staining [2]. In our study, we have examined a significant number of specimens in various pathologies and have elucidated the morphological alterations attributable both to intrinsic modifications of the red blood cells and to alterations of their cell metabolism [18,21,30]. In ischemia, the reduced oxygen supply plays an important role since, by decreasing the energy reserve, it leads to an inefficient activity of the membrane pumps, which cannot restore the calcium ion to physiological levels. The ions accumulate in the cytosol, causing an increased rigidity of the membrane through the lack of phosphorylation of spectrine and the formation of polymers between the cytoplasm and the membrane itself [5,7,8,11,20]. In diabetes, the shortage of insulin does not seem to be determinant in the control of metabolism of the red blood cell. In fact, in the non-vasculopathic diabetic patients, we did not find major alterations of the erythrocyte morphology with respect to normal subjects. In the vasculopathic diabetic patients, in addition to chronic hypoxia, the glycosilation of the membrane proteins and the accumulation of sorbitol also contribute to a rheological worsening [18,19,26]. We found the most important morphological alterations in the patients with chronic hepatopathies with cholestasis. These are due to the reduction of the fluidity of the cell membrane caused by different factors, among which the modification of the phospholipid-cholesterol ratio and the degree of saturation of the fatty acids bound to glycerol in the phospholipids [3,25].

Acknowledgements

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References


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