Evaluation of erythrocyte morphology as deformability index in patients suffering from vascular diseases, with or without diabetes mellitus: correlation with blood viscosity and intra-erythrocytic calcium

Vera Turchetti*, Francesca Leoncini, Caterina De Matteis, Luca Trabalzini, Maurizio Guerrini and Sandro Forconi
Istituto di Semeiotica Medica e Geriatria, Università degli Studi di Siena, Policlinico “Le Scotte”, V. le Bracci, I-53100 Siena, Italy

Abstract. The aim of our study was to evaluate the erythrocytic morphology in vascular patients, with or without diabetes, showing cell alterations correlated to blood viscosity and intra-erythrocytic calcium. We studied 108 subjects: 20 normal subjects, 58 vascular patients (25 suffering from CHD, 19 from CVD, 14 from POAD) and 30 non-insulin-dependent diabetes patients with vascular disease in metabolic compensation (13 CHD, 9 CVD, 8 POAD). Erythrocytic morphology, blood viscosity and intra-erythrocytic calcium were evaluated. Our results show that bowls, the most deformable red cells, decreased significantly in vascular patients and in POAD diabetics, while the discocytes, having a stiffer form, greatly increased in subjects suffering from ischemic disease and in POAD diabetics. The altered red cells (echinocytes and knizocytes) reached a statistical significance in CVD and POAD diabetics. Comparing the percentage of discocytes to intra-erythrocytic calcium content in vasculopathic subjects, we obtained a significant correlation. No evidence of a relationship between discocytes and blood viscosity was found, even if blood viscosity significantly increased in patients affected by ischemic disease. These results suggest that ischemia decreases the deformability of red cells which is supported by the study of red cell morphology, by the erythrocytic morphology index (EMI), which becomes <1, and by the evaluation of cytosolic calcium content.

Keywords: Vascular disease, diabetes, erythrocytic morphology, intra-erythrocytic calcium, blood viscosity

1. Introduction

The study of erythrocyte morphology is of great importance in the field of rheology, since the deformability of the circulating cells has a fundamental influence on the rheological properties of the blood [1–3]. In many pathological situations, this important property is altered, with serious damage to the rheological characteristics of the erythrocyte at the level of the microcirculation [4,5].
In the large vessels, the red blood cell undergoes an ellipsoidal deformation, with the long axis parallel to the direction of the blood flow. However, the most important rheological phenomenon occurs in the arterial microcirculation: the continuous rotation of the membrane about the cytoplasmic contents permits the erythrocyte to assume a parachute shape, which allows it to pass through even the smallest capillaries [6–8]. The filtration of whole blood has been criticised for the excessive influence of extra-erythrocytic factors, such as fibrinogen, the protein composition of the plasma, the viscosity of the plasma, and the mechanical and biological contribution of the leucocytes and their products of activation [9,10]. On the other hand, the filtration of erythrocytes that are washed and suspended again in buffer implies the loss of any connection with the medium in which they live. In addition, with this method, the erythrocytes undergo profound alterations with regard to the cell membrane, which is the main factor involved in their deformability [11–14]. For our study of erythrocyte morphology, we have used a simple and inexpensive method, which consists of the light microscopic observation of the red blood cells fixed in phosphate buffer and suspended in a viscous medium. This permits a detailed evaluation of the single erythrocytes, similar to that obtained with the electron microscope, and a classification based on their morphological appearance [15–17]. The aim of our study was to evaluate erythrocyte morphology, according to the method proposed by Zipursky, in vasculopathic patients with and without diabetes mellitus. In this way, we are able to elucidate any cellular alterations and to correlate them with blood viscosity and intra-erythrocytic calcium, which are important factors connected to the rheological properties of the red blood cell.

2. Materials and methods

We studied 108 subjects: 20 normal subjects (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) of which there were 25 patients with coronary heart disease (CHD), 19 with cerebrovascular disease (CVD) and 14 with peripheral oblitative arterial disease (POAD), and 30 patients with type 2 diabetes mellitus in metabolic compensation, who were affected by vasculopathy, ascertained both clinically and instrumentally (14 males and 16 females, mean age 58.6 ± 10.4 years), of which there were 13 patients with CHD, 9 with CVD and 8 with POAD. The morphology of the erythrocytes was evaluated according to the method of Zipursky: we used 50 μl of anticoagulated venous blood (anticoagulant EDTA-K), immediately after the blood was drawn, with the addition of 0.5 ml of phosphate buffer and 0.5 ml of 3% glutaraldehyde. Evaluation of the specimen was performed, after 40 min, with an immersion light microscope after the addition of 200 mg of glycerol. By varying the focus of the lens, it is possible to make the erythrocytes rotate through 360° and thus to define their three-dimensional morphology. For each specimen, 100 erythrocytes were observed and the percentages of the different morphological classes of red blood cells were calculated (in normal subjects: 55% bowls, 44% discocytes, 1% knizocytes, echinocytes and other pathological forms). Further, we computed the erythrocytic morphology index (EMI) given by the bowls/discocytes ratio as an indication of the degree of cell rigidity [17,18]. The viscosity of the whole blood was determined from samples drawn with 10% EDTA-K stored at 37°C and then measured with a plate-cone rheometer (Carri-Med). This allowed us to obtain the curve of viscosity, by setting the fixed parameters as the initial and final shear stress and the time of the reading: the viscosity was expressed in cPs at 10 s⁻¹ of shear rate [11,18–20]. The intra-erythrocytic calcium was determined by the use of Fura 2-AM fluorescent indicator (Calbiochem). The erythrocytes were washed and centrifuged with phosphate buffer and then incubated with the Fura 2 at 37°C.
for 30 min. The fluorescence was measured with a Perkin-Elmer LS50 spectrofluorimeter; the wave of emission was selected at 505 nm, and the wave of excitation was selected at 335 nm for the Fura 2–Ca$^{++}$ complex and 385 nm for the intracellular free Fura 2. The concentration of cytosolic calcium is expressed as the 335/385 ratio. A similar sample was prepared without the indicator and used as a control [21,22]. The statistical analysis of the data was performed with the Student’s t-test for non-paired data and with linear regression.

3. Results

The results summarised in Table 1 demonstrate that the mean percentage of bowls, the most deformable type, is significantly reduced in the vasculopathic subjects (normal: 56 ± 12%, CHD: 35 ± 23%, CVD: 40 ± 21%, POAD: 38 ± 20%) and in the diabetics with POAD (25.5 ± 18.72). In the meantime, the discocytes, less deformable cells, increased significantly in the ischemic patients (normal: 44 ± 13%, CHD: 63 ± 23%, CVD: 58 ± 21%, POAD: 63 ± 20%) and in the diabetics with POAD (72 ± 19%). The altered erythrocytes (echinocytes and knizocytes) were significantly greater in diabetics with CVD (5.12 ± 4.05%) and with POAD (2.5 ± 2.97%) than in the normal subjects (0.66 ± 0.89%). The EMI is >1 in normal subjects, whereas it is well below 1 in both vasculopathic and diabetic patients (0.70 and 0.61, respectively). On the whole, the value for pathological subjects is 0.65 (Figs 1–4). The intra-erythrocytic calcium is significantly increased in the whole group of vasculopathic patients and in the whole group of diabetics with respect to the normal subjects (Fig. 5) (respective 335/385 values: 2.12 ± 0.79, 2.31 ± 0.65, 1.63 ± 0.42). The same pattern is seen for blood viscosity (respective values: 7.39 ± 1.51, 7.71 ± 1.49, 6.13 ± 0.98 cPs at 10 s$^{-1}$). In the vasculopathic subjects, there is a significant correlation between the percentage of discocytes and the level of intra-erythrocytic calcium (Fig. 6). However, there was no correlation between the percentage of discocytes and blood viscosity, even though the mean value of the latter parameter increased significantly in the ischemic patients. We also noted that in patients suffering from POAD and diabetes, EMI values are worse than those of the other groups.

Table 1

Percentages of different morphological classes of red cells, intra-erythrocytic calcium and blood viscosity values (mean ± SD) in controls and in subjects suffering from vascular diseases, diabetics and non diabetics

<table>
<thead>
<tr>
<th></th>
<th>Bowls</th>
<th>Discocytes</th>
<th>EMI</th>
<th>Knizocytes + echinocytes</th>
<th>Ca$^{++}$</th>
<th>Blood viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 20)</td>
<td>55.46 ± 12.31</td>
<td>43.80 ± 12.60</td>
<td>1.27</td>
<td>0.66 ± 0.89</td>
<td>1.63 ± 0.42</td>
<td>6.13 ± 0.98</td>
</tr>
<tr>
<td>Non-diabetics (n = 58)</td>
<td>37.34 ± 21.53</td>
<td>61.60 ± 21.53*</td>
<td>0.61*</td>
<td>1.89 ± 2.41</td>
<td>2.12 ± 0.79*</td>
<td>7.39 ± 1.51*</td>
</tr>
<tr>
<td>CHD (n = 25)</td>
<td>35.12 ± 23.15</td>
<td>63.44 ± 22.80*</td>
<td>0.55*</td>
<td>1.28 ± 2.24</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CVD (n = 19)</td>
<td>39.57 ± 21.31</td>
<td>57.89 ± 21.46*</td>
<td>0.68*</td>
<td>2.40 ± 2.81</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>POAD (n = 14)</td>
<td>38.28 ± 19.90</td>
<td>63.35 ± 20.12*</td>
<td>0.60*</td>
<td>2.00 ± 2.18</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Diabetics (n = 30)</td>
<td>40.03 ± 20.84</td>
<td>57.34 ± 22.55*</td>
<td>0.70*</td>
<td>3.12 ± 3.29</td>
<td>2.31 ± 0.65*</td>
<td>7.71 ± 1.49*</td>
</tr>
<tr>
<td>CHD (n = 13)</td>
<td>44.84 ± 23.92</td>
<td>54.07 ± 26.77*</td>
<td>0.81*</td>
<td>1.76 ± 2.86</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CVD (n = 9)</td>
<td>46.75 ± 8.32</td>
<td>48.12 ± 9.17</td>
<td>0.95</td>
<td>5.12 ± 4.05*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>POAD (n = 8)</td>
<td>25.50 ± 18.72</td>
<td>71.87 ± 19.07*</td>
<td>0.35*</td>
<td>2.50 ± 2.97*</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*p < 0.05 Student’s t-test.
Fig. 1. Erythrocytic morphology at the microscope in normal subject. Percentages of the different morphological classes of red cells: discocytes 45%, bowls 55%, EMI = 1.22.

Fig. 2. Erythrocytic morphology at the microscope in a subject suffering from peripheral obliterative arterial disease (POAD). Percentages of the different morphological classes of red cells: discocytes 68%, bowls 32%, EMI = 0.47.
Fig. 3. Erythrocytic morphology at the microscope in a subject suffering from coronary heart disease (CHD) with diabetes. Percentages of the different morphological classes of red cells: discocytes 60%, bowls 39%, knizocytes 1%, EMI = 0.65.

Fig. 4. Erythrocytic morphology at the microscope in a subject suffering from cerebrovascular disease (CVD) with diabetes. Percentages of the different morphological classes of red cells: discocytes 50%, bowls 48%, echinocytes 2%, EMI = 0.96.
4. Discussion

In the ischemic pathologies, rheological alterations involving an increase in blood viscosity and a reduction of the deformability of the erythrocytes have been known for some time now [23–27].
There are many factors that worsen the rheological characteristics of the erythrocytes. These cells are influenced both by extracellular factors, such as the plasma concentration of cholesterol, fibrinogen and gamma-globulins, and by factors connected with the structural conformation of the cell membrane and by the intracellular levels of ATP, which are inversely proportional to the cytosolic concentration of calcium [28,29]. The alterations of the red blood cell induced by ischemia cause a depletion of energy, which translates into a worsening of the activity of the ATP-dependent membrane pumps, which are then not able to bring the concentration of the calcium ion to physiological levels [30,31]. The ion accumulates in the cytosol, causing an increased rigidity of the membrane through the lack of phosphorylation of spectrine or the formation of polymers between the cytoplasm and the membrane itself [32–34]. The alteration of cell deformability is seen in the study of erythrocyte morphology as an inversion of the EMI. The significant correlation that we found between intracellular calcium and the percentage of discocytes is testimony to how the latter, even though they are physiological cells, are less deformable than the bowls, which are much more susceptible to the formation of rouleaux with an increase of their aggregability [35]. There was no significant correlation between blood viscosity and any of the parameters that indicate increased cell rigidity. This can be explained by the numerous rheological factors that determine the viscosity of the blood; in addition to the erythrocyte as an individual cell, also important are the hematocrit, fibrinogen and the other plasma proteins, the regional osmolarity, the pH and, naturally, the calibre of the vessel and velocity of flow [23,28,35]. In diabetes associated with vasculopathy, the increased percentage of discocytes is not always statistically significant, even though the ischemic pathology is more severe and generalised [36]. However, the ischemia is accompanied by numerous metabolic alterations, such as an increase of 2,3-diphosphoglycerate caused by the hypoxia and the accumulation of sorbitol, an intermediate product of the polyols pathway, which slowly diffuses through the membrane and is thus responsible for osmotic damage. All this is confirmed by a severe rheological compromise, with a very large increase of the mean value of cytosolic calcium and of blood viscosity [37]. With regard to erythrocyte morphology we note, in spite of a worse rheological pattern, a lower percentage of discocytes compared to those of vasculopathic patients without diabetes, and a significant increase of the altered forms (echinocytes and knizocytes) compared to normal subjects. This fact can be explained by damage occurring in younger erythrocytes, thus causing a reduction of red cell mean life and a subsequent reduction of discocytes, forms that are typical of older erythrocytes. Since this is true both for older erythrocytes and for younger ones, this reduced fluidity does not arise gradually throughout the life of the red blood cells, but develops immediately after their entry into the circulation [38,39]. This finding may indicate the responsibility of the external environment (principally the microcirculation) in causing the increase of the altered forms of erythrocytes [29,34,35].

References


