Altered behaviour of erythrocytes in scleroderma

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Summary
1. Filterability (deformability) of erythrocytes of patients with Raynaud's phenomenon together with progressive systemic sclerosis (PSS) was decreased compared with the filtration of erythrocytes from normal subjects.
2. PSS erythrocytes showed lower electrophoretic velocity and about 23% less neuraminidase-removable sialic acid density on their surface than the normal erythrocytes.
3. PSS erythrocytes showed more adherence to cultured endothelial cells than the control normal erythrocytes.
4. It is concluded that the increased rigidity and adherence of PSS erythrocytes may have pathological significance in the mechanism of vascular abnormalities in PSS.

Key words: adhesion, deformability, erythrocyte, scleroderma, surface charge.

Abbreviations: PBS, phosphate-buffered saline; PSS, progressive systemic sclerosis.

Introduction
Scleroderma (progressive systemic sclerosis, PSS) is a connective tissue disorder with characteristic excessive collagen deposition. The abnormal metabolism of all components of connective tissue has been revealed by extensive studies [1-4]. Besides connective tissue involvement, vascular abnormalities, both morphological (intimal proliferation) and functional (microcirculatory insufficiency), are distinctive features of the disease [1]. The mechanism of these vascular abnormalities in PSS or the link between the unbalanced metabolism of connective tissue components and vascular involvement is unknown. Hyperviscosity of whole blood, increase in acute phase reactants, reduced fibrinolytic activity and minor activation of platelets have been found in a heterogeneous group of patients who exhibited Raynaud's phenomenon [5]. Scleroderma serum was cytotoxic to cultured endothelial cells [6]. To our knowledge, no data have been published on the behaviour of erythrocytes.

Altered haemo-rheological properties is thought to be causally related to different thrombotic diseases [7] and abnormalities in erythrocyte behaviour have been found in diseases with severe vascular complications, such as sickle-cell anaemia [8-10] and diabetes mellitus [11]. We have therefore studied those functional characteristics of erythrocytes which are major determinants of the rheological properties of the blood, i.e. deformability and endothelial adherence in patients with PSS and Raynaud's disease. Our present findings raise the possibility that changes in behaviour of erythrocytes might be related to the vascular complications in scleroderma.

Subjects and methods
General
Blood samples were obtained from patients with Raynaud's phenomenon together with PSS, aged 28-78 years (mean 38). They had symptoms of the disease for 1-24 years (mean 8). Control blood samples were obtained from normal healthy volunteers aged from 21 to 65 years (mean 40). All PSS patients fulfilled the preliminary diagnostic criteria set for PSS [12].

Filterability of erythrocytes
Erythrocytes were separated from heparinized venous blood (Heparin sodium without preserva-
tive, Weddel Pharmaceuticals, London; 20 units/ml of blood), the buffy coat was carefully removed and the cells were washed in Dulbecco’s phosphate-buffered sodium chloride solution (154 mmol/l: saline), pH 7.4, containing 0.25% (v/v) human albumin. Packed cell volume was determined from a concentrated suspension (about 50%) and then adjusted to 10% (v/v) with phosphate-buffered saline (PBS)—albumin. This final erythrocyte suspension was passed through a polycarbonate sieve (Nucleopore Corp., Pleasanton, CA, U.S.A.; 13 mm diameter) of pore diameter 3 μm. The calculated cell/pore ratio, per ml of suspension filtered, was 800/ml. A vacuum of -10 cm water pressure was used. After 1 ml of cell suspension had flowed through the filter, the filtration time per millilitre volume was registered up to 10 ml and the slope was calculated from the linear segment of the curve plotted. A new filter from the same batch was used for each measurement and the filters were selected by measuring the flow rate of PBS-albumin (only filters with a flow rate of 9-9.5 ml/min were used). Triplicate determination were performed on each sample of blood and the difference between the triplicate measurements was usually less than 5%. The assay was performed within 2 h of venepuncture at room temperature.

Elimination of leucocytes

By repeated measurements the leucocyte count was around 100/μl of the washed erythrocyte suspension. In addition, in one experiment, blood was centrifuged against Hypaque–Ficoll mixture [13]. This resulted in an erythrocyte suspension practically free of leucocytes (less than 10/μl).

Electrophoretic velocity of erythrocytes

Electrophoretic motility of washed erythrocytes was measured by the moving boundary electrophoresis technique described in detail by Streichman et al. [14].

Removal and measurement of sialic acid from erythrocytes

To 200 μl of washed erythrocyte suspension (15%, v/v) 200 μl of neuraminidase (0.1 unit/ml; Clostridium perfringens, type V, Sigma, dissolved in Dulbecco’s PBS, pH 7.4) was added and incubated for 60 min at 37°C. A portion (100 μl) of the supernatant was used for sialic acid determination.

Sialic acid was determined by a fluorimetric procedure described by Hess & Rolde [15].

Endothelial adherence measurement

Adhesion of erythrocytes to cultured endothelium was measured by the technique described by Hebbel et al. [10]. Endothelial cells were obtained from pig aorta and grown to confluency by the method of Pearson et al. [16]. A portion (0.2 ml) of 51Cr-labelled erythrocyte suspension (25%, v/v) layered on endothelium which was grown on a plastic plate closely fitted into a 6 mm diameter tissue culture well and was allowed to undergo static incubation at 37°C for 30 min. Non-adherent cells were decanted and, after being removed from the well with a fine forceps, the plastic plate with the endothelium was rinsed six times in saline and finally dropped into distilled water to lyse the remaining cells. The radioactivity of this final lysate (two plates combined) was measured. Erythrocyte suspensions from individual patient were tested in 6–12 wells and simultaneously normal erythrocytes were also tested under identical conditions. Adherence to endothelium was expressed as a ratio (adherence ratio) = radioactivity from PSS erythrocytes/radioactivity from normal erythrocytes.

Statistical treatment of data

The values are given as means ± SEM and statistical comparisons were made by Student’s paired t-test, except the filterability of erythrocytes, where a two-tailed signed-rank (non-parametric) test was used.

Results

Filterability of PSS erythrocytes was significantly lower than that of cells from normal, healthy subjects (Table 1). The difference in the flow rate between the PSS and control erythrocytes was less striking, when filters of 5 μm pore diameter instead of 3 μm were used. There was a considerable variation among the patients, but for individual patients the measured filtration rates were surprisingly constant and reproducible at different times. The low leucocyte count was a reassurance that ‘clogging’ was not a significant factor in the measurements. Additionally, in one experiment we used erythrocyte suspension, separated with density gradient centrifugation practically free of leucocytes. By using this suspension, flow rates similar to those from the washed cells of normal subjects were obtained.

Moving boundary electrophoretic velocity of PSS erythrocytes was significantly lower than that of normal cells (Table 1). The difference in the velocity between the PSS and normal erythrocytes
Erythrocytes in scleroderma

TABLE 1. Filterability, surface sialic acid density and moving boundary electrophoretic velocity of erythrocytes of normal subjects and patients with Raynaud’s phenomenon and progressive systemic sclerosis (PSS)

Mean values ± SEM are shown.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th></th>
<th>PSS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean</td>
<td>No.</td>
<td>Mean</td>
</tr>
<tr>
<td>Filterability (ml/min)</td>
<td>30</td>
<td>0.471 ± 0.02</td>
<td>30</td>
<td>0.220 ± 0.01*</td>
</tr>
<tr>
<td>Sialic acid (nmol/10^9 cells)</td>
<td>15</td>
<td>42.0 ± 1.1</td>
<td>15</td>
<td>35.6 ± 1.2*</td>
</tr>
<tr>
<td>Electrophoretic mobility</td>
<td>15</td>
<td>(A) 0.501 ± 0.08</td>
<td>15</td>
<td>0.459 ± 0.06*</td>
</tr>
<tr>
<td>(mm/min)</td>
<td></td>
<td>(D) 0.689 ± 0.10</td>
<td></td>
<td>0.535 ± 0.07*</td>
</tr>
</tbody>
</table>

* Difference from the controls is significant: P < 0.001.
† (A) Ascending, (D) descending boundaries.

was approximately the same, when either descending boundaries which move faster, or the ascending ones, were compared.

In PSS erythrocytes 23% less neuraminidase-susceptible sialic acid density per cell was found, than in the control cells (Table 1).

Added in Hank’s solution (containing albumin) PSS erythrocytes showed more adherence to pig confluent endothelial cells than cells from healthy volunteers (Fig. 1). In two experiments, addition of a small amount of the patient’s own plasma further increased the adherence of PSS erythrocytes (data are not shown as there were insufficient for statistical comparison).

Discussion

Earlier, in a limited number of patients, we reported a decreased filterability of erythrocytes in PSS and the coincidence of the clinical improvement with the increased filterability of cells in response to prostaglandin infusions [17]. This paper is an extension of our preliminary findings and in a larger number of patients the decreased filterability of erythrocytes is confirmed.

Because of its simplicity, the filtration technique is widely used to assess flexibility of erythrocytes and has proved to be sensitive enough to detect changes in behaviour of cells in a number of diseases with vascular involvement [17, 18]. However, the filtration technique, even when used as recommended [19] (using washed cells and minimizing the error of pore obstruction by leucocytes), is merely an approximation of the more complex phenomenon, deformability, which occurs when the cell passes through capillaries [20]. During this passage, the erythrocyte not only changes its shape but the adjacent membranes of erythrocytes and endothelial cell come into close contact over a relatively large area. The surface properties of the membranes therefore play a decisive role in determining the transit times in the microcirculation. Electrostatic repulsion between the negatively

FIG. 1. Adhesion of 51Cr-labelled erythrocytes from sclerodermic patients (n = 10) to cultured endothelial cells. Distribution of adherence ratios (adhesion ratio = radioactivity of PSS erythrocytes/radioactivity of normal erythrocytes).
charged membranes of erythrocytes and endothelial cells is an important determinant of the interaction. Thus decrease of surface charge could induce cell adhesion and, hence, microvascular insufficiency.

N-Acetylneuraminic (sialic) acid moieties of the membrane glycoproteins are responsible for the bulk net negative charges at the cell surface. Measurement of the enzymatically removable sialic acid content from PSS erythrocytes showed significantly less density than the matched control cells, and this was in accordance with a decreased electrrophoretic mobility of PSS erythrocytes.

Surface sialic acid density of different cells in the circulation was found to be a critical determinant of the life-span. As low as 5% decrease of total surface sialic acid density was an efficient signal for the specific receptors to remove thrombocytes from the circulation [21]. In subjects with sialo-glycoprotein deficient erythrocytes, however, there was no evidence of reduced life-span [22] and hence it was concluded that changes in surface charge do not determine the survival of erythrocytes [23]. This agrees with the lack of reports of altered erythrocyte life-span in PSS. However, removal of sialic acid from the surface of blood cells [24, 25] or endothelial cells [26] did induce cell adhesion, a phenomenon of great pathological significance. PSS erythrocytes with lower sialic acid and charge densities adhered in much higher numbers to the cultured endothelial cells than cells from normal controls. Increased adhesion of erythrocytes to endothelial cells has been reported in sickle-cell anaemia [9, 10] and diabetes mellitus [11]. In both diseases filterability of erythrocytes was decreased [7, 18], and adherence of cells to the endothelium was considered to be related to the vascular complications.

The cause of the increased endothelial adherence of PSS erythrocytes may be caused by an increased activity of lysosomal glycosidase in scleroderma serum [2], an enzyme which can split off the carbohydrate chains of membrane glycoproteins. Furthermore, decrease in neuraminidase-removable sialic acid does not necessarily mean less sialic acid moieties on the cell surface. It has been pointed out that adsorbed plasma proteins, mainly γ-globulins, mask negatively charged sites on the erythrocyte glycocalyx [27]. Hypergammaglobulinaemia has been a frequent finding in patients with PSS and increases in γ-globulins were reported to run parallel with the extent of cutaneous and visceral disease [28]. Being masked by the adsorbed plasma proteins, those sialic acid moieties lose their contribution to the surface charge and are not necessarily accessible to the enzyme. This possibility is supported by our present observation that, in two cases, addition of a small quantity of the patient's own plasma increased the endothelial adhesiveness of erythrocytes. A similar observation was made on sickled erythrocytes by Hebbel et al. [10].

Increased rigidity (decreased filterability), increased endothelial adhesiveness and decreased surface charge of PSS erythrocytes are related to the disease and they may be of pathological importance by providing a possible explanation of the rheological abnormalities and vascular manifestations of PSS.

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References


