Sickle cell disease, pathophysiology and clinical complications

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Sickle cell patients are characterized by a reduced glycocalyx volume

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Abstract

Recently, the importance of the glycocalyx as a protective anti-inflammatory and anti-adhesive barrier at the luminal side of endothelial cells has been established. The mean glycocalyx volume was significantly reduced in patients with HbSS/HbSβ0 (median 0.47L, inter quartile range 0.27-0.66) and HbSC/HbSβ+ (0.23L, 0.0-0.58) as compared to controls (1.09L, 0.52-1.77) (p=0.03).
Background and objectives

The vaso-occlusive process of sickle cell disease (SCD) leads to accumulating ischemic organ damage and a decreased life expectancy. Vaso-occlusion in SCD was primarily attributed to the mechanical obstruction of the microvasculature by circulating sickled erythrocytes. Over the last decades, it has become clear that endothelial activation and dysfunction play a central role in sickle cell vaso-occlusion.¹ Hypoxia, pro-inflammatory cytokines, thrombin, reactive oxygen species, activated leukocytes, as well as direct mechanical injury by sickled erythrocytes all induce endothelial activation in SCD. Activated endothelium itself contributes to the initiation and progression of vaso-occlusion by releasing pro-inflammatory cytokines, pro-coagulant microparticles and by initiating coagulation with surface tissue factor expression.¹ Endothelial activation is characteristic of SCD, even in the clinically asymptomatic state, with further increments during acute vaso-occlusive complications.²³⁴ Furthermore, the extent of endothelial activation was recently demonstrated to be related to the presence of severe disease related manifestations such as pulmonary hypertension.⁵

New insights into endothelial biology have demonstrated that, in the quiescent state, the endothelium is shielded from circulating blood cells and proteins by the glycocalyx, a highly hydrated cell free mesh of membrane-associated proteoglycans, glycosaminoglycans, glycoproteins and glycolipids located at the endothelial surface.⁶ With a thickness ranging from 0.5 to 3.0 μm, it exceeds the intra-luminal size of most endothelial adhesion molecules, thereby potentially preventing interactions of the endothelium with blood constituents.⁷⁸ The glycocalyx volume is regulated by numerous factors and rapid degradation has been observed during ischemia, hypoxia, and exposure to both tumor necrosis factor alpha and oxidized low density lipoproteins.⁹¹⁰¹⁴ Therefore, in conditions of ischemia and inflammation, which occur continuously in SCD, a reduced glycocalyx volume may facilitate interactions of activated endothelial cells with blood cells and proteins. Given the important role of endothelial activation in the pathophysiology of SCD related vaso-occlusion, we set out to investigate whether the glycocalyx volume is reduced in patients with SCD.
Design and methods

Patients and controls
Consecutive adult sickle cell patients (HbSS, HbSβ+/−-thalassemia or HbSC, confirmed with high performance liquid chromatography) visiting the out-patient clinic of the Academic Medical Center in Amsterdam were eligible for inclusion in this study. Exclusion criteria were a history of an acute vaso-occlusive episode (painful crisis, acute chest syndrome, stroke, splenic- or liver sequestration) or blood transfusion 4 weeks prior to sample collection; diabetes mellitus; hemorrhagic retinopathy; hemorrhagic disorders or hypertension (systolic and diastolic blood pressure >140 mm Hg or >90 mm Hg, respectively). For data analysis patients with the most severe genotypes (HbSS and HbSβ+/−-thalassemia) were grouped together, as were patients with the relatively milder genotypes (HbSC and HbSβ+−-thalassemia). Age, sex and race matched individuals heterozygous for hemoglobin C or S (HbAS and HbAC, confirmed by high performance liquid chromatography) served as healthy controls. All patients and controls gave written informed consent and this study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Measurement of systemic glycocalyx volume
The glycocalyx volume can be estimated by comparing circulating blood volume with the intravascular distribution volume of a glycocalyx-permeable tracer such as neutral dextran 40 (molecular weight 40 kDa) as has been described previously. To determine the intravascular distribution volume of dextran 40 at the time of injection was estimated by exponential fitting of the measured dextran 40 concentrations. The intravascular distribution volume of labeled, autologous erythrocytes was used to quantify circulating blood volume. Blood was drawn into heparin collector tubes and centrifuged at 1,330 rpm for 5 min. Subsequently, 250 mg/ml of sodium fluorescein was added to the erythrocyte fraction for 5 min. After washing with water (5% sodium chloride), labeled erythrocytes were re-suspended in saline to the initial volume and re-infused into the patients/controls. Subsequently, blood was drawn at 4, 5, 6, and 7 min after infusion. The fraction of labeled erythrocytes compared with total erythrocyte pool was used to estimate circulating erythrocyte volume. Pre-injection unlabeled erythrocytes (t = −1) served as negative controls. Labeled erythrocytes were measured using a FACScan (FACSCalibur, Becton Dickinson, Mountain View, CA), during which at least 100,000 cells were counted to measure the circulating fraction of labeled erythrocytes. Circulating plasma volume was calculated from circulating erythrocyte volume (Vrbc) and systemic hematocrit (Hsys) by the following formula: circulating plasma volume = ([1 − Hsys] x Vrbc)/Hsys.  

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Dextran 40 was used as a probe to estimate total intravascular volume including the glycocalyx compartment.\textsuperscript{15,17,20} A bolus of 10 ml dextran 1 (Promiten; NPBI International, Emmercompascuum, the Netherlands) was injected to attenuate the risk for anaphylactic reactions. At least 1 h later, 100 ml dextran 40 (Rheomacrodex; NPBI International) was administered intravenously, followed by repeated blood sampling at 5, 7, 10, 15, 20, and 30 min. The dextran 40 concentration was calculated by measuring the increase in glucose concentration in the post infusion samples after hydrolyzation of dextran 40 glucose polymers.\textsuperscript{20} The glucose concentration per time point was assessed in duplicate using the hexokinase method (Gluco-quant, Hitachi 917; Hitachi). To determine the intravascular distribution volume of dextran 40, the concentration of dextran 40 at the time of injection was estimated by exponential fitting of the measured dextran 40 concentrations. To further evaluate the relation between glycocalyx volume and other parameters the groups of patients were split into halves; a small glycocalyx volume-group and a large glycocalyx volume-group.

**Analysis of blood samples**

At baseline, blood samples were collected and placed on ice immediately. Hematocrit was measured after centrifugation of heparinized blood at 10,000 rpm for 5 minutes. The remaining blood samples were centrifuged at 3,000 rpm for 15 minutes; plasma was collected and stored at -80°C. Quantitative plasma hyaluronan levels were measured by enzyme-linked immunosorbent assay (Echelon Biosciences, Salt Lake City, UT). Plasma hyaluronidase levels were determined with a previously described assay (intersample coefficient of variation <20%).\textsuperscript{21}

**Statistics**

Data were expressed as medians with corresponding interquartile ranges (IQR) or as stated otherwise. Between group differences were tested with the Kruskal Wallis Test and post-tested to find which groups differ from each other with Mann-Whitney U test. Categorical data are presented as percentages or numbers. Differences between groups of categorical data are tested with the Chi-square test. For correlation studies the Spearman Rank correlation coefficient was determined. Multiple regression analysis with genotype as co-factor was used to correct for genotype. P-values ≤0.05 were considered statistically significant. Statistical analysis was performed by using SPSS 12.0.2 (SPSS Inc, Chicago, IL).
Chapter 6

Results

Patients and controls
Twenty sickle cell patients and 10 individuals with the HbAS (n=9) or HbAC (n=1) were included. The severe genotype group consisted of 9 HbSS and 3 HbSββ-thalassemia patients, and the mild genotype group consisted of 6 HbSC and 2 HbSβ+-thalassemia patients. The baseline patient characteristics and laboratory data are presented in Table 1. Four patients in the severe genotype group used hydroxyurea.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HbSS/HbSββ-thal</th>
<th>HbSC/HbSβ+-thal</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>12 (9/3)</td>
<td>8 (6/2)</td>
<td>10</td>
</tr>
<tr>
<td>Age (y)</td>
<td>33 (22-43)</td>
<td>31 (26-39)</td>
<td>40 (27-42)</td>
</tr>
<tr>
<td>Male/Female</td>
<td>1/11</td>
<td>3/5</td>
<td>3/7</td>
</tr>
<tr>
<td>Systole (mmHg)</td>
<td>110 (104-121)</td>
<td>114 (101-118)</td>
<td>121 (112-139)</td>
</tr>
<tr>
<td>Diastole (mmHg)</td>
<td>68 (65-78)</td>
<td>70 (56-76)</td>
<td>82 (77-92)*</td>
</tr>
<tr>
<td>BMI</td>
<td>21 (20-24)</td>
<td>23 (22-26)</td>
<td>27 (23-31)*</td>
</tr>
</tbody>
</table>

Blood parameters

<table>
<thead>
<tr>
<th></th>
<th>HbSS/HbSββ-thal</th>
<th>HbSC/HbSβ+-thal</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (mmol/L)</td>
<td>5.5 (5.1-6.0)</td>
<td>7.1 (6.3-7.8)*</td>
<td>8.5 (7.3-8.9)**</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>8.8 (6.3-11)</td>
<td>2.8 (2.1-2.9)*</td>
<td>1.1 (0.9-1.7)**</td>
</tr>
<tr>
<td>Leucocyte count (x10⁹/L)</td>
<td>8.0 (6.5-10.1)</td>
<td>7.0 (5.7-8.2)</td>
<td>7.2 (3.3-9)</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/L)</td>
<td>385 (289-570)</td>
<td>220 (181-235)**</td>
<td>171 (164-181)**</td>
</tr>
<tr>
<td>Fetal Hemoglobin(%)</td>
<td>9.4 (4.2,14.7)</td>
<td>2.4 (0.5-3.6)</td>
<td>0.5 (0.5-0.5)**</td>
</tr>
</tbody>
</table>

All numbers are medians (interquartile range). *P<0.05 versus HbSS/HbSββ-thal ** P<0.001 versus HbSS/HbSββ-thal. P-values are based on Mann Whitney U or Chi-square tests.

Glycocalyx volume

The glycocalyx volume was statistically significantly lower in patients as compared to controls (Figure 2, Table 2). Hyaluronan levels, but not hyaluronidase activity, differed significantly between groups (Table 2). There were no statistically significant differences in blood volume, plasma volume and dextran distribution volume between the groups (data not shown). Analysis of bi-variate correlations (Spearman) of all data showed that glycocalyx volume was correlated to plasma levels hyaluronidase (r=-0.431; P=0.017). Correction for genotype did not alter this relation. Use of hydroxyurea did not have influence on glycocalyx volume.
Figure. Glycocalyx volume in sickle cell patients and healthy controls.
The glycocalyx volume differed significantly between the HbSS/HbSβ+thal group (median 0.47 liters (L), IQR 0.27-0.66), the HbSC/HbSβ+thal group (0.23L, IQR 0.0-0.58) and healthy controls (1.09L, IQR 0.52-1.77) (Kruskal Wallis P=0.025). Bars represent medians and error bars the 75th percentile.

Table 2. Glycocalyx and related parameters.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HbSS/HbSβ-thal</th>
<th>HbSC/HbSβ+thal</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycocalyx volume (L)</td>
<td>0.47 (0.27-0.66)</td>
<td>0.23 (0.0-0.58)</td>
<td>1.09 (0.52-1.77)*</td>
</tr>
<tr>
<td>Hyaluronan (ng/mL)</td>
<td>170 (129-299)</td>
<td>96 (89-109)*</td>
<td>120 (84-184)</td>
</tr>
<tr>
<td>Hyaluronidase (U/ml)</td>
<td>118 (70-188)</td>
<td>108 (81-145)</td>
<td>106 (91-242)</td>
</tr>
</tbody>
</table>

All numbers are medians (interquartile range). *P<0.05 versus HbSS/HbSβ-thal (Mann Whitney U test).
Interpretation and conclusion

New insights into endothelial biology have demonstrated an important role of the glycocalyx in vascular homeostasis.\textsuperscript{9} Loss of glycocalyx by acute hyperglycemia in healthy controls results in endothelial dysfunction and glycocalyx loss is associated with microvascular damage in type 1 diabetes.\textsuperscript{15,17} In the present study, we demonstrated that the glycocalyx volume is significantly reduced in patients with SCD as compared to the controls (the latter characterized by glycocalyx volumes comparable to those reported for healthy controls in other studies). The glycocalyx volume in our sickle cell patients was comparable to that reported in patients with diabetes mellitus type 1.\textsuperscript{15,17} The median glycocalyx volume in the HbSS/HbS\textsuperscript{A}+-thal group (0.47L) was slightly higher than the median glycocalyx volume (0.23L) in the HbSC/HbS\textsuperscript{A}+-thal group. This difference was not significant and might be caused by the low number of subjects in the HbSC/HbS\textsuperscript{A}+-thal group or the fact the we may have included patients with HbSC/HbS\textsuperscript{A}+-thal with relatively severe disease.

Hyaluronidase is an enzyme that degrades hyaluronan, the major glycosaminoglycan component of the glycocalyx.\textsuperscript{21} In line with the findings in patients with diabetes type 1, glycocalyx volumes were inversely correlated to hyaluronidase levels in our patients. However, as the hyaluronidase levels were comparable between the different groups of patients and controls this can not explain the difference between these groups in glycocalyx volume. Studies in humans and animals have shown that the glycocalyx volume is reduced after episodes of experimental ischemia, hypoxia and after exposure to oxidized low-density lipoproteins and oxygen radicals. Acute hyperglycemia, which is associated with the formation of reactive oxygen species, results in a profound loss of glycocalyx volume in healthy volunteers.\textsuperscript{22} Continuous tissue ischemia and reperfusion is associated with the formation of reactive oxygen species in SCD which may result in a reduced glycocalyx volume.

A reduction of the glycocalyx could have several important consequences in SCD. Adhesive interactions between sickle red blood cells, leukocytes and the activated endothelium play a pivotal role in the initiation and propagation of SCD related vaso-occlusion. A reduced glycocalyx volume may facilitate such interactions. A reduced glycocalyx volume may also contribute to the hypercoagulable state of SCD by reducing the availability of naturally occurring anticoagulants, such as thrombomodulin and antithrombin, at the vessel wall.

In conclusion, we demonstrate that the presented sickle cell patients are characterized by a reduced glycocalyx volume. We hypothesize that glycocalyx perturbation could be a new factor of bi-directional importance in the complex pathophysiology of SCD, as glycocalyx volume reductions can both result from and contribute to vaso-occlusion.
Acknowledgments

E.J. van Beers performed the research, analyzed the data and wrote the paper. M.Nieuwdorp designed and performed the research and critically reviewed the paper. L.M.Evers and A.J.Duits performed the research and critically reviewed the paper. H.Vink designed the research, analyzed the data and critically reviewed the paper. J.J.B.Schnog analyzed the data and critically reviewed the paper. B.J.Biemond designed the research, analyzed the data and wrote the paper.
Reference List


