Sialic Acid Content in Erythrocyte Membranes From Pregnant Women Affected by Gestational Diabetes

N. Moretti, R.A. Rabini, L. Nanetti, G. Grechi, M.C. Curzi, N. Cester, L.A. Tranquilli, and L. Mazzanti

Sialic acid (SA) content, membrane fluidity, and Na^+/K^+-adenosine triphosphatase (ATPase) activity were determined in erythrocyte membrane from 10 nonpregnant women (HNPW), 16 pregnant women affected by gestational diabetes mellitus (GDM), and 25 healthy pregnant women (HPW). In GDM patients the membrane erythrocyte SA content was significantly increased compared with HNPW and membrane fluidity was significantly increased in comparison with HPW. Erythrocyte membrane Na^+/K^+-ATPase activity was significantly reduced in GDM patients compared both to HNPW and to HPW subjects. A significant inverse correlation was found between 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) anisotropy and erythrocyte membrane SA content in HNPW and in HPW, while this significant correlation was not observed in GDM. The present results indicate that in comparison with normal pregnancy GDM is characterized by deep alterations of the erythrocyte plasma membrane physicochemical properties (increased fluidity) and functional activities (reduced Na^+/K^+-ATPase activity). These modifications might be at the basis of the altered blood viscosity and placental perfusion observed under such conditions. Moreover, these results show that in physiological pregnancy and in the nonpregnant state, the erythrocyte surface membrane fluidity is inversely correlated with SA content, while in GDM there is an unbalance of this relation, which might be associated with the microcirculatory abnormality present in this disease.

ERYTHROCYTE AGGREGATION is one of the main determinants influencing blood circulation at low shear rates by increasing blood viscosity and inducing "sludging" in the capillary bed. Aggregation of red blood cells (RBC) is a reversible process that occurs when the bridging force due to the adsorption of macromolecules onto adjacent cell surface exceeds the disaggregation forces caused by electrostatic repulsion, membrane strain, and mechanical shearing. An increase in erythrocyte membrane aggregation was found to be associated with cardiovascular risk factors such as diabetes, hypertension, and hyperlipoproteinemia, and in clinical situations such as myocardial ischemia, thromboembolic states, and retinal venous occlusion.[1-5]

Blood viscosity is determined by the chemical and physical properties of corpuscles and plasma components, and is the main limiting factor on flow in the microcirculation, particularly in the placental microvessels.[6] The density of RBC and their deformability are important in determining blood viscosity. The determinants of RBC deformability are mainly the ratio between area and volume, the internal viscosity, that is the density of haemoglobin packing within the cell, and the membrane properties. When RBC deformability is decreased, blood viscosity is significantly increased. This may be associated with a tendency toward reduced placental perfusion, as well as a shortened cell lifespan in maternal circulation.[7,8]

Sialic acid (SA) is a family of acetylated or glycosylated derivatives of neuraminic acid that are present in many membrane glycoproteins and glycolipids. Membrane-bound SA with its carboxyl groups contributes to the majority of the negative surface charge of the erythrocytes.

Type 1 and type 2 diabetes mellitus are characterized by abnormal blood flow patterns in the microcirculation.[9] Gestational diabetes mellitus (GDM) is also associated with increased whole blood viscosity and reduced erythrocyte deformability, which might cause microcirculatory alterations even in the presence of a good metabolic control and of a short disease duration.[10] The purpose of this investigation was to examine in pregnancies complicated by GDM the RBC membrane SA content, which is a determinant of aggregation,[11] and RBC membrane Na^+/K^+-adenosine triphosphatase (ATPase) activity and fluidity, which might be related to the SA content[12] and to cell deformability.[13] Na^+/K^+-ATPase is also a marker of membrane function, being an integral membrane protein that greatly depends for its activity on the physicochemical properties of the membrane microenvironment where it is embedded.[14,15] Moreover, previous studies by our group reported a significant alteration of Na^+/K^+-ATPase activity and fluidity of the cellular membranes in GDM patients.[16]

PATIENTS AND METHODS

We studied 10 healthy nonpregnant women (HNPW; age, 30 ± 4 years), 25 healthy pregnant women (HPW; age, 28 ± 5 years), and 16 pregnant women who satisfied criteria for the diagnosis of GDM (GDM subjects; age, 29 ± 6 years).[17] All of the pregnant women were comparable for gestational age (28 to 32 weeks). GDM women were under dietetic control (1,800 kcal/d; 50% carbohydrates) and none was receiving insulin at the time of sampling. All of the subjects were normoalbuminuric and normotensive, with plasma lipid levels and body mass index within the normal range. The patients affected by GDM were in good glycemic control (fasting glycemia = 4.5 ± 0.6 mmol/L for HPW and 5.2 ± 0.7 mmol/L for GDM patients, HbA_{1c} levels = 5.0% ± 0.6% *v* 4.6% ± 0.5%). All of the subjects gave written informed consent before inclusion in the study. Blood was drawn in the fasting state and the SA content, Na^+/K^+-ATPase activity and fluidity of erythrocyte membranes were determined as described below.

From the Institute of Biochemistry and Department of Gynaecology Obstetrics, School of Medicine, University of Ancona; Department of Diabetology, INRCA, Ancona; and the Department of Obstetrics and Gynaecology, Jesi and Senigallia Hospitals, Italy.

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Address reprint requests to Professor Laura Mazzanti, Institute of Biochemistry, School of Medicine, University of Ancona, Via Ranieri 65, Ancona, Italy.

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Erythrocyte Membrane Preparation

Heparinized blood samples were centrifuged (4,500 × *g*) to remove plasma. RBC were washed twice with isotonic NaCl, lysed hypotonically in 5 mmol/L ice-cold phosphate buffer solution (pH 8), and processed in a Kontron centrifuge (Zurich, Switzerland) at 20,000 × *g* for 20 minutes at 4°C to obtain the membranes. These were then washed with phosphate buffer of decreasing molarity to remove the hemoglobin completely according to the method of Burton et al.[18]

Determination of SA Content

SA content of RBC membranes was determined by the periodate-thiobarbituric acid method of Denny at al.[19] Briefly, membranes (1 mg membrane proteins/mL) were first hydrolyzed in 0.05 mol/L H_2SO_4 in a final volume of 1.0 mL for 1 hour at 80°C to release SA. Both standards and samples were incubated with 0.25 mol/L periodate solution (0.025 mol/L periodic acid in 0.25 mol/L HCl) at 37°C for 30 minutes. After reduction of excess periodate with 0.25 mL 0.32-mol/L sodium thiosulfate, the reaction was completed by addition of 1.25 mL 0.1-mol/L thiobarbituric acid. The samples were heated at 100°C for 15 minutes and cooled to room temperature. The product was extracted with acidic butanol and colorimetrically assayed with a Kontron spectrophotometer at 549 nm.

Na^+/K^+-ATPase Activity

The Na^+/K^+-activated Mg_2^+-dependent ATPase activity was determined in RBC plasma membranes by the method of Kitao and Hattori.[20] The ATPase activity was assayed by incubating the membranes (0.1 mg membrane proteins) at 37°C in 1 mL of medium containing $MgCl_2$ (5 mmol/L), NaCl (140 mmol/L), and KCl (14 mmol/L) in 40 mmol/L Tris-HCl, pH 7.7. The ATPase reaction was started by the addition of 3 mmol/L Na_2 ATP and stopped 20 minutes later by the addition of 1 mL of 15% trichloracetic acid. Inorganic phosphate (P_i) hydrolized from reaction was measured as previously described.[21] Enzyme activity was expressed as the difference in inorganic phosphate released in the presence and absence of 1 mmol/L ouabain. The ATPase activity assayed in the presence of ouabain was subtracted from the total Mg_2^+-dependent ATPase activity to calculate the activity of the ouabain-sensitive Na^+/K^+-ATPase. The results are expressed as micromoles P_i/(mg membrane proteins × 60 min). Protein concentration was determined as described by Lowry et al[22] using serum albumin as a standard.

Fluorescence Studies

The fluorescent probe 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was incubated with the RBC membranes at 37°C for 5 minutes, following the procedures described by Shinitzky and Barenholz.[23] The probe concentration was chosen in respect to a final probe/phospholipid ratio always less than 1:1,000. The measurements were carried out on a Perkin-Elmer LS 50B spectrofluorometer (Norwalk, CT) equipped with fluorescence polarization accessory and a controlled temperature cell holder. The excitation and emission wavelenghts were 360 nm and 430 nm, respectively. The fluorescence anisotropy (r) was calculated by the following equation:

$$r = (I_vG - I_h)/(I_vG + 2I_h)$$

where G is the instrumental factor that corrects the r value for an equal detection of vertically (I_v) and horizontally (I_h) polarized light.[24]

Fluorescence anisotropy is a quantitative index of the freedom of rotation of the probe; a decrease in the r value indicates a higher mobility of TMA-DPH in the site where it is located, ie, increased membrane fluidity.

Statistical Analysis

Statistical analyses were performed using analyis of variance (ANOVA). To reduce the probability of significant differences arising by chance, Bonferroni correction was applied to the data following ANOVA. Linear regression analysis was used to evaluate the correlation between the parameters studied. *P* values less than .05 were considered significant.

RESULTS

As shown in Table 1, in GDM patients the membrane erythrocyte SA content was significantly increased compared with HNPW, while it was not changed if compared with HPW.

As concerns the steady-state fluorescence results (Table 1), HPW did not show significant erythrocyte membrane fluidity alterations in respect to the nonpregnant state. In GDM subjects membrane fluidity was significantly increased in comparison to HPW.

Erythrocyte membrane Na^+/K^+-ATPase activity was significantly reduced in GDM patients compared to HNPW and HPW subjects, while no changes were observed between HNPW and HPW (Table 1).

A significant inverse correlation was found between TMA-DPH anisotropy and erythrocyte membrane SA content in HNPW and in HPW ($r = -0.863$, $P < .001$; $r = -0.673$, $P < .001$, respectively). This significant correlation was not observed in GDM ($r = 0.343$, $P = .193$).

No significant relation was observed between Na^+/K^+-ATPase activity and either SA content and TMA-DPH anisotropy in any of the groups studied (data not shown).

Table 1. Erythrocyte Membrane SA Content, Na^+/K^+-ATPase Activity, and Fluidity Evaluated as Anisotropy of the Probe TMA-DPH

	SA Content (μg/mg protein)	TMA-DPH Anisotropy (r)	Na/K-ATPase activity (μmol P_i/mg protein/60 min)
HNPW (n = 10)	42.33 ± 15.85	0.252 ± 0.043	1.71 ± 0.18
HPW (n = 25)	96.30 ± 34.20*	0.276 ± 0.041†	1.60 ± 0.14
GDM (n = 16)	81.29 ± 41.80*	0.223 ± 0.044‡	1.31 ± 0.09§

NOTE. Values are mean ± SD.

Abbreviations: HNPW, healthy nonpregnant women; HPW, healthy pregnant women; GDM, gestational diabetes mellitus.

*$P < .001$ *v* HNPW.

†$P < .05$ *v* GDM.

‡$P < .05$ *v* HPW.

§$P < .001$ *v* HNPW and HPW.

DISCUSSION

SA plays a central role in the functioning of biological systems, being commonly positioned at the terminal positions of complex carbohydrates. Multiple factors affecting the sialylation and desialylation of glycoproteins and glycolipids may change the SA content in plasma and in different body compartments. Recent studies found significant elevations in serum SA during pregnancy, which persisted 12 weeks postpartum,[25] but the mechanisms underlying this increase were unclear. In the present work a marked increase in the SA content of erythrocyte membranes was observed in normal pregnancies compared to the nonpregnant status. Such elevation might be dependent on the concomitant increase in serum total SA and it might be hypothesized that these modifications in SA content during pregnancy are related with the sialyl transferase activity found in human placenta.[26]

During GDM the erythrocyte membrane SA content was not changed in comparison to normal pregnancies, confirming previous data reporting the lack of modifications in this parameter in young type 1 diabetic subjects compared to age-matched controls.[12] On the contrary, the RBC membrane SA content was previously found to be increased in elderly type 2 diabetic patients compared to age-matched healthy subjects.[12] It might be suggested therefore that the SA content in erythrocyte membranes shows a different behavior in the diabetic condition in an age-dependent manner, being significantly increased only in older subjects.[12]

The data obtained by the study of TMA-DPH anisotropy indicate that the fluidity of the erythrocyte membrane external leaflet is significantly higher in GDM patients compared both tonon pregnant women and to pregnant controls. These results might be dependent on the decreased cholesterol-phospholipids plasma membrane ratio previously observed in erythrocyte membranes from GDM patients,[16] while no relation might be hypotesized with the SA content in GDM on the basis of the lack of statistical correlation between these 2 parameters. On the contrary, in physiological conditions (both in normal pregnancy and in the nonpregnant status) the fluidity of the superficial part of the membrane is significantly related with the SA content. An increase in membrane SA content is associated with reduced TMA-DPH anisotropy, ie, with higher fluidity of the membrane surface.

The action of the SA content on fluidity has been previously demonstrated by other studies, reporting that the fluidity of the intestinal brush-border membranes increases by desialylation by neuraminidase treatment[27-29] and that desialylation also decreases the molecular order in low-density lipoproteins.[30] Moreover, in alcohol-dependent patients a statistically significant relation was found between the decreased SA content of erythrocyte membranes and the abnormal membrane fluidization evaluated by TMA-DPH.[31] Our results in normal pregnancy and in the non pregnant status are consistent with these previous studies.

The study of the plasma membrane Na^+/K^+-ATPase activity in GDM is relevant as its decrease has been suggested to play a role in the pathophysiology of the chronic complications of diabetes.[32] The present study demonstrates that the Na^+/K^+-ATPase activity was significantly reduced in GDM patients compared to both control groups, confirming our previous reports.[19,32] The lack of a significant correlation between Na^+/K^+-ATPase activity and SA content is in disagreement with the behavior observed in insulin-dependent and non–insulin- dependent patients and in healthy subjects aged 18 to 88 years.[12] However, it must be underlined that all the groups studied in the present work were formed of young women ranging in age between 18 and 40 years, so that differences depending on the subjects' age might not become evident.

In conclusion, the present results indicate that in comparison with normal pregnancy GDM is characterized by deep alterations of the erythrocyte plasma membrane physicochemical properties (increased fluidity) and functional activities (reduced Na^+/K^+-ATPase activity). These modifications might be at the basis of the altered blood viscosity and placental perfusion observed under such conditions. Moreover, these results indicate that in physiological pregnancy and in the non pregnant state the erythrocyte surface membrane fluidity is inversely correlated with SA content, while in GDM there is an unbalance of this relation, that might be associated with the microcirculatory abnormality present in this disease.

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