Sialic Acid, Diabetes, and Aging: A Study on the Erythrocyte Membrane

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Sialic acid (SA) content and Na^+/K^+-ATPase activity of red blood cell (RBC) membranes were studied in 26 normoalbuminuric patients with insulin-dependent diabetes mellitus (IDDM), 25 normoalbuminuric patients with non–insulin-dependent diabetes mellitus (NIDDM), and 40 healthy nondiabetic subjects with a negative family history for diabetes. A decrease in RBC membrane SA content and Na^+/K^+-ATPase activity was observed in older control subjects compared with younger controls. A significant correlation between age, Na^+/K^+-ATPase activity, and SA content was also found. No difference was observed in RBC membrane SA content between IDDM and NIDDM subjects, but Na^+/K^+-ATPase activity was significantly lower in IDDM patients. SA content was increased in NIDDM subjects compared with healthy subjects of similar age, whereas Na^+/K^+-ATPase activity was significantly lower in both IDDM and NIDDM subjects compared with controls. In NIDDM, Na^+/K^+-ATPase activity was significantly correlated with age, whereas both Na^+/K^+-ATPase activity and SA content were significantly correlated in IDDM and NIDDM patients. Hemoglobin A_{1c} (HbA_{1c}) levels did not show any significant correlation either with Na^+/K^+-ATPase or with SA content in diabetic patients. The modified SA content and Na^+/K^+-ATPase activity in elderly subjects described in the present study indicate a similar behavior of the erythrocyte membrane during both RBC senescence and aging of subjects.

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SIALIC ACID (SA)—a family of acetylated or glycosylated derivatives of neuraminic acid—is a component of many glycoproteins and glycolipids. In the erythrocyte membrane, it is mainly contained in the SA-rich glycophorins comprising three major proteins involved in cytoskeletal interactions: glycophorin A, glycophorin B, and glycophorin C.[1] Red blood cell (RBC) aging is accompanied by desialylation of membrane glycoconjugates caused by cleavage of terminal SA residues or by removal of sialoglycoconjugates.[2-5] This phenomenon might trigger the clearance of senescent RBCs by unmasking crypto-antigens.

Recent evidence indicates that desialylated, but not sialylated, low-density lipoprotein subfractions induce massive cholesterol accumulation in cells cultured from human aortic intima, and might therefore contribute to age-dependent atherosclerosis development.[6] However, little information is available on the role of SA content in plasma membranes during human aging.

A decreased SA content in glycophorin A has been observed in erythrocyte membranes obtained from diabetic patients. As the main determinant of negative charge on the cell surface, SA has been associated with increased RBC aggregation.[7] An accelerated aging of erythrocytes has been described in human diabetes mellitus.[8] It might be hypothesized that this phenomenon is dependent on abnormal recognition of the signal identifying senescent cells. However, data on the behavior of RBC membrane SA content in relation to cellular and human senescence are still lacking in diabetes mellitus. The aim of the present investigation was to study RBC membrane SA content in healthy and diabetic subjects at various ages to detect a possible relation between RBC membrane SA content and human age in normal and pathological conditions. Na^+/K^+-ATPase activity of the RBC membrane was also studied as a marker of membrane function affected by age and diabetes mellitus.

SUBJECTS AND METHODS

Twenty-six normoalbuminuric insulin-dependent diabetes mellitus (IDDM) patients and 25 normoalbuminuric non–insulin-dependent diabetes mellitus (NIDDM) patients were recruited from the outpatient clinic of INRCA. Forty healthy nondiabetic subjects with a negative family history for diabetes served as controls and were divided into two groups according to age: C_1 consisted of 25 younger controls comparable to the IDDM subjects, and C_2 contained 15 older controls comparable to the NIDDM subjects. Characteristics of the subjects are shown in Table 1. All subjects provided informed consent to participate in the study.

Hemoglobin A_{1c} (HbA_{1c}) level was measured by high-performance liquid chromatography according to the method of Akai.[9]

Erythrocyte Membrane Preparation

Heparinized blood samples (10 mL) collected after overnight fasting were centrifuged (4,500 × *g*) to remove plasma. RBCs were washed twice with NaCl 0.9% isotonic solution, lysed hypotonically in 5 mmol/L ice-cold phosphate buffer solution (pH 8), and processed in a Kontron (Milano, Italy) centrifuge at 20,000 × *g*. The resulting membranes were washed with phosphate buffer of decreasing molarity to completely remove the hemoglobin.[10] The membrane yield was similar in all groups studied (~2 mg membrane proteins).

Na^+/K^+-ATPase Assay

Na^+,K^+-activated Mg^{2+}-dependent ATPase activity was determined in RBC plasma membranes by the method of Kitao and Hattori.[11] ATPase activity was assayed by incubating the membranes (0.1 mg membrane proteins) at 37°C in 1 mL medium containing $MgCl_2$ (5 mmol/L), NaCl (140 mmol/L), and KCl (14 mmol/L) in 40 mmol/L Tris hydrochloride, pH 7.7. The ATPase reaction was started by addition of 3 mmol/L Na_2ATP, and was stopped 20 minutes later by addition of 1 mL 15% trichloracetic acid. Inorganic phosphate (P_i) hydrolyzed from the reaction was assayed as previously described.[12] Enzyme activity was expressed as the difference in inorganic phosphate released in the

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Table 1. Age and Sex Characteristics of the Subjects

Group	No. of Subjects	Age (yr) Range	Mean ± SD	Females	Males	HbA_{1c} (%)
Controls	40	18-88				
Young (C_1)	25	18-52	32.8 ± 10.1	13	12	4.9 ± 0.9
Old (C_2)	15	55-88	73.2 ± 10.9	8	7	5.0 ± 0.8
IDDM	26	19-61	40.3 ± 13.2	14	12	10.2 ± 1.7
NIDDM	25	48-84	64.5 ± 9.7	13	12	9.4 ± 1.6

presence and absence of 1 mmol/L ouabain. ATPase activity assayed in the presence of ouabain was subtracted from total Mg^{2+}-dependent ATPase activity to calculate the activity of ouabain-sensitive Na^+/K^+-ATPase. The results are expressed as micromoles P_i per milligram membrane proteins per 60 minutes. Assays were performed in triplicate. Protein concentration was determined as previously described.[13]

Determination of SA Content

SA content of RBC membranes was determined by the periodate–thiobarbituric acid method of Denny et al.[14] Briefly, membranes (1 mg membrane proteins/mL) were first hydrolyzed in 0.05-mol/L H_2SO_4 in a final volume of 0.1 mL for 1 hour at 80°C to release SA.[15] Both standards and samples were incubated with 0.25 mL 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.

Statistical Analysis

Student's unpaired *t* test was used to determine differences between diabetic and control subjects. Linear regression analysis was used to evaluate correlations between the different parameters. Statistical analysis was performed with StatView software for MacIntosh (Cupertino, CA).

RESULTS

A decrease in RBC membrane SA content was observed in older control subjects compared with younger controls (Table 2; $P < .005$). The study of the relation between subject age and SA content showed a significant correlation in C_1 and C_2 groups evaluated together (Table 3; $r = -.79$, $P < .001$).

Na^+/K^+-ATPase activity was significantly lower in older controls than in younger ones (Table 2; $P < .005$). A significant negative correlation was observed between age and Na^+/K^+-ATPase activity in the control groups evaluated together (Table 3; $r = -.75$, $P < .001$). Na^+/K^+-ATPase activity was significantly related to SA content in healthy subjects (Table 3; $r = .63$, $P < .001$).

No difference was observed in RBC membrane SA content between IDDM and NIDDM subjects, whereas Na^+/K^+-ATPase activity was significantly lower in IDDM (Table 2; $P < .005$).

SA content was increased in NIDDM subjects compared with healthy subjects of similar age (Table 2; $P < .005$). No difference was observed between C_1 and IDDM subjects. Na^+/K^+-ATPase activity was significantly lower both in IDDM subjects compared with C_1 and in NIDDM subjects compared with C_2 (Table 2; $P < .001$).

No significant correlation was observed between age and Na^+/K^+-ATPase activity or SA content in IDDM patients. In NIDDM, Na^+/K^+-ATPase activity was significantly correlated with age ($r = -.573$, $P < .005$). Na^+/K^+-ATPase activity and SA content were negatively correlated in IDDM ($r = -.512$, $P < .005$) and in NIDDM ($r = -.477$, $P < .01$). HbA_{1c} levels were not significantly correlated with Na^+/K^+-ATPase or with SA content in diabetic patients.

Table 2. RBC Membrane SA Content (μg/mg protein) and Na^+/K^+-ATPase Activity (μmol P_i /h/mg protein) in Healthy and Diabetic Subjects (mean ± SD)

Group	SA Content	Na^+/K^+-ATPase Activity
C_1, younger controls (n = 25)	73.29 ± 9.04*	1.49 ± 0.25‡§
C_2, older controls (n = 15)	45.12 ± 14.43*†	1.21 ± 0.15‡‖
IDDM (n = 26)	73.68 ± 20.44	0.74 ± 0.14§¶
NIDDM (n = 25)	65.45 ± 20.21†	0.97 ± 0.15‖¶

*$P < .005$, C_1 *v* C_2.
†$P < .005$, NIDDM *v* C_2.
‡$P < .005$, C_1 *v* C_2.
§$P < .001$, C_1 *v* NIDDM.
‖$P < .001$, C_2 *v* NIDDM.
¶$P < .001$, IDDM *v* NIDDM.

DISCUSSION

A decreased erythrocyte membrane SA content was found in the present study in elderly subjects compared with the young, and a significant negative correlation was found between erythrocyte SA content and subject age.

Sialylated glycoproteins are responsible for the negative charge of the erythrocyte membrane surface.[16] Therefore, the reduction in SA content might lead to decreased intercellular electrostatic repulsion and enhanced erythrocyte aggregation in the elderly. The correlation observed between SA content and Na^+/K^+-ATPase activity in controls might be the result of the dependence of both parameters on age.

In diabetic subjects, a completely different behavior of Na^+/K^+-ATPase and SA content has been observed. In fact, Na^+/K^+-ATPase activity was significantly reduced both in NIDDM and in IDDM patients compared with control groups of similar age. The correlation between age and enzymatic activity was present only in NIDDM patients. One might therefore hypothesize that diabetes mellitus affects Na^+/K^+-ATPase activity in the same way as aging, in this case being the cause of

Table 3. Results of Regression Analysis Showing Statistical Significance for Na^+/K^+-ATPase Activity, SA Content, HbA_{1c}, and Age

Comparison	Normal Regression	IDDM Regression	NIDDM Regression
Na^+/K^+-ATPase *v* age	−.75 ($P < .001$)	NS	−.57 ($P < .005$)
SA *v* age	−.79 ($P < .001$)	NS	NS
SA *v* Na^+/K^+-ATPase	.63 ($P < .001$)	−.51 ($P < .005$)	−.48 ($P < .01$)
SA *v* HbA_{1c}	NS	NS	NS
Na^+/K^+-ATPase *v* HbA_{1c}	NS	NS	NS

early aging. In contrast, RBC membrane SA content was increased in NIDDM subjects compared with elderly controls. The increased SA content in NIDDM subjects might suggest increased erythropoiesis in NIDDM subjects compared with nondiabetic subjects of similar age. No modification in SA content was observed in IDDM subjects compared with young controls.

Previous studies in NIDDM and IDDM with incipient nephropathy demonstrated increased serum total SA,[17,18] which is claimed to be a strong predictor of cardiovascular mortality.[19,20] The mechanism of this increase is still unknown, although acute-phase proteins or serum triglyceride levels have been supposed to be involved in the elevation of serum SA.[19,20] It is noteworthy that in the present study the alteration in erythrocyte SA content was present only in NIDDM patients, who are known to present elevated serum SA,[21] whereas normoalbuminuric IDDM subjects, who are reported to show only a slight elevation in serum SA,[18] had no modification in erythrocyte SA content.

The increase in membrane SA observed in NIDDM might be due to either enhanced SA synthesis or decreased sialidase activity. Further studies are in progress to elucidate the mechanisms of this alteration. However, a role of glycemic control might be excluded on the basis of the lack of correlation between SA and HbA_{1c}.

In diabetic patients, there was a negative correlation between Na^+/K^+-ATPase activity and SA content. Previous studies by our group suggested that the alterations of erythrocyte Na^+/K^+-ATPase activity in diabetes might be caused by a modified membrane fluidity.[22] It might therefore be hypothesized that membrane SA content affects Na^+/K^+-ATPase activity in diabetes through a modification of membrane fluidity.

In conclusion, NIDDM affects some features of the RBC membrane, such as SA content, in a way opposite to that of physiological aging, whereas other membrane functions (Na^+/K^+-ATPase) are altered by IDDM and NIDDM in a way similar to aging. The altered RBC membrane SA content in NIDDM might be due to a more generalized derangement in SA metabolism also involving serum SA, a possible marker of predisposition to cardiovascular disease.

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